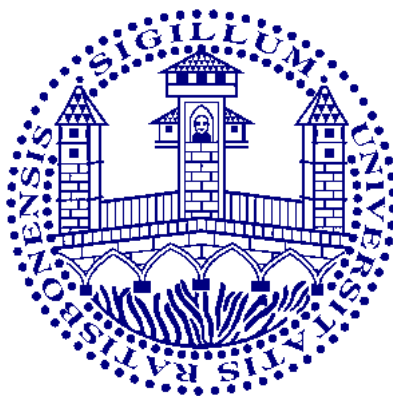


Label-free Sensing Strategies to Monitor Disease Models of Glaucoma In Vitro



DISSERTATION

Zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.) der
Fakultät für Chemie und Pharmazie

der Universität Regensburg

vorgelegt von

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im Jahr

2023

Promotionsgesuch eingereicht am: 09.11.2023

Promotionskolloquium: 30.01.2024

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1 Introduction

1.1 Glaucoma

1.1.1 Ocular Anatomy and Aqueous Humor Outflow

The eyes are organs enabling the vision of our environment and are highly sensitive. Human eyes are very similar to those of other mammals, although there are some differences, for example, eyes of cats are working with a contrast that is ten times better than that of human eyes (Campbell *et al.*, 1973). We gather most of the information about our environment through our eyes, which makes the visual sense the most important one to us. However, this thesis does not cover optics and the processes of how our vision is generated, but instead, the principle of aqueous humor production and outflow, which is impaired in glaucoma, is addressed. In glaucoma, which is a set of eye diseases, mechanobiological alterations in the proper function of the eye lead to progressing peripheral vision loss until complete blindness (for more information see section 1.1.2). The main reason for this is considered an increased intraocular pressure (IOP), which in succession damages the visual nerve (or also called optic nerve) (Gordon *et al.*, 2002). These relationships are explained based on Figure 1.1, which demonstrates the anterior cavity of a human eye.

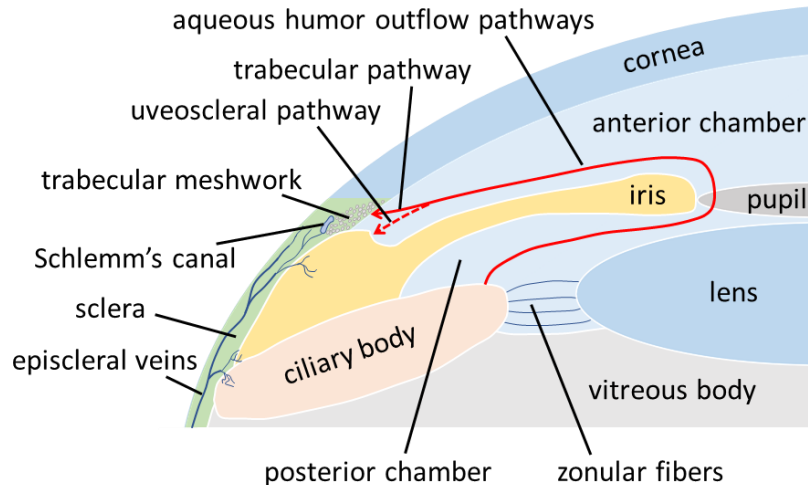


Figure 1.1 Schematic illustration demonstrating a cross-section through the anterior cavity of the human eye with its main components and aqueous humor dynamics. Aqueous humor is produced in the ciliary body and flows from the posterior chamber through the pupil to enter the anterior chamber. From there, drainage of aqueous humor occurs mainly by the trabecular pathway through the trabecular meshwork and Schlemm's canal (red arrow with solid line), while only a small fraction is drained out by the uveoscleral pathway (red arrow with dashed line). The illustration was redrawn according to published literature (Kronfeld, 1962; Mietzner & Breunig, 2019). Drawings not to scale.

In contrast to the posterior cavity, filled with vitreous body, a gel-like material for maintaining the eye's shape, in the anterior cavity, the IOP is generated, which results from the balance between production and outflow of aqueous humor. This aqueous humor is a fluid produced in the ciliary body (Van Alphen & Macri, 1966) providing the iris, lens, and cornea with nutrients. It is a transparent substitute for blood vessels avoiding light reflection and scattering. After its secretion by the ciliary body, the aqueous humor crosses the posterior chamber (not to be confused with the posterior cavity) and the pupil, and finally reaches the anterior chamber. After nutrient supply, it leaves the eye via the iridocorneal angle, which occurs by two distinct outflow pathways. The main route is the trabecular outflow pathway (or also called conventional pathway) (indicated by the red arrow with solid line in Figure 1.1), where the aqueous humor traverses the trabecular meshwork (TM) and the Schlemm's canal and flows into the bloodstream via the episcleral veins (Tamm, 2009). The second route is the uveoscleral outflow pathway (or also called unconventional pathway) (indicated by the red arrow with dashed line in Figure 1.1), where the aqueous humor leaves the eye by a route through the iris, ciliary body, suprachoroidal space, sclera and finally reaching episcleral tissues (Bill & Phillips, 1971). The suprachoroidal space is located internal to the sclera and external to the choroid, which in turn is the innermost part of the shell of the eyeball and covered by the retina in the back regions of the posterior cavity (Kronfeld, 1962). The fraction of the uveoscleral outflow of total outflow in human eyes is less than 15% (Bill & Phillips, 1971). An increase in IOP can be caused by an enhanced production of aqueous humor in the ciliary body or by an increased resistance to its outflow. IOP generation and maintenance were found to be only dependent on the trabecular outflow pathway and independent of the uveoscleral outflow pathway (Johnson & Erickson, 2000; Johnson, 2006). Although the IOP originates from the production and outflow of aqueous humor, IOP itself also influences the outflow capacity, where the uveoscleral outflow pathway is less sensitive to IOP changes (Johnson & Erickson, 2000). The IOP is typically measured by applanation tonometry, which was developed in the late 19th century (Imbert, 1885). Normal values, which are on average 15 mmHg – 16 mmHg, help maintain the eye's shape, while a value above 21 mmHg is considered an elevated IOP (Shiose, 1990). IOP is dependent on race (Klein & Klein, 1981), blood pressure (Klein & Klein, 1981), and obesity (Mori *et al.*, 2000), while influences of age alone were not discovered yet, since systemic factors like blood pressure and obesity are typically coupled with age. Considering the increase in the occurrence of high blood pressure and obesity with age, a decrease in IOP with age was even found in one study (Shiose, 1990). IOP is often increased in glaucoma, the mechanism of which is not completely resolved, but structural changes in the TM seem to be most likely responsible for an increased resistance to aqueous humor outflow, leading to an increased IOP (Tamm & Fuchshofer, 2007). The TM is a spongy tissue located at the junction region of the sclera and cornea (Kronfeld, 1962) consisting of three parts with characteristically different structures. The inner uveal meshwork is located closest to the anterior chamber and consists of lamellae of flat endothelial cells with the spaces in between filled with extracellular collagen fibers leaving trapezoidal or rhomboidal openings with sizes of 25 μm – 75 μm (Flocks, 1956). The corneoscleral meshwork exhibits a similar

lamellar structure, but with elliptical openings with smaller sizes of 5 μm – 50 μm (Flocks, 1956). The juxtacanalicular tissue (JCT) (or also called cribriform meshwork) lies directly adjacent to the inner wall endothelium of Schlemm's canal and is composed of rather flat cells embedded in a very loosely packed extracellular matrix (ECM) (Vegge, 1967). Due to the porous character of the inner uveal meshwork and the corneoscleral meshwork, these parts of the TM do not significantly contribute to outflow resistance (Tamm & Fuchshofer, 2007). Aqueous humor passes the TM by mainly flowing through spaces between TM cells and ECM fibers, so it flows around the cells and does not cross the cell bodies (Tamm, 2009). In the healthy eye, outflow resistance is mainly generated by the JCT region of the TM and the inner wall endothelium of Schlemm's canal (Johnson, 2006). In the glaucomatous eye, there is still debate about where the increased outflow resistance exactly originates from, but the JCT region of the TM is mostly considered to be the main factor (Tamm & Fuchshofer, 2007). In a healthy state, an expansion in this region allows an increased outflow of aqueous humor to reduce IOP, which is probably impaired in glaucoma (O'Callaghan *et al.*, 2017).

1.1.2 The Pathophysiology of Glaucoma

Blindness typically appears in adults aged 50 years and older with 34 million cases of global blindness in this group, where glaucoma is one of the main reasons beside cataract, undercorrected refractive error, age-related macular degeneration, and diabetic retinopathy (Steinmetz *et al.*, 2021). Due to the increasing average age of the global population, especially in the western industrial nations, this number is expected to increase, which makes ophthalmology a topic of increasing interest and importance. Glaucoma is a set of eye diseases, all in common characterized by progressing loss of the peripheral visual field caused by damage to the visual nerve. Barkan was the first who started the classification of glaucoma into two major groups, by differently wide openings of the iridocorneal angle (Barkan, 1938). Nowadays, glaucoma is divided into three classes, open-angle glaucoma (or also called wide-angle glaucoma), closed-angle glaucoma (or also called narrow-angle glaucoma or angle closure glaucoma), and normal-tension glaucoma (or also called low-tension glaucoma). Open-angle glaucoma is characterized by a normal opening of the iridocorneal angle, while an increased resistance to aqueous humor outflow is generated by the filtration apparatus itself (TM and Schlemm's canal), leading to an increased IOP (Chandler & Trotter, 1955). In contrast to that, closed-angle glaucoma comprises different subtypes, all in common characterized by repeated episodes of closure of the iridocorneal angle by contact between iris and TM, which prevents the aqueous humor from leaving the eye by the two outflow pathways with the result of an increased IOP (Chandler & Trotter, 1955). Normal-tension glaucoma is a somewhat different type of glaucoma since it shows the same disease pattern as the two previously mentioned types but with a normal IOP (Killer & Pircher, 2018). The appearance of normal-tension glaucoma is explained as a complex syndrome consisting of a variety of pathological pathways, among them a reduced IOP tolerance. Equivalent to other diseases, glaucoma can be separated into primary and

secondary forms, where secondary glaucoma means that it emerges as a result of another issue, like an eye injury, inflammation, or long-time cortisone medication, whereas primary forms are not correlated with other health issues. Primary open-angle glaucoma (POAG) is the most prevalent form of glaucoma. About 69 million people in the world are affected by POAG, which corresponds to a percentage of about 0.9%, with a much higher percentage of about 2.4% in the population aged 40 years and older (Zhang *et al.*, 2021). Mostly both eyes are affected but it is typically stronger pronounced in one eye. Although its pathophysiology is not completely understood yet, the most important risk factor of POAG is an increased IOP (Gordon *et al.*, 2002) and treatments lowering IOP can stop the progression of this disease (Weinreb & Khaw, 2004). In the context of POAG, an increased IOP results from a reduced outflow of aqueous humor due to an elevated resistance in the outflow pathways. Reasons for this elevated resistance have been unclear for a long time but the amount and quality of the ECM in the JCT region of the TM are considered to be responsible (Tamm & Fuchshofer, 2007). Recently, it was discovered that stiffening of cells and ECM in the TM, in terms of an increased Young's modulus (for more information about Young's modulus see section 3.3.1), plays an important role in POAG (Last *et al.*, 2011; Wang *et al.*, 2018). Since it is known that the stiffness of cells is mainly determined by the cytoskeleton, with actin filaments as the major contributor (Wang, 1998), the role of cytoskeletal changes in TM cells of glaucomatous eyes was investigated in the past few years. Structural reorganizations like intracellular formation of tangled actin filaments and crosslinked actin networks (Read *et al.*, 2007) and accumulation of ECM (Tamm & Fuchshofer, 2007) were identified in the TM of glaucomatous eyes. Accordingly, a cytoskeleton-induced increase in TM stiffness is directly correlated with an increased outflow resistance and, thus, an increased IOP. Furthermore, an increase in TM stiffness with senescence was figured out (Morgan *et al.*, 2015), which would explain the more frequent occurrence of POAG in people of advanced age. The progressing peripheral vision loss is a direct consequence of the increased IOP since the visual nerve head (or also called optic disc) and retinal ganglion cells are damaged by the increased mechanical stress (Quigley *et al.*, 1980). In normal-tension glaucoma, the reduced IOP tolerance leads to the same result of visual nerve damage. The peripheral vision loss will extend to complete blindness when untreated (glaucoma treatment see in section 1.1.3). Therefore, early recognition and treatment are of highest importance but the asymptomatic and painless course of POAG in the early stage makes an early recognition difficult. It is diagnosed by ophthalmoscopy (or also called funduscopy) and visual field assessment using perimetry (Crick, 1982).

A connection between POAG and the transforming growth factor- β 2 (TGF- β 2) was identified in a couple of studies. Under physiological conditions, TGF- β 2, as a member of the transforming growth factor- β (TGF- β) protein family, is known to regulate cellular differentiation and proliferation in many important functions all over the human body. It elicits these effects by binding to its corresponding growth factor receptor. But in POAG, an increased concentration of TGF- β 2 was found in the aqueous humor of eyes affected by this disease (Tripathi *et al.*, 1994). TGF- β 2 was discovered to induce an increased expression of

ECM proteins in cell culture (Zhao *et al.*, 2004) and eye perfusion studies (Gottanka *et al.*, 2004). A significant role of TGF- β proteins was also identified in other diseases correlated with an accumulation of ECM, like liver fibrosis (Bataller & Brenner, 2005) and kidney fibrosis (Liu, 2006). Connective tissue growth factor (CTGF) is a downstream mediator of TGF- β 2 because CTGF is highly up-regulated in TM cells after TGF- β 2 treatment (Fuchshofer *et al.*, 2007). In physiological concentrations, CTGF is known to play an important role in biological processes like cell adhesion, proliferation, and migration. CTGF is also expressed in the TM (Tomarev *et al.*, 2003). A specific receptor for CTGF has not been identified so far, it can perform its functions through binding to a variety of different receptors and also to integrins, many of them also found in TM cells (Junglas *et al.*, 2009). Since CTGF is a downstream mediator of TGF- β 2, and TGF- β 2 is abundant in the aqueous humor of glaucomatous eyes, Junglas *et al.* concluded that CTGF levels may be increased in the TM in POAG (Junglas *et al.*, 2012). Artificially increased concentrations of CTGF, either by direct addition of CTGF or by adenoviral gene transfer of a vector encoding for CTGF, influence the actin cytoskeleton of TM cells in such a way that the IOP increases and the visual nerve gets damaged (Junglas *et al.*, 2012).

1.1.3 Glaucoma Treatment Strategies

There are different methods to treat glaucoma with the common aim of reducing IOP to prevent or, at least, decelerate the progression of the disease after diagnosis. The risk of proceeding visual damage and the risk of getting completely blind is reduced, but already acquired damage of the visual nerve is irreversible and not curable. Glaucoma treatment strategies comprise medication and surgical methods, either incisional or by laser. Generally, medication is the first-choice type of treatment, where many different drugs are available on the market, which are typically applied as eye drops. The selection of the proper drug depends on many factors, among them the age of the patient, current IOP, the extent of visual nerve damage, and the type of glaucoma. Parasympathomimetic drugs like pilocarpine cause a reduction in pupil size and an opening of new capillary districts in the outflow pathways by a vasodilatation effect, leading to an increase in aqueous humor outflow (Cristini, 1949). Prostaglandin analogs like latanoprost enhance the uveoscleral outflow by increasing the permeability of the tissues involved in this outflow pathway due to a relaxation effect (Bito *et al.*, 1993). An additional enhancement of the trabecular outflow by prostaglandins was discovered later and explained by the disassembly of actin stress fibers in Schlemm's canal cells (Bahler *et al.*, 2008). Parasympathomimetic drugs and prostaglandins can only be used in open-angle glaucoma. β -Adrenergic antagonists (or also called β -blockers) like timolol decrease the production of aqueous humor in the ciliary body by blocking β -adrenergic receptors (Zimmerman & Kaufman, 1977). Carbonic anhydrase inhibitors like dorzolamide also decrease the production of aqueous humor in the ciliary body, but due to the inhibitory effect on human carbonic anhydrase isoenzyme II involved in the ciliary processes (Lippa *et al.*, 1992). Different drugs can also be combined. For example,

timolol and dorzolamide are combined in the drug Cosopt®, which is available as eye drops on the German market. α 2-Adrenergic agonists like brimonidine decrease the production of aqueous humor in the ciliary body by the inhibition of adenylate cyclase and additionally enhance the uveoscleral outflow by binding to imidazoline receptors in the ciliary body (Toris *et al.*, 1995). If medication alone is not able to stably reduce IOP or side effects are obtained, a treatment by surgical methods is necessary. Surgery in open-angle glaucoma aims at the ciliary body or the TM (cyclodialysis, trabeculectomy, trabeculoplasty), whereas closed-angle glaucoma requires the iris to be treated (iridectomy, iridoplasty). In cyclodialysis, heat is applied to detach the ciliary body from the sclera, leading to a reduction of aqueous humor production in the ciliary body and simultaneously to an increase in the uveoscleral outflow (Alvis, 1958). Trabeculectomy (or also called goniotomy) describes the surgical removal of parts of the TM to increase the trabecular outflow (Sugar, 1961). An alternative to trabeculectomy is trabeculoplasty, a laser treatment of the TM to increase the trabecular outflow, which is explained by heat-induced shrinkage of collagen fibers or by tissue contraction at the burn sites (Wise & Witter, 1979). Surgical treatments of closed-angle glaucoma comprise iridectomy, a surgical removal of parts of the iris to open the iridocorneal angle for the drainage of aqueous humor (von Graefe, 1859), and iridoplasty, an application of laser pulses to the iris to widen the iridocorneal angle (Cohen *et al.*, 1982). In severe cases of glaucoma, an implantation of an artificial drainage device may be required to create an alternative pathway of aqueous humor outflow from the anterior chamber to lower the IOP, with many different types available regarding design and material (Chaudhry *et al.*, 2012). These conventional glaucoma treatment strategies comprising medication and surgical methods exhibit several disadvantages, especially for the treatment of POAG. A poor permeability of the cornea and fast drainage with the tear fluid limits the intraocular bioavailability of the drugs applied as eye drops. Side effects often occur because the drugs do not directly treat the pathological changes in the TM, which are responsible for the increased IOP in POAG. Additionally, patients may have trouble with the correct application of eye drops and often forget or even ignore application. Surgical methods are painful and maybe lead to scar formation and other complications. Thus, searching for new glaucoma treatment strategies, which more specifically target the origin of IOP increase, is of great interest in recent research and also in the future. Several different compounds and their potential IOP lowering effects have been investigated recently, for example, cannabinoids (Oltmanns *et al.*, 2008), melatonin receptor agonists (Alarma-Estrany *et al.*, 2011), adenosine receptor agonists (Crosson *et al.*, 2005), serotonin receptor agonists (Sharif, 2010), dopamine receptor agonists (Bucolo *et al.*, 2012), hydroxysteroid dehydrogenase-1 inhibitors (Rauz *et al.*, 2003), nitric oxide synthase inhibitors (Kiel *et al.*, 2001), and small interfering RNA (Comes & Borrás, 2007). Since evidence was found for the correlation between POAG and the cytoskeleton-related increase in TM stiffness, research was focused on actin cytoskeleton modulators for the treatment of this disease. The actin cytoskeleton disrupting agents cytochalasin B (Kaufman & Bárány, 1977), cytochalasin D (Johnson, 1997), latrunculin A (Peterson *et al.*, 1999), and latrunculin B (Peterson *et al.*, 2000) were already demonstrated to have positive effects on aqueous humor outflow capacity.

More recently, the focus of research was turned on Rho-associated protein kinase (ROCK) inhibitors, which also show actin cytoskeleton regulation. To understand the effect of ROCK inhibitors, one must first have a look at ROCKs and the Ras homolog (Rho) family itself. As a subfamily of the Ras superfamily of proteins, Rho comprises different G proteins, which are not membrane-bound and are therefore called small G proteins. They are abundant in cells all over the human body. Rho proteins are also called Rho GTPases and are inactive when bound to GDP, whereas binding to GTP activates them (Figure 1.2).

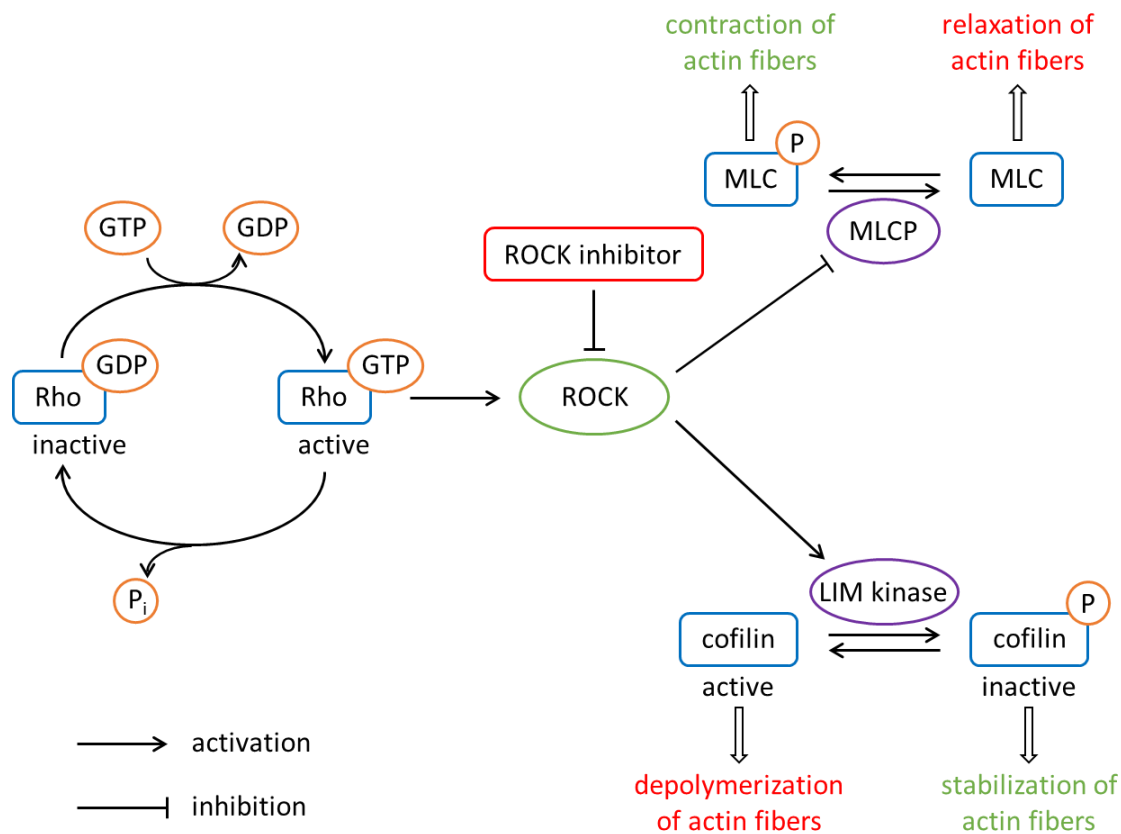


Figure 1.2 Schematic illustration of the most important Rho-associated protein kinase (ROCK) signaling pathways. A GDP-GTP switch provides the active form of a Rho GTPase, which can activate ROCKs. ROCKs inhibit myosin-light-chain phosphatase (MLCP) and activate LIM kinases, finally leading to contraction and stabilization of actin fibers (green). The function of ROCKs can be inhibited by ROCK inhibitors, leading to relaxation and depolymerization of actin fibers (red). The diagram was summarized from published literature (Nakagawa *et al.*, 1996; Uehata *et al.*, 1997; Yang *et al.*, 1998; Mietzner & Breunig, 2019).

There are many different Rho proteins, among them the most common forms RhoA, RhoB, and RhoC, regulating many aspects of actin cytoskeleton dynamics, especially the formation of stress fibers and focal adhesions. These functions are elicited by ROCKs, which are intracellular proteins and are activated by the active forms of the Rho GTPases (Figure 1.2). There are ROCK I and ROCK II, which play distinct roles in a tissue-specific manner (Nakagawa *et al.*, 1996), but the most important effects are inhibition of myosin-light-chain phosphatase (MLCP) (Uehata *et al.*, 1997) and activation of LIM kinases (Yang *et al.*, 1998)

(Figure 1.2). MLCP dephosphorylates the myosin-light-chain (MLC) in smooth muscles causing their relaxation. Thus, MLCP inhibition by ROCKs leads to a sustained contraction of actin fibers. ROCKs phosphorylate LIM kinases, thereby activating their ability to phosphorylate and inactivate cofilin, which is an actin-depolymerizing protein (for more information about cytoskeletal filaments see section 1.2). Thus, the activation of LIM kinases by ROCKs leads to actin filament stabilization and accumulation. Accordingly, inhibition of Rho GTPases or inhibition of ROCKs leads to relaxation, destabilization, and depolymerization of actin fibers (Figure 1.2). Since the TM tissue was shown to express Rho GTPases (Thieme *et al.*, 2000) and ROCKs (Fukiage *et al.*, 2001), the application of Rho GTPase inhibitors or ROCK inhibitors may reduce outflow resistance and, consequently, also reduce IOP in glaucomatous eyes. Although crystal structure studies have revealed the low selectivity of ROCK inhibitors for one specific kinase (Breitenlechner *et al.*, 2003), multiple studies have shown positive effects of ROCK inhibitors on outflow capacity and IOP, thus, opening a new way of glaucoma treatment by ROCK inhibitors (Rao & Epstein, 2007). The ROCK inhibitor Y-27632 increases aqueous humor outflow capacity in perfused eyes (Rao *et al.*, 2001) and shows IOP reduction in rats (Uehata *et al.*, 1997) and rabbits (Waki *et al.*, 2001). The probably most prominent and most investigated ROCK inhibitor is fasudil (or also called HA1077 or AT877), which was also the first protein kinase inhibitor approved for medical use. Fasudil was discovered as an arterial vasodilator (Asano *et al.*, 1989) and was initially utilized for the treatment of cerebral vasospasm (Shibuya *et al.*, 1992). It was approved for this use in 1995 by the National Medical Products Administration in China and the Pharmaceuticals and Medical Devices Agency (PMDA) in Japan, but it was not approved by the Food and Drug Administration (FDA) in the USA and the European Medicines Agency (EMA) in the EU. The function of fasudil as a potent ROCK inhibitor was identified later (Asano *et al.*, 1998). Fasudil was found to increase blood flow in the visual nerve head and to lower IOP in rabbits (Sugiyama *et al.*, 2011). In 2017, fasudil was tested for its potential IOP lowering effect for the first time in human glaucomatous subjects, with a positive result of IOP reduction, revealing its promising application for glaucoma treatment (Pakravan *et al.*, 2017). Furthermore, fasudil can also prevent retinal ganglion cell death in rats (Zhang *et al.*, 2020). In another context, fasudil was shown to suppress tumor progression by slowing down tumor cell migration (Ying *et al.*, 2006). But a disadvantage of many ROCK inhibitors like fasudil for the treatment of glaucoma is its high hydrophilicity and consequently low intraocular bioavailability after its injection into the vitreous body, which can be overcome by a depot formulation, as it was recently shown by the use of intravitreally injectable poly(lactic-co-glycolic acid) (PLGA) microspheres containing fasudil (Mietzner *et al.*, 2020). Fasudil is released from the microspheres over a prolonged period of time, diffuses through the vitreous body, and reaches its target tissue, the TM, with the flow of aqueous humor. Several other ROCK inhibitors have been studied in the past few years, among them the compound H-1152P, a fasudil analogue, which was proven to reduce IOP in normotensive and hypertensive rabbits (Nishio *et al.*, 2009). More recently, two novel compounds were discovered to act as ROCK inhibitors. Ripasudil, a fasudil analogue, showed an IOP reduction in patients after one year of application (Tanihara *et al.*, 2016) and additionally prevents

retinal ganglion cell death in mice (Akaiwa *et al.*, 2018), which motivated the PMDA in Japan for its approval. Netarsudil was discovered to reduce IOP in rabbits (Sturdivant *et al.*, 2016), which is attributed to the finding that it inhibits ROCK I and ROCK II and disrupts actin stress fibers and focal adhesions in TM cells (Lin *et al.*, 2018). The FDA in the USA and the EMA in the EU recently approved netarsudil as a medication to treat open-angle glaucoma. Thus, ROCK inhibitors have proven to be a promising class of drugs allowing a causative therapy of POAG with only little to no side effects, where conjunctival hyperemia is the most reported one. All these discoveries and studies might help find new drugs in the future, inhibiting ROCK-signaling or influencing other pathways, with possibly even better efficacy and safety profiles and no side effects. Special focus is directed to injectable depot formulations with long-time release of drugs for a target-specific delivery, increasing bioavailability, and reducing application frequency.

1.2 Cytoskeleton

Since there is a known correlation between POAG and the cytoskeleton-related stiffness of the TM, in this section some information is given about the cytoskeleton with its different subtypes with characteristic structures and functions. The cytoskeleton is a complex network of filamentous proteins ensuring cell stability and many other cellular functions. Three different types of filaments can be distinguished: microtubules, intermediate filaments, and actin filaments (or also called microfilaments), with each having a distinct structure and functions. This is demonstrated in Figure 1.3.

The structure of microtubules (Stephens & Edds, 1976; Hawkins *et al.*, 2010) is characterized by linear protofilaments, which are formed out of heterodimers of α - and β -tubulin (Figure 1.3 A). One single microtubule is formed by the tubular arrangement of typically thirteen protofilaments with the hollow lumen filled with cytosol. The microtubules are the cytoskeletal filaments with the biggest size with a mean outer diameter of (23 – 25) nm. They show polarity with their so-called plus end growing towards the cell periphery and their so-called minus end anchored at the centrosome (or also called microtubule-organizing center). Microtubule dynamics are characterized by dynamic instability, which describes the coexistence of polymerizing and depolymerizing microtubules, which can transit into each other, where both processes are occurring on the plus ends (Desai & Mitchison, 1997). This ensures high flexibility. Microtubule functions encompass the separation of chromosomes in the process of cell division, maintenance of cell shape, intracellular particle and vesicle transport, secretion of particles, and formation of cilia, which are thin and flexible evaginations of the plasma membrane for capturing nutrient particles or transporting particles extracellularly within a whole organism.

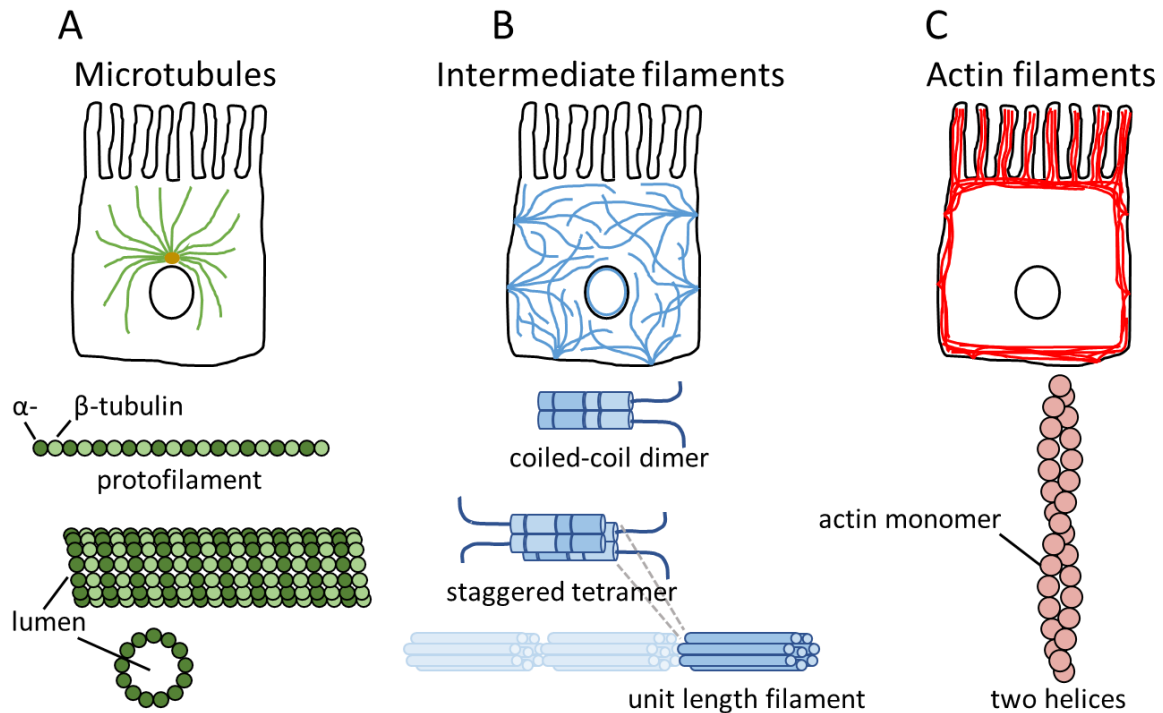


Figure 1.3 Schematic illustration showing the distribution over the cell and structure of the three different types of cytoskeletal filaments. **(A)** Microtubules are growing from the centrosome towards the cell periphery. Their structure is based on a tubular arrangement of thirteen protofilaments consisting of alternating α - and β -tubulin units. **(B)** Intermediate filaments appear in stiff, but highly organized, networks distributed over the complete cell body and anchored at desmosomes (cell-cell) and hemidesmosomes (cell-ECM) in the plasma membrane. They also make up the nuclear lamina lining the inner nuclear membrane. Intermediate filaments are composed of homopolymers of different filament-forming proteins. Two coiled-coil dimers form one staggered tetramer, eight staggered tetramers form one unit length filament, several unit length filaments in succession form one intermediate filament. **(C)** Actin filaments appear in different structures close to the plasma membrane: microvilli, stress fibers anchored at focal contacts (cell-ECM), and cell cortex anchored at adherens junctions (cell-cell). Actin filaments are homopolymers of actin composed of two interlaced helices of actin strands. Illustrations were redrawn according to published literature (Hanson & Lowy, 1963; Hawkins *et al.*, 2010; Dominguez & Holmes, 2011; Etienne-Manneville, 2018).

The second type of cytoskeletal filaments is the intermediate filaments (Fuchs & Weber, 1994; Etienne-Manneville, 2018), which comprise different filament-forming proteins sharing similarities in amino acid sequence and protein structure, subcategorized into six cytoplasmic classes, with the exception of the nuclear lamina lining the inner nuclear membrane (Figure 1.3 B). Intermediate filament structure is based on homopolymers, while staggered tetramers are formed out of two coiled-coil dimers. Eight of these tetramers are compacted into a rope, where seven of them encircle one in the middle, forming one unit length filament. Intermediate filaments result from longitudinal association of unit length filaments. With an average total diameter of 10 nm, they are sized between microtubules and actin filaments, leading to their denotation as intermediate filaments. They do not show polarity and are considered the stiffest of the three cytoskeletal filament types. Their main function of ensuring mechanical stability of the cell is fulfilled by forming highly organized

intermediate filament networks. They also contribute to the maintenance of 2D and 3D cell structures by binding to anchoring points in the plasma membrane, called desmosomes, which are cell-to-cell junctions and mainly consist of cadherins (Green & Jones, 1996). Intermediate filaments also bind to hemidesmosomes in the plasma membrane, which consist of several different proteins, among them one integrin (Green & Jones, 1996; Walko *et al.*, 2014), connecting the cytoskeletal filaments to the ECM, thus, intermediate filaments also participate in cell adhesion.

The third type of cytoskeletal filaments is the actin filaments (or also called microfilaments) (Hanson & Lowy, 1963; Dominguez & Holmes, 2011), which are polymers of actin and interact with several actin-binding proteins in the cell, some of them described below. Globular actin (or also called G-actin), how actin is called in its monomeric form, assembles into filaments with an average diameter of (5 – 8) nm composed of two helical, interlaced strands of actin, where actin is then denoted as filamentous actin (or also called F-actin) (Figure 1.3 C). Actin filaments show polarity with a treadmilling polymerization, which means that polymerization occurs at the plus end and depolymerization at the minus end leading to a constant length in a steady state (Brenner & Korn, 1979). Two different arrangements of actin filaments were identified, bundles and networks, depending on the actin-binding proteins associated with it (Matsudaira, 1991). Bundles with all plus ends directed to the same end of the bundle appear in filopodia and lamellipodia for cell migration and in microvilli for increasing the cell surface area for a more efficient nutrient absorption. Bundles with a bidirectional orientation of the filaments are obtained in stress fibers, where the bundles are crosslinked by myosin II. Stress fibers bind to focal contacts (or also called focal adhesions) in the plasma membrane for connection to the ECM, so they are important for cellular contractility, cell adhesion, and migration (Wong *et al.*, 1983). Focal contacts are multi-protein complexes with integrins as the main component linking the actin cytoskeleton to the ECM (Dogic *et al.*, 1999). Networks of actin filaments appear in the cell cortex (or also called actin cortex) lining the inner face of the plasma membrane, where actin is then often denoted as cortical actin (Chugh & Paluch, 2018). The cell cortex, which binds to adherens junctions in the plasma membrane, thus, participating in the formation of cell-to-cell junctions, is responsible for cell shape control and formation of a contractile ring to divide the cytoplasm in the process of cell division. The linkage between actin filaments of the cell cortex and adherens junctions is facilitated by the actin-binding proteins α -catenin and vinculin (Thomas *et al.*, 2013). There are many further actin-binding proteins playing important roles in the regulation of actin filaments, for example, cofilin, which boosts actin filament depolymerization for their reorganization (Yang *et al.*, 1998). Another example is the force generation in muscle cells, which is facilitated by the interaction between actin filaments and myosin II filaments (Allen & Walsh, 1994). Two other actin-binding molecules, which are extensively utilized by biochemists, are phalloidin and the class of cytochalasins. Phalloidin stabilizes actin filaments and inhibits their depolymerization (Estes *et al.*, 1981), whereas cytochalasins inhibit polymerization by binding to the plus ends of the filaments leading to their degradation (Brown & Spudich, 1981).

1.3 Water Permeability and Aquaporins

As it was discussed in detail in section 1.1, glaucoma is strongly associated with IOP regulation. Key parameters are the extent of aqueous humor production in the ciliary body and its drainage by the TM and Schlemm's canal. The correlation between IOP regulation and certain water channels, called aquaporins (AQPs) (for more information about AQPs see further down in this section), was described by Verkman (Verkman, 2003). AQPs are expressed all over the human body and especially in the eye (Verkman, 2003). The ciliary epithelium expresses AQP1 and AQP4, where the release of aqueous humor into the posterior chamber is enhanced by these two AQPs (Zhang *et al.*, 2002). Deletion or inhibition of AQP1 and/or AQP4 reduces aqueous humor production in the ciliary body and, consequently, reduces IOP (Zhang *et al.*, 2002). The TM and Schlemm's canal express AQP1, whereas the reason for this expression still remains unclear (Verkman, 2003). Although the flow of aqueous humor through the cell bodies does not play a significant role in its drainage (Tamm, 2009), water flux (or also called water transport) across the plasma membranes can be utilized for IOP regulation because water flux is correlated with cell volume. Cell volume reduction was shown to increase the permeability of TM cell monolayers and is expected to also do so in the in-vivo TM (O'Donnell *et al.*, 1995), with a potential enhancement of aqueous humor outflow and correlated decrease in IOP.

Generally, water flux can be divided into water flux across the entire cell layer (transepithelial) and water flux across one plasma membrane (transmembrane). Transepithelial water flux is only accessible by using transwell filters to generate an apical and basal compartment. The water flux can then be measured by the fluorophore dilution method (Levin *et al.*, 2006), by using special capillary tubes associated with either the apical or basal compartment (Dorr *et al.*, 1997), or by impedance measurements with laterally resolved detection by an increased number of electrodes (Urban, 2019). No significant net cell volume changes occur here because water entering the cell body by the apical membrane leaves it again by the basal membrane or vice versa. In contrast, transmembrane water flux is associated with cell volume changes, so, this type of water flux can be measured by cell volume assessment techniques: cytometry (O'Donnell *et al.*, 1995), light microscopy (Farinas *et al.*, 1997), interferometry of light beams transmitted through the cells (Farinas & Verkman, 1996), light scattering (Soveral *et al.*, 1997; Niemietz & Tyerman, 2002), fluorescence (Hamann *et al.*, 2002; Mitchell *et al.*, 2002), radio labeling (O'Donnell *et al.*, 1995), extracellular impedance analysis (Kimmelberg *et al.*, 1992) or surface plasmon resonance (SPR) (Robelek & Wegener, 2010). Disregarding this discrimination of transepithelial and transmembrane water flux, water flux can be hydrostatically driven or osmotically induced. While in the former a hydrostatic pressure gradient is artificially generated leading to water flux along this gradient, osmotic water flux is induced by hypertonic and hypotonic solutions leading to differences in osmolality/osmolarity across the plasma membrane (for more information about osmolality/osmolarity see section 4.2.2). In the context of transmembrane water flux, the osmotic pressure generated by hypertonic

conditions outside the cell triggers water molecules to leave the cell body. Thus, extracellular hypertonic stimulation is associated with cell shrinking. Having a reverse osmotic pressure gradient by hypotonic conditions outside the cell leads to water molecules entering the cell body, thus, causing a cell swelling. Both processes occur until equilibrium of osmolalities is reached while changing the cell volume. Three different ways for water to cross the plasma membrane exist. The first way is passive diffusion of the polar water molecules across the nonpolar plasma membrane, where the entrance of water molecules into the membrane is facilitated by defects in the bilayer arising from thermal fluctuations, which is therefore associated with a high activation energy (Deamer & Bramhall, 1986). The second way of water flux is only part of the transepithelial water flux and occurs on the paracellular route, where claudin-2 as part of the tight junction complex can form a water channel (Rosenthal *et al.*, 2010). In contrast to the loosely packed TM, in common epithelia, this way of water flux only accounts for a very small fraction of the overall transepithelial water flux due to the generally watertight character of tight junctions. The third way occurs especially in tissues where a high extent of water flux is needed, like in the kidney epithelium. This is achieved by aquaporins (AQPs), which are transmembrane proteins forming water-selective channels, enabling an enhanced water flux across the plasma membrane by reducing its activation energy. AQPs were discovered in 1992, where the first one identified was CHIP28, which is nowadays known as aquaporin-1 (AQP1) (Preston *et al.*, 1992). In mammals, 13 different AQPs have been identified by now, AQP0 to AQP12, which are divided into three subcategories: the conventional water-selective AQPs, with AQP1 belonging to this category, the aquaglyceroporins, which show permeability to glycerol and other small molecules additionally to water, and the mostly intracellular superaquaporins with mainly unknown functions (Madeira *et al.*, 2014). Most AQPs are located in the plasma membrane, while their structure is based on homotetramers with each monomer having an independent water pore (Verkman & Mitra, 2000). Monomers exhibit an average molar mass of 30 kDa consisting of six transmembrane domains. A common feature of all AQPs is their high permeability to water, with some of them more selective than others. AQP1 also shows permeability to Na⁺, K⁺, and Cs⁺ additionally to water (Yool *et al.*, 1996), which is attributed to a cation channel located in the center of the tetramer (Yu *et al.*, 2006). Beside the permeability to these small cations, AQP1 exhibits a very high selectivity for water, which originates from the diameter of about 3 Å at the narrowest part of the monomer pores, while water molecules exhibit a diameter of about 2.8 Å (Murata *et al.*, 2000), preventing the passage of larger solutes and ions. This high selectivity for water makes AQP1 perfectly suited for the investigation of water flux.

After an osmotically triggered water flux, either combined with shrinking or with swelling, the cells are left in a non-physiological state due to alterations in morphology. The cells respond to this impairment by a two-phase mechanism (McCarty & O'Neil, 1992). The first phase occurs on a millisecond to second time scale and is therefore largely unknown, but the cytoskeleton was found to play a critical role in this phase. The second phase occurs on a minute time scale and is characterized by osmolyte redistribution followed by water flux to

regain the original volume. This is called *regulatory volume change* (RVC) (or also called cell volume regulation or cell volume regulatory mechanism) and is divided into two different types, depending on the direction of the osmotic gradient, which initially induced the water flux. After hypertonically induced cell shrinking until equilibrium of osmolalities is reached, the cell takes up osmolytes temporarily leading to an increased osmolality inside the cell, which is compensated by a water flux to the cell inside. Thus, the original loss in cell volume is partially or even completely balanced by water influx, which is called *regulatory volume increase* (RVI). Conversely, after hypotonically induced cell swelling, the cell releases osmolytes followed by a water outflux, which is called *regulatory volume decrease* (RVD). Osmolyte uptake and release are implemented by certain ion channels. RVI processes are mainly accomplished by the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter and the Na^+/H^+ exchanger, whereas mainly K^+ channels and Cl^- channels are involved in RVD processes (McCarty & O'Neil, 1992; Lang *et al.*, 1998). The water flux, which is triggered by the osmolality change generated by these ion transport processes, occurs through AQPs, thus, AQPs are also involved in RVC processes. In addition to RVC processes, a variety of different metabolic pathways, for example, glycogen synthesis, glycolysis, or lipogenesis, can contribute to the regeneration of cell volume after osmotically triggered water flux since these metabolic pathways can be affected by cell volume changes and influence the intracellular concentrations of several metabolites, leading to osmolality changes and, accordingly, to a water flux (Lang *et al.*, 1998).

Beside the abovementioned purposes for IOP regulation or enhancing water flux in tissues like the kidney, AQPs fulfill several more important physiological functions. Secretion of gland fluids, skin hydration, neuroexcitation, fatty acid metabolism, and cell migration belong to the most important functions of AQPs (Verkman, 2012). In tumor cells, AQP1 was found to enhance their migration and spread, potentially explaining the overexpression of AQPs in most tumor cells, and suggesting AQPs as novel targets in cancer therapy (Hu & Verkman, 2006). Due to the plenty of physiological tasks related to proper AQP function, there are several diseases associated with AQP dysfunction, like nephrogenic diabetes insipidus caused by loss of AQP2 function, or neuromyelitis optica caused by serum autoantibodies directed against extracellular epitopes on AQP4 (Verkman, 2012). This emphasizes the importance of AQPs and introduces a new class of drugs and therapies based on AQP modulation. This involves both AQP activation and AQP inhibition. Enhancing AQP function is typically achieved by compounds up-regulating AQP expression, and may, for example, help treat the two abovementioned diseases nephrogenic diabetes insipidus and neuromyelitis optica. Further possible applications of AQP expression up-regulators are the reduction of fat mass in obesity and the acceleration of wound healing (Verkman, 2012). AQP inhibitors are expected to reduce tumor migration and spread, thus, this class of drugs is investigated for its potential use in cancer therapy (Verkman, 2012). While the correlation between AQP inhibition in the ciliary body and IOP reduction was already demonstrated (Zhang *et al.*, 2002), modulating AQP1 function in the TM and Schlemm's canal may help find out their purpose of expression and potential correlation with IOP regulation.

Quite a few AQP inhibitors have already been identified, where no relevant compound addressing aquaporins with high enough inhibitory effect and water solubility was discovered so far (Salman *et al.*, 2022). Most compounds target the conventional AQPs because most of the research is focused on them, especially AQP1. Heavy metal ions turned out to be a promising class of AQP inhibitors, where HgCl_2 was the first one identified, which potently inhibits AQP1 (Preston *et al.*, 1992) and AQP4 (Yukutake *et al.*, 2008). Zinc(II) (as ZnCl_2) and copper(II) (as CuCl_2) were described to inhibit AQP4 (Yukutake *et al.*, 2009). Silver(I) (as AgNO_3 or silver sulfadiazine) and gold(III) (as HAuCl_4) were found to be even better AQP inhibitors than HgCl_2 (Niemi *et al.*, 2002). AQP inhibition by heavy metal ions is based on the interaction with cysteine residues in pore-forming regions of the AQP monomers, with the disadvantage of low selectivity and toxic side effects. These limitations can be overcome by using gold(III) coordination complexes, which provide the opportunity of fine-tuning by the use of selected ligands for increasing activity and reducing side effects (De Almeida *et al.*, 2014). The tetraethylammonium ion was examined for a potential inhibitory effect on AQP1, which resulted in a disagreement because some studies found an AQP1 inhibition (Detmers *et al.*, 2006; Müller *et al.*, 2008) and others did not (Yang *et al.*, 2006; Sogaard & Zeuthen, 2008). While there is still debate about the possible inhibition of AQP1 by tetraethylammonium, its effect on K^+ channels is undisputed, where it inhibits both voltage-dependent K^+ channels (Hille, 1967) and Ca^{2+} -dependent K^+ channels (Latorre *et al.*, 1982). Due to the fact that RVD processes depend on K^+ channels and Cl^- channels, they could be inhibited by tetraethylammonium (Mitchell *et al.*, 2002). The Cl^- channel inhibitor 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB) and the K^+/Cl^- cotransport inhibitor [(dihydroindenyl)oxy]alkanoic acid (DIOA) were also able to inhibit RVD processes (Mitchell *et al.*, 2002). RVI inhibition was achieved by the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport inhibitors bumetanide and ethacrynic acid (O'Donnell *et al.*, 1995). In total, research on AQPs and AQP modulators is gaining increasing interest due to the great impact on drug development (Salman *et al.*, 2022). Thus, investigating water flux with the aim of finding new and efficient AQP modulators is of paramount importance.

1.4 Hydrogels

Hydrogels are already known for more than 100 years and are best described by hydrophilic polymer networks, which do not dissolve in water but swell by absorbing water. These networks are formed either chemically (covalent bonds, ionic bonds, or hydrogen bonds between linear polymer chains) or physically (entanglement of linear polymer chains). Examples of materials capable of forming hydrogels are encountered several times in this thesis. The vitreous body, which was introduced in section 1.1.1, is a soft, flexible, and transparent hydrogel located in the posterior cavity of the eye for maintaining the eyeball's shape (Kronfeld, 1962). It contains a lot of inorganic ions, like sodium and chloride, proteins, like collagens and albumin, glucose, lactate, hyaluronic acid, and several more components dissolved in about 99% water (Kokavec *et al.*, 2016; Murphy *et al.*, 2016; Velpandian, 2016).

Although the collagen content in the vitreous body of 300 mg/L and the hyaluronic acid content of (42 – 400) mg/L is rather low, these two components are responsible for the gel-like nature of the vitreous body due to their high molar mass and the strongly pronounced ability to absorb water (Kronfeld, 1962). The structure of the vitreous body is described by a dilute network of collagen fibrils surrounded by hyaluronic acid (Murphy *et al.*, 2016). The hydrogel network is formed spontaneously by hydrogen bonds between polymer chains. In research and clinical practice, the vitreous body is often substituted by alternatives for reasons of availability, whereby hydrogels formed by natural or synthetic polymers constitute a promising approach (Mondelo-García *et al.*, 2021). For example, agar was used as a simple and cheap alternative to collagen, which provides, in combination with hyaluronic acid, elasticity to the gel (Kummer *et al.*, 2007). Responsible for the gelation of agar is its main component agarose, a natural biopolymer, where the network is also formed spontaneously by hydrogen bonds between polymer chains. Agarose gels are conventionally used in gel electrophoresis for the separation of nucleic acids since agarose was found to be perfectly suited for this purpose (Hjertén, 1962). Two further hydrogels addressed in this thesis are formed by the synthetic polymers polyacrylamide (PAAm) and polyethylene glycol (PEG). They are used in this thesis to mimic cells according to similar behavior in terms of stiffness and viscoelasticity (for more information about viscoelasticity see section 3.3.2). To be able to form a hydrogel network, PAAm and PEG have to be chemically modified first (for more information see section 4.3). PAAm gels, which are typically used for gel electrophoretic separation of proteins, were first used for zone electrophoresis, where linear chains of acrylamide were crosslinked covalently by *N,N'*-methylenebisacrylamide (Raymond & Weintraub, 1959). Ammonium persulfate (APS) as radical initiator (Raymond & Wang, 1960) and tetramethylethylenediamine (TEMED) as catalyst (McAllister *et al.*, 1963) for accelerating the polymerization were introduced later. PEG gels are extensively used as drug delivery systems (Peppas *et al.*, 1999) and cell carriers for tissue engineering (Bryant & Anseth, 2002) due to their excellent biocompatibility. Using functionalized PEG chain ends and network formation via crosslinking by a Diels-Alder reaction turned out to be a promising approach (Kirchhof *et al.*, 2013). A common feature of all hydrogels is their ability to swell by absorbing water when put in contact with an aqueous solution. The mechanism of swelling is assigned to water molecules entering and penetrating the polymeric network through pores, which is driven by electrostatic forces, osmotic forces, and capillary forces (Ganji *et al.*, 2010). This swelling effect of hydrogels is utilized in superabsorbents in diapers and bandages. Further, water retention in the hydrogels provides a constant drug diffusion pathway, which enables the use of hydrogels as drug delivery systems, as already mentioned above for PEG. The diffusion of a drug through the hydrogel network is influenced by the average pore size, pore size distribution, and pore interconnections, which are in turn determined by the composition of the polymer chains and the crosslinking density (Hoffman, 2012). Further critical factors are the size of the drug and the type and strength of interactions with the polymer chains. Moreover, hydrogels are often used to mimic soft tissues and ECM in tissue engineering and cell culture due to similar mechanical and viscoelastic properties (Huang *et al.*, 2012; Chaudhuri, 2017). Since this similarity between

hydrogels and extracellular tissue is also valid between hydrogels and the cell bodies themselves, this also suggests the possibility to use hydrogels as a simplified model for cells. In total, hydrogels are a promising material class that can be used for several important biotechnological purposes.

1.5 Cell-Based Assays and Transducer Principles

A bioassay is a method or procedure, where a biological recognition element, like an enzyme or antibody, in direct contact with a transducer translates physicochemical properties of an analyte into a measurable output signal to determine the presence, concentration, or effect of the analyte. Assays with cells as the biological recognition element are called cell-based assays and are considered as a compromise between animal experiments and the interaction analysis of isolated biomolecules. Cell-based assays can be divided into label-free and label-based approaches, where label-based assays need an additional substance to report on cellular changes by, for example, radioactivity or fluorescence. One example of a cell-based assay with addition of a fluorescent label used in this thesis is the PrestoBlue® assay, which is described in detail in section 3.5. The drawback of label-based assays is their endpoint detection, which can be overcome by using label-free techniques enabling time-resolved monitoring of the cellular reaction. The most common transducer principles used in label-free cell-based assays are piezoelectric, electrochemical, and optical. Two transducer principles, which are the instrumental basis of this thesis, are shortly described below and examples of application are given: the quartz crystal microbalance (QCM) as a piezoelectric transducer (section 1.5.1) and the Electric Cell-Substrate Impedance Sensing (ECIS) as an electrochemical transducer (section 1.5.2). The theoretical principles of these two techniques are shortly summarized in Figure 1.4.

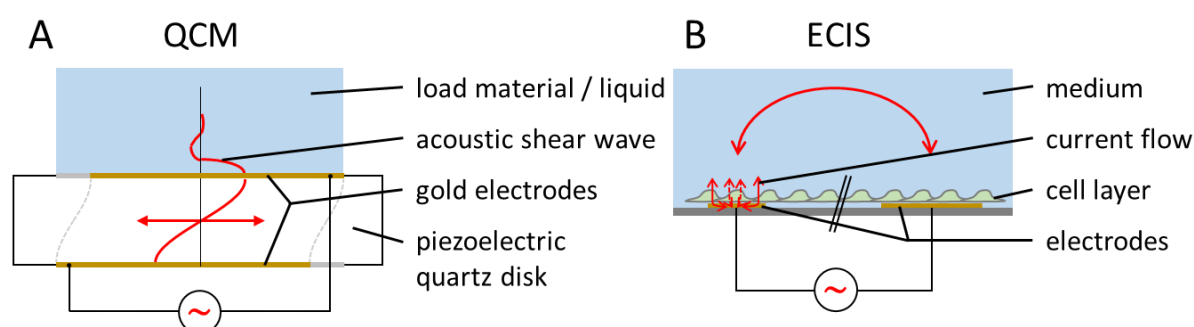


Figure 1.4 Schematic illustration showing the transducer principles of the two techniques mostly applied in this thesis for cell-based biosensing. **(A)** The QCM is based on the piezoelectrically induced oscillation of a quartz disk and its acoustic shear wave and is sensitive to mass changes and viscoelastic changes of the load material (more information in section 3.2). **(B)** ECIS is based on the insulating properties of cells covering the electrodes and is sensitive to anything capable of changing the current flow through the cell layer (more information in section 3.4). Drawings not to scale.

Since optical transducers, like the surface plasmon resonance (SPR), are not used in this thesis, they are not explained in detail, but combinations of different transducer principles and their implementations are demonstrated (section 1.5.3).

1.5.1 Quartz Crystal Microbalance (QCM)

A label-free transducer regularly used in cell-based assays is the QCM. The underlying theoretical principles are explained in detail in section 3.2 and sketchy demonstrated in Figure 1.4 A. Condensed, it is based on acoustic shear waves, the inverse piezoelectric effect, electromechanical coupling, and impedance spectroscopy. Quartz disks with one gold electrode on each side can be brought to high-frequency oscillation by an external electric field due to their piezoelectric nature. More than 60 years ago Sauerbrey discovered the linear correlation between resonance frequency shift and mass change of an adsorbed load material (Sauerbrey, 1959), which led to the QCM's first important application field as a mass sensor for deposition of thin rigid films with nanogram sensitivity. The first applications as a mass sensor were the determination of adsorption isotherms of gases (Slutsky & Wade, 1962) and vapor detection by using coated quartz crystals (King, 1964). The main application of the Sauerbrey equation for QCM instruments is metal deposition from gas phase (Deakin & Melroy, 1988; Stavila *et al.*, 2012). This principle is nowadays still applied, for example, in sputter coaters, where the layer thickness can be determined from the resonance frequency change of the quartz resonator. The second important application field of the QCM is interaction analysis of biomolecules in liquid phase, which was enabled by more powerful electronics capable of operating in a viscous environment. One binding partner is immobilized on the quartz surface and the other is present in the surrounding liquid, while a binding event correlates with a shift in resonance frequency. For example, the resonance frequency change was used to detect the deposition and lipase-induced digestion of a lipid film on the quartz surface (Joyce *et al.*, 2016) and to detect salivary α -amylase in a sandwich enzyme-linked immunosorbent assay (Della Ventura *et al.*, 2017). The DNA polymerase catalyzed synthesis of double-stranded DNA, while a primer was immobilized on the quartz surface, was also followed by observation of the resonance frequency (Matsuno *et al.*, 2001). The third important application field of the QCM was introduced in the 1990s when cell biologists started to grow living adherent cells on the quartz surface as the growth substrate. Due to the cells' non-rigid and viscoelastic behavior and their influence on the resonance frequency by additional energy dissipation, quantification of mass adsorption by the Sauerbrey equation is no longer possible. The QCM's real power was revealed as a monitor for cell attachment and spreading, as well as for following changes in their micromechanics. The first studies demonstrating the successful monitoring of cell attachment by the QCM investigated platelet adhesion (Matsuda *et al.*, 1992) and osteoblast attachment (Redepenning *et al.*, 1993) and were based on recording the resonance frequency shift. The QCM response was found to be cell type-specific (Wegener *et al.*, 1998). A more comprehensive study on cell adhesion revealed an integral QCM response, which

could be separated by applying impedance analysis (for more information about impedance see section 3.1), with the result that only cellular parts involved in cell-substrate interactions are contributing to the QCM response (Wegener *et al.*, 2000c). As already explained in section 1.2, cytoskeletal mechanics are highly correlated to adhesion processes. Thus, any kind of stimulus that is influencing the cytoskeleton, may be detected by QCM readings. This includes especially receptor activation (Chen *et al.*, 2011) and cytotoxicity studies, for example, on the microtubule-binding drugs nocodazole and Taxol (Marx *et al.*, 2007), the fixative glutaraldehyde (Kang & Muramatsu, 2009), or the detergent Triton X-100 (Fattison *et al.*, 2011). Further application fields of the QCM as a sensor for micromechanics include cytocompatibility testing of substrate materials (Michaelis *et al.*, 2012) and drug discovery (Hug, 2003). Together, the QCM technology has proven to be an extremely useful and powerful tool for monitoring mass depositions, biomolecular interactions, and cell-based assays with huge advantages over other techniques due to its label-free, non-invasive, and time-resolved nature.

1.5.2 Electric Cell-Substrate Impedance Sensing (ECIS)

Another label-free transducer with numerous applications in cell-based assays is ECIS. Like the QCM, it is also based on impedance spectroscopy, but on electrochemical instead of piezoelectric principles, which is explained in detail in section 3.4 and sketchy demonstrated in Figure 1.4 B. It was introduced as a biosensor for the morphology of mammalian cells (Giaever & Keese, 1984) and utilizes the insulating properties of cells sitting on electrodes, which has led to many cell-based applications sensitive to changes of cell shape or cell-substrate interactions. More precisely, every process capable of influencing the current flow through the cell layer can be detected by ECIS readings. But this also causes the disadvantage that the same signal change is generated by different cellular processes, which can be overcome by adding a structural resolution by modeling the data (Giaever & Keese, 1991 + erratum). Applications of ECIS include the observation of cell adhesion (Wegener *et al.*, 2000b), proliferation (Stolwijk *et al.*, 2012), migration and wound healing triggered by electrical wounding (Keese *et al.*, 2004), micromotion (Giaever & Keese, 1991 + erratum), cell death (Stolwijk *et al.*, 2012), cytotoxicity testing (Opp *et al.*, 2009), barrier function testing (Martin & Jiang, 2012), receptor activation (Stolwijk *et al.*, 2019) and electroporation (Stolwijk, 2011). According to this huge variety of cell-based assays, where ECIS has been applied successfully as a label-free, non-invasive, and time-resolved technique with a great potential for parallelization, it seems quite obvious that ECIS is considered to become as important for cell biology as microscopy in the future.

1.5.3 Combinations of Different Sensing Techniques

The two label-free transducer principles of QCM and ECIS introduced above can be combined in one experimental setup to yield a measurement technique capable of simultaneously sensing both micromechanics and morphological properties of one cell layer, which is then called ECIS-QCM (or also called QCM-ECIS). The first approach for this combination was based on using the conventional QCM setup combined with an additional electrode dipping into the liquid reservoir serving as the counter electrode (CE) in ECIS mode measurements (Janshoff *et al.*, 1996). In a more recent approach, ECIS-QCM measurements were performed by using two co-planar electrodes in a special double-quartz design (Oberleitner, 2018) (see Figure 4.18). Two QCM sensor spots enabled the generation of two individual QCM signals. Current flow between a small uncoated region on the coated sensor spot serving as the working electrode (WE) and the uncoated sensor spot serving as the CE facilitated ECIS mode measurements simultaneous to QCM readings.

Optical transducers are often combined with piezoelectric or electrochemical transducers in one experimental setup. For example, surface plasmon resonance (SPR) has been used a couple of times in combination with QCM or ECIS. A detailed description of the theoretical principles of SPR can be found elsewhere (Homola *et al.*, 1999; Englebienne *et al.*, 2003), but shortly explained, it is based on the generation of surface plasmons by the total internal reflection of an incident light beam at an interface between two media of different refractive index. Thus, SPR is capable of sensing molecular adsorption and desorption reactions at this interface. The combined measurement technique QCM-SPR allows to study biomolecular interactions, while simultaneously monitoring resonance frequency and refractive index changes, which is an advantage over using one of the techniques alone. But this requires a quartz oscillation in an SPR environment. In one study, this was achieved by etching the quartz disk surface to create a surface corrugation grating and covering it with a gold layer, which serves as the top electrode of the quartz resonator and simultaneously as the SPR coupling layer (Plunkett *et al.*, 2003). In another approach, a quartz disk was brought in contact with a hemispherical prism with a refractive index matching oil in between, enabling the quartz crystal's oscillation (Kim *et al.*, 2010). The lower side of the quartz disk was coated with indium tin oxide (ITO) and the upper side with consecutive layers of ITO, titanium, and gold. ECIS and SPR were combined in one experimental setup (ECIS-SPR) for simultaneous sensing of morphological changes and dynamic mass redistribution in the same cell layer. This was achieved by preparing a sensor chip from a high refractive index glass slide, equipping it with two WEs and one CE by photolithography for ECIS readings, and mounting it on a high refractive index prism for simultaneous SPR readings (Michaelis *et al.*, 2013). The same approach was used to investigate the stimulation of G protein-coupled receptors utilizing the complementary nature of impedimetric and optical readouts (Lieb *et al.*, 2016).

2 Objectives

As an improvement and optimization of conventional glaucoma treatment strategies by eye drop medication and surgery, new treatment strategies with more target-specific approaches have gained increasing interest in the past few years. Especially Rho-associated protein kinase (ROCK) inhibitors, like fasudil, are considered to have promising prospects to provide a causative therapy for primary open-angle glaucoma (POAG), the most prevalent form of glaucoma. They target the actual cause of an increased intraocular pressure (IOP), which was identified as cytomolecular alterations of trabecular meshwork (TM) cells in the aqueous humor outflow pathway, by relaxation and destabilization of actin fibers with the consequence of enhancing outflow and reducing IOP. The aim of this thesis was to contribute to the development of new therapeutic interventions addressing glaucoma by culturing 2D cell layers of the TM and using different cell-based assays to record drug-induced cytomolecular changes. The two non-invasive approaches of the quartz crystal microbalance (QCM) as a sensor for cytomoleculars and Electric Cell-Substrate Impedance Sensing (ECIS) as a sensor for cell morphology changes were considered suitable to reveal cellular changes associated with the pathogenesis and treatment of POAG. First, using hydrogels as a model for cells and correlating their mechanical parameters with QCM readout parameters should enable a mechanical characterization of cells by the QCM in terms of elastic and viscoelastic moduli. Glaucomatous eyes were simulated by the addition of connective tissue growth factor (CTGF) to the appropriate immortalized cell line and their treatment was simulated by the addition of fasudil. Monitoring the concomitant cellular changes by QCM and ECIS should provide a deeper understanding of the mode of action of CTGF and fasudil, while structural cytoskeletal changes should be documented by confocal laser scanning microscopy (CLSM). This was expected to help understand the underlying mechanisms of the pathogenesis and treatment of POAG. Since ROCK inhibitors are typically applied via injection into the vitreous body, fasudil should be studied with respect to its diffusion through the vitreous body, for which a new assay with a transwell filter setup had to be developed. The assay development, which included self-fabrication of electrode arrays, should be directed to an online detection of fasudil migration through the vitreous body and its subsequent impact on TM cells. Since certain coherences between IOP regulation and water flux have been identified previously, osmotically triggered water flux should be investigated comprehensively to figure out if water flux itself can be utilized for new glaucoma treatment strategies and if the QCM might facilitate future screening for aquaporin modulators.

3 Theoretical Background

In this chapter, the theoretical basics of the techniques used in this thesis are addressed. The fundamental principles and equations describing the most important issues are presented and discussed. Firstly, impedance spectroscopy is explained (section 3.1), which constitutes the basis of the quartz crystal microbalance (QCM) technique and the Electric Cell-Substrate Impedance Sensing (ECIS) technique. Both these impedance-based techniques were extensively used in this thesis and are described in this chapter later on (QCM: section 3.2, ECIS: section 3.4). Furthermore, the principle of the PrestoBlue® assay is shown (section 3.5) and relevant information on mechanical moduli is given, that have been used to describe the mechanical properties of cells and hydrogels (section 3.3).

3.1 Impedance Spectroscopy

Impedance spectroscopy is a very versatile method applied in many advanced techniques. Many researchers have published scripts dealing with its theory and applications, for example, Barsoukov and Macdonald (Barsoukov & Macdonald, 2005). The most important aspects are summarized here. Impedance spectroscopy is a measurement principle for gaining information about the electrical impedance of a test system under excitation with an alternating current (AC) voltage. The following sections cover general mathematical expressions regarding the complex impedance (section 3.1.1) and impedance elements in equivalent circuits (section 3.1.2).

3.1.1 The Complex Impedance

The impedance is the resistance in an AC circuit and so it is dependent on the frequency. The electrical resistance R of a direct current (DC) circuit is given by the ratio of the applied voltage U to the resulting electrical current I , which is known as Ohm's law:

$$R = \frac{U}{I} \quad (1)$$

The resistance is given in the unit Ω ($\Omega = \frac{V}{A}$). In DC circuits, voltage and current are constant over time leading to a frequency-independent resistance, which is also called ohmic resistance. In contrast to that, impedance is frequency-dependent because AC circuits are characterized by the application of voltages that change periodically over time. The voltage typically displays a sinusoidal shape, which induces a sinusoidal current (Figure 3.1).

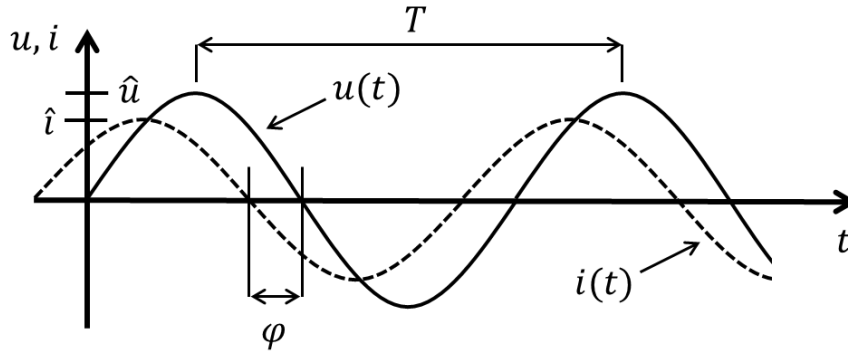


Figure 3.1 Sinusoidal AC voltage $u(t)$ and corresponding sinusoidal AC current $i(t)$, characterized by their amplitudes $\hat{u}$ and $\hat{i}$, respectively, and the period T , which is the same for both voltage and current. Accordingly, the frequency f ($f = \frac{1}{T}$) is also the same. The phase shift φ between voltage and current is caused by capacitive and/or inductive components in the system.

Both voltage and current are functions of time and frequency (indicated by small symbols) with quite small amplitudes enabling non-invasive investigation of biological samples. The sinusoidal AC voltage $u(t)$ can be mathematically expressed in the form:

$$u(t) = \hat{u} \cdot \sin(\omega t) \quad (2)$$

with its amplitude $\hat{u}$ and the angular frequency $\omega = 2\pi f$, where f is the frequency of the applied AC voltage. As it is shown in Figure 3.1, the resulting current $i(t)$ has also a sinusoidal form of the same frequency, but with a phase shift φ (also called phase angle) with respect to the voltage, caused by capacitive and/or inductive components in the system:

$$i(t) = \hat{i} \cdot \sin(\omega t - \varphi) \quad (3)$$

where $\hat{i}$ is its amplitude. The direction and extent of the phase shift depend on the exact proportions of capacitive and inductive components (see section 3.1.2). Analogous to Ohm's law (Eq. (1)), the impedance Z of an AC circuit is given by the ratio of the applied voltage to the resulting electrical current, but with the difference that both quantities are functions of time:

$$Z = \frac{u(t)}{i(t)} \quad (4)$$

Thus, just like the resistance, the impedance is given in Ω . The impedance describes on the one hand the ratio of both amplitudes of voltage and current, and on the other hand the phase shift between both quantities. Both aspects are condensed by characterizing the impedance as a complex quantity (indicated by underlined symbols):

$$\underline{Z} = \frac{\underline{u(t)}}{\underline{i(t)}} \quad (5)$$

with the complex voltage:

$$\underline{u}(t) = \hat{u} \cdot (\cos(\omega t) + j \cdot \sin(\omega t)) = \hat{u} \cdot e^{j\omega t} \quad (6)$$

and the complex current:

$$\underline{i}(t) = \hat{i} \cdot (\cos(\omega t - \varphi) + j \cdot \sin(\omega t - \varphi)) = \hat{i} \cdot e^{j(\omega t - \varphi)} \quad (7)$$

with the imaginary unit $j = \sqrt{-1}$. Combining Eq. (5), Eq. (6) and Eq. (7) leads to:

$$\begin{aligned} \underline{Z} &= \frac{\underline{u}(t)}{\underline{i}(t)} = \frac{\hat{u}}{\hat{i}} \cdot e^{j\varphi} \\ &= |Z| \cdot e^{j\varphi} \\ &= |Z| \cdot (\cos\varphi + j \cdot \sin\varphi) \end{aligned} \quad (8)$$

The complex impedance $\underline{Z}$ is fully characterized by its magnitude $|Z| = \frac{\hat{u}}{\hat{i}}$ and the phase shift φ between voltage and current. Like every other complex quantity, the impedance can also be described by its real part $Re(\underline{Z})$ and imaginary part $Im(\underline{Z})$:

$$\begin{aligned} \underline{Z} &= Re(\underline{Z}) + j \cdot Im(\underline{Z}) \\ &= |Z| \cdot \cos\varphi + j \cdot |Z| \cdot \sin\varphi \\ &= R + j \cdot X \end{aligned} \quad (9)$$

The real part of the complex impedance is denoted as the resistance R , but with the difference to ohmic resistances that they are frequency-independent, whereas the real part of the complex impedance may be frequency-dependent. It represents energy losses and dissipation in the system. The imaginary part of the complex impedance is denoted as the reactance X , which is induced by capacitive and/or inductive components. It is frequency-dependent and represents energy storage in the system. The complex impedance can be displayed as a vector in the complex plane, in which the imaginary part (y-axis) is plotted against the real part (x-axis) (Figure 3.2). This vector can be described either by the Cartesian coordinates, resistance R and reactance X , or by the polar coordinates, the vector length representing the magnitude of impedance $|Z|$ and the angle of the vector with respect to the x-axis representing the phase shift φ .

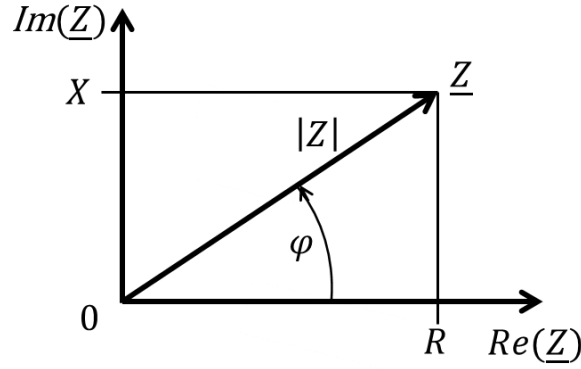


Figure 3.2 Geometric representation of the Impedance $\underline{Z}$ as a vector in the complex plane with the real part on the x-axis and the imaginary part on the y-axis. The impedance is fully described either by the Cartesian coordinates, resistance R and reactance X , or by the polar coordinates, magnitude of impedance $|Z|$ and phase shift φ .

Thus, both representations of the complex impedance can be converted into each other according to trigonometry. Polar coordinates can be calculated from Cartesian coordinates by:

$$|Z| = \sqrt{\text{Re}(\underline{Z})^2 + \text{Im}(\underline{Z})^2} = \sqrt{R^2 + X^2} \quad (10)$$

$$\varphi = \arctan\left(\frac{\text{Im}(\underline{Z})}{\text{Re}(\underline{Z})}\right) = \arctan\left(\frac{X}{R}\right) \quad (11)$$

Vice versa, Cartesian coordinates can be calculated from polar coordinates by:

$$R = |Z| \cdot \cos\varphi \quad (12)$$

$$X = |Z| \cdot \sin\varphi \quad (13)$$

3.1.2 Impedance Elements in Equivalent Circuits

There are several models describing biological systems and their impedance, all of them are based on the three basic circuit elements known from electrical engineering: ohmic resistor, capacitor, and inductor. In the following discussion, an ideal behavior is assumed for these three devices, which means that, for example, a capacitor only possesses capacitive properties and no resistive properties, which would not be the case for a real capacitor. The three basic elements and, additionally, the constant phase element, which is a non-ideal and empiric circuit element, and their impedance contributions are discussed in detail.

Ohmic Resistor

An ohmic resistor is synonymous with an ideal resistor. By applying an AC voltage an ohmic resistor only influences the amplitude of the current and does not cause a phase shift φ between voltage and current. An ohmic resistor is independent of the AC frequency. The impedance of an ohmic resistor $\underline{Z}_R$ is only resistive and has no imaginary part:

$$\underline{Z}_R = R + j \cdot 0 = R \quad (14)$$

Using Eq. (8) and Eq. (14) and with $|\underline{Z}_R| = R$, this confirms the phase shift φ to be zero:

$$\underline{Z}_R = R \cdot e^{j\varphi} = R \rightarrow \varphi = 0 \quad (15)$$

In the complex plane, the impedance vector of an ohmic resistor is a straight line along the x-axis.

Capacitor

For ideal capacitors in AC circuits the current is proportional to the change of the voltage with time with the capacitance C (with the unit $F = \frac{S}{\Omega}$) as the proportionality factor:

$$\underline{i}(t) = C \cdot \frac{d\underline{u}(t)}{dt} \quad (16)$$

The capacitance C of an ideal capacitor is frequency-independent. By using Eq. (6) this gives:

$$\underline{i}(t) = C \cdot \underline{u}(t) \cdot j\omega \quad (17)$$

With this and Eq. (5), the impedance of an ideal capacitor $\underline{Z}_C$ is given by:

$$\underline{Z}_C = \frac{\underline{u}(t)}{\underline{i}(t)} = \frac{1}{j\omega C} = -j \cdot \frac{1}{\omega C} \quad (18)$$

Thus, the impedance of an ideal capacitor has no real part, but only an imaginary part, which consists of capacitive contributions without inductive contributions. The impedance of an ideal capacitor is indirectly proportional to the (angular) frequency ω and the capacitance C . An ideal capacitor induces a phase shift φ between voltage and current of:

$$\varphi = -\frac{\pi}{2} \quad (19)$$

which means that the sinusoidal AC current is negatively shifted by $\frac{\pi}{2}$ with respect to the voltage (see Eq. (6) and Eq. (7)). In the complex plane, the impedance vector of an ideal capacitor is a straight line along the y-axis in negative direction. A non-ideal capacitor exhibits a phase shift of $-\frac{\pi}{2} < \varphi < 0$.

Inductor

The inductor is the third basic element in AC circuits. For ideal inductors in AC circuits the voltage is proportional to the change of the current with time with the inductance L (with the unit $H = \Omega \cdot s$) as the proportionality factor:

$$\underline{u}(t) = L \cdot \frac{d\underline{i}(t)}{dt} \quad (20)$$

The inductance L of an ideal inductor is frequency-independent. By using Eq. (7) this gives:

$$\underline{u}(t) = L \cdot \underline{i}(t) \cdot j\omega \quad (21)$$

With this and Eq. (5), the impedance of an ideal inductor $\underline{Z}_L$ is given by:

$$\underline{Z}_L = \frac{\underline{u}(t)}{\underline{i}(t)} = j\omega L \quad (22)$$

Similar to the ideal capacitor, the impedance of an ideal inductor has no real part, but only an imaginary part, which consists of inductive contributions without capacitive contributions. The impedance of an ideal inductor is directly proportional to the (angular) frequency ω and the inductance L . An ideal inductor induces a phase shift φ between voltage and current of:

$$\varphi = \frac{\pi}{2} \quad (23)$$

which means that the sinusoidal AC current is positively shifted by $\frac{\pi}{2}$ with respect to the voltage (see Eq. (6) and Eq. (7)). In the complex plane, the impedance vector of an ideal inductor is a straight line along the y-axis in positive direction. A non-ideal inductor exhibits a phase shift of $0 < \varphi < \frac{\pi}{2}$.

In real systems, there is always a combination of capacitors and inductors, resulting in a final phase shift depending on the exact proportions of capacitors and inductors.

Constant Phase Element (CPE)

Such ideal behavior, as described for the circuit elements ohmic resistor, ideal capacitor, and ideal inductor, is not enough for modeling real electrochemical systems, since they are typically characterized by non-ideal behavior due to inhomogeneity or electrode roughness. Therefore, empirical elements are needed to describe real electrochemical systems, like the constant phase element (CPE), which is a non-ideal circuit element constituting mixed resistive and capacitive behavior. It is typically used for describing the interface impedance of electrodes in contact with electrolytes. This situation is described by non-ideal behavior because one side of the interface (electrode) is charged with electrons and the other side

(electrolyte) is charged with ions. The area-related impedance contribution of a constant phase element $\underline{Z}_{CPE}$ (in $\Omega \cdot \text{cm}^2$) is given by:

$$\underline{Z}_{CPE} = \frac{1}{A_{CPE} \cdot (j\omega)^n} \quad (24)$$

with the real number $n = [0,1]$ and the frequency-independent parameter A_{CPE} , which is not an area, but instead, describes an area-related resistance/capacitance (with the unit $\frac{\text{F} \cdot \text{s}^{n-1}}{\text{cm}^2}$). The CPE induces a phase shift φ between voltage and current of:

$$\varphi = -n \cdot \frac{\pi}{2} \quad (25)$$

For the first extreme case of $n = 0$, the CPE describes an ohmic resistor with $\underline{Z}_{CPE} = \frac{1}{A_{CPE}}$ (with $A_{CPE} = \frac{1}{R}$ and unit $\frac{1}{\Omega \cdot \text{cm}^2}$) and $\varphi = 0$. For the second extreme case of $n = 1$, an ideal capacitor is described by the CPE with $\underline{Z}_{CPE} = \frac{1}{j\omega A_{CPE}}$ (with $A_{CPE} = C$ and unit $\frac{\text{F}}{\text{cm}^2}$) and a phase shift $\varphi = -\frac{\pi}{2}$. For any other situation of $n =]0,1[$, the CPE shows mixed resistive and capacitive properties.

3.2 Quartz Crystal Microbalance (QCM)

The quartz crystal microbalance (QCM) is a mass-sensitive method, which is based on acoustic waves. Acoustic waves cover the frequency range from about 1 kHz to 1 GHz and can have different origins, but piezoelectric materials, such as quartz, ensure a frequency-stable wave source and a highly sensitive detection device. The QCM is also called bulk acoustic wave sensor due to the acoustic waves oscillating in the quartz bulk and it is also called thickness shear mode resonator due to the shear (transverse) wave generated in the piezoelectric material with the frequency depending on the thickness of the quartz disk. The QCM offers several advantages compared to other transducers: it is label-free, non-invasive, very sensitive, and time-resolved with a multiparametric and integral signal readout. Further practical advantages are the simple, cost-effective, and highly automated performance. Examples of application were already given in section 1.5.1. The following sections focus on the basic principles of the QCM (section 3.2.1) and on how impedance analysis combined with electromechanical equivalent circuits extends the QCM from just a microbalance to a versatile tool, which can, for example, be used as a transducer in biosensors (section 3.2.2).

3.2.1 Basics of the QCM Technique

Piezoelectricity

Piezoelectricity is the fundamental principle the QCM relies on and was discovered in 1880 by Jacques and Pierre Curie (Curie & Curie, 1880). Piezoelectricity finds application in microphones, quartz watches, and inkjet printers. The piezoelectric effect can occur in all crystal systems that lack inversion symmetry. Examples of piezoelectric materials are lithium niobate or zinc oxide, but mostly quartz is used (Grate *et al.*, 1993). Among the large variety of different crystals showing piezoelectricity, only quartz exhibits the optimal combination of chemical, electrical, mechanical, and thermal properties that has led to its widespread use in today's electronics (Bottom, 1982). Therefore, quartz is used in QCM, particularly α -quartz, which has a trigonal crystal system. Quartz disks are prepared by slicing them from a quartz single crystal at an angle of 35.25° with respect to the crystallographic z-axis (optical axis) (Alder & McCallum, 1983). They are called ambient-temperature-cut (AT-cut) quartz crystals because of their constant resonance frequency over a broad temperature range (Bottom, 1982). The piezoelectric effect is explained in detail in Figure 3.3 using the example of quartz. Its crystal system consists of SiO_4 tetrahedrons linked to neighboring tetrahedrons by oxygen atoms, where three tetrahedrons together form a hexagonal unit, which contains three silicon atoms and three oxygen atoms (Figure 3.3 A). The three partially positive charged silicon atoms form a center of positive charges in the middle of the hexagonal unit, which coincides, under normal conditions, with the one formed by the three partially negative charged oxygen atoms, resulting in a balanced overall charge (Figure 3.3 B). The piezoelectric effect describes the phenomenon of electromechanical coupling, which is the transformation between mechanical and electrical energy. One has to distinguish between two forms of the piezoelectric effect, the direct piezoelectric effect and the inverse piezoelectric effect. The direct piezoelectric effect describes the generation of electrical surface charges when mechanical stress is applied to a piezoelectric material (Figure 3.3 C). In detail, a stress applied along the x-axis (electrical axis) leads to a further symmetry reduction and a polarization along the y-axis (mechanical axis) occurs, because the positive and negative charge centers no longer overlap, leading to a charge separation at the crystal surfaces. An AT-cut quartz crystal constitutes the core component of the QCM after two circular metal film electrodes (mostly gold) were deposited on its surfaces, with Si-O-dipoles within the quartz bulk in their natural orientation (Figure 3.3 D). In contrast to the direct piezoelectric effect explained before, the inverse piezoelectric effect describes the generation of a strain within the material upon application of an electric field, where the induced strain is perpendicular to the electric field lines (Figure 3.3 E). When a static electric field is applied to the quartz disk via the electrodes, the Si-O-dipoles are shifted due to attractive or repulsive forces with the direction of deformation depending on the direction of the electric field. This inverse piezoelectric effect is utilized in the QCM.

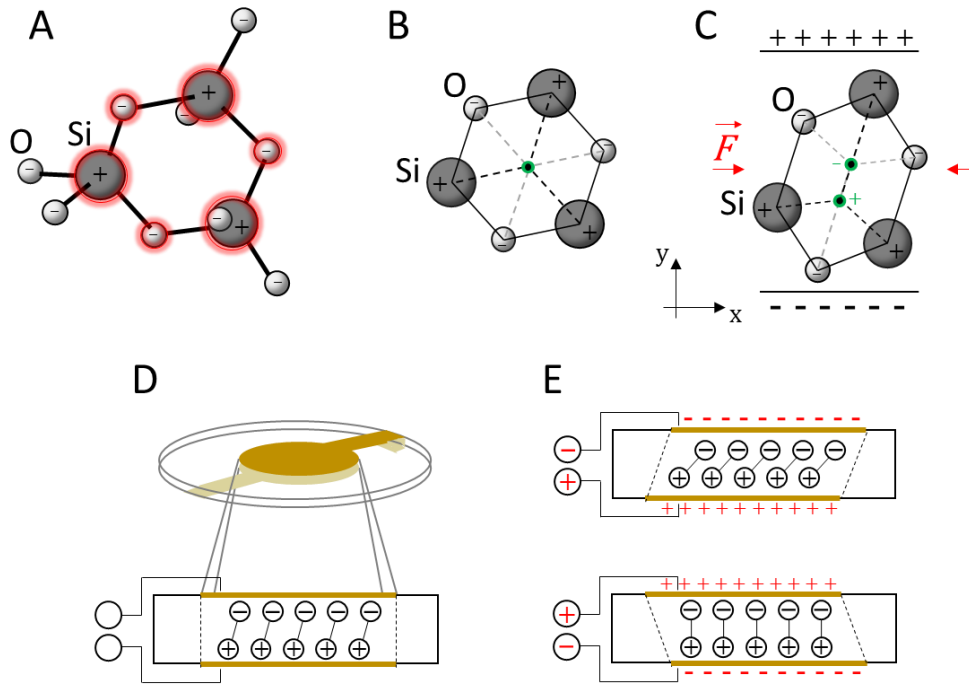


Figure 3.3 Schematic illustration of the electromechanical coupling in piezoelectric quartz resonators. **(A)** Part of the trigonal crystal system of α-quartz with oxygen linked SiO₄ tetrahedrons with the silicon atoms (black) having a partial positive charge and the oxygen atoms (white) having a partial negative charge; a hexagonal unit consisting of three silicon atoms and three oxygen atoms is highlighted in red. **(B)** Simplified scheme of the hexagonal unit with the orientation of charges in its natural state, where the center of positive charges and the center of negative charges overlap resulting in a zero net charge. **(C)** Direct piezoelectric effect: application of mechanical stress along the x-axis causes the separation of both the centers of positive and negative charges along the y-axis leading to the formation of opposing charges on the quartz surfaces. **(D)** Disk-shaped AT-cut quartz crystal with circular gold film electrodes on both sides with the Si-O-dipoles in the natural orientation. **(E)** Inverse piezoelectric effect: application of a static electric field to the quartz via the electrodes causes a transversal displacement of the dipoles and, thus, a mechanical strain within the material. The direction of the deformation is dependent on the direction of the electric field. Illustrations were redrawn according to published literature (Arnau & Soares, 2009).

According to the inverse piezoelectric effect, if the voltage applied to the quartz disk switches polarity back and forth, the particle displacement in the quartz is alternating with the same frequency. So, the quartz disk can be brought to oscillation, which can be described by shear displacements starting from both quartz surfaces propagating as two acoustic waves into the middle of the disk (Figure 3.4 B, green and blue wave). Their direction of propagation occurs parallel to the electric field lines in opposite directions and their direction of oscillation occurs perpendicular to the electric field lines. Accordingly, they are called shear or transverse waves in contrast to longitudinal waves, which exhibit an oscillation in direction of propagation. Due to this feature of being able to oscillate, quartz disks are also called quartz oscillators or quartz resonators. Like any other oscillating system, a quartz disk can show resonance at a resonance frequency f_0 (also called fundamental resonance frequency). If the frequency of the externally applied AC voltage matches the

resonance frequency f_0 of the quartz disk (or any overtone of it), the two propagating shear waves superimpose into a standing shear wave (Figure 3.4 B, red wave). The following condition (resonance condition) must be fulfilled:

$$n\lambda = 2d_q \quad (26)$$

with the non-negative odd integer n ($n = 1, 3, 5, \dots$) as the overtone number, the wavelength λ of the AC voltage, which is also the wavelength of the standing shear wave, and the thickness of the quartz disk d_q . For $n = 1$, this is called fundamental resonance condition, which is shown in Figure 3.4. At these resonance conditions, minimal oscillation impedance is obtained. With its node in the middle of the quartz disk, the maximal amplitude of the standing shear wave is obtained at the quartz surfaces (Figure 3.4 C), which is dependent on the amplitude of the applied voltage. At standard driving voltages of 150 mV (rms) the displacement amplitude of a typical 5 MHz quartz resonator is < 1 nm at any spot (Oberleitner, 2018). Thus, as compared to mammalian cells of about $10 \mu\text{m}$ diameter, the QCM is a highly non-invasive technique. Furthermore, this amplitude is only realized in the center of the electrodes due to a Gaussian-shaped decrease in oscillation amplitude with increasing distance from the electrode center (Figure 3.4 D). Accordingly, QCM readings exhibit the highest sensitivity in the electrode center.

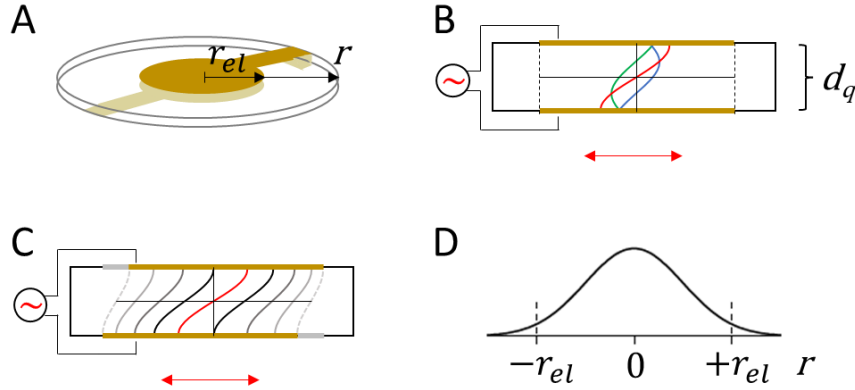


Figure 3.4 Schematic illustration of the oscillation of a quartz resonator. Typical quartz disk dimensions are in the range of 10 mm – 25 mm in diameter and $50 \mu\text{m}$ – $1600 \mu\text{m}$ in thickness. **(A)** Disk-shaped AT-cut quartz crystal of radius r with circular gold film electrodes on both sides of radius r_{el} . **(B)** According to the inverse piezoelectric effect, an application of an AC voltage to the quartz disk of thickness d_q via the electrodes causes a mechanical oscillation of the quartz. It can be described by two acoustic waves (green and blue) that propagate parallel to the electric field lines through the material, but with opposite directions. The direction of oscillation occurs perpendicular to the electric field lines, so they are called shear (transverse) waves. For the resonance condition (applied AC voltage matching the resonance frequency of the quartz crystal), the two shear waves superimpose into a standing shear wave (red), which is shown for the fundamental case ($n = 1$ in Eq. (26)). **(C)** Maximal amplitude of the standing shear wave is obtained at the quartz surfaces and the node is located in the middle of the quartz disk. The standing shear wave loses oscillation amplitude with increasing distance from the electrode center, which is also demonstrated in **(D)**, where the Gaussian-shaped amplitude distribution over the quartz surface is shown with the maximum in the center of the electrodes. Illustrations were redrawn according to published literature (Arnau & Soares, 2009).

The resonance frequency f_0 can simply be correlated with the propagation velocity of shear waves in AT-cut quartz v_q and the wavelength λ , which is described by:

$$f_0 = \frac{v_q}{\lambda} = \frac{v_q}{2d_q} = \frac{\sqrt{\frac{\mu_q}{\rho_q}}}{2d_q} \quad (27)$$

Therein, λ is expressed by twice the thickness of the quartz disk d_q according to Eq. (26) at the fundamental resonance condition. The propagation velocity of shear waves in AT-cut quartz v_q can be determined by the shear modulus of AT-cut quartz μ_q ($2.947 \cdot 10^{10} \frac{\text{kg}}{\text{m} \cdot \text{s}^2}$) and the density of quartz ρ_q ($2.648 \cdot 10^3 \frac{\text{kg}}{\text{m}^3}$) (Reed *et al.*, 1990) and amounts to a value of $3336 \frac{\text{m}}{\text{s}}$. This results with the often-used thickness of the quartz disk d_q of $330 \mu\text{m}$ in a resonance frequency f_0 of about 5 MHz.

Sauerbrey Equation: the QCM as a Mass-Sensitive Technique

If the thickness of the quartz disk d_q increases, its resonance frequency f_0 decreases according to Eq. (27). A similar situation occurs, if an additional foreign mass layer is added to the quartz surface, which exhibits quartz-like behavior (similar shear modulus, similar density). The resonance frequency of the quartz disk decreases upon deposition of an additional layer, even if it is not exactly the same material. Sauerbrey was the first who described this mathematically by a relationship between resonance frequency shift Δf and mass change Δm , which is a consequence of the application of an additional mass layer (Sauerbrey, 1959):

$$\frac{\Delta f}{f_0} = - \frac{\Delta m}{\rho_q \cdot A \cdot d_q} \quad (28)$$

Therein, A is the area of the electrode on the quartz disk. Expressing d_q by the term given in Eq. (27) leads to the following relation, which is known as the Sauerbrey equation/relation:

$$\Delta f = - \frac{2f_0^2}{A \cdot \sqrt{\mu_q \cdot \rho_q}} \cdot \Delta m = -S_f \cdot \frac{\Delta m}{A} \quad (29)$$

with the integral mass sensitivity $S_f = \frac{2f_0^2}{\sqrt{\mu_q \cdot \rho_q}}$ (or also called Sauerbrey constant). For 5 MHz AT-cut quartz resonators the integral mass sensitivity S_f is determined to $5.66 \cdot 10^6 \frac{\text{m}^2}{\text{kg} \cdot \text{s}}$, which translates with commonly achieved frequency resolutions of 1 Hz to a mass sensitivity of $18 \cdot 10^{-8} \frac{\text{kg}}{\text{m}^2} = 18 \frac{\text{ng}}{\text{cm}^2}$. Regarding a typical electrode area of about 0.3 cm^2 , the QCM is working with nanogram sensitivity. A better (lower) mass sensitivity requires a higher integral mass sensitivity S_f , which corresponds to a higher resonance frequency f_0 and, according to Eq. (27), to a smaller quartz disk thickness d_q . Thus, a desirable improvement in

mass sensitivity can be achieved by reducing quartz disk thickness but is limited by the loss in mechanical stability. The quartz disks used in this thesis with a thickness of 330 μm and a resonance frequency of 5 MHz constitute a good compromise between mass sensitivity and mechanical stability. As mentioned above, the Sauerbrey equation (Eq. (29)) is only valid for the deposition of mass layers of a quartz-like material. This includes the feature of rigidity, which means that the foreign mass layer oscillates simultaneously with the quartz disk without any delay caused by viscous properties. Furthermore, the validity of the Sauerbrey equation is restricted to thin and homogeneous mass layers, and the environment has to be free of damping effects, which is only fulfilled in vacuum or air, but not in liquids. If all these conditions are met, the QCM constitutes a mass-sensitive device for the detection of adsorption and desorption of small masses.

The QCM beyond the Sauerbrey Limitations

Nomura and Okuhara were the first reporting on QCM measurements under liquid loading conditions (Nomura & Okuhara, 1982), which has opened up the door to a huge variety of application fields. Even though the Sauerbrey equation has the abovementioned limitations, it can also be applied to molecular adsorptions to the quartz disk from liquid phase with reasonable approximation. But, a quantitative determination of the added mass from the resonance frequency change is hardly possible. Only a semi-quantitative determination based on a proper calibration curve or a qualitative determination is possible. Going one step back to the situation of having just a liquid on the quartz surface, a resonance frequency shift in contrast to the blank resonator is also obtained, but this does not behave like a rigid mass layer, so the Sauerbrey equation is not valid. At the quartz-liquid interface, the shear oscillation of the quartz is transmitted into the liquid bulk due to the dynamic viscosity of the liquid η'_L (for more information about viscosity see section 3.3.2). The first layer of liquid molecules directly attached to the quartz oscillates synchronously with it (no-slip condition), but subsequent layers show a damped oscillation with the amplitude decreasing with increasing distance from the surface. This energy dissipation caused by the dynamic viscosity of the liquid η'_L is, next to pure mass loading, another reason for a resonance frequency shift. Many different models have been developed in the past four decades to quantitatively describe the resonance frequency shift induced by a liquid loading on the quartz resonator. For the quartz oscillation in a purely viscous liquid (Newtonian liquid, for example, water), Kanazawa and Gordon discovered a model with very good accuracy (Kanazawa & Gordon, 1985):

$$\Delta f = -f_0^{\frac{3}{2}} \sqrt{\frac{\eta'_L \cdot \rho_L}{\pi \cdot \mu_q \cdot \rho_q}} \quad (30)$$

where ρ_L is the density of the liquid. Thus, in contrast to the Sauerbrey equation, where the additional mass is the decisive factor, here, the resonance frequency shift originates from

the dynamic viscosity η'_L and the density ρ_L of the liquid loading. The abovementioned damping of the shear wave in the viscous liquid can be mathematically derived from the Navier-Stokes equations (Zhang *et al.*, 2006). It can be described by an exponential decay of its amplitude $a(z)$ at the distance from the quartz surface z :

$$a(z) = a_0 \cdot e^{-bz} = a_0 \cdot e^{-\frac{z}{\delta}} \quad (31)$$

with its amplitude at the quartz-liquid interface a_0 and the decay constant (or propagation constant) b . The decay length (or penetration depth) of the shear wave δ into the liquid, which is the reciprocal of the decay constant b , is the distance from the quartz surface at which the amplitude of the shear wave $a(z)$ has dropped to $\frac{1}{e} \cdot a_0$ and can be determined by (Kanazawa & Gordon, 1985):

$$\delta = \sqrt{\frac{\eta'_L}{\pi \cdot f_0 \cdot \rho_L}} \quad (32)$$

The decay length δ is considered to limit the sensitive volume of the QCM, which means that load material closer to the quartz surface than the decay length will influence the quartz oscillation and can therefore be detected by QCM measurements, and load material beyond this limit will not. The decay length δ and, therefore, also the sensitive volume of the QCM will increase when the dynamic viscosity of the liquid η'_L increases or when the density of the liquid ρ_L decreases. When water at 20 °C ($\eta'_L = 10^{-3} \text{ Pa} \cdot \text{s}$, $\rho_L = 10^3 \frac{\text{kg}}{\text{m}^3}$) is used as a load of a 5 MHz quartz resonator, the decay length is determined to about 250 nm, indicating that the sensitive volume of the QCM is restricted very close to the resonator surface. Using olive oil at 20 °C as a load on the same resonator, a liquid with about 100-fold dynamic viscosity ($\eta'_L \approx 10^{-1} \text{ Pa} \cdot \text{s}$) and slightly smaller density ($\rho_L = 0.914 \cdot 10^3 \frac{\text{kg}}{\text{m}^3}$) compared to water, yields, according to Eq. (32), an about 10-fold bigger decay length ($\delta \approx 2.6 \mu\text{m}$). When the quartz resonator is loaded with a hydrogel, which is no Newtonian liquid but, instead, a viscoelastic material (for more information about viscoelastic materials see section 3.3.2), the situation gets more complicated, but Eq. (32) can also be applied to determine the decay length δ with reasonable approximation (see section 5.1.1). Having animal cells on the quartz surface, a similar situation as for hydrogels is obtained, because they also behave as viscoelastic materials. Elastic properties originate from their cytoskeleton, cell cortex, and lipid membranes, whereas viscous properties are mainly caused by the cytoplasm. Their average dynamic viscosity is close to or slightly higher than that of water (Bicknese *et al.*, 1993) and their average density is also close to or slightly higher than that of water (Wegener *et al.*, 1998). Thus, according to Eq. (32), the decay length of the shear wave δ into cells sitting on the quartz surface is similar to that of water ($\delta \approx 250 \text{ nm}$). In contrast to that, adherent epithelial cell layers exhibit a height of about 6 μm (Breitenstein *et al.*, 2007) or 7 μm (Van Driessche *et al.*, 1993), whereas their cell-surface distances are in the range from 10 nm (Braun & Fromherz, 1997) to 70 nm (Lanni *et al.*, 1985). This leads to the fact that cells can be detected by QCM measurements, but only those parts of the cellular body that are

involved in cell-substrate adhesion are located within the decay length and, therefore, contribute to the overall signal (Wegener *et al.*, 2000c).

As mentioned before, a resonance frequency shift can either be caused by pure mass loading or by changes in dynamic viscosity or density of the liquid loading. In real systems, mostly both factors together come into play. Thus, there is an ambiguity in the interpretation of QCM data as long as the resonance frequency shift is the only parameter of choice. One possible way to separate both factors is achieved by applying the QCM with dissipation monitoring (QCM-D), where the driving voltage is periodically turned on and off (Höök *et al.*, 1999). When the driving voltage is turned off, the oscillation decays exponentially depending on the energy dissipation caused by the load material. This enables the determination of the dissipation factor D , which is defined as the loss of energy divided by the elastically stored energy. Another way of separating pure mass loading from energy dissipation is to apply electrical impedance spectroscopy (see section 3.1), where the electrical impedance of the quartz resonator is determined in a frequency range that closely embraces its fundamental resonance frequency. Equivalent circuit modeling is performed afterwards, where these measured impedance spectra are fitted by an appropriate model. This is done in order to obtain the individual electrical contributions to the impedance, which can be correlated with mechanical properties of the load. This enables simultaneous determination of both pure mass loading and viscoelastic properties. Because this approach is applied in this thesis, this will be demonstrated in the following section by addressing the analogy between the mechanical and electrical description of oscillating systems and discussing the impact of different loadings on the resonator oscillation.

3.2.2 Equivalent Circuit Model and Impedance Analysis

As shown in section 3.2.1 by the direct and inverse piezoelectric effect, mechanical and electrical energy are closely related and can be transformed into each other, known as electromechanical coupling. Accordingly, as piezoelectric material, quartz can be regarded as an electromechanical coupler/transducer. Thus, these electro-acoustical systems, which can be brought to oscillation, can be described by a mechanical or by an electrical model, which are equivalent. In the following, this is firstly described for an unloaded/unperturbed quartz resonator, then impedance analysis for this model is demonstrated and, finally, a few different loading conditions are discussed.

Equivalent Circuit Model for an Unperturbed Resonator

The mechanical oscillation of the unperturbed quartz resonator can be described by the model of a damped harmonic oscillator (Figure 3.5 A). It consists of a mass m in contact with a brake piston (with viscous damping coefficient r) parallel to a spring (with Hooke's

constant k) fixed to a stationary wall. When the system is displaced from its equilibrium position, a restoring force F acts on it, which brings the system into oscillation. More precisely, harmonic oscillations occur between two masses m_1 and m_2 and the reduced mass $\mu = \frac{m_1 \cdot m_2}{m_1 + m_2}$ of these two masses has to be considered. Here, m_2 belongs to the stationary wall, which is regarded to be infinite. Approximately, this leads to $\mu \approx m_1$, so that m_2 can be neglected. Without damping, the system would perform a sinusoidal oscillation with constant amplitude and frequency $f = \frac{1}{2\pi} \sqrt{\frac{k}{m}}$. But damping has to be regarded here, which is expressed by an additional frictional force and is implemented by the viscous damping coefficient r . Depending on the extent of damping, the system can be underdamped, critically damped, or overdamped, which will not be discussed in detail in this thesis. The equation of motion for a damped harmonic oscillator results from the balance of forces (restoring force and frictional force) (Feshbach & Tikochinsky, 1977):

$$F = m \frac{d^2x}{dt^2} = -r \frac{dx}{dt} - kx \quad (33)$$

where x is the displacement. This damped harmonic oscillation is related to the oscillatory displacement of the particles of the quartz crystal structure in the QCM.

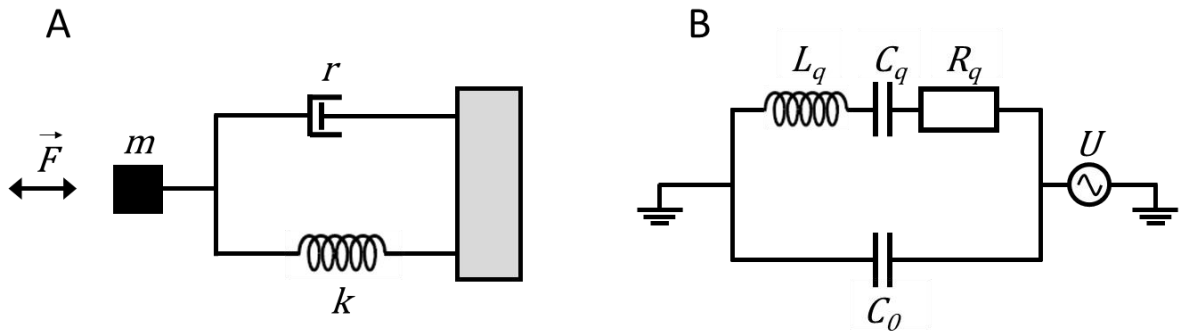


Figure 3.5 Schematic illustrations of the mechanical and electrical model of an electro-acoustical system, like a quartz resonator. **(A)** The mechanical model of the quartz oscillation describes a damped harmonic oscillator, which experiences a restoring force F after the mass m has been displaced, which is connected to a brake piston (with viscous damping coefficient r) parallel to a spring (with Hooke's constant k). **(B)** The electrical equivalent of the quartz oscillation can be described by a Butterworth-Van Dyke (BVD) equivalent circuit, which consists of an inductor (with inductance L_q), a capacitor (with capacitance C_q), and an ohmic resistor (with resistance R_q) in series (called motional arm), with a capacitor (with capacitance C_0) in parallel (called static arm), where an AC voltage U is applied. Illustrations were redrawn according to published literature (Buttry & Ward, 1992).

According to the electromechanical coupling in piezoelectric materials, this mechanical model has an electrical equivalent, which also describes the quartz oscillation. This can be represented by a Butterworth-Van Dyke (BVD) equivalent circuit (Figure 3.5 B). This equivalent circuit consists of an inductor (with inductance L_q), a capacitor (with capacitance

C_q), and an ohmic resistor (with resistance R_q) in series, with a capacitor (with capacitance C_0) in parallel (Buttry & Ward, 1992). An AC voltage U is applied and the system is grounded at both ends. According to Kirchhoff's rules for the addition of voltages in electric circuits, the equation for the series branch (motional arm) of the electrical BVD model is given by:

$$U = L_q \frac{d^2 q}{dt^2} = -R_q \frac{dq}{dt} - \frac{q}{C_q} \quad (34)$$

where q is the charge. The isomorphic structure of Eq. (33) and Eq. (34) underlines the equivalency of both the mechanical and electrical models. The force F in the mechanical model is linked with the AC voltage U in the electrical model and the displacement x is associated with the charge q . Accordingly, each electrical element in the BVD equivalent circuit can be connected to a mechanical analogue in the model of the damped harmonic oscillator. The inductance L_q is linked with the mass m and represents inertia. The capacitance C_q is inversely related to Hooke's constant k and represents mechanical elasticity. Both the inductance L_q and the capacitance C_q contribute to energy storage in the system. The ohmic resistance R_q is linked with the viscous damping coefficient r and represents friction. It contributes to energy loss in the system (energy dissipation). The grounding in the electrical model corresponds to the fixation to a stationary wall in the mechanical model. Thus, the mechanical properties of the quartz oscillation are accessible by analyzing the parameters of the electrical impedance and vice versa (Buttry & Ward, 1992). For the complete description of the BVD equivalent circuit, the static arm with the capacitance C_0 also has to be considered. It represents dielectric properties of the quartz between the electrodes and parasitic contributions from wiring and contacting.

Impedance Analysis of the BVD Model

Impedance spectroscopy (see section 3.1) can be used to measure the impedance of an oscillating quartz crystal as a function of frequency. In detail, an AC voltage with a small amplitude is applied to the system and the resulting current is measured, or a current is applied and voltage is measured. So, the magnitude of impedance $|Z|$ and the phase shift φ between voltage and current are determined in a frequency-dependent manner. With this, spectra of the magnitude of impedance (shortly called impedance spectra) and spectra of the phase shift (shortly called phase spectra) are obtained, from which the electrical circuit parameters can be determined afterwards by fitting the above introduced BVD model to the data. Sometimes, impedance and phase spectra are plotted together in one single diagram, which is called Bode plot. Such Bode plots are shown in Figure 3.6 for simulated data for the completely undamped case ($R_q = 0$) (Figure 3.6 A) and the damped case ($R_q > 0$, here $R_q = 2100 \Omega$) (Figure 3.6 B). They are shown near resonance of a 5 MHz quartz resonator, where two distinct resonance frequencies can be obtained for the completely undamped case (Figure 3.6 A), the resonant frequency f_R and the antiresonant frequency f_A .

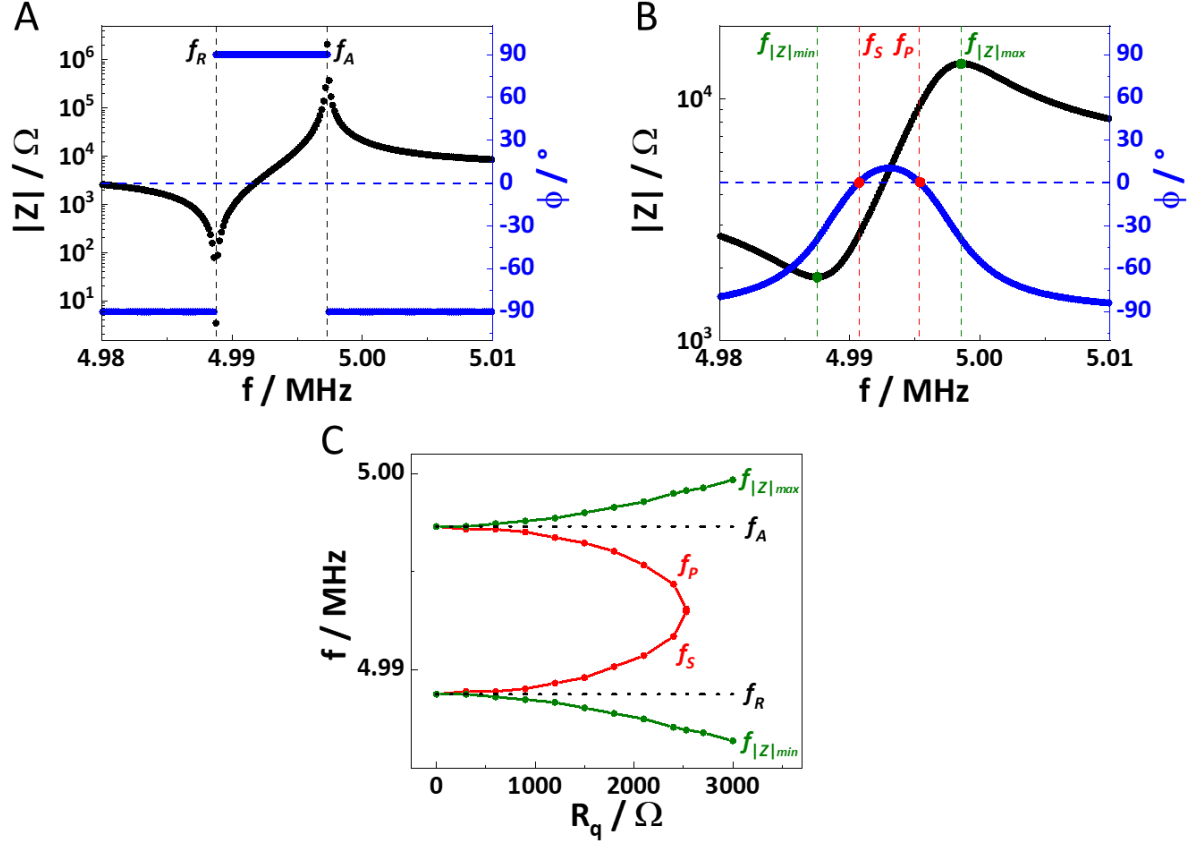


Figure 3.6 Simulated impedance and phase spectra of a BVD equivalent circuit for a 5 MHz quartz resonator near resonance in dependency on the damping (R_q) with constant values for L_q ($4.7 \cdot 10^{-2}$ H), C_q ($2.1655 \cdot 10^{-14}$ F), and C_0 ($6.3 \cdot 10^{-12}$ F). **(A)** Bode plot of the magnitude of impedance $|Z|$ (black) and the phase shift φ (blue) for the undamped case ($R_q = 0$) with the frequencies of zero phase, the resonant frequency f_R and the antiresonant frequency f_A , which coincide with the frequencies of minimal (for f_R) and maximal impedance (for f_A). **(B)** Bode plot of the magnitude of impedance $|Z|$ (black) and the phase shift φ (blue) for the damped case ($R_q > 0$, here $R_q = 2100 \Omega$), where the frequency of minimal impedance $f_{|Z|_{min}}$ and the series resonant frequency f_S (where $\varphi = 0$) no longer overlap. The same is true for the frequency of maximal impedance $f_{|Z|_{max}}$ and the parallel resonant frequency f_P (where $\varphi = 0$). **(C)** Behavior of these characteristic frequencies for increasing damping (R_q). According to B, f_R splits into $f_{|Z|_{min}}$ and f_S , and f_A splits into $f_{|Z|_{max}}$ and f_P , while the difference increases for increasing damping (R_q).

Both f_R and f_A are defined as the frequencies where the phase shift is zero and, accordingly, also the imaginary part of the impedance is zero (compare to Eq. (9)). Between these two frequencies ($f_R < f < f_A$), the phase shift φ displays the value $\frac{\pi}{2} = 90^\circ$ (Figure 3.6 A, blue curve) because the BVD circuit is dominated by the inductance L_q (compare to Eq. (23)). Away from resonance, outside these two frequencies ($f < f_R$ or $f_A < f$), the phase shift φ displays the value $-\frac{\pi}{2} = -90^\circ$ because the BVD circuit is dominated by the parallel capacitance C_0 (compare to Eq. (19)). The magnitude of impedance $|Z|$ shows an antisymmetric Lorentzian curve shape (Figure 3.6 A, black curve). For the completely undamped case, the frequencies of zero phase coincide with the frequencies for the extreme values of the magnitude of impedance. At the resonant frequency f_R , the inductance L_q is in

resonance with the capacitance C_q , which leads to a minimum of the magnitude of impedance (also called minimal impedance $|Z|_{min}$). At the antiresonant frequency f_A , the inductance L_q is in resonance with both the capacitances C_q and C_0 , which leads to a maximum of the magnitude of impedance (also called maximal impedance $|Z|_{max}$). But, even for the description of an unloaded quartz resonator, damping has to be taken into account ($R_q > 0$, here $R_q = 2100 \Omega$) (Figure 3.6 B), which leads to a flattening and broadening of impedance and phase spectrum. The maximal phase shift φ_{max} is decreasing with increasing extent of damping, while the lower limit of the phase shift stays approximately at the value of $-\frac{\pi}{2} = -90^\circ$. The frequencies of zero phase no longer coincide with the frequencies for the minimal and maximal impedance. The resonant frequency f_R is split into the frequency of minimal impedance $f_{|Z|_{min}}$, where, as the name already tells, the magnitude of impedance exhibits its minimum, and the series resonant frequency f_S , where the phase shift and the imaginary part of the impedance are zero. In the same way, the antiresonant frequency f_A is split into the frequency of maximal impedance $f_{|Z|_{max}}$ and the parallel resonant frequency f_P . How exactly these frequencies change for increasing damping, is shown in Figure 3.6 C. The frequency of minimal impedance $f_{|Z|_{min}}$ is decreasing with increasing extent of damping and the series resonant frequency f_S is increasing meanwhile. Equally, the frequency of maximal impedance $f_{|Z|_{max}}$ is increasing with increasing extent of damping and the parallel resonant frequency f_P is decreasing meanwhile. If the damping reaches a certain magnitude (here $R_q \approx 2500 \Omega$), the series resonant frequency f_S and the parallel resonant frequency f_P converge, which results from the maximal phase φ_{max} sitting exactly at zero phase. Increasing damping even further results in a phase spectrum completely being negative and, accordingly, no existing frequency of zero phase.

Perturbing the quartz oscillation with any surface load introduces an additional serial contribution to the motional arm of the BVD equivalent circuit (see further down at: *Impact of Different Loadings on the Resonator Oscillation*). Due to the additivity of serial impedance contributions, an additional contribution to the damping behaves exactly the same as an increase in R_q , so these considerations here are still valid for increasing the damping by an external additional load. But, under common liquid loading conditions, the extent of damping is rather small (500 – 600 Ω), so that the separation of these characteristic frequencies is quite low (Figure 3.6 C). If the inductance L_q would increase, which is related to an increase in the mass m (change of quartz characteristics or additional inductance contribution by a surface load), all the four frequencies would experience a negative shift in accordance with the Sauerbrey equation (see Eq. (29)), which is also described further down.

But, before discussing different loading conditions, the impedance of the unperturbed quartz resonator and the abovementioned characteristic frequencies are described mathematically first. For parallel circuits, it is convenient to use the inverse of the impedance $\underline{Z}$, which is the admittance $\underline{Y}$. According to Kirchhoff's rules for addition of

impedance contributions in electrical circuits (normal addition in series branches, reciprocal addition in parallel branches), the admittance $\underline{Y}$ of the BVD model is given by:

$$\begin{aligned}\underline{Y} &= \frac{1}{\underline{Z}} = \frac{1}{\underline{Z}_m} + \frac{1}{\underline{Z}_s} = \frac{1}{\underline{Z}_R + \underline{Z}_L + \underline{Z}_C} + \frac{1}{\underline{Z}_s} \\ &= \frac{1}{R_q + j\omega L_q - \frac{j}{\omega C_q}} + j\omega C_0\end{aligned}\quad (35)$$

Therein, $\underline{Z}_m$ is the impedance of the motional arm in Figure 3.5 B (R_q , L_q , and C_q in series) and is given by the sum of the individual impedance contributions assigned to R_q , L_q , and C_q , which are described by Eq. (15), Eq. (22), and Eq. (18), respectively. $\underline{Z}_s$ is the impedance of the static arm in Figure 3.5 B only consisting of C_0 , whose impedance contribution is described by Eq. (18). The admittance $\underline{Y}$ is, same as the impedance $\underline{Z}$, a complex quantity, which can be separated into its real part conductance G and imaginary part susceptance B :

$$\underline{Y} = G + jB \quad (36)$$

After a few mathematical steps to get the denominator in Eq. (35) free of imaginary components, the expression for the admittance $\underline{Y}$ can be separated into real and imaginary components, which yields expressions for the conductance G and the susceptance B (Yang & Thompson, 1993):

$$G = \frac{R_q}{R_q^2 + \left(\omega L_q - \frac{1}{\omega C_q}\right)^2} \quad (37)$$

$$B = -\frac{\omega L_q - \frac{1}{\omega C_q}}{R_q^2 + \left(\omega L_q - \frac{1}{\omega C_q}\right)^2} + \omega C_0 \quad (38)$$

By using Eq. (37) and Eq. (38), mathematical expressions for the abovementioned characteristic resonance frequencies (f_R and f_A for the undamped case; $f_{|Z|_{min}}$, f_S , f_P , and $f_{|Z|_{max}}$ for the damped case) can be found. First, this is conducted for the undamped case ($R_q = 0$). Applying this condition to Eq. (37) and Eq. (38) yields $G = 0$ and the expression for B also gets simplified. Using Eq. (36) and $\underline{Z} = \frac{1}{\underline{Y}}$ gives an expression for the impedance of the undamped BVD model:

$$\underline{Z} = j \cdot \frac{\omega^2 L_q C_q - 1}{\omega C_q - \omega C_0 (\omega^2 L_q C_q - 1)} \quad (39)$$

So, for the undamped case, the impedance $\underline{Z}$ is purely imaginary. Searching for the frequencies of zero phase (and, accordingly, zero imaginary part) gives the resonant frequency f_R and the antiresonant frequency f_A with (Yang & Thompson, 1993):

$$f_R = \frac{1}{2\pi} \sqrt{\frac{1}{L_q C_q}} \quad (40)$$

$$f_A = \frac{1}{2\pi} \sqrt{\frac{1}{L_q C_q} + \frac{1}{L_q C_0}} \quad (41)$$

The magnitude of impedance is minimal for f_R and maximal for f_A . Both f_R and f_A are isomorphic to the frequency of an undamped harmonic oscillator $f = \frac{1}{2\pi} \sqrt{\frac{k}{m}}$.

For the damped case ($R_q > 0$), the calculation of the characteristic resonance frequencies gets more complicated because $G \neq 0$. Nevertheless, this calculation can be performed equally to the undamped case, which yields the frequencies of zero phase, the series resonant frequency f_S and the parallel resonant frequency f_P (Yang & Thompson, 1993; Janshoff *et al.*, 2000):

$$f_S = \frac{1}{2\pi} \sqrt{\frac{1}{L_q C_q} \left(1 + \frac{C_0 R_q^2}{2L_q}\right)} = f_R \left(1 + \frac{C_0 R_q^2}{2L_q}\right) \quad (42)$$

$$f_P = \frac{1}{2\pi} \sqrt{\frac{1}{L_q C_q} \left(1 + \frac{C_q}{2C_0} - \frac{C_0 R_q^2}{2L_q}\right)} = f_R \left(1 + \frac{C_q}{2C_0} - \frac{C_0 R_q^2}{2L_q}\right) \quad (43)$$

The second part of Eq. (42) and Eq. (43) results from using Eq. (40). The term $\frac{C_q}{2C_0}$ describes approximately the difference between f_R and f_A , independently of the damping R_q . With this, these equations also show that f_S is originating from f_R , and f_P is originating from f_A , with the separation factor $\frac{C_0 R_q^2}{2L_q}$ describing the deviation, which is increasing with increasing damping (R_q), as already obtained in Figure 3.6 C. The frequencies of minimal impedance $f_{|Z|_{min}}$ and maximal impedance $f_{|Z|_{max}}$ no longer coincide with the respective frequencies of zero phase and are determined to (Yang & Thompson, 1993; Janshoff *et al.*, 2000):

$$f_{|Z|_{min}} = \frac{1}{2\pi} \sqrt{\frac{1}{L_q C_q} \left(1 - \frac{C_0 R_q^2}{2L_q}\right)} = f_R \left(1 - \frac{C_0 R_q^2}{2L_q}\right) \quad (44)$$

$$f_{|Z|_{max}} = \frac{1}{2\pi} \sqrt{\frac{1}{L_q C_q} \left(1 + \frac{C_q}{2C_0} + \frac{C_0 R_q^2}{2L_q}\right)} = f_R \left(1 + \frac{C_q}{2C_0} + \frac{C_0 R_q^2}{2L_q}\right) \quad (45)$$

Thus, $f_{|Z|_{min}}$ originates from f_R and is shifted from it by the separation factor $\frac{C_0 R_q^2}{2L_q}$ (similar to f_S), but in negative direction. $f_{|Z|_{max}}$ originates from f_A and is shifted from it by the separation factor $\frac{C_0 R_q^2}{2L_q}$ (similar to f_P), but in positive direction.

Impact of Different Loadings on the Resonator Oscillation

In the previous two sections, it has been discussed that the unperturbed quartz oscillation can be described by a BVD equivalent circuit, and its impedance and characteristic resonance frequencies can be calculated. However, any additional load on top of the quartz surface influences its oscillation, which is represented by an additional impedance contribution in the BVD equivalent circuit (Figure 3.7) (Buttry & Ward, 1992). It is denoted as the load impedance $\underline{Z}_{Load}$ and complements the motional impedance of the unloaded resonator $\underline{Z}_m^0$ on the series/motional branch to the motional impedance of the loaded resonator $\underline{Z}_m$.

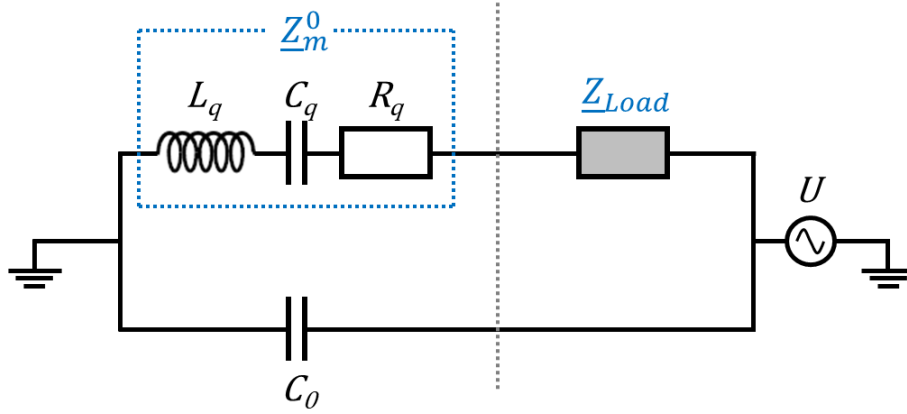


Figure 3.7 Schematic illustration of the BVD equivalent circuit for a loaded quartz resonator. Additional to the circuit elements L_q , C_q , R_q (summarized in the motional impedance $\underline{Z}_m^0$), and C_0 , describing the unloaded quartz resonator (see Figure 3.5 B), the impedance element $\underline{Z}_{Load}$ is implemented, which represents the surface load. The illustration was redrawn according to published literature (Buttry & Ward, 1992).

The load impedance $\underline{Z}_{Load}$ is, of course, also a complex quantity, which can be separated into its real part and imaginary part. The real part can be expressed by the resistance of the load R_L and the imaginary part can be regarded to be only inductive with the inductance of the load L_L without capacitive properties (Bandey *et al.*, 2000):

$$\underline{Z}_{Load} = \text{Re}(\underline{Z}_{Load}) + j \cdot \text{Im}(\underline{Z}_{Load}) = R_L + j\omega L_L \quad (46)$$

The load impedance $\underline{Z}_{Load}$ can also be approximated by (Bandey *et al.*, 2000):

$$\underline{Z}_{Load} \approx \frac{n\pi}{4K^2\omega_s C_0 \sqrt{\mu_q \rho_q}} \cdot \underline{Z}_{sm} \quad (47)$$

where n is the overtone number, K is the electromechanical coupling constant for AT-cut quartz with $K^2 = 7.899 \cdot 10^{-3}$, ω_s is the angular series resonant frequency ($\omega_s = 2\pi f_s$) and $\underline{Z}_{sm}$ is the surface mechanical impedance with the unit $\frac{\text{kg}}{\text{m}^2 \cdot \text{s}}$. Similar to electrical impedances, the surface mechanical impedance $\underline{Z}_{sm}$ is also a complex quantity and will be discussed further down for different loading conditions. The expression for the admittance $\underline{Y}$ in

Eq. (35) has to be extended by the expression for $\underline{Z}_{Load}$, which is given in Eq. (46), which then results in the following term:

$$\begin{aligned}\underline{Y} &= \frac{1}{\underline{Z}} = \frac{1}{\underline{Z}_m} + \frac{1}{\underline{Z}_s} = \frac{1}{\underline{Z}_m^0 + \underline{Z}_{Load}} + \frac{1}{\underline{Z}_s} \\ &= \frac{1}{(R_q + R_L) + j\omega(L_q + L_L) - \frac{j}{\omega C_q}} + j\omega C_0\end{aligned}\quad (48)$$

This additivity of the resistances and inductances emphasizes that an additional contribution by an external loading (R_L/L_L) behaves exactly the same as a change of the quartz characteristics or dimensions (R_q/L_q). Among the huge variety of different surface loading conditions, which are described well in the literature (Bandey *et al.*, 2000; Johannsmann, 2007), the most important ones for this thesis are discussed in the following: ideal mass layer, Newtonian liquid, and semi-infinite viscoelastic body. Combinations of two or more of these loadings are also possible but the calculation gets way more complicated then (Bandey *et al.*, 2000).

Ideal Mass Layer

An ideal mass layer as a load on the quartz disk is characterized by all the conditions that have to be fulfilled for the Sauerbrey equation (see Eq. (29)) to be valid. The mass layer has to be rigid, thin, and homogeneous, with a quartz-like behavior (similar shear modulus, similar density). For this case, the surface mechanical impedance $\underline{Z}_{sm}$ is given by (Bandey *et al.*, 2000):

$$\underline{Z}_{sm} = j\omega d_L \rho_L \quad (49)$$

where d_L is the thickness of the load and ρ_L the density of the load. The absence of viscous properties leads to a synchronous oscillation of quartz disk and foreign mass layer without energy loss. This is also reflected by the fact that $\underline{Z}_{sm}$, and therefore also $\underline{Z}_{Load}$, are purely imaginary ($Re(\underline{Z}_{Load}) = 0$, $R_L = 0$). The additional impedance contribution in the BVD equivalent circuit $\underline{Z}_{Load}$ can be condensed to the inductance of the load L_L . Accordingly, Eq. (46) and Eq. (48) can be simplified since there is only an increase in the inductance, which is related to an increase in the mass m . This leads to a parallel negative shift of all the four characteristic resonance frequencies ($f_{|Z|_{min}}$, f_S , f_P , $f_{|Z|_{max}}$), which is in accordance with the Sauerbrey equation (see Eq. (29)), while the shape of the spectrum is unaffected.

Newtonian Liquid

A Newtonian liquid is a liquid whose dynamic viscosity is constant, independent of the shear stress applied. Examples are water and oil. If such a Newtonian liquid is used as a load on a

quartz resonator, the load no longer oscillates synchronously with the quartz disk, due to the dynamic viscosity of the load η'_L (as already discussed above in section 3.2.1, *The QCM beyond the Sauerbrey Limitations*). This introduces energy dissipation into the surface load and the surface mechanical impedance $\underline{Z}_{sm}$ depends on the density-viscosity product of the load (Bandey *et al.*, 2000):

$$\underline{Z}_{sm} = \sqrt{\frac{1}{2}(\omega\rho_L\eta'_L)(1+j)} \quad (50)$$

The surface mechanical impedance $\underline{Z}_{sm}$, and therefore also $\underline{Z}_{Load}$, possesses equal real and imaginary parts. Energy storage (L_L) and energy dissipation (R_L) are of the same magnitude. Both factors influence the four characteristic resonance frequencies ($f_{|Z|_{min}}$, f_S , f_P , $f_{|Z|_{max}}$). Additional to the negative shift obtained by the mass loading (L_L), increasing damping by R_L causes the same behavior of the resonance frequencies as obtained for increasing R_q (see Figure 3.6 C). So, the combination of both energy storage and energy dissipation for deposition of a Newtonian liquid on a quartz resonator leads to complex alterations of the four characteristic resonance frequencies. It can also be obtained in Figure 3.6 that increasing damping/energy dissipation (R_L) is mirrored in a damping of the spectrum with an increase in the minimal impedance $|Z|_{min}$ and a decrease in the maximal impedance $|Z|_{max}$.

Semi-infinite Viscoelastic Body

Viscoelasticity is explained in detail in section 3.3.2 and can shortly be summarized as the feature of a material of having combined elastic and viscous properties. These materials are characterized by the complex shear modulus G^* (see Eq. (59)). The term ‘semi-infinite’ means that the viscoelastic load material is restricted from below (negative z-dimension) by the quartz disk and is infinite to the positive z-dimension, where infinite actually means that the layer thickness is much bigger than the decay length of the shear wave δ . The surface mechanical impedance $\underline{Z}_{sm}$ for a semi-infinite viscoelastic body is then given by (Bandey *et al.*, 2000):

$$\underline{Z}_{sm} = \sqrt{\rho_L G^*} \quad (51)$$

Typical viscoelastic materials are hydrogels, but also cells can be regarded as viscoelastic bodies. Elastic properties are generated by the cytoskeleton, cell cortex, and the lipid membrane, while viscous properties originate mainly from the cytoplasm and the liquid between cell and quartz. Beside these specific cellular compartments with well-known mechanical contributions, the complexity of the cell body leads to the fact that most of the compartments exhibit a complex and partially unknown mechanical behavior. The complexity is further increased in 2-D monolayers or even more in 3-D structures due to the highly organized interaction between the cells. Thus, there is no model available explaining cellular behavior on the QCM completely (Johannsmann, 2007).

Therefore, an empirical approximation was established in this thesis based on hydrogels modeling the elastic and viscoelastic properties of cells (see section 5). Hydrogels constitute the possibly most important class of viscoelastic materials, which can be compared to cells by their mechanical moduli (see section 3.3). Beside this approach, changes of viscoelastic properties caused by cell adhesion or alterations in the cytoskeleton due to external stimuli translate into impedance changes, which can be detected very accurately with QCM readings. Thus, the QCM can be used as a transducer in whole-cell biosensors to report on changes in viscoelastic properties. The correlation between QCM parameters and viscoelastic properties is also discussed in the following section.

3.3 Mechanical Moduli

As discussed in the previous section (section 3.2), the QCM signal generation is closely related to the mechanical behavior of the load on the quartz surface. Mechanical behavior can be described by mechanical moduli, which can be divided into elastic moduli for purely elastic materials and viscoelastic moduli for materials with additional viscous properties. There is a clear distinction between elastic and viscous materials. Purely elastic materials, such as most metals, respond with a strain (see Eq. (53)) synchronous to the application of a stress (see Eq. (52)) leading to no phase shift φ between both quantities. They deform non-permanently and return to their original shape without any energy losses after removal of the stress. In purely viscous materials, such as water, strain lags behind stress with $\varphi = 90^\circ$. They deform under thermal energy dissipation and remain in their deformed state when stress disappears. Most hydrogels, as well as cells, fall somewhere in between these two boundary situations, which is defined as viscoelastic behavior. They show combined elastic and viscous characteristics. Viscoelastic materials can exhibit very distinct behavior regarding the distribution of elastic and viscous properties (Meddad & Fisa, 1997; Zhang *et al.*, 2018). But many viscoelastic materials (such as polyacrylamide (PAAm)) behave predominantly elastically when only slightly deformed, but viscous properties are gradually growing when the deformation increases (Gu *et al.*, 2017). They show a phase shift between stress and strain of $0^\circ < \varphi < 90^\circ$. Viscoelastic materials can also be characterized by elastic moduli with a certain approximation, as long as they are measured in their predominantly elastic deformation region, which is in most cases $< 10\%$ deformation. In the following sections, the Young's modulus (section 3.3.1), the most often used elastic modulus, and the complex shear modulus (section 3.3.2), the modulus of choice for describing viscoelastic materials, are discussed in detail.

3.3.1 Young's Modulus

The application of a stress to a material leads to a strain within this material. Stress σ is defined as the pressure causing a deformation:

$$\sigma = \frac{F}{A} \quad (52)$$

where F is the force acting on the area A . Accordingly, the stress σ is given in the unit Pa. Strain ε describes the percentual deformation along the direction of the applied force and can be determined by:

$$\varepsilon = \frac{|l - l_0|}{l_0} \quad (53)$$

where l_0 is the original length along the direction of the applied force and l is the length after application of the force. Accordingly, the strain ε is dimensionless. By continually increasing the stress applied to a material and measuring the resulting strain, a so-called stress-strain curve can be determined to describe the relationship between both parameters. In Figure 3.8, a stress-strain curve is demonstrated for a viscoelastic polymer (PAAm), showing predominantly elastic behavior (linear) for low strain (about 0% – 20%) and viscoelastic behavior (non-linear) for higher strain, where the viscous part can no longer be neglected.

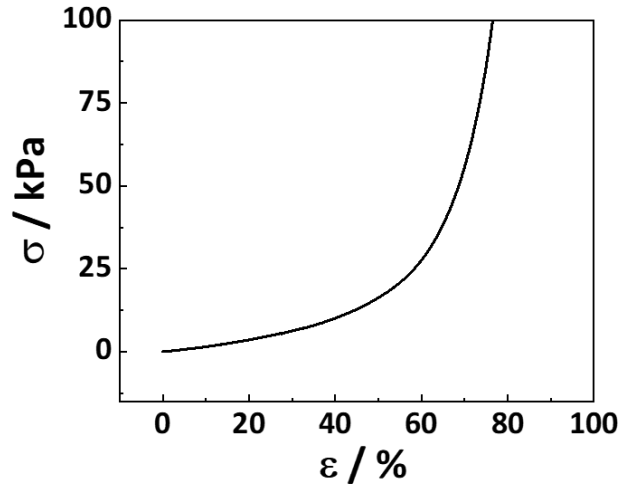


Figure 3.8 Typical stress-strain curve of a polyacrylamide (PAAm) hydrogel measured by compression testing (see section 4.4.1) as an example of a viscoelastic polymer with linear correlation between stress σ and strain ε for low strain (about 0% – 20%), reflecting predominantly elastic behavior, and non-linear behavior for higher strain, reflecting a significant viscous part.

An elastic modulus is defined as the slope of the stress-strain curve in the predominantly elastic deformation region. Different types of elastic moduli can be distinguished by the

direction of the application of stress. The Young's modulus E is the most important one and is often referred to as the elastic modulus. It describes the deformation along one certain axis, in contrast to, for example, a deformation in all dimensions at the same time, which is described by the bulk modulus. The Young's modulus E can be determined by applied stress σ divided by resulting strain ε , showing an isomorphic structure to Hooke's law:

$$E = \frac{\sigma}{\varepsilon} \quad (54)$$

with the unit Pa. Since a stiff material needs a higher stress σ to show the same strain ε , it exhibits a higher Young's modulus E . Thus, the Young's modulus can be regarded as a measure of stiffness. Isotropic materials (equal extent of deformation in all dimensions), for example, most hydrogels, are characterized by one single Young's modulus, whereas anisotropic materials exhibit different Young's moduli depending on the direction of the applied stress. The Young's modulus is commonly determined by compression testing (see section 4.4.1), but a determination by extension is also possible. Another approach for determination of the Young's modulus of soft hydrogels utilizes the bending of the hydrogel by gravity (Peng *et al.*, 2009).

The impact of the stiffness of the quartz loading on the QCM signal has been investigated and proven a couple of times in the literature, but without finding any specific correlation between stiffness and QCM parameters (Heitmann *et al.*, 2007; Zhou *et al.*, 2015). In this thesis, a correlation of the Young's modulus E (as a parameter for stiffness) with QCM parameters was established for PAAm hydrogels, which was then applied to cells.

3.3.2 Complex Shear Modulus

As discussed above, linearity in the stress-strain curve (see Figure 3.8) indicates predominantly elastic behavior, which can be described by the Young's modulus E . But this is not possible in the non-linear regions of the stress-strain curve, which are caused by viscoelastic behavior. As already mentioned above, viscoelastic materials show mixed elastic and viscous properties. One important parameter to describe viscous and viscoelastic materials is the dynamic viscosity η' , sometimes just called viscosity. The dynamic viscosity η' can be summarized as the concept of the existence of friction between adjacent material layers under relative motion. Figure 3.9 demonstrates the dynamic viscosity and its effect on liquids under shear stress. In contrast to normal stress, where the force is perpendicular to the material cross-section (see Eq. (52)), shear stress τ means that the force is parallel to the material cross-section with:

$$\tau = \frac{F}{A} \quad (55)$$

The outer layers of liquid particles are represented by the plates with area A . This shear stress acts on the upper plate inducing its motion with velocity v in direction of the shear

stress and perpendicular to the y -dimension. Due to the liquid's dynamic viscosity, only the first layer of liquid particles (the upper plate itself) moves with the velocity v , the layers below lose velocity with increasing distance from the upper plate down to zero at the fixed lower plate. This decrease in particle velocity v is linear with increasing distance y from the upper surface for Newtonian liquids, as shown in Figure 3.9, so the ratio $\frac{v}{y}$ is constant at every point. But for more complex liquids, this may not be linear, so in general, the partial derivative $\frac{\partial v}{\partial y}$ has to be regarded, which is called the local shear velocity. The exact magnitude of the local shear velocity $\frac{\partial v}{\partial y}$ at every point is determined by the dynamic viscosity η' at this point and is proportional to the applied shear stress τ with the dynamic viscosity η' as the proportionality factor:

$$\tau = \eta' \cdot \frac{\partial v}{\partial y} \quad (56)$$

Accordingly, the dynamic viscosity η' is given in the unit $\text{Pa} \cdot \text{s}$. Eq. (56) also shows that Newtonian liquids with their constant local shear velocity $\frac{\partial v}{\partial y}$ exhibit a constant dynamic viscosity η' .

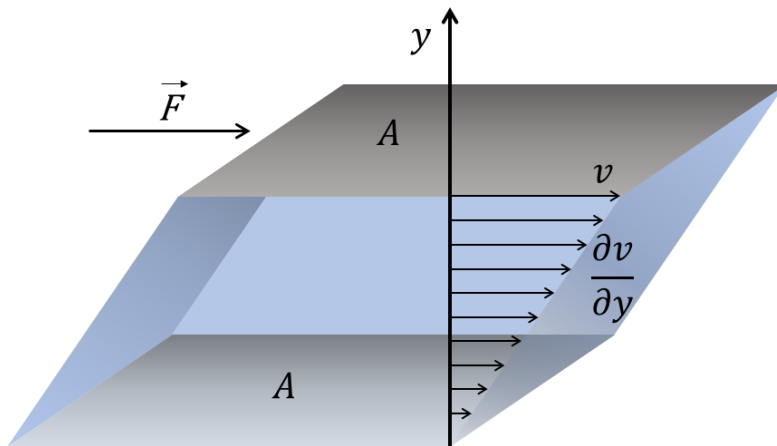


Figure 3.9 Schematic illustration of the dynamic viscosity of a Newtonian liquid between two parallel plates representing the outer layers of liquid particles. If the upper plate experiences a parallel force F , while the lower plate is fixed, the liquid particles move with the velocity v perpendicular to the y -dimension. The liquid's dynamic viscosity gives rise to friction between adjacent liquid layers, which causes a decrease in velocity v with increasing distance from the upper plate down to zero at the lower plate. The ratio $\frac{v}{y}$ (for Newtonian liquids), or in general, the partial derivative $\frac{\partial v}{\partial y}$ for liquids with a non-linear decrease in particle velocity (non-Newtonian liquids) is proportional to the applied shear stress τ with the dynamic viscosity η' as the proportionality factor (Eq. (56)).

Instead of the dynamic viscosity η' , sometimes also the kinematic viscosity ν is used, which is calculated by dividing the dynamic viscosity η' by the density ρ of the material:

$$\nu = \frac{\eta'}{\rho} \quad (57)$$

Accordingly, the kinematic viscosity ν is given in the unit $\frac{\text{m}^2}{\text{s}}$. These viscous properties explained here are combined with elastic properties (see section 3.3.1) in viscoelastic materials. In this case, the dynamic viscosity η' has to be generalized to the complex viscosity η^* , a complex quantity with the dynamic viscosity η' as the real part and the so-called storage viscosity η'' as the imaginary part:

$$\eta^* = \eta' + j \cdot \eta'' \quad (58)$$

Closely related to the complex viscosity η^* is the complex shear modulus G^* . It is the commonly used modulus for describing viscoelastic materials. Similar to the Young's modulus E (see Eq. (54)), the complex shear modulus G^* is the ratio of stress to strain, so it is also given in the unit Pa, but with an oscillatory stress applied. Accordingly, stress and strain have to be regarded as complex quantities, and, thus, also the complex shear modulus G^* is a complex quantity. It can be separated into its real part called storage modulus G' , representing the elastic part (energy storage), and its imaginary part called loss modulus G'' , representing the viscous part (energy loss):

$$G^* = G' + j \cdot G'' \quad (59)$$

Typically, the magnitude of the complex shear modulus $|G^*|$ is used as a measure to describe viscoelasticity with:

$$|G^*| = \sqrt{G'^2 + G''^2} \quad (60)$$

To fully describe the material of interest, additional to the magnitude, the phase shift φ between stress and strain needs to be given, or the material is described by storage modulus G' and loss modulus G'' . Sometimes both types of representation (Cartesian and polar) are mixed up when the complex shear modulus G^* is given by its magnitude $|G^*|$ and the storage modulus G' , for example. The connection between complex viscosity η^* and complex shear modulus G^* is given by:

$$\eta^* = \frac{G^*}{j\omega} \quad (61)$$

where ω is the angular frequency of the oscillatory stress. Combining Eq. (58), Eq. (59) and Eq. (61) leads to the following relationship:

$$\eta' + j \cdot \eta'' = \eta^* = \frac{G^*}{j\omega} = \frac{G''}{\omega} + j \cdot \left(-\frac{G'}{\omega} \right) \quad (62)$$

According to this, the dynamic viscosity η' corresponds to $\frac{G''}{\omega}$ and the storage viscosity η'' corresponds to $-\frac{G'}{\omega}$. So, the dynamic viscosity η' can be calculated from the loss modulus

G'' , which is, beside the storage modulus G' , typically obtained from rheological measurements (see section 4.4.2).

If QCM measurements are performed with a semi-infinite viscoelastic material as a load on the quartz surface, the material's complex shear modulus G^* is the main parameter influencing the load impedance $\underline{Z}_{Load}$ (as already discussed above at: *Impact of Different Loadings on the Resonator Oscillation*). Using Eq. (46), Eq. (47), and Eq. (51) and expressing the complex shear modulus G^* by its magnitude $|G^*|$ and the storage modulus G' yields expressions for the real part of the load impedance $Re(\underline{Z}_{Load})$ and the imaginary part of the load impedance $Im(\underline{Z}_{Load})$ (Bandey *et al.*, 2000):

$$\begin{aligned} Re(\underline{Z}_{Load}) &= \frac{n\pi}{4K^2\omega_s C_0 \sqrt{\mu_q \rho_q}} \sqrt{\frac{1}{2} \rho_L (|G^*| + G')} \\ &= M_q \sqrt{\frac{1}{2} \rho_L (|G^*| + G')} \end{aligned} \quad (63)$$

$$\begin{aligned} Im(\underline{Z}_{Load}) &= \frac{n\pi}{4K^2\omega_s C_0 \sqrt{\mu_q \rho_q}} \sqrt{\frac{1}{2} \rho_L (|G^*| - G')} \\ &= M_q \sqrt{\frac{1}{2} \rho_L (|G^*| - G')} \end{aligned} \quad (64)$$

Therein, the constant factor M_q summarizes the constant material properties of quartz. For a 5 MHz AT-cut quartz resonator in fundamental resonance, it is determined to $0.104 \frac{\Omega \cdot m^2 \cdot s}{kg}$. Based on Eq. (63) and Eq. (64), the magnitude of the complex shear modulus $|G^*|$ and the storage modulus G' can be calculated from the real and imaginary part of the load impedance:

$$|G^*| = \frac{1}{\rho_L} \left[\left(\frac{Re(\underline{Z}_{Load})}{M_q} \right)^2 + \left(\frac{Im(\underline{Z}_{Load})}{M_q} \right)^2 \right] \quad (65)$$

$$G' = \frac{1}{\rho_L} \left[\left(\frac{Re(\underline{Z}_{Load})}{M_q} \right)^2 - \left(\frac{Im(\underline{Z}_{Load})}{M_q} \right)^2 \right] \quad (66)$$

With this, properties of viscoelastic materials, especially cells and hydrogels, can be approximated from readings of $Re(\underline{Z}_{Load})$ and $Im(\underline{Z}_{Load})$, parameters obtained from standard QCM measurements. This demonstrates that the QCM is, beside a mass-sensitive sensor on the one hand, a sensor for viscoelastic properties and stiffness on the other hand. When cells are grown on the quartz surface, the QCM can be used as a sensor for cytomechanics.

3.4 Electric Cell-Substrate Impedance Sensing (ECIS)

Electric Cell-Substrate Impedance Sensing (ECIS) was developed by Giaever and Keese in 1984 as a biosensor for the morphology of mammalian cells, whereby they found that the cells and their movement influence the impedance signal (Giaever & Keese, 1984). Nowadays ECIS is a well-established technique with many different cell-based applications published until now, which range from the observation of cell-specific processes like cell adhesion or proliferation, over barrier function and cytotoxicity testing, up to cell wounding and electroporation. Examples of application were already given in section 1.5.2. The merit and success of ECIS are mainly attributed to the fact that it is a supplement or even surrogate for microscopy. A similarity to QCM is that ECIS is also based on impedance spectroscopy (see section 3.1), which causes many similar advantageous properties: label-free, non-invasive, very sensitive, time-resolved, multiparametric, simple to perform, highly automated, and with integral signal readout. The possibility for parallelization (typically 8-well or 96-well) is an advantage over QCM, so ECIS is also suitable for high-throughput screening. But the big difference to QCM is that ECIS is not based on piezoelectricity, but instead, on electrochemical principles. This is demonstrated in Figure 3.10, which gives an overview of the basics of the ECIS technique. A dish with a bottom made of a cell culture-compatible polymer (typically polyethylene terephthalate (PET) or polycarbonate (PC)) is used as a container for the culture medium, which is an electrolyte. Two coplanar electrodes (mostly consisting of gold, typical thickness 50 nm – 100 nm) are deposited on top of the culture substrate, one very small serving as the working electrode (WE, area $A \approx 5 \cdot 10^{-4} \text{ cm}^2$) and the other one with a 100 – 500-fold bigger area serving as the counter electrode (CE, $A \approx 5 \cdot 10^{-2} \text{ cm}^2 - 2.5 \cdot 10^{-1} \text{ cm}^2$). If an AC voltage (small amplitude of about 10 mV (rms) for non-invasive sensing) is applied to the electrodes, the current can flow in a closed circuit, because the current can cross the electrode-electrolyte interface and pass through the culture medium. So, the impedance of the system is obtained, either by determination of its magnitude and phase shift or by determination of real and imaginary parts. If cells are seeded, attached, and spread on the electrodes, they impede the current flow. In Figure 3.10, the cells are depicted as cylindrical disks because they are approximated as such in the ECIS model, which is explained below. Although the cell bodies behave like insulators, the current flow is not completely blocked, but the current can cross the cell layer in two distinct ways (paracellular and transcellular), which depends on the frequency f of the applied AC voltage.

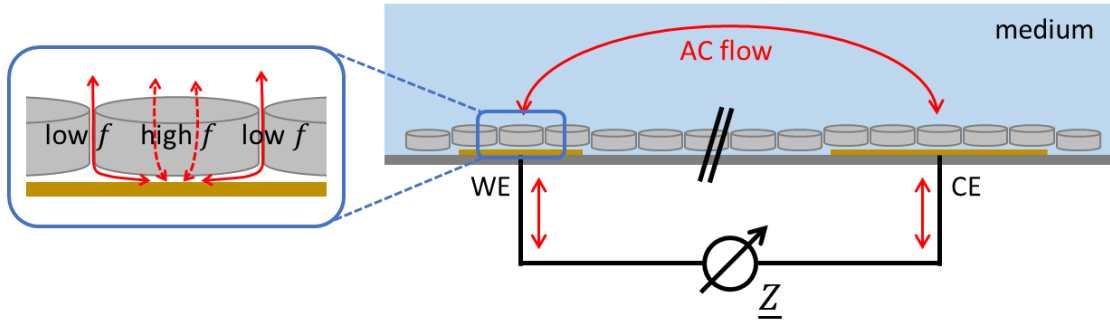


Figure 3.10 Schematic illustration of the ECIS measurement principle. In the ECIS model, cells are approximated as cylindrical disks, which cover the culture substrate and the coplanar electrodes (mostly gold) deposited on its surface. The working electrode (WE) and counter electrode (CE, 100 – 500-fold bigger area than WE) are electrically connected outside the wells and the applied alternating current (AC) can pass through the culture medium, but is impeded by the insulating cells. Accordingly, the dielectric nature of the cells is revealed by impedance measurements. The current pathway is dependent on the AC frequency f , which is shown in the small zoom-in at the left. Low-frequency current flows on paracellular pathways beneath and between the cells (red arrows with solid line), whereas high-frequency current flows on transcellular pathways through the cell bodies (red arrows with dashed line). Drawings not to scale.

The frequency dependency of the current flow can be explained by the fact that the paracellular pathway can be approximated as resistor R_c and the transcellular pathway as capacitor C_c (Giaever & Keese, 1991 + erratum), which is shown in the equivalent circuit in Figure 3.11 A. The impedance of an ideal capacitor $\underline{Z}_C$ is given by Eq. (18), so, accordingly, its magnitude is given by:

$$|\underline{Z}_C| = \sqrt{0^2 + \left(\frac{1}{\omega C_c}\right)^2} = \frac{1}{\omega C_c} = \frac{1}{2\pi f C_c} \quad (67)$$

whereas the impedance contribution of the resistor R_c is not frequency-dependent (see Eq. (14)). According to Eq. (67), at low frequencies ($f \approx 400$ Hz), the impedance $|\underline{Z}_{C_c}|$ of the capacitor C_c is high in comparison to the impedance of R_c . Because the current flows in the way of the lowest resistance/impedance, it avoids the membrane and flows on paracellular pathways, which means it flows in the gap between cells and electrode and in the paracellular cleft between the cells. The paracellular cleft with the tight junctions between the cells is responsible for the cellular barrier function. If cellular changes occur in these regions, impedance changes are detected very sensitively, so barrier function is accessible with ECIS at low frequencies. At high frequencies ($f \approx 40$ kHz), the impedance $|\underline{Z}_{C_c}|$ is low in comparison to the impedance of R_c , so the current preferably crosses the cell membranes and flows through the cytoplasm. The measured capacitance linearly increases with the electrode area A due to the definition of the capacitance C of a parallel-plate capacitor:

$$C = \epsilon_0 \epsilon_r \cdot \frac{A}{d} \quad (68)$$

with the vacuum permittivity ε_0 , the relative permittivity of the dielectric medium ε_r , and the plate distance d . Thus, the more of the electrode is covered with cells (cell-free fraction of the total electrode area decreases), the lower is the total capacitance C , and, according to Eq. (67), the higher is the total impedance. Thus, measuring at high frequencies enables ECIS to sense cell spreading and electrode coverage, where recording of the capacitance is preferred over the impedance due to the linear correlation with the cell-free electrode area. But these two ways of current flow are not strictly separated, they are always mixed up, so even at a rather low frequency, there is always some portion of the current flowing through the cells and vice versa. At the mid-frequency range, there is an almost equal contribution of both current ways, which can be used for simultaneous sensing of both barrier function and cell spreading. The exact frequency ranges are, of course, dependent on the electrode area and on R_c and C_c , so they are dependent on the cell type, but 400 Hz is in most cases a good frequency to monitor barrier function. Cell spreading and electrode coverage are usually investigated at 40 kHz. Therefore, it is obvious that all the cellular processes mentioned above like cell adhesion, proliferation, migration, and so on, as well as cell integrity testing, can be observed by ECIS measurements since they influence the current pathways due to changes of electrode coverage, cell shape, or cell contacts. Accordingly, impedance changes can be recorded. In particular, cytotoxicity of test substances causes damage or detachment of cells from the electrode leading to an impedance decrease.

The big difference in the area of the two electrodes leads, according to Eq. (67) and Eq. (68), to the fact that the total impedance is dominated by the small working electrode (small area A , low capacitance C , high impedance $|\underline{Z}_c|$) and wounding or electroporation also occur mainly at the working electrode because the biggest fraction of the voltage drops there. The sensitivity of ECIS measurements can therefore be increased by reducing the area of the working electrode with the disadvantage that fewer cells are covering the electrode then leading to a result that is maybe less representative for the whole cell layer. Thus, the working electrode area always has to be chosen as a good compromise between sensitivity and representativity and it has to be adjusted to the requirements of each experiment.

The total impedance $\underline{Z}$ of the system is described by Eq. (9), but its imaginary part, which is the reactance X , possesses no inductive properties, it is purely capacitive. Thus, the impedance is described by the resistance R as the real part and the capacitance C as the imaginary part. By measuring in a wide frequency range (typically 10^0 Hz – 10^6 Hz) and recording impedance, resistance, and capacitance, much information about the cell layer under investigation is collected at the same time. For most experiments, this is enough information for discussion of the cellular processes occurring. But, for example, in cytotoxicity testing, there is no information about the biological origin of impedance changes. This structural resolution is partially gained by establishing an appropriate model and fitting the model to the experimental data. For this purpose, Giaever and Keese established the ECIS model in 1991, which is shown in Figure 3.11 (Giaever & Keese, 1991 + erratum). An equivalent circuit of the cell-covered electrode (Figure 3.11 A) was extended to the ECIS model, which is demonstrated in Figure 3.11 B with its most important parameters.

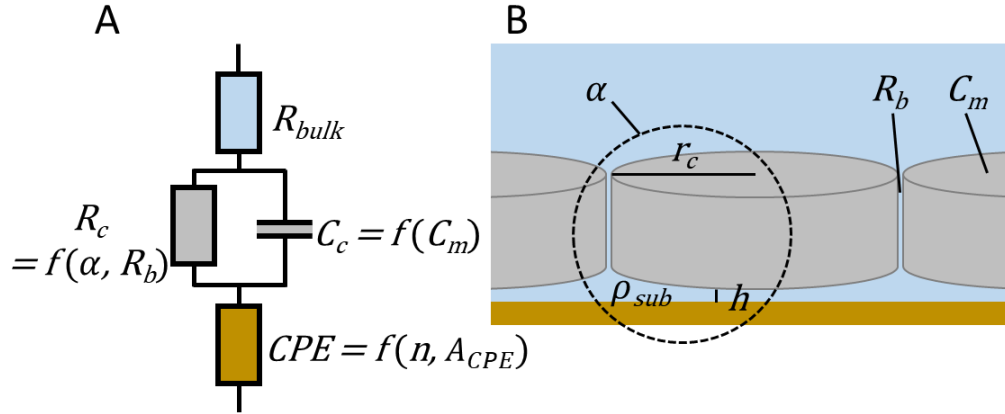


Figure 3.11 Schematic illustration of the ECIS model according to Giaever and Keese (Giaever & Keese, 1991 + erratum). The CPE was not part of the original ECIS model, it was added later (Wegener *et al.*, 2000a). **(A)** Non-redundant equivalent circuit for a cell-covered electrode illustrating the ECIS model with R_{bulk} (ohmic resistor) representing the resistance of the culture medium (blue), a CPE representing the interface impedance of the electrode in contact with medium (golden), and a parallel circuit of the cell-dependent contributions R_c (not an ohmic resistor) and C_c representing the paracellular (R_c) and transcellular (C_c) current pathways (grey). **(B)** In the ECIS model, the cells covering the electrodes are approximated as cylindrical disks of uniform dimensions (r_c) and distance from the electrode (h). These two parameters are, together with the specific resistivity beneath the cell layer ρ_{sub} , condensed to the parameter α , which is next to the paracellular resistance R_b and the membrane capacitance C_m part of the impedance originating from the cell layer.

In an equivalent circuit representation, the cell-free electrode (only medium on top of the electrode) is regarded as an ohmic resistance R_{bulk} in series to a constant phase element (CPE) (see section 3.1.2), where the CPE was not part of the original ECIS model, it was added later (Wegener *et al.*, 2000a). R_{bulk} represents the resistance of the electrolyte and the CPE represents the interface impedance of the electrode in contact with the electrolyte. The impedance contribution of a CPE is given in Eq. (24) with the real number $n = [0,1]$ and the frequency-independent parameter A_{CPE} . For gold-film electrodes in contact with physiological electrolytes, the number $n \approx 0.96$, so the CPE is more like a capacitor with little resistive contributions. When the equivalent circuit is to be established for the cell-covered system, many more circuit elements (resistances and capacitances) are added to describe the different ways of current flow. This can be condensed to a non-redundant equivalent circuit, which possesses the minimal number of circuit elements without information loss (Figure 3.11 A). This is, additionally to R_{bulk} and the CPE describing the cell-free electrode, achieved by the frequency-dependent cell resistance R_c (paracellular pathway) and the cell capacitance C_c (transcellular pathway). So, the cell layer itself is described by a parallel circuit of R_c and C_c , which was already mentioned above in the discussion of the different current pathways. The cells constitute extra impedance contributions, so the total impedance for a cell-covered electrode is higher than for a cell-free one. In detail, R_c describes the integral resistance of the cell layer summarizing the resistance beneath the cells (α in the ECIS model) and the resistance created by the paracellular clefts (R_b in the ECIS model). C_c describes the integral capacitance of the cell layer (C_m in the ECIS model).

This equivalent circuit representation is, of course, just a rough approximation, which is improved by extending it to the ECIS model (Figure 3.11 B) (Giaever & Keese, 1991 + erratum). In this model, the cells are approximated as cylindrical disks of uniform cell radius r_c and distance from the electrode h . These geometrical details become important due to the dependency on the exact location where the current enters and exits the electrode and the cell. This is done by assuming a radially symmetric current flow beneath the cells and fragmenting the cells into infinitesimal parts. Differential equations based on the balance of potentials are formulated for this specific geometry. The impedance contribution arising from the cell layer is described by three new cell-related parameters (α , R_b , and C_m), which cannot be represented by classical impedance elements as known from electrical engineering and are discussed in the following. α describes the frequency-dependent resistance beneath the cells and is dependent on the cellular dimensions (r_c , h) and the specific resistivity beneath the cell layer ρ_{sub} (with unit $\Omega \cdot \text{cm}$, not to be confused with the density):

$$\alpha = r_c \cdot \sqrt{\frac{\rho_{sub}}{h}} \quad (69)$$

with the unit $\sqrt{\Omega} \cdot \text{cm}$. For a given cell type, r_c and ρ_{sub} are approximately constant, so α mainly depends on h . R_b describes the paracellular barrier resistance, which correlates with the ion permeability through the paracellular cleft and is indirectly proportional to it. So, a high permeability of a leaky cleft means a low R_b value, which is given in the unit $\Omega \cdot \text{cm}^2$. Both these parameters α and R_b are not completely independent of each other, most cell types with a high R_b value also exhibit a high α value. C_m describes the average cell membrane capacitance of one membrane (basal or apical). The bigger the surface area of the membranes (for example, by the presence of microvilli on the apical membrane), the higher is the C_m value of the respective cell type. But C_m is the parameter with the smallest variance (of α , R_b , and C_m) because cells with smooth membranes exhibit a C_m value of around $1 \frac{\mu\text{F}}{\text{cm}^2}$ and it only rarely exceeds a value of $3 \frac{\mu\text{F}}{\text{cm}^2}$, whereas there might be huge differences in the resistance beneath the cells (α) and the paracellular barrier resistance (R_b). Finally, the total impedance of a cell-covered electrode depends on six parameters (α , R_b , C_m , n , A_{CPE} , and R_{bulk}) and two Bessel functions of the first kind of order 0 and 1 (Giaever & Keese, 1991 + erratum). These parameters can be obtained by fitting the ECIS model to the experimental data of a certain experiment. Typically, a set of impedance spectra recorded over time is subjected to fitting, where each individual impedance spectrum is analyzed by the model. First, values of $|Z_{CPE}|$ and $|Z_{R_{bulk}}|$ describing the cell-free electrode are extracted, so the remaining parameters are only cell-related (α , R_b , and C_m), which determine the cellular contribution to the impedance of the cell-covered electrode. A numerical optimization by the least squares method is performed to obtain very precise values for α , R_b , and C_m for a certain cell type. With this, time-resolved changes of α , R_b , and C_m are obtained enabling the assignment of impedance contributions to cellular structures, which is not possible by just looking at the total impedance. The ECIS model is a

very appropriate and approved approach for describing cell-covered electrodes, but it is still just a model not describing cell-covered electrodes in all situations completely correct.

In Figure 3.12, simulated impedance spectra for the cell-free situation (black) and a cell-covered electrode (different red tones) are shown for typical parameters of R_{bulk} , n , A_{CPE} , α , R_b , C_m , and the electrode area A given in the description. An electrode area of $5 \cdot 10^{-3} \text{ cm}^2$ is used, a 10-fold bigger area compared to the typical WE area of $5 \cdot 10^{-4} \text{ cm}^2$, according to an electrode pattern with ten electrodes in one well (so-called 8W10E). To demonstrate their influence on the shape of the spectrum, the three cell-related parameters α , R_b , and C_m are systematically varied, while all the other parameters are kept constant. The typical curve shape of the impedance spectra of the cell-free and cell-covered electrode cannot exactly, but roughly, be explained by the equivalent circuit in Figure 3.11 A and the frequency dependency of the single impedance contributions. $|Z_{C_c}|$ and $|Z_{CPE}|$ are indirectly proportional to the frequency f/ω (see Eq. (18) and Eq. (24)), whereas $|Z_{R_{bulk}}|$ is independent of the frequency. $|Z_{R_c}|$ is dependent on the frequency, but the extent of this frequency dependency depends on the value of α . As already mentioned above, the cell-free situation corresponds to a series circuit of R_{bulk} and CPE, which is dominated by the CPE at low frequencies ($10^0 \text{ Hz} - 10^3 \text{ Hz}$) because $|Z_{CPE}|$ is then higher than $|Z_{R_{bulk}}|$. This is mirrored in the impedance spectrum by the straight line with a slope of $-n = -0.96$ reflecting the indirect proportionality of $|Z_{CPE}|$ to the frequency. At high frequencies ($10^4 \text{ Hz} - 10^6 \text{ Hz}$), R_{bulk} is dominating because $|Z_{CPE}|$ is then lower than $|Z_{R_{bulk}}|$, which is consistent with the horizontal line reflecting the independency of the frequency. Impedance spectra of cell-covered electrodes overlap with impedance spectra of cell-free electrodes in the very low-frequency range ($10^0 \text{ Hz} - 10^1 \text{ Hz}$) and the very high-frequency range ($10^5 \text{ Hz} - 10^6 \text{ Hz}$) because the CPE and R_{bulk} , respectively, are dominating the impedance of the cell-covered electrode as well in these frequency regions. The exact frequencies of these regions, of course, depend on the single parameters. Spectra of cell-covered and cell-free electrodes differ from each other in the mid-frequency range ($10^1 \text{ Hz} - 10^5 \text{ Hz}$), where the cells dominate the impedance spectrum. So, the parallel circuit of R_c and C_c has to be regarded additionally to explain the shape of the impedance spectrum. At lower frequencies ($10^1 \text{ Hz} - 10^3 \text{ Hz}$), $|Z_{C_c}|$ is higher than $|Z_{R_c}|$, so the current avoids C_c and goes through R_c , which reflects the paracellular pathway. Thus, the impedance spectrum is dominated by R_c , which correlates with α and R_b in the ECIS model. Depending on these two parameters, the spectrum shows a more or less pronounced plateau. For the case of a dominating R_b value, which has a frequency-independent impedance contribution in contrast to α , there is a strongly pronounced plateau (see for low α values in Figure 3.12 A or high R_b values in Figure 3.12 B). But, for an increasing α value in contrast to R_b , the plateau evolves into a more frequency-dependent segment, which correlates with the frequency dependency of the impedance contribution of α (see for high α values in Figure 3.12 A or for low R_b values in Figure 3.12 B). Additionally, by increasing α or R_b , this plateau/segment is shifted towards higher impedance values. At higher frequencies ($10^3 \text{ Hz} - 10^5 \text{ Hz}$), $|Z_{C_c}|$ is lower than $|Z_{R_c}|$,

so the current goes preferably through C_c , which reflects the transcellular pathway. Corresponding to the capacitive behavior, the impedance spectrum shows a frequency dependency here with a straight line with a slope of approximately -1 , which is in accordance with Eq. (67). Also, according to Eq. (67), the impedance is decreasing for increasing capacitance C , which is shown for increasing C_m in Figure 3.12 C.

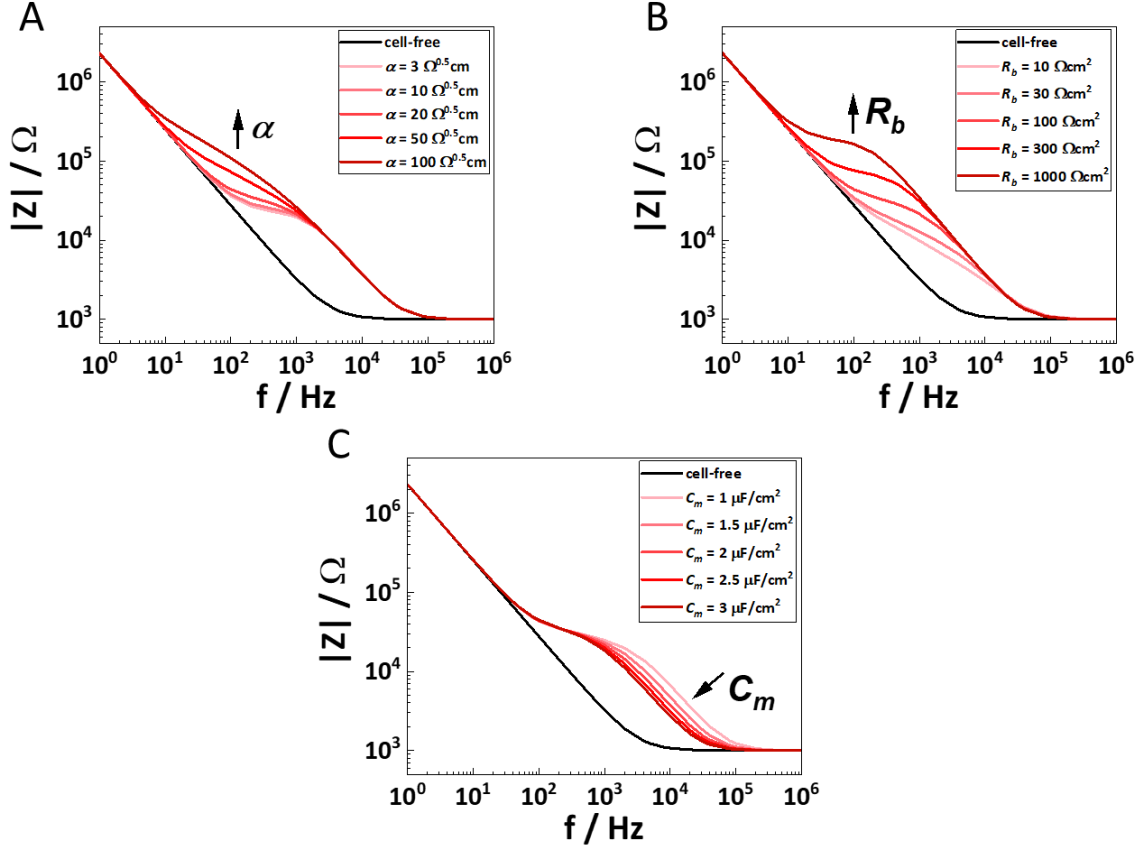


Figure 3.12 Simulated impedance spectra of a cell-covered gold-film electrode for systematical variation of the cell-related parameters α (A), R_b (B), and C_m (C) (different red tones) in contrast to a spectrum of a cell-free electrode (black). The arrows indicate the direction the parameter values increase. Except for the parameter under investigation, all other parameters are kept constant: $R_{bulk} = 1000 \Omega$ (typical value of 8W1E), $n = 0.96$, $A_{CPE} = 1.5 \cdot 10^{-5} \frac{F \cdot s^{n-1}}{cm^2}$, $\alpha = 20 \sqrt{\Omega} \cdot cm$, $R_b = 100 \Omega \cdot cm^2$, $C_m = 2 \frac{\mu F}{cm^2}$, and electrode area $A = 5 \cdot 10^{-3} cm^2$.

In total, ECIS, as a very versatile technique, enables sensing of the morphology of cells adherently grown on electrode arrays by measuring their impedance over time. It has numerous applications in cell-based assays with huge advantages over other techniques due to its non-invasive, time-resolved, and very sensitive nature. Modeling the data enables to distinguish between different origins of impedance changes.

3.5 PrestoBlue® Assay

Beside the abovementioned possibility to observe a potential toxicity of chemicals in cell-based assays with ECIS, there are several more assays available assessing cytotoxicity. The lactate dehydrogenase (LDH) assay and the ethidium homodimer assay can be used when the integrity of the cell membrane is investigated. Sensing for enzymatic activity can be performed with the calcein AM assay. A functional metabolism is tested by the MTT assay and the PrestoBlue® assay. The latter especially detects mitochondrial activity by the presence of NADH or NADPH (Lall *et al.*, 2013). The PrestoBlue® assay utilizes the fact that both NADH and NADPH are continually produced within an intact cell metabolism. After the cells were exposed to the possibly cytotoxic test substance for a pre-defined period of time, a resazurin solution is added to the cells. Resazurin is blue-colored, shows very weak fluorescence, and is membrane-permeable. Inside the cells, it is reduced to resorufin, which is pink-colored and highly fluorescent. This reduction is illustrated in Figure 3.13 and relies on the presence of NADH or NADPH, which are both only available in cells with intact metabolism. Thus, if the test substance showed a toxic effect leading to an impairment of the cell metabolism, the cells are incapable of providing enough NADH or NADPH for the complete reduction of resazurin. The test result is quantified by a measurement of the fluorescence intensity, whereby low cytotoxicity leads to a high fluorescence intensity due to NADH- or NADPH-based reduction of resazurin to the highly fluorescent resorufin. Vice versa, high cytotoxicity leads to low fluorescence intensity. Like in ECIS measurements, the PrestoBlue® assay is easily parallelizable, where typically a 96-well format is used. But in contrast to ECIS, the PrestoBlue® assay is label-based and invasive due to the resazurin solution, which has to be added additionally. A further drawback in comparison to ECIS is the non-time-resolved behavior of the PrestoBlue® assay because it is an endpoint assay, which means that the result of the test is not observed continuously over time, but instead, only at a selected time point.

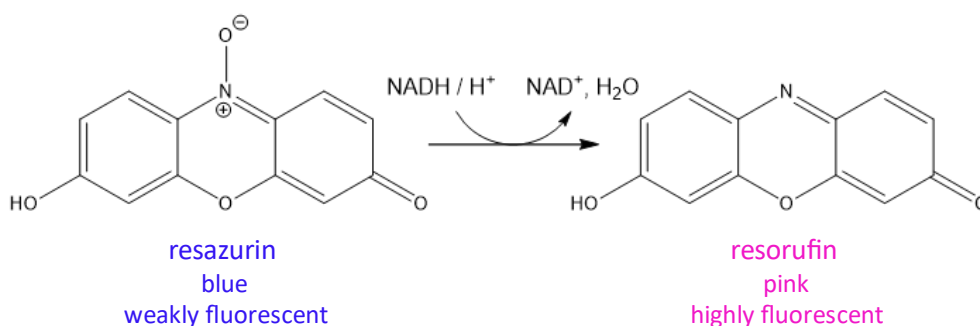


Figure 3.13 Chemical reaction occurring in the PrestoBlue® assay for detection of cytotoxicity. The membrane-permeable resazurin (blue-colored, weakly fluorescent) is reduced to resorufin (pink-colored, highly fluorescent) by NADH or NADPH in cells with intact metabolism.

In addition to different concentrations of the substance under study, three types of controls have to be investigated in the PrestoBlue® assay in parallel: (1) The blind control serves to correct for background fluorescence from the vessel and resazurin molecules and only contains the resazurin solution without cells. (2) The negative control contains the same as the test solutions, but without any test substance, not inducing any cytotoxicity. (3) For the positive control, the detergent Triton X-100 is added to lyse the cells and shut down their metabolism by membrane permeabilization leading to maximal cytotoxicity. Correction for background fluorescence is performed by calculating the corrected fluorescence intensity I_{corr} by the subtraction of all the measured fluorescence intensities I minus the measured fluorescence intensity of the blind control $I(blind\ ctrl)$:

$$I_{corr} = I - I(blind\ ctrl) \quad (70)$$

Due to the indirect proportionality between the extent of cytotoxicity and fluorescence intensity, in many cases the cytotoxicity index CI is used instead, which is directly proportional to the extent of cytotoxicity and is determined by:

$$CI = 1 - \frac{I_{corr}}{I_{corr}(neg.\ ctrl)} \quad (71)$$

where $I_{corr}(neg.\ ctrl)$ is the corrected fluorescence intensity of the negative control, which represents the maximal value of all measured fluorescence intensities due to no cytotoxicity. Thus, highly cytotoxic substances and the positive control show CI values close to 1, but substances with no or only very little cytotoxic effect show CI values close to 0.

4 Materials and Methods

In this chapter, all the experimental details are given regarding the handling of cells, dimensions of substrates and their fabrication (if not purchased commercially), volume or mass of chemicals, incubation times, temperature, ingredients of reaction mixtures, material information, workflows, measurement settings, and information about data acquisition and processing. The commercial suppliers of solutions and their office locations are summarized in the appendix section (section 11.4). Devices are denoted with their exact product number. Their commercial suppliers and respective office locations are also summarized in the appendix section (section 11.5).

4.1 Cell Culture Techniques

In the following, all cell culture-related work is described in detail, which comprises the general description of the cell lines used in this thesis (section 4.1.1), their specific requirements in cell culture (section 4.1.2), the protocol for their sub-cultivation (section 4.1.3) and their preparation for measurements (section 4.1.4).

4.1.1 Cell Lines

The cells mainly used in this thesis are the immortalized human trabecular meshwork (HTM-N) cells, which were thankfully received from Prof. Dr. Fuchshofer (University of Regensburg, Germany) and used to model the trabecular meshwork (TM) of a human eye. A microscopic image of these cells is depicted in Figure 4.1, which shows that they grow in a monolayer with cobblestone-like morphology. The HTM-N cells were derived from primary cells (human trabecular meshwork cells, HTM cells), which were previously isolated from the TM of a human eye and immortalized afterwards by transfection with a simian virus 40 (SV40). The procedure of SV40 immortalization and the preliminary characterization of the HTM-N cells were described by Pang in 1994 (Pang *et al.*, 1994). For some experiments, the Madin-Darby canine kidney cells strain I (MDCK-I) and strain II (MDCK-II) and normal rat kidney (NRK) cells were used. MDCK-I and MDCK-II cells were received from the biochemistry institute of the university of Münster and NRK cells were obtained from *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ). The MDCK cells are epithelial cells, while the two strains differ in their exact origin. MDCK-I cells were isolated from the distal tubule of the kidney, whereas the MDCK-II cells were isolated from the proximal tubule of the kidney. The NRK cells are epithelial-like and were cloned from a mixed culture, from which the clone 52E was used as a very stable cell line. One further cell line was used in this thesis, which is derived from MDCK-I cells: the Madin-Darby canine kidney cells strain I with overexpression of human aquaporin-1 (MDCK-I-AQP1). They were

kindly provided by Prof. Dr. Pohl (University of Linz, Austria) and were previously transfected with AQP1 by the calcium-phosphate precipitation method to enhance water flux across the plasma membrane (Deen *et al.*, 1997). All of the cells used in this thesis grow in monolayers.

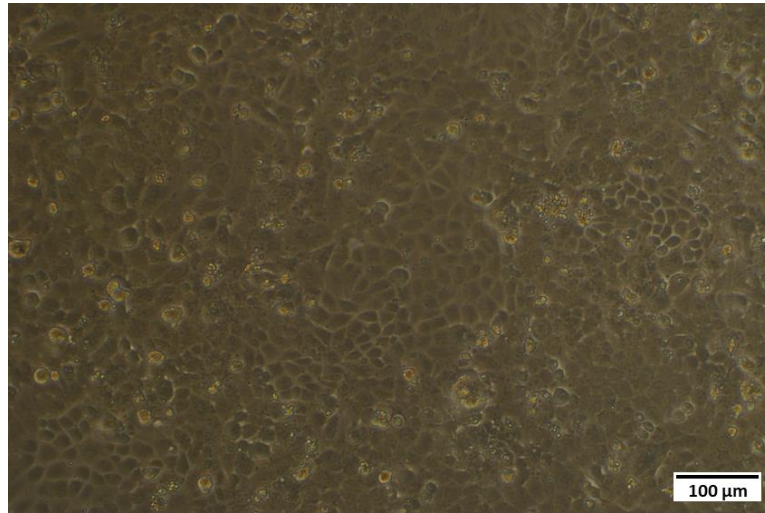


Figure 4.1 Phase contrast microscopic image of immortalized human trabecular meshwork (HTM-N) cells grown on the bottom of a cell culture flask.

4.1.2 Cell Culture Conditions

All cell culture work was performed in a laminar flow hood HERAsafe KS/KSP class II biological safety cabinet, which was disinfected with 70% (v/v) EtOH in deionized water before and after usage to maintain sterile conditions. All equipment and containers brought inside the laminar flow hood were spray-disinfected with EtOH. The sterility of the equipment was achieved by autoclaving at 120 °C in an autoclave DX-45 unless the materials were supplied sterile by the manufacturer. Non-sterile solutions were filtered inside the laminar flow hood through Filtropur S filters with a pore size of 0.2 μm diameter. The cells were seeded onto the bottom of sterile 25 cm² polystyrene culture flasks with 4 mL of culture medium and kept in an incubator Heraeus BB 15 at 37 °C, with 5% carbon dioxide (CO₂) and water vapor saturation. This amount of culture medium is enough to completely cover the cell layer and to provide the cells with nutrients for a few days. All solutions were warmed up to 37 °C in a water bath TW12 before they get in contact with cells. Solutions and materials contaminated by cells were autoclaved at 120 °C before disposal. The five different cell lines mentioned in section 4.1.1 required different culture media, which is summarized in Table 4.1. The basic medium (Dulbecco's Modified Eagle's Medium (DMEM) or Minimum Essential Medium Eagle (MEM)) contained various salts, vitamins, amino acids, sugars, and the pH-indicator phenol red, with the product number of Sigma Aldrich revealing the exact amount of each ingredient. L-Glutamine (L-Gln) and fetal bovine serum (FBS) were added, which were both important for cell proliferation and growth. Penicillin and streptomycin were added additionally to avoid bacterial growth. In the case of MDCK-I-AQP1 cells, hygromycin B was added to the medium at a final concentration of about 80 mg/L –

100 mg/L each time fresh medium was added to the cells. This compound inhibits protein synthesis in all cells without hygromycin resistance gene. Since the MDCK-I-AQP1 cells transfected with AQP1 carry this gene, hygromycin B induces selection pressure in favor of these cells and kills those cells that do not carry AQP1.

Long-time cell storage was performed by deep-freezing in a cryogenic tank GC – BR 2150M filled with liquid N₂, ensuring a constant temperature of –196 °C. For cryoconservation of cells, a special freezing medium was used, which consists of normal medium with 10% dimethyl sulfoxide (DMSO) preventing ice crystals. Freezing the cells was carried out in a first cooling step by filling the cell suspension in cryovials, which were placed in a container filled with isopropyl alcohol and stored in a freezer at –80 °C for 24 h. This ensured a slow and constant cooling rate of 1 °C/min, before the cryovials were placed in the cryogenic N₂ tank. Thawing of the cells was performed by placing a cryovial with frozen cell suspension into a freezer at –20 °C for 30 min, ensuring gentle thawing conditions. Afterwards, it was warmed up to about room temperature (RT) in a heated water bath. Removal of the DMSO was achieved by slowly adding an excess of normal medium at 37 °C, centrifuging in a Heraeus Multifuge 1S-R at 110 *g* for 10 min, aspirating the supernatant, and subsequent carefully re-suspending the pellet in normal medium. For cell cultivation, the cell suspension was seeded into culture flasks. Freshly thawed cells were not directly used for experiments, but instead, only after two weeks of sub-cultivation (section 4.1.3). Cells were used for experiments for up to six months in sub-cultivation, then fresh cells were thawed to avoid phenotypic changes over time and to ensure constant experimental conditions.

Table 4.1 Compositions of cell culture media for the five different cell types used in this thesis. The cell culture medium consisted of basic medium Dulbecco's Modified Eagle's Medium (DMEM) or Minimum Essential Medium Eagle (MEM) with the corresponding product number of Sigma Aldrich giving the exact composition. They were additionally supplemented with fetal bovine serum (FBS), L-glutamine (L-Gln), penicillin, and streptomycin with the exact amounts depending on cell type. For the MDCK-I-AQP1 cells, the selection antibiotic hygromycin B was added to the medium at a final concentration of about 80 mg/L – 100 mg/L before it was added to the cells.

Ingredient	HTM-N	NRK	MDCK-I	MDCK-I-AQP1	MDCK-II
basic medium	DMEM D6546	DMEM D5671	MEM M2279	MEM M2279	MEM M2279
+ FBS	10% (v/v)	5% (v/v)	5% (v/v)	5% (v/v)	5% (v/v)
+ L-Gln	0.5846 g/L	0.2923 g/L	0.5846 g/L	0.5846 g/L	0.5846 g/L
+ penicillin	0.1 g/L	0.1 g/L	0.1 g/L	0.1 g/L	0.1 g/L
+ streptomycin	0.1 g/L	0.1 g/L	0.1 g/L	0.1 g/L	0.1 g/L

4.1.3 Sub-Cultivation of Cells

The cells seeded onto the bottom of the 25 cm² polystyrene culture flasks grow and proliferate until the complete bottom of the flask is covered by the cells without any free spaces, which is then called a confluent cell layer. Beyond that, the cell density can increase slightly by reducing the individual cell size. For maintaining a continued proliferation, the cells were detached from the bottom and a fraction of them was seeded into new culture flasks. This process is called sub-cultivation or passaging. The sub-cultivation of the cells was done according to a standardized protocol. The cell-cell and cell-substrate contacts are dependent on divalent cations. To weaken these contacts, the supernatant medium in the culture flask was aspirated and the cells were washed twice with 4 mL of phosphate buffered saline without Ca²⁺ and Mg²⁺ (PBS⁻, see Table 4.4). This step also serves to remove dead cells and cell residues. After aspiration of PBS⁻, the detachment of the cells is enhanced by two further steps, the addition of a solution of ethylenediaminetetraacetate (EDTA) and the addition of a trypsin solution. The exact implementation of these two steps depends on the cell line used, which is summarized in Table 4.2. EDTA improves the removal of divalent cations by chelating them with very high affinity and trypsin cuts proteins like cell-cell and cell-substrate contacts. 4 mL of EDTA solution were added to the culture flask and incubated at 37 °C and 5% CO₂ for 10 min (twice for MDCK-I cells, not applied to HTM-N cells). After aspiration of EDTA, 1 mL of trypsin solution was added (2.5 g/L for HTM-N, MDCK-I, and MDCK-I-AQP1 cells, 0.5 g/L for NRK and MDCK-II cells) and incubated at 37 °C and 5% CO₂ for 10 min to completely detach the cells from the bottom of the culture flask. To stop the trypsinization process, the cells were suspended in 10 mL medium (9 mL for HTM-N cells) and centrifuged in a centrifugation tube in a Heraeus Multifuge 15-R at RT and 110 *g* for 10 min. The supernatant medium was aspirated and the pellet was re-suspended in 3 mL – 5 mL fresh medium. To obtain a confluent cell layer one week later, a 1:20-dilution (1:15 for MDCK-I-AQP1 cells) of this cell suspension was seeded into a new culture flask together with fresh medium (4 mL in total cell suspension plus medium). Typically, two identical flasks were prepared with one of them serving as a backup flask. To avoid turbulences within the cell suspension caused by the abrupt temperature change from RT to 37 °C potentially leading to a non-uniform distribution of cells over the surface of the culture flask, the cells were allowed to settle down by keeping the culture flasks for 10 min at RT inside the laminar flow hood before they were placed in the incubator at 37 °C. Each of the cell lines was sub-cultivated once a week and fed once in between (3 d – 4 d after sub-cultivation) by exchanging the culture medium. Another fraction of the detached cells was seeded to the growth substrates that were used for experiments (section 4.1.4).

Table 4.2 Summary of the sub-cultivation protocols for the five different cell types. Ethylenediaminetetraacetate (EDTA) was dissolved in phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS^{-}), the ingredients of which are given in Table 4.4. Both the differently concentrated trypsin solutions contained 0.2 g/L EDTA additionally. Both the EDTA and trypsin incubation steps were performed in the incubator Heraeus BB 15 at 37 °C and 5% CO_2 .

Procedure	HTM-N	NRK	MDCK-I	MDCK-I-AQP1	MDCK-II
EDTA	–	10 min, 0.4162 g/L	2 x 10 min, 0.4162 g/L	10 min, 0.4162 g/L	10 min, 0.4162 g/L
trypsin	10 min, 2.5 g/L	10 min, 0.5 g/L	10 min, 2.5 g/L	10 min, 2.5 g/L	10 min, 0.5 g/L

4.1.4 Cell Preparation for Experiments

During sub-cultivation, after re-suspending the cells with fresh medium, a cell suspension of unknown cell concentration (cell number per mL) was obtained, which was used for seeding cells to a variety of substrates used for experiments (Table 4.3). Due to different growth characteristics and different sizes of the cells, a confluent cell layer means different areal cell densities (cell number per cm^2) for each single cell line. This is for the HTM-N cells 200 000 cells/ cm^2 , for the NRK cells 250 000 cells/ cm^2 and for the MDCK cells (all kinds) 450 000 cells/ cm^2 . This means that seeding a cell suspension of these areal cell densities leads, after attachment and spreading, to a substrate surface completely covered by the respective cells. With the growth area of the substrates and the volume of solution that perfectly fitted into the respective substrates, the intended cell concentrations were calculated from the required areal cell densities (see Table 4.3). For maintaining these areal cell densities constant over all experiments, after re-suspension, the cells in the cell suspension were counted in a Bürker chamber with a phase contrast microscope Diaphot. This was achieved by adding 25 μL of the cell suspension to the Bürker chamber, which was sufficient to completely fill the space between both glass slides. An integrated grid with known dimensions enabled it to scale up the cell number counted with the microscope to the cell concentration in the cell suspension, which is, of course, only a rough approximation of the real cell concentration. This estimated cell concentration was then diluted with medium to the intended cell concentration and seeded to the respective substrate, which was sterilized before by treatment with argon (Ar) plasma for 30 s – 1 min at the highest energy level in an argon plasma cleaner PDC 32G-2, if not delivered sterile by the manufacturer. The cells were allowed to settle down for 10 min at RT. The substrates were kept at 37 °C and 5% CO_2 for 2 d (3 d for the transwell arrays and glass rings), so that the cells could adhere and form a confluent monolayer with intact cell contacts and barrier function, with feeding of the cells 1 d before the measurement. This feeding step was a bit different to the one in the culture flasks because here the medium added only contained 0.35% FBS or was even serum-free. This little amount of 0.35% FBS is used when the physiological situation in the human eye is to be simulated (see section 7), and serum-free

medium is used either when the influence of growth factors on the cells is investigated, which would be disturbed by serum (see section 6), or when the measurement takes place in another serum-free buffer (see section 8). How exactly the cells were treated directly before and during measurement, this is explained in subsequent sections. When cell adhesion was to be investigated, directly after settling down for 10 min at RT, the sensor substrate was connected to the measurement instrument and the cells' adhesion to the substrate was followed in real time by the measurement.

Table 4.3 Summary of the different substrates used for experiments, their respective growth area and appropriate volume of solution (of one well for the case of an array), and, based on that, the calculated cell concentration needed in the cell suspension, exemplary for HTM-N cells with a required areal cell density of 200 000 cells/cm². The calculation is performed by: areal cell density · area / volume, which can also be applied to NRK cells with a required areal cell density of 250 000 cells/cm² and to MDCK cells (all kinds) with a required areal cell density of 450 000 cells/cm².

Substrate	(Well) area / cm ²	Volume / mL	HTM-N cell concentration / cells/mL
QCM disk (section 4.6) / double-quartz-disk (section 4.7) / microscopy chamber (section 4.10.1)	1.13	1	226 000
ECIS array (section 4.8.1)	0.8	0.4	400 000
transwell array (section 4.8.2) / glass rings (section 4.10.3)	2	1	400 000
96-well plate for PrestoBlue® (section 4.9)	0.34	0.2	340 000
Petri dish for CLSM (section 4.10.3)	20.43	10	408 600

4.2 Solutions and Materials

4.2.1 Composition of Buffers and Solutions

When a test solution was added to cells during measurement, in most cases a 50:50-addition (or also called 2x-addition) was performed to minimize impairment of the cells, where half of the supernatant liquid is replaced by the test solution, which is then needed double-concentrated (2x). In this thesis, this type of addition of solutions is called half replacement method. The full replacement method, where the complete liquid is exchanged with the test solution, is applied for all measurements without cells and for water flux experiments (description of experiments see section 4.6.3, results see section 8) because there it would be difficult to prepare solutions that yield the exact intended osmolality when diluted by a factor of two. Controls were always investigated in parallel, in order to check for unspecific

reactions of the cells. For this, solutions were prepared consisting of exactly the same as the test solution, but without the actual test substance. Test solutions and controls were always prepared by dilution from stock solutions to the final intended concentration (for the full replacement method) or double-concentrated (for the half replacement method) 1 d – 3 d before the experiment. In the following, if not yet described before, all the buffers and solutions used in this thesis are presented with their preparation, purpose of use, concentration, and storage.

Double-distilled water (dd-H₂O) was often used as a solvent, which was prepared by redistillation of regular distilled water and was stored at RT. Phosphate buffered saline without Ca²⁺ and Mg²⁺ (PBS⁻), which is mainly needed for sub-cultivation of cells (see section 4.1.3), and phosphate buffered saline with Ca²⁺ and Mg²⁺ (PBS⁺⁺), which is especially needed for the cytochemical fluorescence staining protocol (see section 4.10.3), were both purchased ready to use and were stored at 4 °C. Their exact compositions are given in Table 4.4.

Table 4.4 Compositions of phosphate buffered saline without Ca²⁺ and Mg²⁺ (PBS⁻) and phosphate buffered saline with Ca²⁺ and Mg²⁺ (PBS⁺⁺), both purchased ready to use, with the mass concentration β given for six different salts dissolved in distilled water, while PBS⁻ did not contain Ca²⁺- and Mg²⁺-salts, but PBS⁺⁺ did.

Buffer	β (NaCl) / g/L	β (KCl) / g/L	β (Na ₂ HPO ₄) / g/L	β (KH ₂ PO ₄) / g/L	β (CaCl ₂ ·2H ₂ O) / g/L	β (MgCl ₂ ·6H ₂ O) / g/L
PBS ⁻	8	0.2	1.15	0.2	–	–
PBS ⁺⁺	8	0.2	1.15	0.2	0.133	0.1

For the water flux experiments, the buffer PBS⁺⁺/glucose (PBS⁺⁺/Glc) was used, which was prepared by adding D-glucose at a concentration of 1 g/L to PBS⁺⁺, to obtain an isotonic buffer solution. Isotonic means the same osmolality as the physiological osmolality of the cells, which is in the range of 260 mosmol/kg – 320 mosmol/kg (Freshney, 2010). Based on this PBS⁺⁺/Glc buffer a variety of hypertonic solutions were prepared by adding sucrose at the following concentrations: 20 mM, 100 mM, 200 mM, 300 mM, 400 mM, and 800 mM. The preparation of hypotonic solutions had to be performed differently. For this, firstly a basic buffer (Table 4.5) had to be prepared, which was then used as the solution with the lowest osmolality (hypo 1). By addition of sucrose at a concentration of 58 mM a second hypotonic solution with a higher osmolality (hypo 2) was obtained and adding 169 mM sucrose to the basic buffer led to an isotonic solution. The specific conductivity of the hypertonic and hypotonic solutions was adjusted to the value of the respective isotonic solution, which was achieved by adding different amounts of a sodium chloride solution (see section 4.2.2). This solution was prepared by dissolving NaCl in dd-H₂O at a concentration of 1.5 M. The osmolalities of the isotonic, hypertonic, and hypotonic solutions were measured afterwards (described in section 4.2.2) and they were stored at 4 °C.

Table 4.5 Composition of the basic buffer needed for the preparation of hypotonic solutions. The ingredients were dissolved in distilled water at the given concentration c and the pH value was adjusted to 7.4 with NaOH.

Buffer	c (KCl) / mM	c (CaCl ₂) / mM	c (KH ₂ PO ₄) / mM	c (MgSO ₄) / mM	c (D-glucose) / mM	c (HEPES) / mM
basic buffer	3.3	1.3	1.2	0.4	10	25

The membrane-permeable ROCK inhibitor fasudil (see section 1.1.3) was the most intensively examined substance in this thesis, as its influence especially on the HTM-N cells was investigated. A stock solution of fasudil HCl (Figure 4.2) of 10 mM or 2.5 mM in PBS⁻ was thankfully received from Dr. Mietzner / Prof. Dr. Göpferich (University of Regensburg, Germany), which was diluted with the respective medium to the respective concentration of interest, where 100 μ M and 25 μ M were mostly used. For comparison, so-called *released* fasudil was used. This was also provided by Dr. Mietzner / Prof. Dr. Göpferich, who prepared poly(lactic-co-glycolic acid) (PLGA) microspheres containing fasudil, which was then released under constant shaking at 37 °C for several days in PBS⁻ additionally containing 0.02% (v/v) Tween 20. The solution was sterile filtered and the fasudil concentration was determined afterwards by high performance liquid chromatography (HPLC). Quantification of *released* fasudil by HPLC was performed using a 1260 Infinity II LC system equipped with a reverse phase C₁₈ column and a diode array detector for measuring the absorbance of fasudil at a wavelength λ of 320 nm. Detailed information on the elution protocol of the HPLC measurements, the preparation of the PLGA microspheres, and the release of fasudil is given elsewhere (Mietzner *et al.*, 2020). All fasudil solutions were stored at -20 °C.

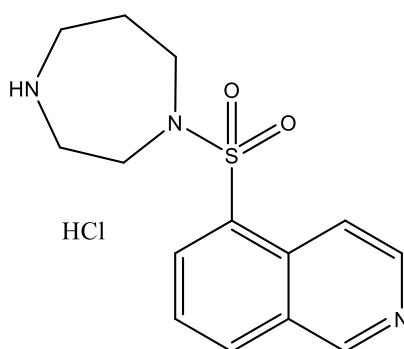


Figure 4.2 Structural formula of the ROCK inhibitor fasudil HCl.

Cytochalasin D was used as an actin filament binding drug, which inhibits their polymerization, so the filaments are decomposed over time (Casella *et al.*, 1981). A stock solution was prepared by dissolving cytochalasin D in DMSO at a concentration of 9.85 mM, which was diluted with serum-free medium to the standard concentration of 5 μ M, but lower concentrations (0.5 μ M and 0.05 μ M) were also tested. The cytochalasin D solutions were stored at -20 °C.

A stock solution of the connective tissue growth factor (CTGF) (see section 1.1.2) of 50 mg/L in PBS⁻ was thankfully received from Dr. Frömel / Prof. Dr. Fuchshofer (University of Regensburg, Germany). This stock solution was diluted with serum-free medium to obtain the standard concentration of 50 µg/L, but 100 µg/L was also used once. All the CTGF solutions were stored at -20 °C.

Bovine serum albumin (BSA), beside its role as the main constituent in FBS, was used as a blocker to prevent binding of proteins like CTGF to the plastic walls of cell culture vessels (Wedge & Svenneby, 1986). A BSA solution was prepared by dissolving BSA in serum-free medium at a concentration of 10 g/L. When CTGF was to be tested in the presence of BSA, the CTGF stock solution was diluted with this BSA solution instead of pure serum-free medium. For the half replacement method, the concentration of BSA is then halved to 5 g/L by the addition to the cells. The BSA solution was stored at 4 °C, but the CTGF-BSA solutions were stored at -20 °C.

In some experiments, the effect of fasudil was tested after the cells were pre-stimulated by CTGF. In order not to dilute the CTGF concentration in the wells by the addition of fasudil (half replacement), the fasudil solutions contained the same concentration of CTGF as it was present in the wells. These solutions were prepared by diluting the fasudil stock solution with serum-free medium and additionally adding CTGF at a concentration of 50 µg/L, which were stored at -20 °C until usage.

The AQP inhibitor hydrogen tetrachloroaurate trihydrate (HAuCl₄·3H₂O) (see section 1.3) was dissolved in the PBS⁺⁺/Glc buffer to yield a stock solution with a concentration of 1 mM. Isotonic, hypertonic, and hypotonic solutions containing HAuCl₄·3H₂O were prepared by adding this stock solution at a standard concentration of 50 µM. Both stock solution and test solutions were stored at 4 °C.

The Na⁺/K⁺/2Cl⁻ cotransport inhibitor bumetanide (see section 1.3) was dissolved in DMSO to yield a stock solution with a concentration of 40 mM. Isotonic, hypertonic, and hypotonic solutions containing bumetanide were prepared by adding this stock solution at a standard concentration of 10 µM. The stock solution was stored at -20 °C, whereas the test solutions containing bumetanide were stored at 4 °C.

The K⁺ channel inhibitor tetraethylammonium chloride (Et₄NCl) (see section 1.3) was dissolved in the PBS⁺⁺/Glc buffer to yield a stock solution with a concentration of 225 mM. Isotonic, hypertonic, and hypotonic solutions containing Et₄NCl were prepared by adding this stock solution at a standard concentration of 7.5 mM. Both stock solution and test solutions were stored at 4 °C.

Paraformaldehyde (PFA) is a well-established fixation agent (Biberfeld *et al.*, 1974) and was used during sample preparation prior to cytochemical fluorescence staining (see section 4.10.3). PFA was dissolved in PBS⁺⁺ at a concentration of 40 g/L, which took about

two weeks under constant stirring at RT due to the low water solubility. The PFA solution was stored at -20°C .

The detergent Triton X-100 (Miozzari *et al.*, 1978) was used as the positive control in the PrestoBlue[®] assay and for membrane permeabilization in the cytochemical fluorescence staining protocol. For both purposes, a Triton X-100 solution was diluted in PBS⁺⁺, while it was diluted to 0.5% (v/v) for the PrestoBlue[®] assay and to 0.2% (v/v) for the cytochemical fluorescence staining. Both Triton X-100 solutions were stored at 4°C .

TRITC-phalloidin is a conjugate of the fluorophore tetramethylrhodamine isothiocyanate (TRITC) and the cytotoxin phalloidin, which binds to actin filaments with high affinity, while TRITC makes them visible under fluorescence microscopes like the confocal laser scanning microscope (CLSM) (Bereiter-Hahn & Kajstura, 1988). Thus, it was used for the cytochemical fluorescence staining of actin filaments. A stock solution of TRITC-phalloidin was prepared by dissolving it in PBS⁺⁺ at a concentration of 0.3 g/L, which was further diluted with PBS⁺⁺ to the standard concentration of use of 6 mg/L. The stock solution was stored at -20°C , whereas the final solution was stored at 4°C , but both were stored protected from light.

4',6-Diamidino-2-phenylindole (DAPI) is a fluorescent label for DNA (Morikawa & Yanagida, 1981), so it was used to stain the nuclei of fixed cells for cytochemical fluorescence staining. A stock solution was obtained by dissolving DAPI 2HCl in PBS⁺⁺ at a concentration of 100 $\mu\text{g/L}$, which was further diluted with PBS⁺⁺ to the standard concentration of use of 10 $\mu\text{g/L}$. Both solutions were stored protected from light, the stock solution at -20°C and the final solution at 4°C .

Resazurin was used as a reagent to be converted in the PrestoBlue[®] assay (Lall *et al.*, 2013). Resazurin was dissolved in PBS⁺⁺ at a concentration of 100 mg/L, which served as a stock solution. This stock solution was diluted with PBS⁺⁺ to 10 mg/L before use, while both resazurin solutions were stored at 4°C .

An acrylamide (AAm) solution was used for the preparation of polyacrylamide (PAAm) gels and was purchased ready to use at a concentration of 400 g/L in distilled water, which was stored at 4°C . The concentrations of application and their preparation are given in section 4.3.2.

Crosslinking of PAAm gels was achieved by using a solution of *N,N'*-methylenebisacrylamide (BAAm), which was purchased ready to use at a concentration of 20 g/L in distilled water and stored at 4°C . The concentrations of application and their preparation are given in section 4.3.2.

Ammonium persulfate (APS) served as a radical initiator for the radical polymerization of PAAm gels. A solution of APS was prepared by dissolving it in dd-H₂O at a concentration of 150 g/L, which was aliquoted and stored at -20°C . One aliquot of the APS solution was used for maximal two weeks while storing at 4°C . The final concentration of application was 1.2 g/L (see section 4.3.2).

Tetramethylethylenediamine (TEMED) was used as a catalyst for the radical polymerization of PAAm gels and was purchased as a ready-to-use liquid ($\geq 99\%$ p.a.), which was stored at RT. TEMED was diluted in the final gel mixtures to 0.15% (v/v) (see section 4.3.2).

Sodium bisulfate (NaHSO_4) was used as a buffer in the gel mixtures, a solution of which was obtained by dissolving it in dd- H_2O at a concentration of 1.32 g/L, which was stored at 4 °C. The final concentration of application was 0.12 g/L (see section 4.3.2).

For the polymerization of polyethylene glycol (PEG) hydrogels, two macromonomers were needed, the tetrazine-functionalized 8armPEG with a molecular weight of 10 kDa (8armPEG10k-Tz) (see section 4.3.3) and the norbornene-functionalized 8armPEG with a molecular weight of 10 kDa (8armPEG10k-Nb) (see section 4.3.3), both kindly provided by Dr. Ziegler / Prof. Dr. Göpferich (University of Regensburg, Germany), who synthesized both compounds, as previously described (Ziegler *et al.*, 2021). Both macromonomers were received in crystalline form and stored at -20 °C protected from light. The preparation of aqueous solutions, their concentrations, and the polymerization reaction are described in section 4.3.3.

The adhesion promoter TI Prime (1-methoxy-2-propanol acetate) was used when quartz resonators were coated with photopolymer (see section 4.5.2), where a layer of the adhesion promoter facilitated a strong adhesion between quartz disk and photopolymer. TI Prime was purchased ready to use and stored at 4 °C.

The photopolymer AZ® ECI 3027, a mixture of ethyl (S)-2-hydroxypropanoate and *N*-butyl acetate, was used for photolithography (see section 4.5). It was purchased ready to use and stored at 4 °C protected from light.

A sodium hydroxide (NaOH) solution was used to remove ultraviolet-exposed photopolymer in the photolithographic processes (see section 4.5). This solution was prepared by dissolving NaOH in distilled water at a concentration of 7 g/L directly before use.

A solution of potassium iodide (KI) with iodine (I_2) (KI/I_2) was used to etch gold layers when new sensors were fabricated by photolithography (see section 4.5.1). This solution was prepared by dissolving KI in distilled water at a concentration of 82.9 g/L and adding I_2 at a concentration of 67.1 g/L. The KI/I_2 solution was stored at 4 °C protected from light.

A trypan blue solution was used for diffusion experiments of a colored molecule through the vitreous body and agarose gels (see section 4.2.5). For this purpose, a commercially purchased trypan blue solution was diluted with distilled water to a final concentration of 0.5 g/L, which was stored at RT.

A polyvinylpyrrolidone-iodine solution was used for disinfection of porcine eyes prior to vitreous body extraction (see section 4.2.3). It was purchased ready to use at a concentration of 75 g/L and stored at RT.

An Elma Clean 10 solution was used to clean quartz disks and contaminated pieces of the Teflon® QCM chambers. This solution was purchased, diluted to 5% (v/v) with distilled water, and stored at RT.

A mixture of sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) was used to clean quartz disks under very harsh conditions. For this, H_2SO_4 (95% – 97%) and H_2O_2 (30%) were mixed in a ratio of 3:1 directly before use.

4.2.2 Determination of Specific Conductivity and Osmolality

The specific conductivity γ (typically given in mS/cm), which is the reciprocal of the specific resistivity ρ (typically given in $\Omega \cdot \text{cm}$), is higher for solutions with higher concentrations of charged molecules. The specific conductivity decreases by the addition of non-conductive molecules, for example, sucrose. Thus, the hypotonic, isotonic, and hypertonic solutions prepared with different concentrations of sucrose for water flux experiments (preparation see section 4.2.1, description of experiments see section 4.6.3, results see section 8) differed in their specific conductivity, which might induce unspecific cell responses. To avoid that, all the solutions were adjusted to be iso-conductive in comparison to the corresponding isotonic solution (the solution called *iso* for hypotonic solutions, $\text{PBS}^{++}/\text{Glc}$ for hypertonic solutions, preparation see section 4.2.1) with the help of a 1.5 M NaCl solution (Table 4.6). The measurements were performed with an electrical conductivity meter inoLab Multi 720 at 25 °C, while small amounts of the NaCl solution were added stepwise until the specific conductivity reached a value close to the one of the corresponding isotonic solution. The specific conductivity of the $\text{PBS}^{++}/\text{Glc}$ solution was determined to about 16 mS/cm without addition of NaCl solution and the hypertonic solutions were adjusted to approximately that value, whereas the specific conductivities of the hypotonic solutions and the corresponding isotonic solution (*iso*) were adjusted to a value of about 4 mS/cm. The differences in the finally measured values were very small (Table 4.6), thus, no influence on the cells' behavior regarding the specific conductivity was expected.

The osmolality π is typically given in mosmol/kg (please note the difference to osmolarity in mosmol/L) and describes the molality (mol/kg) of a solution, while taking into account all dissolved molecules that contribute to the solution's osmotic pressure. When two solutions of different osmolalities are separated by a semipermeable membrane (like the plasma membrane of cells), solvent molecules (H_2O) will diffuse from the solution with lower osmolality through the membrane to the solution with higher osmolality until equilibrium of osmolalities is reached. This principle was utilized in the water flux experiments, where a high osmolality of the solution surrounding the cells triggers water to flow out of the cells, and vice versa for low osmolality. Osmolality differences between cytosol and extracellular fluid were obtained by using different concentrations of sucrose, as described in

section 4.2.1. The osmolalities of the hypotonic, isotonic, and hypertonic solutions were measured by a cryoscopic osmometer Osmomat 030 by freezing the solutions to a temperature of -7°C and determining the melting point. The osmolality of each solution was determined by measuring two individual samples and calculating the mean. Additional to the specific conductivity γ , Table 4.6 summarizes the osmolality π of the hypotonic, isotonic, and hypertonic solutions. It is also expressed as the osmolality difference $\Delta\pi$ to the corresponding isotonic solution, which is only correct for the full replacement method. For the half replacement method, the osmolalities had to be separately determined by 1:1-dilution with the respective isotonic solution, but where roughly half the values of the full replacement method.

Table 4.6 Summary of the specific conductivity γ (measured at 25°C) and osmolality π of hypotonic, isotonic, and hypertonic solutions used for water flux experiments. The solutions were prepared as described in section 4.2.1. The specific conductivities of the hypotonic solutions (hypo 1 and hypo 2) and the corresponding isotonic solution (iso) were adjusted to a value of about 4 mS/cm with a 1.5 M NaCl solution. For setting up the specific conductivity of the hypertonic solutions (named by the sucrose concentration), the specific conductivity of the corresponding isotonic solution ($\text{PBS}^{++}/\text{Glc}$) was measured, whereupon the hypertonic solutions' specific conductivities were adjusted to approximately the same value. The osmolality π was determined as the mean out of the measurements of two individual samples. It is also expressed as the difference $\Delta\pi$ to the corresponding isotonic solution, which is only correct for the full replacement method. For the half replacement method, the osmolalities had to be separately determined by 1:1-dilution with the respective isotonic solution. $\Delta\pi$ of the 800 mM sucrose solution for the half replacement method was not determined because this solution was only added by the full replacement method. Since all these solutions had to be prepared several times and the specific conductivity and osmolality were impossible to be adjusted to exactly the same values every time, these values given here are the mean values of all solutions prepared.

Solution	$\gamma / \text{mS/cm}$	$\pi / \text{mosmol/kg}$	$\Delta\pi / \text{mosmol/kg}$ (full replacement)	$\Delta\pi / \text{mosmol/kg}$ (half replacement)
hypo 1	3.98	95	-194	-103
hypo 2	3.88	159	-130	-66
iso	3.95	289	0	0
$\text{PBS}^{++}/\text{Glc}$	15.92	298	0	0
20 mM	15.86	322	+24	+16
100 mM	15.89	406	+108	+57
200 mM	15.93	544	+246	+116
300 mM	15.87	648	+350	+170
400 mM	15.89	782	+484	+233
800 mM	15.90	1400	+1102	-

After preparation and measurements these solutions were filtered sterile inside the laminar flow hood through Filtropur S filters, which allowed a long-time storage. Nevertheless, the solutions were freshly prepared about twice a year, and specific conductivity and osmolality

showed slight differences each time they were prepared. Thus, the values given in Table 4.6 are mean values of all solutions prepared. The two isotonic solutions exhibited values slightly beneath 300 mosmol/kg, and the osmolality was observed to be strongly dependent on the sucrose concentration with a higher sucrose concentration yielding a higher osmolality. For the water flux experiments, the inhibitors $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, bumetanide, and Et_4NCl were also investigated. These inhibitors were used in one hypotonic solution (hypo 1) and one hypertonic solution (200 mM sucrose) and the respective isotonic solutions (iso, $\text{PBS}^{++}/\text{Glc}$). To investigate a potential influence of the inhibitors on the osmolality of the solutions, the osmolalities of these solutions were measured with and without inhibitors and compared to each other, with the results demonstrated in section 8.1.3. The same was performed for 100 μM fasudil in serum-free HTM-N medium in comparison to pure serum-free HTM-N medium without fasudil, to investigate if water flux may contribute to the cell response to a fasudil treatment (result demonstrated in section 6.3).

4.2.3 Extraction of Vitreous Body

In the transwell experiments (description of experiments see section 4.8.2, results see section 7), vitreous body was needed to study the diffusion of fasudil in an in-vitro disease model of glaucoma. Mostly, the vitreous body of porcine eyes is used as a model for the human one, because the vitreous of both pigs and humans are, despite some differences, very similar regarding composition and mechanical properties (Weber *et al.*, 1982). Thus, for all the transwell experiments, the vitreous body was obtained from porcine eyes, thankfully received from the local slaughterhouse Freihart (Sinzing, Germany). After slaughter, the eyes were kept on ice and maximally 3 h later their dissection was started (Figure 4.3). First, remaining tissue directly attached to the eyes was removed by scissors, followed by disinfection in polyvinylpyrrolidone-iodine solution for a few minutes. Eyes were then cleaned in PBS^- for a few minutes (Figure 4.3 A), which led to clean porcine eyes ready for dissection, as demonstrated in Figure 4.3 B. Dissection of the eyes was achieved by a razor blade, which allowed to scrape out the vitreous body with a spatula (Figure 4.3 C). Care had to be taken in order not to scrape out the lens and parts of the retina. The yield of vitreous body from one eye was about 1 mL. In this manner, the vitreous body of one to two eyes was extracted, merged, and directly used for experiments. The vitreous body was only used on the day of extraction. Pipetting of vitreous body was possible by using cropped pipet tips (Figure 4.3 D). For QCM investigations on the vitreous body, it was directly added to quartz disks in a volume of 500 μL – 1000 μL . For transwell experiments, transwell filter inserts with a circular membrane made of PC with 6.5 mm diameter, a pore density of 10^8 pores per cm^2 and 0.4 μm pore diameter were used, where the vitreous body was added in a volume of 200 μL , which led to a height of the vitreous body in the insert of 6 mm (Figure 4.3 E).

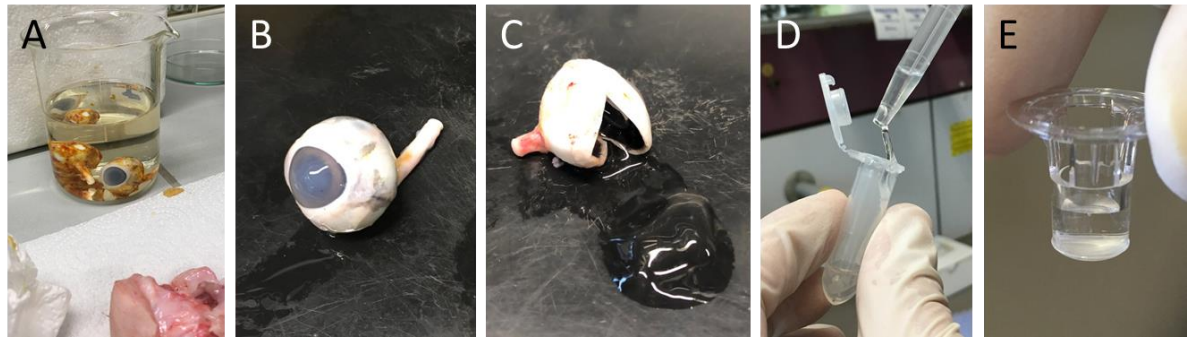


Figure 4.3 Images showing the porcine eye dissection and vitreous body extraction. **(A)** Cleaning of eyes in PBS[−] after removal of tissue and after disinfection in polyvinylpyrrolidone-iodine solution. **(B)** Porcine eye after cleaning and prior to dissection. **(C)** Porcine eye after dissection with a razor blade with the vitreous body scraped out. **(D)** Vitreous body in a micro test tube capable of being pipetted through a cropped pipet tip. **(E)** Vitreous body in a transwell filter insert.

4.2.4 Preparation of Agarose Gels

As described in the previous section (section 4.2.3), vitreous body was extracted from porcine eyes and used for the diffusion studies of fasudil in transwell experiments. It is an ongoing challenge in ophthalmology to find new and better substitutes for the human vitreous body, where, beside gases and liquids, especially hydrogels are in the focus of recent research (Mondelo-García *et al.*, 2021). Thus, agarose gels of different concentrations were tested in this thesis as substitutes for the vitreous body. Three different concentrations of agarose were used: 15 g/L (1.5% (w/v)), 10 g/L (1% (w/v)) and 5 g/L (0.5% (w/v)). To prepare the gels, agarose was dissolved in dd-H₂O in the intended concentration in a glass beaker and boiled up 3 – 4 times in a microwave oven OMW 210-S with mixing a couple of times in between. Agarose gels solidified by cooling to RT leading to hydrogels, which could not be pipetted anymore. Thus, the gels were pipetted in hot state to the transwell filter inserts (see Figure 4.3 E) in a volume of 200 µL before they cooled down to RT.

4.2.5 Diffusion Tests

In the transwell experiments (description of experiments see section 4.8.2, results see section 7), the diffusion of fasudil through porcine vitreous body and agarose gels was investigated. The basics of diffusion were discovered by Fick in 1855 (Fick, 1855). Diffusion results from random Brownian motion of particles and is, without external effects like convection and differences in temperature or pressure, only driven by the concentration gradient. The particles tend to move from highly concentrated regions to lower concentrated regions, to compensate for this concentration gradient, which will lead to a uniform distribution of the particles with constant concentration after an unlimited time of diffusion. Since the diffusion of fasudil is not visible by eye, in preliminary experiments, a

trypan blue solution (blue colored) was used instead of fasudil for visualization of the diffusion through the vitreous body and agarose gels. Although fasudil and trypan blue are not really similar in terms of molar mass and water solubility, these experiments helped roughly estimate the duration of diffusion. The vitreous body or agarose gels (1.5% (w/v), 1% (w/v) and 0.5% (w/v)) were added to transwell filter inserts (see Figure 4.3 E) in a volume of 200 μL (6 mm height). One single drop of the trypan blue solution (10 μL – 20 μL) was added on top and its diffusion through the vitreous body was observed for 1 h and through agarose gels for 3 h during incubation at 37 °C. A couple of images were taken in between to monitor the progress of diffusion over time. Additionally, a calculation of the duration of diffusion was performed. When the random walk model, a mathematical model to describe stochastic processes, is applied to diffusion, this gives in the three-dimensional case the following expression for the average duration of diffusion t (Berg, 1984):

$$t = \frac{s^2}{6D} \quad (72)$$

with the mean displacement s and the diffusion coefficient D , which depends on the diffusing molecule and the medium of diffusion. This model describes the random movement of particles without regarding concentration gradients. Diffusion coefficients can be calculated by the Stokes–Einstein equation:

$$D = \frac{k_B T}{6\pi\eta' r} \quad (73)$$

where k_B is Boltzmann's constant, T the temperature, η' the dynamic viscosity of the diffusion medium, and r the radius of the diffusing molecule. Using Eq. (73) for the calculation of the diffusion coefficient D of fasudil in the vitreous body is just an approximation because fasudil has to be considered as a spherical particle. But, since diffusion coefficients of fasudil in the vitreous body or similar media are unknown, this calculation is supposed to be a good approximation for this purpose. This calculation was performed in section 7.1 with previously published values for η' of the vitreous body and r of fasudil. With this, Eq. (72) can be used to calculate the average duration of diffusion t of fasudil in the vitreous body.

4.3 Preparation of Hydrogels

4.3.1 General Gel Polymerization Conditions

Two types of hydrogels were investigated in this thesis, polyacrylamide (PAAm) gels and polyethylene glycol (PEG) gels. Their preparation is described in section 4.3.2 (PAAm gels) and section 4.3.3 (PEG gels), respectively. QCM measurements (see section 4.6.1), compression testing (see section 4.4.1), and rheological measurements (see section 4.4.2) were performed with these hydrogels, and the results are described in section 5. Table 4.7

summarizes the experimental conditions along the workflow of polymerization and measurement, and the temperature during the three different measurement types applied to the two different hydrogel types.

Table 4.7 Summary of the experimental conditions in QCM measurements, compression testing, and rheological measurements with polyacrylamide (PAAm) and polyethylene glycol (PEG) hydrogels. Compression testing was not performed for PEG hydrogels. RT = room temperature.

Hydrogel type	QCM (section 4.6.1)	Compression testing (section 4.4.1)	Rheology (section 4.4.2)
PAAm (section 4.3.2)	online measurement of the polymerization, RT	polymerization before measurement, RT	online measurement of the polymerization, 37 °C
PEG (section 4.3.3)	online measurement of the polymerization, 37 °C	–	online measurement of the polymerization, 37 °C

Since compression testing on PAAm hydrogels was only possible at room temperature (RT) (see section 4.4.1), the QCM measurements on PAAm gels were also performed at RT. All remaining measurements were conducted at 37 °C. To investigate the influence of temperature and to ensure comparability, one QCM experiment on PAAm gels was performed for variable temperature (results see section 5.1.1). All QCM measurements and all rheological measurements were performed as time-resolved measurements in the respective device, thus, the hydrogel polymerization progress could be observed in real time by the measurement. For compression testing, which was only applied to PAAm, gel samples with completed polymerization had to be used. Thus, for this purpose, PAAm gels were pre-polymerized in specially designed Teflon® vessels (Figure 4.4) mimicking the QCM chambers (see Figure 4.14) in terms of size and stored in dd-H₂O until the measurement. Beside this purpose of use, these Teflon® vessels were also used for monitoring of HTM-N cell adhesion by phase contrast microscopy (description of experiments see section 4.10.1, results see section 6.1). The assembly of these Teflon® vessels is shown in Figure 4.4 and described in the following. A first rubber ring (Figure 4.4 B) was placed into the Teflon® base (Figure 4.4 A). A circular glass slide with 14 mm diameter served as the substrate for PAAm gel polymerization and was placed on top of the first rubber ring (Figure 4.4 C). A second rubber ring was added on top (Figure 4.4 D). Thus, the glass slide was sandwiched between two rubber rings in order to tightly close the gap between glass slide and cylindrical Teflon® frame placed on top (Figure 4.4 E) serving as the lateral limitation of polymerization. The vessel was sealed by a Teflon® plug (Figure 4.4 F).

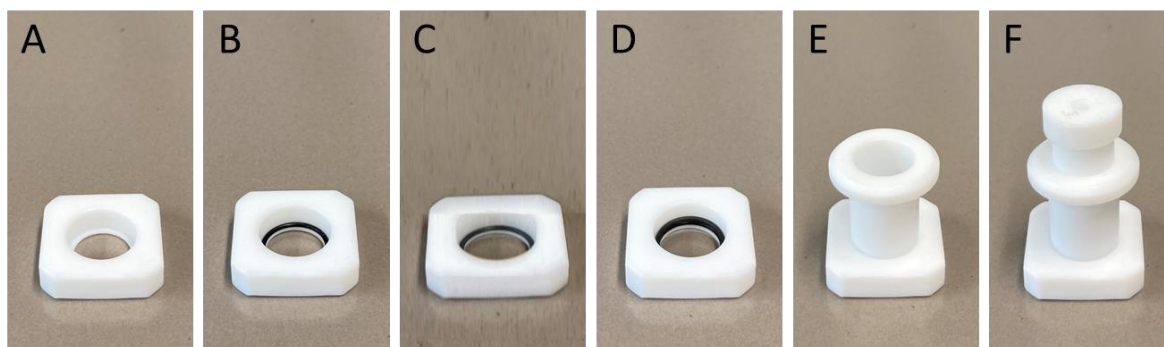


Figure 4.4 Images showing the stepwise assembly of the Teflon® chambers used for polymerization of PAAm hydrogels prior to compression testing and for phase contrast microscopic monitoring of HTM-N cell adhesion. Teflon® base (A), first rubber ring (B), glass slide with 14 mm diameter (C), second rubber ring (D), and cylindrical Teflon® frame (E) were assembled yielding a liquid container, which could be sealed by a Teflon® plug (F).

Five differently concentrated PAAm gels and two differently concentrated PEG gels were prepared. For obtaining good statistics with representative results, the measurements on the PAAm and PEG hydrogels were repeated several times. For the QCM measurements, two chambers could be recorded at the same time (see section 4.6.1), thus, one gel mixture batch was freshly prepared (see sections 4.3.2 and 4.3.3) and distributed into the two chambers. Data were not only averaged between two gels studied in parallel, but also between individual measurements on different days. Compression testing was performed for five samples of each PAAm gel composition (see section 4.4.1). Thus, after a gel mixture batch was prepared, it was distributed into five Teflon® vessels for parallel polymerization. After polymerization and storage in dd-H₂O until measurement, the five samples were successively measured by compression testing and averaged. Rheological measurements (see section 4.4.2) could only be performed for one gel at the same time, so, each gel sample had to be prepared separately. This was done five times for each of the different PAAm gels and three times for each of the two different PEG gels. The hydrogels polymerized in the QCM chambers and the Teflon® vessels (same inner dimensions) yielded cylindrical gels with a diameter of 12 mm. The height of the gels was adjusted by the volume of the gel mixture added. For the QCM measurements, this was a standard height of 2 mm (226 μ L), while the influence of the height (1 mm – 5 mm) was investigated in one measurement, and for compression testing, a gel height of 7 mm was used. In the rheological measurements, the dimensions of the device determined the dimensions of the gels (diameter of 25 mm, height of 0.5 mm), and accordingly the volume (245 μ L).

4.3.2 Polyacrylamide (PAAm) Hydrogels

Polyacrylamide (PAAm) hydrogels were prepared by radical polymerization out of the monomers acrylamide (AAm) and *N,N'*-methylenebisacrylamide (BAAm), while the latter

served as a crosslinker between linear AAm-AAm-chains to form a hydrogel network (Figure 4.5). The stiffness of the hydrogel network can be tuned by the concentrations of AAm and BAAm. The reaction was accelerated by the radical initiator ammonium persulfate (APS) and the catalyst tetramethylethylenediamine (TEMED). Different to conventional preparation methods of PAAm gels, a solution of sodium bisulfate (NaHSO_4) was added to the mixture additionally, which served as a buffer. Because the radical initiation by APS generated sulfate (SO_4^{2-}) ions, the mixture was buffered by the presence of bisulfate (HSO_4^-) ions.

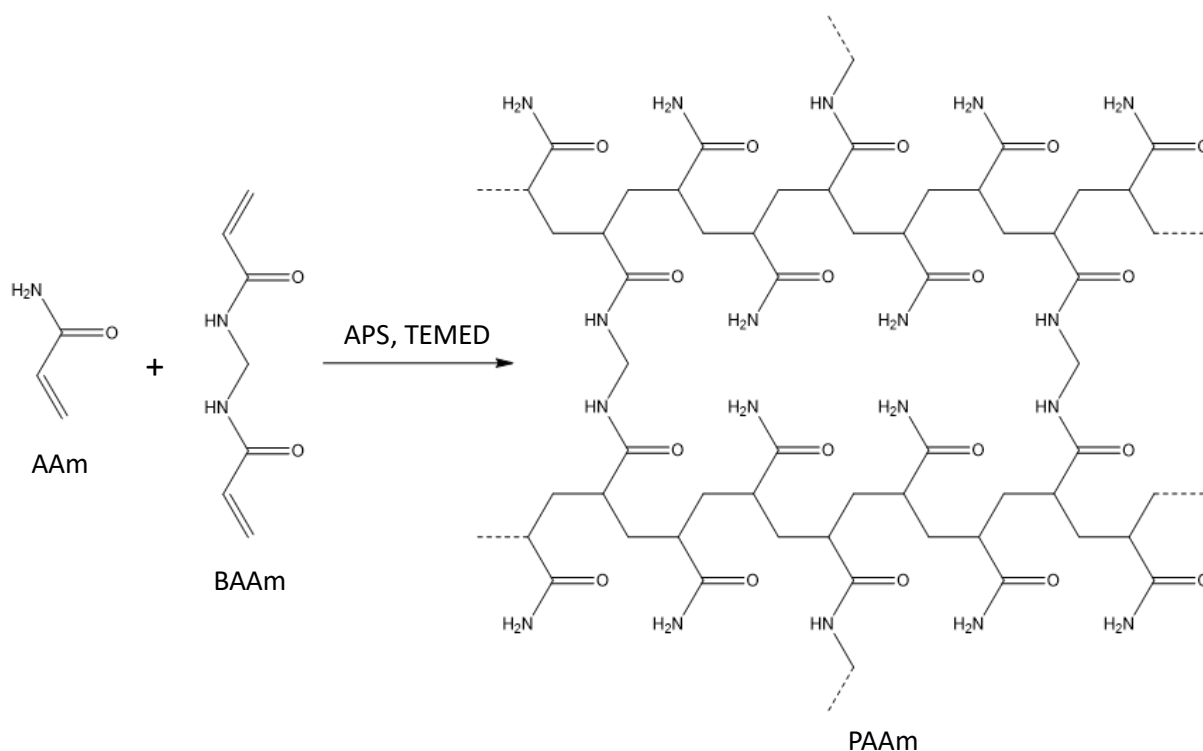


Figure 4.5 Chemical reaction during the preparation of polyacrylamide (PAAm) hydrogels by radical polymerization from the monomers acrylamide (AAm) and *N,N'*-methylenebisacrylamide (BAAm), which served as a crosslinker, with radical initiation by ammonium persulfate (APS) and catalysis by tetramethylethylenediamine (TEMED).

In detail, PAAm hydrogels were prepared by mixing a 400 g/L AAm solution, a 20 g/L BAAm solution, and dd- H_2O in different ratios to obtain five different PAAm hydrogels A – E of varying compositions (Table 4.8). Going from PAAm gel A to PAAm gel E, the concentrations of AAm and BAAm decreased and the ratio BAAm/AAm also decreased. Thus, PAAm gel A was expected to be the stiffest gel, whereas PAAm gel E was expected to be the softest gel. A 1.32 g/L NaHSO_4 solution was added to yield a final concentration of 0.12 g/L. A 150 g/L APS solution was added to yield a final concentration of 1.2 g/L. TEMED ($\geq 99\%$ p.a.) was added in a dilution of 0.15% (v/v). The final concentrations of NaHSO_4 , APS, and TEMED were the same for all PAAm gels. After all the components were added together and mixed at RT, the polymerization process immediately started, where the mixtures reached a solid state already after about 3 min, especially for PAAm gel A. Therefore, the mixture had to be

distributed into the respective vessels/device very quickly. QCM measurements in corresponding QCM chambers (see section 4.6.1), pre-polymerization in Teflon® vessels and subsequent compression testing (see section 4.4.1), and rheological monitoring of the polymerization (see section 4.4.2) were performed with these PAAm gels A – E.

Table 4.8 Preparation of polyacrylamide (PAAm) hydrogels. Different mass concentrations β of acrylamide (AAm) and *N,N'*-methylenebisacrylamide (BAAm) yielded PAAm hydrogels A – E of different compositions, while all of them contained the same amounts of 0.12 g/L NaHSO₄, 1.2 g/L APS, and 0.15% (v/v) TEMED. For better comparison to literature, the mass percentage is also given. Components were dissolved in dd-H₂O. In the order from PAAm gel A to E, the stiffness was expected to decrease.

PAAm gel type	β (AAm) / g/L	β (BAAm) / g/L
A	150 (15% (w/v))	10 (1% (w/v))
B	120 (12% (w/v))	2.4 (0.24% (w/v))
C	75 (7.5% (w/v))	1.2 (0.12% (w/v))
D	50 (5% (w/v))	0.6 (0.06% (w/v))
E	30 (3% (w/v))	0.3 (0.03% (w/v))

4.3.3 Polyethylene Glycol (PEG) Hydrogels

Polyethylene glycol (PEG) hydrogels were prepared according to a method previously described (Ziegler *et al.*, 2021), which is demonstrated in Figure 4.6. Hydrogels consisted of macromonomers with a hexaglycerol-based backbone and eight PEG side chains (Figure 4.6 A). The molecular weight of the macromonomers was adjusted by the number n of ethylene glycol units to about 10 kDa, thus, their basic structure is abbreviated by 8armPEG10k. One type of macromonomer was modified by functionalizing the PEG chain ends with tetrazine to yield a tetrazine-functionalized 8armPEG with a molecular weight of 10 kDa (8armPEG10k-Tz) (Figure 4.6 B) and another type of macromonomer was obtained by functionalizing the PEG chain ends with norbornene (8armPEG10k-Nb) (Figure 4.6 C). 8armPEG10k hydrogels (or short: PEG hydrogels) were prepared by crosslinking these two macromonomers via inverse electron demand Diels-Alder (iEDDA) reactions, while a hydrogel network is created by the plenty of possibilities the macromonomers can be combined (Figure 4.6 D). The iEDDA reaction occurred under release of N₂ spontaneously at 37 °C.

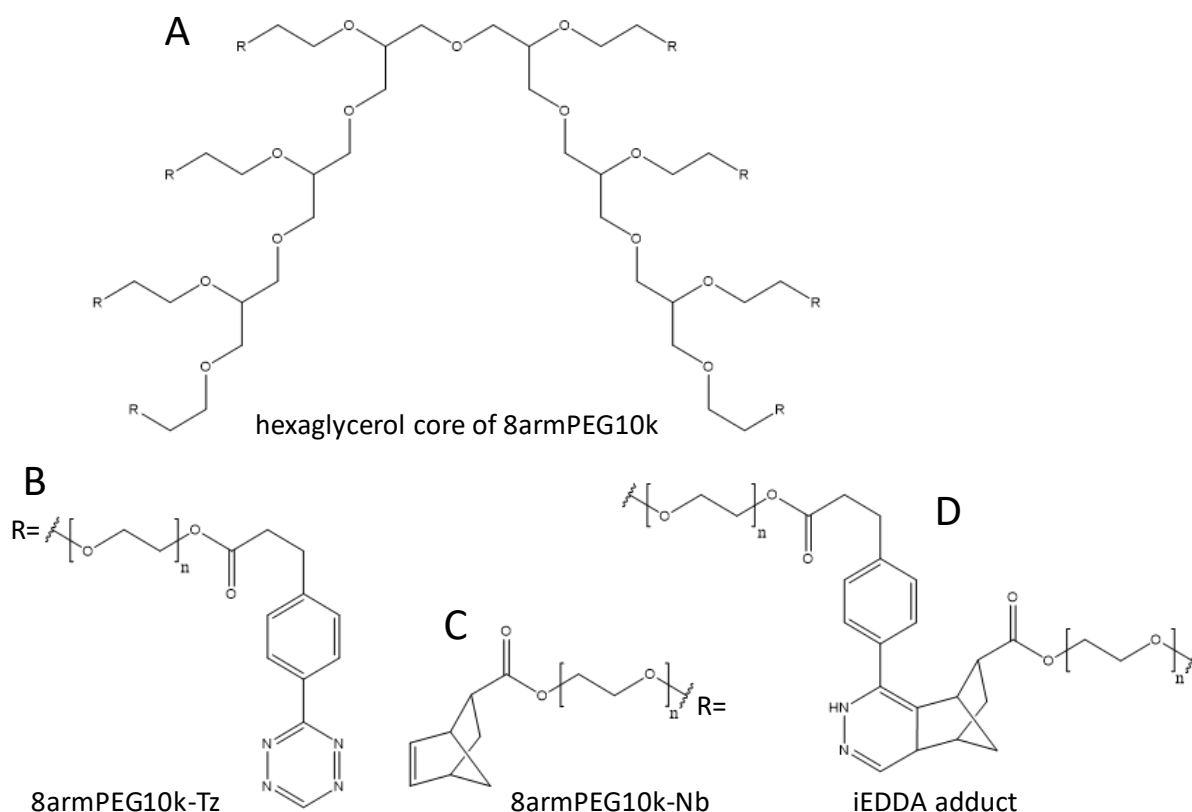


Figure 4.6 Preparation of polyethylene glycol (PEG) hydrogels (Ziegler *et al.*, 2021). **(A)** Hexaglycerol-based backbone of 8armPEG macromonomers with eight PEG side chains. The molecular weight was adjusted by the number n of ethylene glycol units to about 10 kDa. **(B)** PEG side chains were functionalized at the end with tetrazine (exactly: by esterification with 3-(4-(1,2,4,5-tetrazine-3-yl)phenyl)propanoic acid) to yield 8armPEG10k-Tz macromonomers. **(C)** PEG side chains were functionalized at the end with norbornene (exactly: by esterification with exo-5-norbornenecarboxylic acid) to yield 8armPEG10k-Nb macromonomers. **(D)** Hydrogel network creation by multiple formations of inverse electron demand Diels-Alder (iEDDA) adducts out of 8armPEG10k-Tz and 8armPEG10k-Nb macromonomers under release of N_2 .

For the hydrogel preparation, both macromonomers were separately dissolved in dd- H_2O (including centrifugation in a centrifuge 5415 R at 6100 g for 30 s) and mixed in a 1:1-ratio. Two different PEG hydrogels were prepared by dissolving the macromonomers in two different concentrations: PEG gel A with monomer concentrations of 50 g/L (5% (w/v)) and PEG gel B with monomer concentrations of 30 g/L (3% (w/v)). Accordingly, PEG gel A was expected to be stiffer than PEG gel B. After the two macromonomer solutions were added together and mixed at RT, the mixture was distributed into the respective vessels/device very quickly. QCM measurements in corresponding QCM chambers (see section 4.6.1) and rheological measurements (see section 4.4.2) were performed with these PEG gels A – B, while both types of measurements were conducted at 37 °C and under water vapor saturation.

4.4 Mechanical Characterization of Hydrogels

In this section, compression testing (section 4.4.1) and rheological measurements (section 4.4.2) used for mechanical characterization of the PAAm and PEG hydrogels are described.

4.4.1 Compression Testing

Compression testing of hydrogels was performed to obtain their Young's modulus E (see section 3.3.1), a parameter describing the stiffness of materials in their elastic deformation region. These measurements were conducted for PAAm gels A – E, but not for PEG gels, because PEG gels would not withstand a compression due to their foamy consistency. Compression testing could not be performed during the polymerization process, but only after hydrogel polymerization. Thus, the preparation of PAAm gels (see section 4.3.2) was conducted in Teflon® vessels (see Figure 4.4) prior to the measurements. From each of the five differently composed PAAm gels A – E, five samples were prepared. After 90 min of polymerization at RT, the gels were demounted from the Teflon® vessels and stored in Petri dishes filled with dd-H₂O at RT in order not to let them dry out, but maximal 1 h in order not to let them swell (examination of gel swelling see section 5.1.1). Compression testing was performed at RT using the material testing system Instron 5542 (Figure 4.7). The polymerization in the Teflon® vessels yielded cylindrical gels with a diameter of about 12 mm and the gel height was adjusted to about 7 mm by the volume of gel mixture added. The exact dimensions of the gel samples were measured with a caliper because they were crucial for the accurate determination of the Young's modulus. They were placed onto the lower plate of the compression testing system (Figure 4.7), while the PAAm gel E was directly poured out of the Teflon® vessel onto the lower plate after polymerization due to its very low stiffness. The movement of the upper plate occurred with a constant velocity of 1 mm/min compressing the hydrogel in the elastic deformation region (0% – 10% compressive strain). Thus, compression was applied by a given strain and the stress in the material was measured by the force counteracting against the movement of the upper plate. The resulting stress-strain curve enabled the determination of the Young's modulus by its slope. Each gel sample was measured once yielding a five-fold determination of the Young's modulus for each of the differently composed PAAm gels A – E. Statistical analysis regarding arithmetic mean μ and standard deviation σ was performed, where σ was calculated by:

$$\sigma = \sqrt{\frac{1}{x-1} \sum_{i=1}^x (a_i - \mu)^2} \quad (74)$$

with a_i as the single parameter values, which are the calculated values of the Young's modulus in this case, and x as the total number of replicates, which is 5 for these measurements.



Figure 4.7 Material testing system Instron 5542 used for the determination of the Young's modulus E of PAAm gels A – E by compression testing. Cylindrical hydrogels were placed onto the fixed lower plate after their polymerization and compressed by the movement of the upper plate.

4.4.2 Rheological Measurements

Rheological measurements on hydrogels were performed to obtain their complex shear modulus G^* (see section 3.3.2), a parameter reflecting viscoelastic properties. These measurements were conducted for PAAm gels A – E and PEG gels A – B in a rheometer Kinexus lab+ (Figure 4.8). Freshly prepared gel mixtures (see sections 4.3.2 and 4.3.3) were added to the lower plate of the device with the exception of PAAm gel E being pre-polymerized for 20 min – 30 min due to its very low stiffness. The dimensions of the hydrogels were adjusted to a diameter of 25 mm and a height of 0.5 mm by the upper plate, which applied the oscillatory stress with a distortion frequency of 1 Hz during gel polymerization. With this, the measurements were performed during polymerization and the complex shear modulus G^* was monitored over time, with a time resolution of 1 s – 5 s depending on gel stiffness. A temperature of 37 °C was ensured by heating the lower plate of the device and water vapor saturation was achieved by a solvent trap. In detail, an oscillatory stress was applied and the resulting phase-shifted strain was measured by the rheometer, yielding the magnitude of the complex shear modulus $|G^*|$, the storage modulus G' , the loss modulus G'' , and the phase shift φ . These measurements were conducted five times for each of the five different PAAm gels A – E and three times for each of the two different PEG gels A – B. Because baseline recording was not possible and the measurements were started by hand after addition of freshly prepared gel mixtures, each individual measurement exhibited a different start point of data recording during the polymerization

process of the gels. This led to the disadvantage that these measurements could not be normalized and averaged. But instead, plateau values reflecting the polymerized state of hydrogels were extracted from the respective end of measurement and statistical analysis was performed afterwards by means of arithmetic mean and standard deviation (see Eq. (74)).

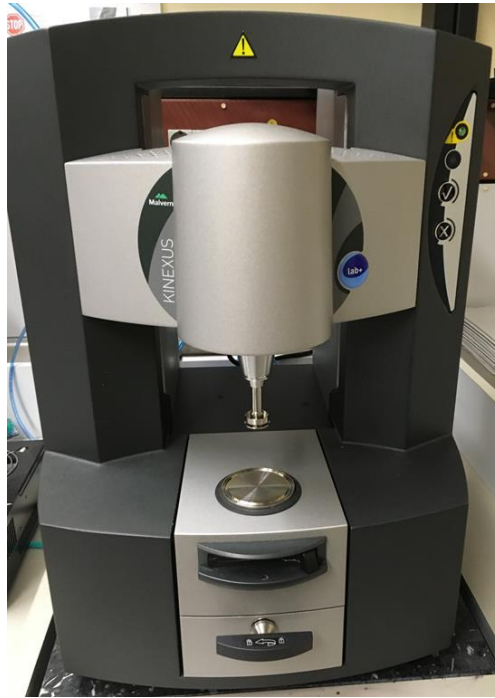


Figure 4.8 Rheometer Kinexus lab+ used for determination of the complex shear modulus G^* of PAAm gels A – E and PEG gels A – B. Freshly prepared hydrogel mixtures were added to the fixed and heatable lower plate. The rotatable upper plate defined hydrogel dimensions and applied an oscillatory stress during polymerization.

4.5 Sensor Fabrication

For most of the measurements applied in this thesis, sensors (quartz disks, ECIS arrays) were purchased from commercial sources. But for the transwell experiments (description of experiments see section 4.8.2, results see section 7), arrays were self-fabricated in an enlarged format (section 4.5.1) completed by an 8-well-corpus serving as a holder for the transwell filter inserts. Furthermore, for the QCM experiments with double-quartz-disks (description of experiments see section 4.7, results see section 8), commercially available quartz disks with a two-electrode-setup were processed by spin coating and photolithography (section 4.5.2).

4.5.1 Production of ECIS Arrays in Enlarged Format

The diffusion of fasudil through porcine vitreous body and agarose gels was investigated by means of transwell experiments, while the vitreous body or agarose gels were placed into transwell filter inserts, as already shown in Figure 4.3 E. The arrangement of eight parallelly investigated transwell filter inserts did not fit for the commercial ECIS arrays because the arrays were too small. Thus, arrays were self-fabricated in an enlarged format by photolithography (Figure 4.9). First, a Lexan® (a type of PC) plate was coated with a gold layer of 100 nm thickness, which was achieved by sputtering for 10 min in an Ar plasma generated in a sputter coater Balzers SCD 050 equipped with a gold sputtering target (Figure 4.9 A). After that, the gold-coated Lexan® plate was covered with the photopolymer AZ® ECI 3027 by spin coating at 2000 rpm for 1 min in a spin coater WS-650-23B yielding a uniformly distributed layer of the photopolymer (Figure 4.9 B), followed by soft-baking at 100 °C for 30 min in a drying cabinet Heraeus T6 for stabilization of the photopolymer (Figure 4.9 C). The photopolymer-coated substrate was illuminated by ultraviolet (UV) light for 2 min through a specific mask (see further down in Figure 4.10) in a UV exposure device BEL14002 in order to destabilize the photopolymer at intended spots (Figure 4.9 D). Subsequently, the UV-irradiated photopolymer was removed by treatment with NaOH solution for 20 s, followed by washing twice with distilled water and drying in nitrogen (N₂) stream (Figure 4.9 E). At these spots, where the photopolymer was removed, the gold layer was etched by flushing several times with a KI/I₂ solution, followed by washing twice with distilled water and drying in N₂ stream, generating the intended gold electrode layout on the Lexan® plate (Figure 4.9 F). Remaining photopolymer covering the gold electrode layout was removed by UV illumination for 2 min without a mask and subsequent NaOH treatment, washing, and drying (Figure 4.9 G). The Lexan® plate with the intended gold electrode layout was spin coated again with photopolymer at 2000 rpm for 1 min (Figure 4.9 H), followed by stabilization by soft-baking at 100 °C for 30 min (Figure 4.9 I). UV illumination for 2 min through a second mask (Figure 4.9 J) and subsequent NaOH treatment, washing, and drying (Figure 4.9 K) yielded a gold electrode layout on the Lexan® plate partially covered by photopolymer depending on the two masks (see further down in Figure 4.10) used during UV illumination. The gold electrode layout was determined by the mask used in the first UV illumination step (Figure 4.9 D) and the pattern of photopolymer coating was determined by the mask used in the last UV illumination step (Figure 4.9 J). The electrodes themselves and the pads for contacting with the measurement instrument were not covered by photopolymer, whereas the electrical supply lines were insulated by the photopolymer coating. The photolithographic fabrication of the sensor substrate was completed by hard-baking at 118 °C for 2 h in order to obtain long-time stability of the photopolymer coating (Figure 4.9 L).

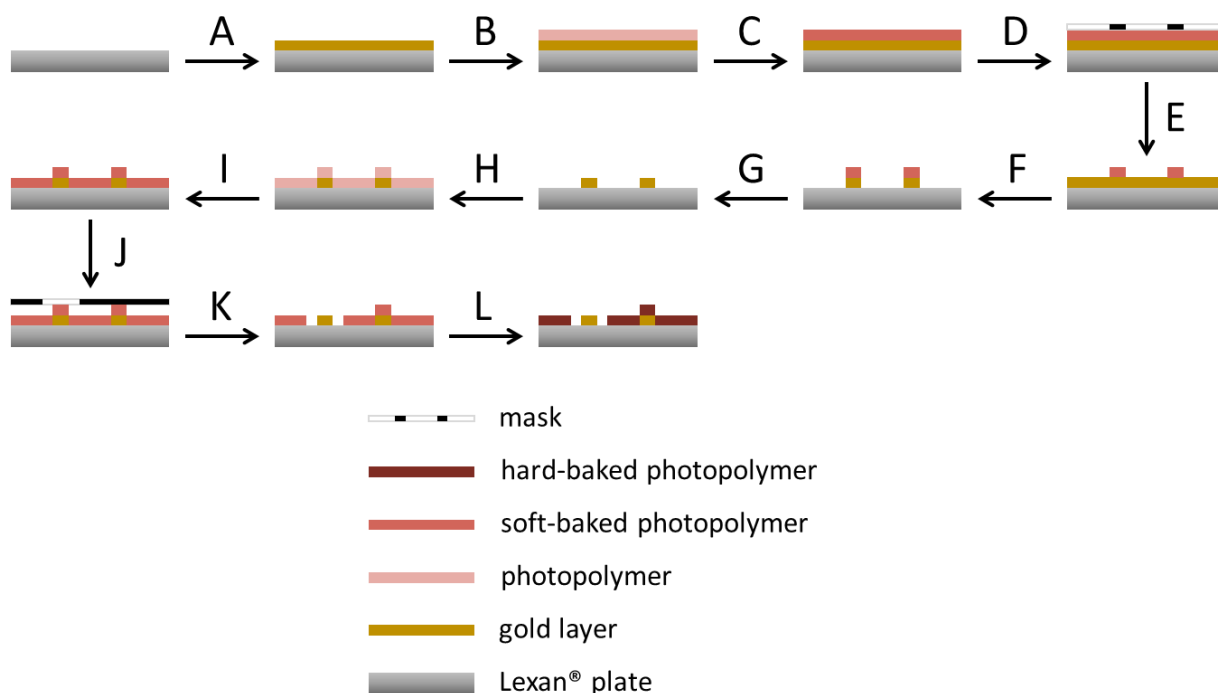


Figure 4.9 Schematic illustration of the photolithographic sensor fabrication for transwell experiments. **(A)** Sputter coating of a gold layer onto a Lexan® plate. **(B)** Spin coating with photopolymer. **(C)** Soft-baking at 100 °C. **(D)** Destabilization of photopolymer by UV illumination through a specific mask (see Figure 4.10). **(E)** Removal of destabilized photopolymer by NaOH. **(F)** Etching of gold by KI/I₂. **(G)** Destabilization of photopolymer by UV illumination without a mask and subsequent removal of destabilized photopolymer by NaOH. **(H)** Spin coating with photopolymer. **(I)** Soft-baking at 100 °C. **(J)** Destabilization of photopolymer by UV illumination through a second mask (see Figure 4.10). **(K)** Removal of destabilized photopolymer by NaOH. **(L)** Hard-baking at 118 °C, finally yielding naked gold spots serving as electrodes and contact pads, and photopolymer-covered gold spots serving as electrical supply lines insulated by the photopolymer. Drawings not to scale.

Three different electrode layouts were investigated in the transwell experiments, each generated by using different masks during UV illumination in the photolithographic fabrication process (Figure 4.10). These masks were drawn by the presentation program Microsoft PowerPoint and printed with black inkjet dye onto transparent inkjet films by an inkjet printer SureColor SC-P400. All three array types were characterized by one common CE in the middle of the array reaching into each well.

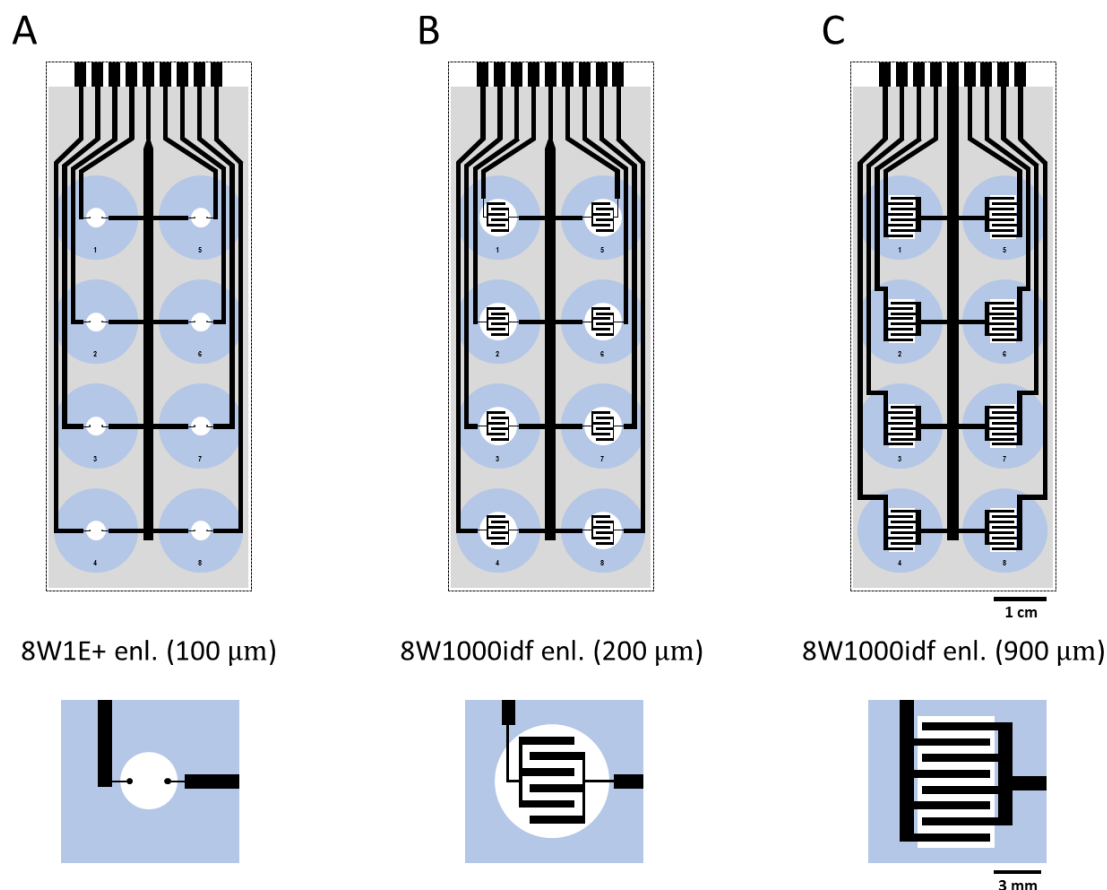


Figure 4.10 Drawings of the masks used for UV illumination in the photolithographic fabrication of the three different sensor layouts for transwell experiments with a closer look at the electrode structure of one well. Black areas correspond to the first mask used in the first UV illumination step (see Figure 4.9 D) defining the gold electrode structure. White areas define photopolymer-free regions, and blue areas define the wells' cell growth area. Grey and blue areas together correspond to the second mask used in the last UV illumination step (see Figure 4.9 J) defining the photopolymer-coated regions. **(A)** 8W1E+ enl. (100 μm) array: 8-well array with two circular electrodes (WE and CE) of 400 μm diameter in each well and electrical supply lines with 100 μm width adjacent to the electrodes and 900 μm width more distant to the electrodes. **(B)** 8W1000idf enl. (200 μm) array: 8-well array with inter-digitated finger configuration of WE and CE with three fingers each with 500 μm width and 500 μm distance (1000 μm pitch in total) in each well and electrical supply lines with 200 μm width adjacent to the electrodes and 900 μm width more distant to the electrodes. **(C)** 8W1000idf enl. (900 μm) array: 8-well array with inter-digitated finger configuration of WE and CE with four fingers each with 500 μm width and 500 μm distance (1000 μm pitch in total) in each well and electrical supply lines with 900 μm width all over the array.

The first transwell array type fabricated by photolithography was called 8W1E+ enl. (100 μm) array (Figure 4.10 A) according to the eight wells each possessing one circular WE and one circular CE with 400 μm diameter each. The abbreviation "enl." stands for "enlarged", according to a bigger size of these arrays compared to the commercial ECIS arrays. Since the three array types differed with respect to the width of the electrical supply lines adjacent to the electrodes, this dimension is given in brackets in the array name. This was for this array type 100 μm , whereas the width of the electrical supply lines more distant

to the electrodes was 900 μm for all the array types. Furthermore, two array types with an inter-digitated finger (idf) configuration of WE and CE in each well were fabricated and investigated. The 8W1000idf enl. (200 μm) array (Figure 4.10 B) showed three fingers for both WE and CE with 500 μm width and 500 μm distance. The pitch is defined as the sum of width and distance, which resulted in a pitch of 1000 μm in this case. The width of the electrical supply lines adjacent to the electrodes was 200 μm . This 8W1000idf enl. (200 μm) array was characterized by a bigger total electrode area, an electrode pattern more distributed over the well surface, and a bigger photopolymer-free region compared to the 8W1E+ enl. (100 μm) array. The third array type was the 8W1000idf enl. (900 μm) array (Figure 4.10 C), which exhibited four fingers for both WE and CE with 1000 μm pitch and 900 μm width of the electrical supply lines all over the array. In contrast to the 8W1000idf enl. (200 μm) array, the 8W1000idf enl. (900 μm) array was characterized by an even bigger electrode area, an electrode pattern even more distributed over the well surface, and a rectangular photopolymer-free region, leading to the situation that only the electrode fingers and no supply lines were uncoated. If these three electrode layouts are compared to commercial 8W1E or 8W10E ECIS arrays regarding their electrode layout and area, one important difference can be found. The three electrode layouts fabricated in this thesis do not possess a CE, which was manyfold bigger in area than the WE. Instead, both electrodes were equal in size, which led to the fact that both WE and CE can actually act as WE. Thus, the total electrode area was determined instead of the WE area, which was in each well $3.31 \cdot 10^{-3} \text{ cm}^2$ for the 8W1E+ enl. (100 μm) array, $1.26 \cdot 10^{-1} \text{ cm}^2$ for the 8W1000idf enl. (200 μm) array, and $1.84 \cdot 10^{-1} \text{ cm}^2$ for the 8W1000idf enl. (900 μm) array.

The fabrication of the transwell arrays was completed by gluing an 8-well-corpus shaped from Plexiglas® (a type of poly(methyl methacrylate)) to the respective sensor substrate fabricated by photolithography, which is shown for the 8W1000idf enl. (900 μm) array in Figure 4.11. This was achieved by sticking the 8-well-corpus to the sensor substrate (Figure 4.11 A) with silicone glue followed by drying for 2 d – 3 d in air, which yielded the final 8-well arrays with a well diameter of 1.6 cm and a well height of 1.9 cm (Figure 4.11 B and C). The wells served as liquid containers for the cell culture medium covering the cells growing over the electrodes, while the 8-well-corpus enabled it to place transwell filter inserts into the wells, as can be seen for a few wells in Figure 4.11 C. After testing for tightness with pure cell culture medium for about 1 d, the arrays were ready to use.

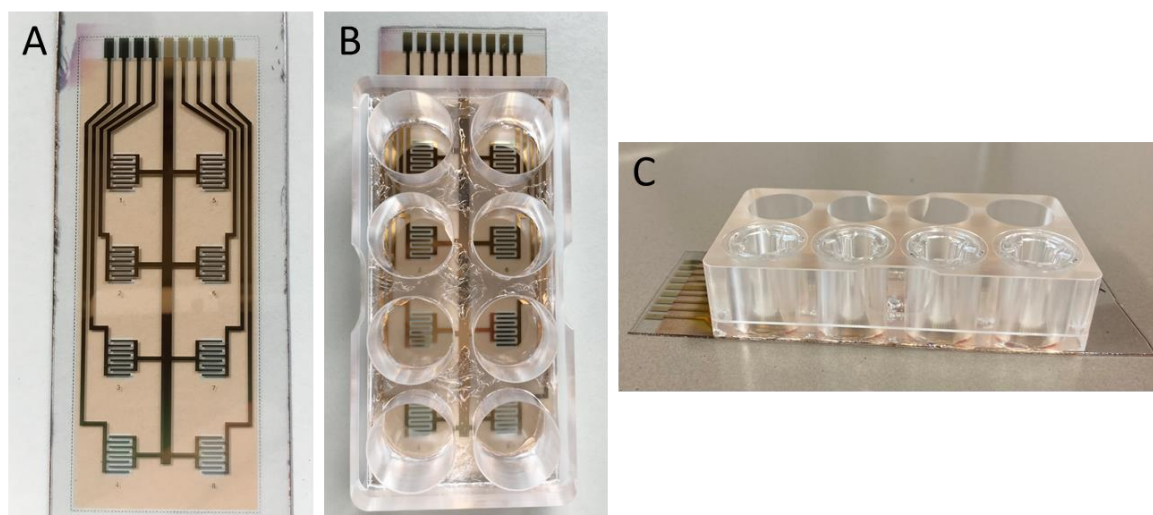


Figure 4.11 Images of the 8W1000idf enl. (900 μm) array. **(A)** Lexan® plate with gold electrode layout and photopolymer layer fabricated by photolithography (according to Figure 4.9), while using the mask from Figure 4.10 C. Electrode fingers and contact pads were uncoated. **(B)** Top view of the array completed by sticking an 8-well-corpus to the Lexan® plate with silicone glue yielding wells with a diameter of 1.6 cm (area of 2 cm²). **(C)** Side view of the array showing the height of the 8-well-corpus of 1.9 cm with transwell filter inserts placed into some wells.

4.5.2 Spin Coating and Photolithography on Quartz Disks

For QCM experiments performed with double-quartz-disks (description of experiments see section 4.7, results see section 8), resonators were purchased commercially with two pairs of electrodes. For the quartz disk side facing the liquid, one electrode had to be covered by photopolymer (Figure 4.12, conventional quartz disks with one pair of electrodes see Figure 4.13). The reason why this was done is explained in section 4.7. The half-sided photopolymer coating is generated by spin coating and photolithography, as already demonstrated for the transwell arrays in Figure 4.9. Before that, a commercial double-quartz-disk (Figure 4.12 A) was cleaned from contaminations by washing with acetone and isopropyl alcohol, followed by heating to 120 °C for 30 min in order to remove solvent residues. Prior to spin coating of the photopolymer, the adhesion promoter TI Prime was applied, which facilitated a strong adhesion between quartz disk and photopolymer by converting the primarily hydrophilic quartz surface into a more hydrophobic one. The adhesion promoter was spin coated onto the cleaned quartz disk at 3000 rpm for 20 s, which was thermally activated at 130 °C for 10 min afterwards. After that, the quartz disk was spin coated with photopolymer at 3000 rpm for 1 min, which was soft-baked at 100 °C for 30 min (Figure 4.12 B). UV illumination for 2 min through a mask covering half of the quartz disk and subsequent NaOH treatment, washing, and drying yielded a double-quartz-disk with one half of the top side covered by photopolymer, which was completed by hard-baking at 118 °C for 2 h for long-time stability (Figure 4.12 C).

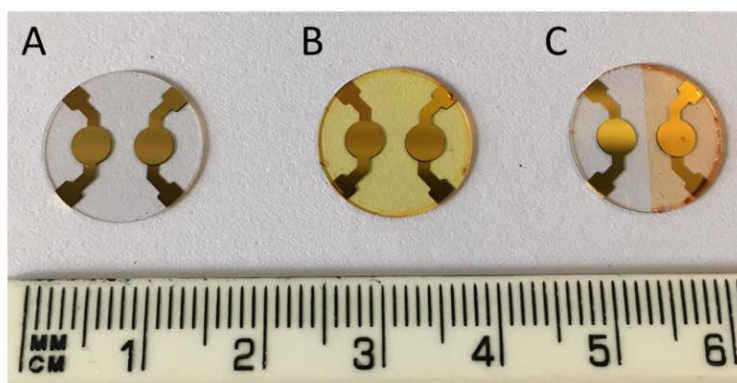


Figure 4.12 Image showing double-quartz-disks along their workflow including spin coating and photolithography. **(A)** Commercial double-quartz-disk. **(B)** Double-quartz-disk spin coated with adhesion promoter and spin coated with photopolymer and subsequently soft-baked at 100 °C. **(C)** Ready-to-use double-quartz-disk, which was illuminated by UV light through a mask covering half of the quartz disk, followed by NaOH treatment and hard-baking at 118 °C. Spin coating and photolithography steps were performed according to Figure 4.9.

4.6 Experimental Design of QCM Measurements

Viscoelastic monitoring of cells and hydrogels was performed by using the QCM. The basic principles were explained in section 3.2. In the following, general issues regarding quartz handling and mounting as well as settings and conditions of the QCM measurements (section 4.6.1), the most important parameters obtained from QCM measurements (section 4.6.2), some special aspects of the water flux experiments (section 4.6.3) and data processing and presentation (section 4.6.4) are described.

4.6.1 General Setup, Conditions, and Performance of QCM Measurements

In this thesis, all the QCM measurements were performed with 5 MHz AT-cut quartz disks with a resonance frequency f_0 of about 5 MHz resulting from their thickness d_q of 330 μm (Figure 4.13). They exhibited a diameter of 14 mm. With the exception of some measurements with double-quartz-disks (see section 4.7), they possessed one pair of circular gold film electrodes, one centered on each side with a diameter of 6 mm. Electrical supply lines led from the electrodes in the center to the disk edge, conveyed by a gold film around the disk edge to a contact pad on the other side. Thus, both electrodes could be electrically contacted from the same side, by contacting one electrode directly by the electrical supply line near the disk edge and the other electrode by the respective contact pad. Since the quartz disks were equal on both sides, the electrical connection to the measurement device arbitrarily could be performed from one side, while the other side was then used for cell growth or hydrogel polymerization. By repeatedly using the quartz disks, the gold film at the

disk edge was sometimes damaged, which was renewed by applying a drop of silver conductive paint followed by drying in N₂ stream.

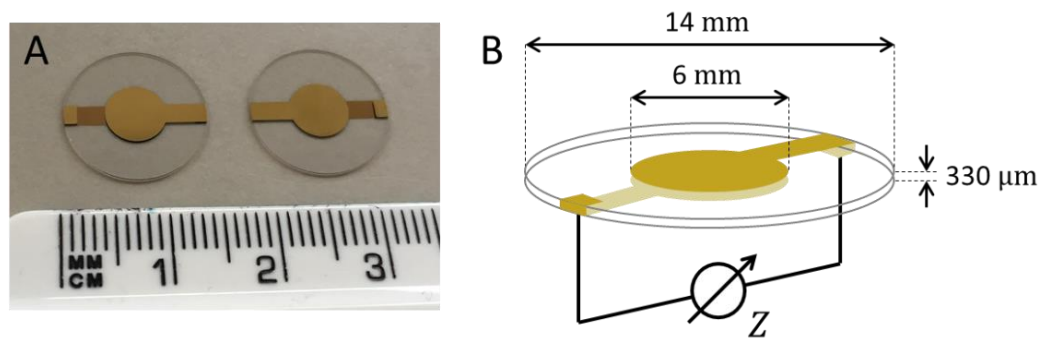


Figure 4.13 Image (A) and schematic illustration (B) of the 5 MHz AT-cut quartz disks used in this thesis with a diameter of 14 mm and a thickness of 330 μm and circular gold film electrodes on both sides with a diameter of 6 mm. Quartz disks were equal in appearance on both sides with electrical supply lines from the electrodes in the center to the disk edge conveyed by a gold film around the disk edge to a contact pad on the other side. This enabled electrical contacting of both electrodes from the same side, as it is demonstrated for the lower side in (B), and cell growth or hydrogel polymerization on the top side, which would also be possible vice versa. Both electrodes were connected to the measurement device and the impedance Z was measured.

The quartz disks had to be fixed for a constant and stable oscillation, and a liquid reservoir on top of the quartz disks was necessary for the cell culture medium or the gel polymerization mixture. Both requirements were met by mounting the quartz disks into Teflon® QCM chambers, which are shown in Figure 4.14. Figure 4.14 B demonstrates the main components of a QCM chamber with incorporated quartz disk. The cylindrical Teflon® frame above the quartz disk served as the lateral limitation of the liquid reservoir with a diameter of 12 mm (area of 1.13 cm²). The Teflon® contact piece beneath the quartz disk served for contacting both electrodes from the lower side with its two included gold pins. The quartz disk was sandwiched between two rubber rings and each Teflon® component for tensionless fixation of the quartz disk on the one hand, and tight sealing of the liquid reservoir on the other hand. The stepwise mounting of a quartz disk into a Teflon® QCM chamber is demonstrated in Figure 4.14 A, where the whole QCM chamber is upside-down in the images A1 – A4. A flexible metal spring was placed into a Teflon® cabinet to prevent shattering of the quartz disk (Figure 4.14 A1). The cylindrical Teflon® frame was inserted into the cabinet and a rubber ring was placed on top (Figure 4.14 A2). The quartz disk was placed in the middle of the cylindrical Teflon® frame on top of the rubber ring (Figure 4.14 A3). Fixation of the quartz disk was achieved by the Teflon® contact piece, which was placed on top and fixed to the cabinet by the two screws, while the two gold pins were aligned to contact both electrodes (Figure 4.14 A4). The chamber was inverted, yielding a liquid reservoir on top of the quartz disk generated by the cylindrical Teflon® frame, which was sealed by a Teflon® plug with two small holes dipping into the liquid (Figure 4.14 A5). This was done to minimize the area of the liquid-air interface, which might reflect longitudinal waves with potentially disturbing effect (Lin & Ward, 1995), and to minimize evaporation of

liquid from the reservoir ensuring a relatively constant liquid height. Air exchange between the liquid reservoir and the surrounding atmosphere was enabled by the small holes in the plug. Furthermore, the plug ensured sterility to a certain extent, which was enhanced by placing a Petri dish on top. The QCM chamber was placed on a socket, which was connected to the measurement device.

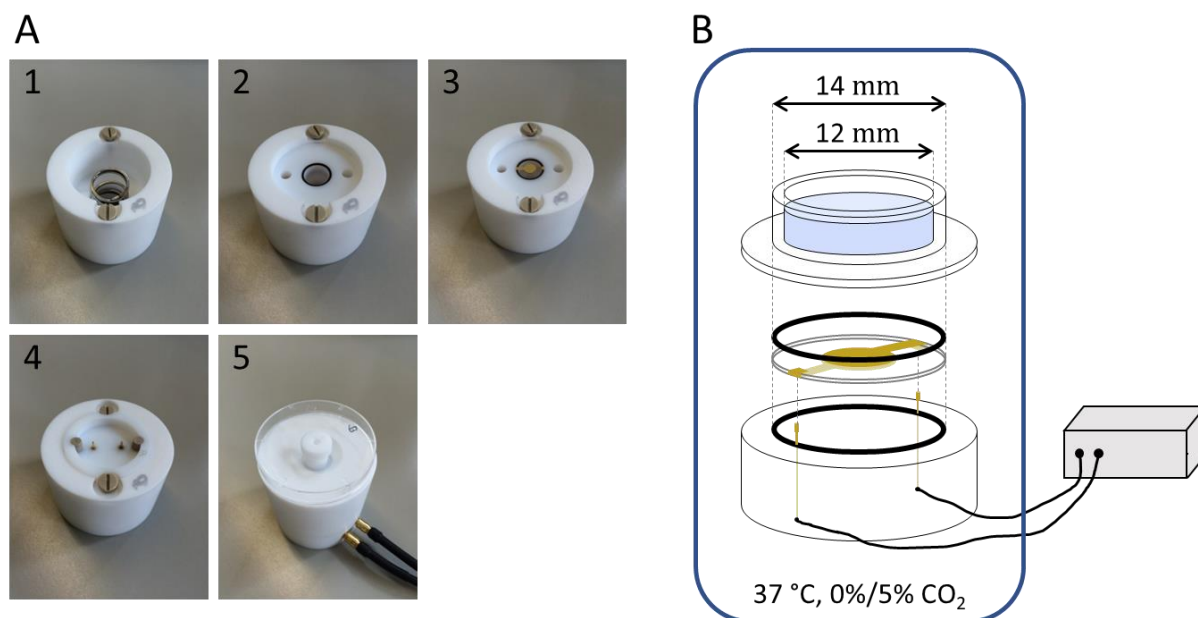


Figure 4.14 Experimental setup of QCM measurements. **(A)** Images showing the stepwise mounting of a quartz disk into a Teflon® QCM chamber. Into a cabinet with a spring **(A1)**, a cylindrical frame and a rubber ring were inserted **(A2)**. The quartz disk was placed on the rubber ring **(A3)**, which was subsequently fixed by the contact piece with two gold pins simultaneously enabling electrical contacting of both electrodes from the same side **(A4)**. The QCM chamber was inverted, generating a liquid reservoir on top of the quartz disk, which was sealed by a Teflon® plug and a Petri dish, and placed on a socket, which was connected to the measurement device **(A5)**. **(B)** Schematic illustration of the fixation of a quartz disk in a Teflon® QCM chamber and electrical connection to the measurement device. The liquid reservoir on top of the quartz disk with a diameter of 12 mm was generated by the cylindrical frame. The QCM chamber was placed in an incubator New Brunswick Galaxy 48 S at 37 °C with water vapor saturation and adaptable CO₂ content. Drawings not to scale.

The QCM measurement device consisted of an impedance analyzer 1260A, a computer, and a relay. The impedance analyzer applied a sinusoidal driving voltage with a small amplitude of 150 mV (rms) in a frequency range of 4.97 MHz – 5.04 MHz to the electrodes and measured the resulting current, thus, the magnitude of impedance $|Z|$ and the phase shift φ between voltage and current were determined in a frequency-dependent manner (spectra). These settings were controlled by the computer with a LabVIEW-based QCM measurement software to record impedance and phase spectra over time. Simulated impedance and phase spectra were already demonstrated in Figure 3.6, and typically measured impedance spectra are shown further down in Figure 4.15. The relay served for switching between two QCM chambers, which were measured alternately. Thus, the first QCM chamber was analyzed,

starting with a frequency of 5.04 MHz and measuring impedance and phase spectrum simultaneously with 150 equally spaced frequencies. When the frequency reached a value of 4.97 MHz, the relay automatically switched to the second QCM chamber, which was then measured in the same frequency range. This was repeated successively several times and the minimal impedance $|Z|_{min}$ was extracted from the spectra by the measurement software during measurement, enabling time-resolved recording of the minimal impedance. The parallel measurement of two chambers resulted in a time resolution of 2.24 min. There is the possibility to reduce time resolution down to a few seconds by reducing the frequency range measured around the minimal impedance, but with the restriction that no further parameters beside the minimal impedance can be extracted then. This was performed in the so-called fast-mode (in contrast to the conventional full-spectrum-mode), which was only applied for the water flux experiments (description of experiments see section 4.6.3, results see section 8) because a faster time resolution was needed there to follow the relatively fast response of the cells to osmolality changes. A frequency range covering 6.3 kHz centered around the frequency of minimal impedance with 14 equally spaced frequencies was applied there, resulting in a time resolution of 16.5 s. For all the other experiments followed by QCM measurements, the time resolution of 2.24 min was good enough with the advantage that multiple parameters could be extracted from the measurements afterwards (see section 4.6.2). In the results section, information on the mode of measurement is only given when the fast-mode was applied. No information is given, when the conventional full-spectrum-mode was applied.

Before starting the actual measurement, the proper oscillation of the quartz disk was checked by measurements in air and under liquid loading, where at least one full spectrum was measured each. Furthermore, the measurement under liquid loading served for calculating the contribution of the liquid to the load impedance $\underline{Z}_{Load}$ by a LabVIEW-based QCM fitting software (see section 4.6.2). For this measurement under liquid loading, the volume of the liquid was adapted to the volume used in the actual measurement. This was 1 mL of culture medium for measurements on cells and 226 μ L of dd-H₂O for measurements on hydrogels, except for one measurement for investigation of the influence of the gel height, where the respective intended gel height (1 mm – 5 mm) defined the volume (113 μ L – 565 μ L). Measurements on hydrogels were performed by measuring a short baseline (about 15 min) with dd-H₂O, followed by completely exchanging the dd-H₂O with the freshly prepared gel solution (PAAm or PEG) (full replacement method). The polymerization occurred on the quartz disks, while recording the change in minimal impedance in real time, reflecting the progress of polymerization. As already described in section 4.3.1, QCM measurements on PAAm hydrogels were conducted at RT (outside of incubator) and QCM measurements on PEG hydrogels were performed in an incubator New Brunswick Galaxy 48 S at 37 °C, with 0% CO₂ and water vapor saturation. In one measurement for investigation of the influence of temperature on the PAAm gels, the QCM chambers were relocated from the outside of the incubator (RT) to the inside (37 °C) during the measurement. As already explained above in section 4.1.4, QCM measurements on cells

were conducted with confluent cell layers or during cell adhesion. Confluent cell layers were established after two days of incubation in the incubator Heraeus BB 15 at 37 °C and 5% CO₂ after seeding, with feeding of the cells on the day in between, where the medium (1 mL) is exchanged with serum-free medium (1 mL). The measurements were performed in the incubator New Brunswick Galaxy 48 S at 37 °C, with 5% CO₂ and water vapor saturation. After a stable baseline was obtained with serum-free medium covering the cells (minimum 2 h), the test solution was added by the half replacement method, where 500 µL of the serum-free medium was removed and replaced by 500 µL of the double-concentrated test solution. The cells' reaction to the test solution was observed for at least 24 h. Water flux experiments were performed a bit differently, which is described below (see section 4.6.3). For monitoring cell adhesion, the QCM chambers with freshly seeded cells were connected to the measurement device directly after passaging (including the 10 min storage in the laminar flow hood for settling down of cells), and the cells' adhesion to the quartz disks was followed for about 60 h in the incubator New Brunswick Galaxy 48 S at 37 °C, with 5% CO₂ and water vapor saturation. Because the QCM measurements took at least 24 h, the number of experimental replicates, where cells of the same passage number were used, was limited to two (two measurement chambers in parallel). In one experiment, vitreous body was characterized regarding its viscoelastic properties (section 7.1). For this, vitreous body was added to the quartz disks in a volume of 500 µL – 1000 µL and the minimal impedance was recorded for a few minutes at 37 °C.

Since quartz disks were used several times, they had to be cleaned thoroughly after usage to re-establish pure sensor surfaces with constant oscillation properties. Quartz cleaning after measurements on hydrogels was achieved by 70% (v/v) EtOH and after measurements on cells by washing with PBS⁻ and subsequent trypsinization (2.5 g/L trypsin for all cell types) under incubation at 37 °C for 10 min to completely detach the cells from the quartz surface, followed by two further washing steps with PBS⁻. After trypsinization, quartz disks and those pieces of the Teflon[®] QCM chambers that were possibly in contact with cells (cylindrical Teflon[®] frame, upper rubber ring, and Teflon[®] plug) were cleaned by ultrasonic treatment in an ultrasonic bath Sonorex Digitec DT 31 H under incubation with 5% (v/v) Elma Clean 10 at 75 °C for 15 min. This ultrasonic treatment was repeated twice with distilled water, followed by drying in N₂ stream. Drying was completed in a drying cabinet Memmert BE400 at 37 °C for 1 h or in air over night. Before a new cell type was used on the quartz disks, they were additionally cleaned under very harsh conditions by incubating in a 3:1-mixture of H₂SO₄ and H₂O₂ for 5 min. Re-establishment of sterility was achieved by treatment with Ar plasma for 1 min before seeding a new cell suspension.

4.6.2 Description of Measurement Parameters

As already explained in section 3.1, the impedance is a complex quantity. The magnitude of impedance $|Z|$ and the phase shift φ between voltage and current are the output

parameters of the measurements. For the sake of convenience, the magnitude of impedance is just called impedance, for example, the parameter $|Z|_{min}$ is called minimal impedance. Typical impedance spectra, as they were recorded during measurements of 5 MHz AT-cut quartz resonators, are demonstrated in Figure 4.15. As already mentioned in the previous section (section 4.6.1), they were measured in the frequency range from 4.97 MHz – 5.04 MHz at 150 discrete frequencies, but here they are shown for a smaller region of 4.98 MHz – 5.01 MHz showing the resonance frequency region with a minimum at about 4.99 MHz and a maximum at about 5.00 MHz. As already discussed in sections 3.2 and 3.3, the QCM is sensitive to viscoelasticity and stiffness, which can also be observed here. Upon increasing the viscoelasticity of the quartz loading in the order from an almost undamped oscillation in air (Figure 4.15, black curve), over a liquid loading (Figure 4.15, red curve) up to an adherent cell layer (Figure 4.15, green curve), the minimum is shifted towards lower frequencies, which was already observed in Figure 3.6 for simulated spectra. At the same time, the minimal impedance $|Z|_{min}$ increases. So, this parameter correlates with the extent of the viscoelastic loading. Furthermore, an increase in cell stiffness, which is closely related to viscoelasticity, would lead to a further increase in the minimal impedance.

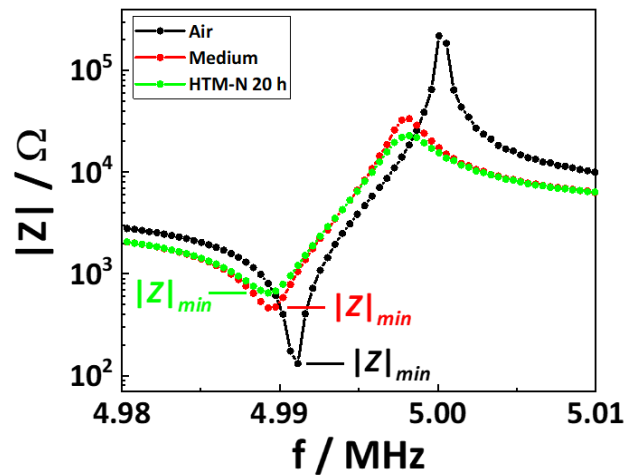


Figure 4.15 Impedance spectra of typical QCM measurements of the oscillation of a 5 MHz AT-cut quartz resonator in air (black), under loading with cell culture medium (red), and loading with HTM-N cells after 20 h of adhesion surrounded by medium (green) with the respective minimal impedance $|Z|_{min}$ highlighted.

During the measurements, the minimal impedance was extracted from the recorded impedance spectra and monitored in real time, reflecting the progress of gel polymerization or the cell response to a certain stimulus. Later, after a measurement has been terminated, the minimal impedance was determined much more precisely with the help of a spline function consisting of 8000 data points fitted to the impedance spectra by a LabVIEW-based QCM fitting software. Furthermore, this fitting software enabled the calculation of many more parameters from the spectra by modeling the spectra according to the BVD equivalent circuit model (see Figure 3.7). Therein R_q , L_q , and C_0 were fitted, while C_q was fixed to

$2.1655 \cdot 10^{-14}$ F representing constant material properties of quartz. Those parameters obtained by the fitting software and used for evaluation of experiments are described in the following.

The load impedance $\underline{Z}_{Load}$ was already introduced before by Eq. (46) and describes the impedance contribution of the quartz loading to the total impedance. Here, its magnitude $|Z|_{Load}$ was obtained by the fitting software. Furthermore, this software was able to calculate the difference to the surrounding liquid loading by additionally using one spectrum of the quartz disk just loaded with liquid, which was always measured in advance. Thus, the fitting software enabled the calculation of the impedance contribution originating just from the load additionally to the liquid. This parameter is denoted as $\Delta|Z|_{Load}$ and is also just called load impedance. For the example of cells surrounded by medium, this parameter describes the impedance contribution of the cell layer and is zero before cell adhesion. For the example of hydrogel polymerization, the load impedance $\Delta|Z|_{Load}$ is zero before polymerization start and increases in the progress of polymerization. Although the load impedance $\Delta|Z|_{Load}$ of a pure liquid loading is theoretically zero, this was sometimes not the case due to signal fluctuations, thus, normalization to a certain time point was performed as for every other parameter (as will be explained in section 4.6.4) leading to the change of the load impedance $\Delta\Delta|Z|_{Load}$. The load impedance $\Delta|Z|_{Load}$ is, similar to the minimal impedance $|Z|_{min}$, correlated with changes of viscoelasticity and stiffness. As also demonstrated by Eq. (46), the load impedance can be separated into its real part $Re(\underline{Z}_{Load})$ and imaginary part $Im(\underline{Z}_{Load})$. The real part represents energy dissipation, whereas the imaginary part represents energy storage. Similar to the load impedance $\Delta|Z|_{Load}$ calculated by the fitting software as the difference to the liquid loading, this was also done for its real and imaginary parts, but by hand. The respective values for subtraction were extracted from the spectrum of the quartz disk just loaded with liquid. Same as for the load impedance, these two parameters were normalized, although they should be zero for a pure liquid loading.

The conductance G was introduced in Eq. (36) as the real part of the admittance $\underline{Y}$. Figure 4.16 displays simulated conductance spectra, while the equivalent circuit elements were taken from fitting real measurements of a 5 MHz quartz resonator loaded with water and a PAAm hydrogel, respectively. The conductance spectra show a peak at resonance with maximal conductance G_{max} . This peak can be characterized by the frequency of maximal conductance $f(G_{max})$ and the half-width-half-maximum of maximal conductance Γ . Upon a change from water (Figure 4.16, red curve) to PAAm gel (Figure 4.16, green curve) the frequency of maximal conductance $f(G_{max})$ is slightly shifted towards lower frequencies, which is due to an increase in mass, and the peak is broadened due to an increase in viscoelasticity, mirrored in an increase in its half-width-half-maximum Γ .

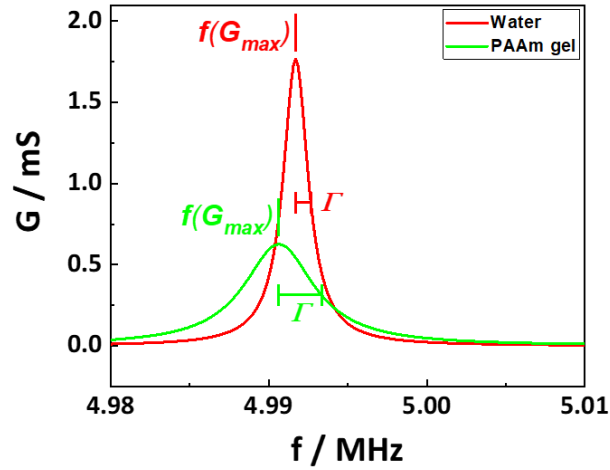


Figure 4.16 Simulated conductance spectra for the oscillation of a 5 MHz AT-cut quartz resonator under loading with water (red) and under loading with a PAAm hydrogel (green). The conductance displays a peak at resonance, the maximal conductance G_{max} , with its frequency $f(G_{max})$ and half-width-half-maximum Γ .

Shortly summarized, the parameters minimal impedance $|Z|_{min}$, load impedance $\Delta|Z|_{Load}$, and half-width-half-maximum of maximal conductance Γ are expected to increase, when viscoelasticity or stiffness is increasing, and the frequency of maximal conductance $f(G_{max})$ is expected to decrease upon mass deposition.

Beside these QCM parameters described above, there are many further parameters with similar interpretations, which can be obtained from QCM readings and used for evaluation of experiments, but they have not been investigated in this thesis. Examples are the maximal phase shift φ_{max} , the two frequencies of zero phase, the frequency of minimal impedance $f_{|Z|_{min}}$, the maximal impedance $|Z|_{max}$ and its frequency $f_{|Z|_{max}}$, and the dissipation factor D (which is defined as $\frac{2\Gamma}{f(G_{max})}$).

4.6.3 Transmembrane Water Flux Experiments

The possibility to monitor osmotically triggered water flux into or out of cells by the QCM was first discovered in 2000 (Wegener *et al.*, 2000c), which was from then on successfully applied several times (Zhou *et al.*, 2015; Chen *et al.*, 2021). Thus, the QCM as a rather established technique in this application field was used in this thesis as a screening tool for water flux experiments. Water flux experiments (results see section 8) were performed with MDCK-I cells, MDCK-I-AQP1 cells, MDCK-II cells, and HTM-N cells, while the presence of AQP1 in cells recombinantly expressing (MDCK-I-AQP1) and naturally expressing this protein (HTM-N) (Liton *et al.*, 2006) was expected to enhance water flux. The water flux was triggered osmotically, while investigating both hypertonic and hypotonic conditions (by

different amounts of sucrose, see sections 4.2.1 and 4.2.2). In contrast to a transepithelial water flux, which means a water flux across the complete cell layer including both basal and apical membranes, typically obtained in transwell filter-based experiments, here, a transmembrane water flux across the apical membrane was obtained, since the basal membrane was not accessible due to the adhesion to the quartz disk. The measurements slightly differed in performance from the normal QCM measurements described above (see section 4.6.1). About 30 min before measurement start, the serum-free medium (1 mL) covering the cells on the quartz disk was exchanged with 1 mL of the corresponding isotonic solution. For hypotonic solutions, the corresponding isotonic solution was the solution called *iso*, and for hypertonic solutions, this was the PBS⁺⁺/Glc buffer (see sections 4.2.1 and 4.2.2). For the MDCK-I-AQP1 cells, hygromycin B was present until this solution change to the isotonic solution, where it was fine for this selection agent to be no longer present during the measurements because they took maximally 3 h. Different kinds of inhibitors (HAuCl₄·3H₂O, bumetanide, and Et₄NCl) were also investigated regarding their influence on the water flux. They were present in the hypertonic and hypotonic test solutions and the isotonic solutions. All the water flux experiments were performed in the incubator New Brunswick Galaxy 48 S at 37 °C, with 0% CO₂ and water vapor saturation. After this incubation with isotonic solution for about 30 min, the measurement was started. After recording a stable baseline (about 15 min), the hypertonic or hypotonic solutions were added, while performing the full replacement method in most of the experiments, where the 1 mL of isotonic solution was completely exchanged with 1 mL of the test solution of interest. In one experiment, the half replacement method was tested. Beside individual additions, which means one addition per cell layer, also serial additions were performed, where the osmolality was stepwise increased/decreased by consecutive additions to the same cell layer. Since the water flux measurements took maximally 3 h, the cells were seeded in parallel in up to six QCM chambers, which were measured sequentially (three times two chambers) for investigation of a variety of conditions or for obtaining experimental replicates. As already mentioned in section 4.6.1, the fast-mode was applied, thus, the minimal impedance $|Z|_{min}$ was the only parameter obtained from the measurements. Upon an osmolality change of the medium surrounding the cells, an osmotic gradient to the cell inside is established, which is compensated by water flux across the apical membrane. The direction of water flux depends on the direction of the osmotic gradient, which is demonstrated in Figure 4.17. A hypertonic medium surrounding the cells triggers water to flow from the inside of the cells to the outside, which leads to shrinking of the cells (Figure 4.17 A). This is associated with a stiffening of the cells since a correlation between a hypertonic stimulation and a cytoplasmic viscosity increase was found (Periasamy *et al.*, 1992). According to the behavior of the minimal impedance $|Z|_{min}$ described in the previous section (section 4.6.2), this parameter was expected to increase upon hypertonic stimulation. The inverse was expected to happen for the hypotonic situation: water flux from the outside of the cells to the inside leading to swelling and, accordingly, softening of the cells (Figure 4.17 B). The minimal impedance $|Z|_{min}$ was expected to decrease meanwhile. A second phenomenon was identified in literature to contribute to the overall

QCM response to osmotic stimulations, which is especially relevant for epithelial cells with their strongly expressed tight junctions, preventing sucrose from diffusing through the paracellular clefts and reaching the narrow liquid compartment between the basal membrane and the quartz surface (Wegener *et al.*, 2000c). Accordingly, the osmotic gradient influences not only the cell bodies, but also this narrow liquid compartment beneath the cell layer, leading to an additional water flux across the basal membrane associated with an alteration of the cell-substrate distance, known to be a decisive factor influencing the composite result of QCM measurements (Wegener *et al.*, 2000c). Furthermore, there is evidence for a correlation between the magnitude of the osmotic gradient and the extent of water flux (McCarty & O'Neil, 1992). Thus, this was expected to be reflected in a bigger shift of the minimal impedance $|Z|_{min}$ for the addition of solutions with higher osmotic gradients relative to the isotonic solution. Additionally, RVI and RVD processes were investigated in this thesis and the influence of the compounds hydrogen tetrachloroaurate trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), bumetanide, and tetraethylammonium chloride (Et_4NCl) were examined, described to elicit inhibitory effects on certain aspects of water flux.

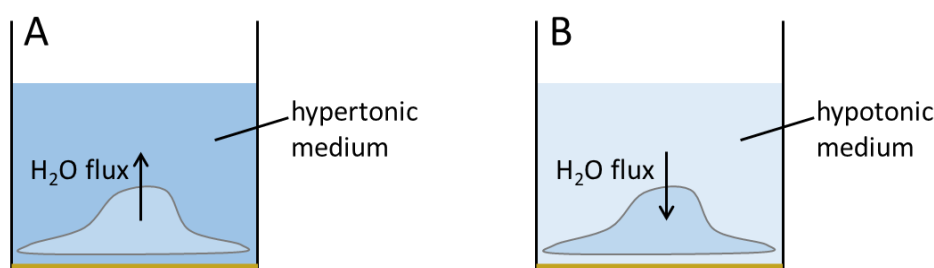


Figure 4.17 Schematic illustrations of the transmembrane water flux across the apical membrane osmotically induced in the water flux experiments. **(A)** The osmotic gradient induced by hypertonic extracellular stimulation triggers a water flux from the inside of the cells to the outside leading to cell shrinking and stiffening. **(B)** Inverse osmotic gradient induced by hypotonic extracellular stimulation triggers a water flux from the outside of the cells to the inside leading to cell swelling and softening. Drawings not to scale.

4.6.4 Data Processing and Presentation

After the minimal impedance $|Z|_{min}$ has been determined by splining and further parameters (load impedance $\Delta|Z|_{Load}$, half-width-half-maximum of maximal conductance Γ , frequency of maximal conductance $f(G_{max})$) were extracted from the measurements by the fitting software, they were plotted as functions of time by the data analysis and graphing program Origin. These time courses were presented normalized to a certain time point, which is mostly the time point of addition of the (first) test solution. In this case, normalization means that the parameter value at this time point was subtracted from all the parameter values leading to a delta value ($\Delta|Z|_{min}$, $\Delta\Delta|Z|_{Load}$, $\Delta\Gamma$, $\Delta f(G_{max})$) of zero at the time point of addition. Although magnitudes of complex quantities can actually not be subtracted, this is common practice and was done for simplification reasons. This was also performed for the time, resulting in a start of exposure at time point zero. To correct for

random signal fluctuations, measurements were mostly repeated several times for statistical analysis by means of arithmetic mean μ and standard deviation (SD) σ (see Eq. (74)), which is then denoted for each diagram in the form: mean $\pm$ SD. When cells were analyzed by QCM readings, it was differentiated between experimental replicates (same passage number) and biological replicates (different passage number). QCM measurements on cells were averaged over both experimental and biological replicates, where the number of replicates is given for each diagram in the form: mean $\pm$ SD, $n = x(y)$, with x as the total number of replicates and y as the number of biological replicates. For QCM measurements on hydrogels, the number of replicates is given for each diagram in the form: mean $\pm$ SD, $n = x$, with x as the total number of replicates. There were some experiments with the total number of replicates $x = 2$ because of limited time, however, the standard deviation was also calculated for these experiments, although it is actually not appropriate because it does not summarize any information. The standard deviation is displayed as error bars into both positive and negative directions. Error bars are given for each single data point, but only up to a total number of 60. If there are more than 60 data points shown in a diagram, there will not be an error bar for each single data point. Due to the mostly high number of replicates, especially for measurements on hydrogels, and the high sensitivity of the QCM technique, outlier curves or curves with huge outliers sometimes occurred. Those were identified by personal judgement and removed from the dataset before averaging.

4.7 QCM Measurements with Double-Quartz-Disks

For a few water flux experiments (see section 8.2.1), QCM measurements were performed with AT-cut double-quartz-disks (Figure 4.18) instead of normal AT-cut quartz disks (see Figure 4.13). These double-quartz-disks were developed by Dr. Oberleitner (Oberleitner, 2018) and exhibited two pairs of electrodes instead of one for measuring the oscillation impedance of the same cell layer at two individual sensor spots. The diameter of the quartz disks was the same as for the normal ones (14 mm), while the same thickness of 330 μm resulted in the same resonance frequency of about 5 MHz. The electrode diameter was 3.5 mm each with electrical supply lines and contact pads enabling electrical contacting of all four electrodes from the lower side. Both sensor spots would show resonance at the exact same frequency leading to a coupled resonance signal. Suppression of this resonance interference between the two sensor spots was achieved by covering one of them with a rigid layer, which was in accordance with the Sauerbrey equation (see Eq. (29)) because loading the quartz with an additional rigid mass reduces the resonance frequency. A photopolymer layer served as the rigid mass layer, which was generated by spin coating and photolithography (see section 4.5.2). One half of the top side was coated with the photopolymer, covering one sensor spot and reducing its resonance frequency, which is

denoted as QCM1. The uncoated sensor spot is denoted as QCM2. Their respective oscillation impedances (Z_{QCM1} and Z_{QCM2}) were recorded individually.

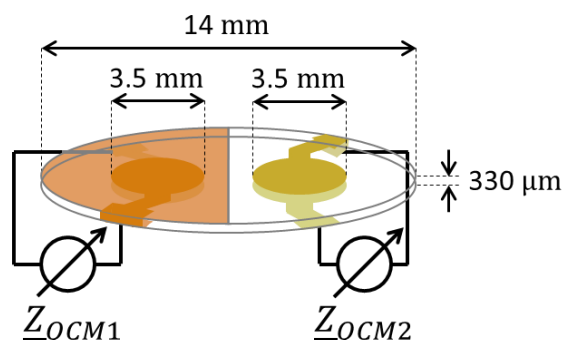


Figure 4.18 Schematic illustration of the 5 MHz AT-cut double-quartz-disks used in this thesis with a diameter of 14 mm and a thickness of 330 μm and two pairs of electrodes with a diameter of 3.5 mm each designed by Dr. Oberleitner (Oberleitner, 2018). Both pairs of electrodes exhibited electrical supply lines and contact pads, just like normal quartz disks, but in duplicate on each side, enabling electrical contacting of all four electrodes from the lower side, whereas one half of the top side was covered by photopolymer for resonance decoupling (here the left one), as described in section 4.5.2. The oscillation impedances of both the photopolymer-coated sensor spot (Z_{QCM1}) and the uncoated sensor spot (Z_{QCM2}) were recorded individually and alternatingly.

These double-quartz-disks were mounted into Teflon® QCM chambers, which were similar to the ones used for the normal quartz disks (see Figure 4.14). The only difference was that the contact piece exhibited four gold pins instead of two for simultaneously contacting all four electrodes and the socket also had four contacts instead of two. The same measurement device comprising impedance analyzer, computer, and relay was used with an extended version of the LabVIEW-based QCM measurement software for controlling the relay to switch alternatingly between the two sensor spots. The test measurements in air and under liquid loading were measured in a frequency range of 4.96 MHz – 5.04 MHz with 150 equally spaced frequencies, whereas for all the measurements on cells (MDCK-I and MDCK-I-AQP1), the fast-mode was applied, as described in section 4.6.1. Narrow frequency ranges covering 6.3 kHz each centered around the respective frequency of minimal impedance with 14 equally spaced frequencies was used here. Impedance spectra (Figure 4.19) and phase spectra (not shown) were measured simultaneously, showing a successful resonance decoupling of the photopolymer-coated sensor spot (Figure 4.19, ●) from the uncoated one (Figure 4.19, ○), since the coated sensor spot showed resonance at a smaller frequency. Upon increasing viscoelastic loading from an almost undamped oscillation in air (Figure 4.19, black curves), over a liquid loading (Figure 4.19, red curves) up to an adherent cell layer (Figure 4.19, green curves), the same shift of the minimum was obtained, as already observed for the normal quartz disks in Figure 4.15. The resonance frequency decreases and minimal impedance $|Z|_{min}$ increases for both sensor spots. The alternating measurement of the two sensor spots in this narrow frequency range around the respective minimum was repeated successively several times and the minimal impedance was extracted from the spectra by

the measurement software during measurement. This enabled a time-resolved recording of the minimal impedance with the same time resolution of 16.5 s as in the fast-mode of regular QCM measurements.

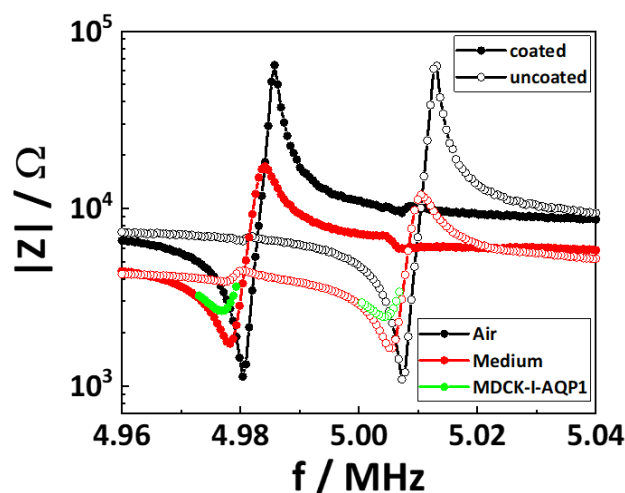


Figure 4.19 Impedance spectra of typical QCM measurements of the oscillation of a 5 MHz AT-cut double-quartz-disk with decoupled resonance of the two sensor spots, one coated by photopolymer (●) and the other one uncoated (○). Measurements in air (black) and under loading with cell culture medium (red) were performed for the full frequency region of 4.96 MHz – 5.04 MHz, whereas measurements on cells (here MDCK-I-AQP1, green) were conducted in frequency ranges covering 6.3 kHz centered around both minima (fast-mode).

The advantage over conventional QCM measurements is to measure the response of one cell layer at two individual sensor spots instead of measuring two cell layers in parallel. The performance of water flux experiments with double-quartz-disks was the same as with normal quartz disks (see section 4.6.3), while MDCK-I and MDCK-I-AQP1 cells were used. Due to the fast-mode applied the only parameter for evaluation of experiments was the minimal impedance $|Z|_{min}$. Splining of the minimal impedance with the fitting software and data normalization and presentation were performed equally to regular QCM measurements. Due to damaging of the photopolymer coating in the cleaning process of the quartz disks, they were only used for up to two consecutive experiments before their photopolymer coating was removed by rinsing with acetone and a new layer of photopolymer was applied.

Additional to the simultaneous QCM measurements of the two QCM sensor spots, an ECIS measurement is possible by measuring the electrical impedance between the two electrodes on the top side (Oberleitner, 2018). For this, a small photopolymer-free region with a diameter of 250 μm – 2 mm is needed on the top electrode of the photopolymer-coated sensor spot (QCM1) to enable the current to cross the electrode-electrolyte interface, which then serves as the smaller WE. The top electrode of the uncoated sensor spot (QCM2) serves as CE. These measurements were not performed in this thesis, although they would enable

the combination of the two non-invasive techniques of QCM and ECIS for simultaneous sensing of both viscoelastic and morphological properties of one cell layer.

4.8 Experimental Design of ECIS Measurements

ECIS was used to electrically monitor morphological changes of HTM-N cells. The basic principles were explained in section 3.4. In the following, array handling as well as settings and conditions of the ECIS measurements (section 4.8.1), differences in conditions and performance for measurements in transwell format (section 4.8.2), and data processing and presentation (section 4.8.3) are described.

4.8.1 General Setup, Conditions, and Performance of ECIS Measurements

Most of the ECIS measurements in this thesis were performed in transwell format (description of experiments see section 4.8.2, results see section 7), but for a few experiments, ECIS was also applied in the regular setup. For this, commercial 8W10E arrays were used (Figure 4.20), which exhibited eight wells with ten small WEs in each well. WE diameter was $250\text{ }\mu\text{m}$, leading to a total WE area of about $4.9 \cdot 10^{-3}\text{ cm}^2$. One common CE reached into each of the wells for measuring the impedance between WEs and CE with an integrated signal over the ten WEs obtained in each well. The substrate of the arrays and the walls of the wells consisted of PET, a material well-suited for cell culture. Electrodes including electrical supply lines were made of gold, where the specific WE pattern was generated by photopolymer coating, which also insulated the supply lines.



Figure 4.20 Image of an 8W10E array used for ECIS experiments with eight wells each having ten small WEs ($d = 250\text{ }\mu\text{m}$ each, total WE area $A \approx 4.9 \cdot 10^{-3}\text{ cm}^2$) with one common CE in the middle of the array reaching into each well. Nine contact pads served for electrical connection of the electrodes (eight WEs and one common CE) to the measurement device. Substrate and wells were made of PET and electrodes were made of gold. Electrical insulation of electrical supply lines was achieved by photopolymer coating.

The array was electrically connected to the measurement device by nine contact pads (eight WEs and one common CE). The measurement device was composed of the measurement instrument ECIS® Z θ , a computer, and a holder. The holder served for the electrical connection of the arrays by the contact pads and enabled the measurement of two arrays in parallel. The measurement instrument ECIS® Z θ applied a constant current in a frequency range of 20 Hz – 96 kHz at 10 – 16 individually selected frequencies to the electrodes and measured the corresponding voltage drop, thus, the impedance of the system is obtained, by determination of its real part (resistance R) and imaginary part (reactance X). This was controlled by the computer with the ECIS software, which enabled it to monitor spectra of impedance magnitude $|Z|$, resistance R , and capacitance C over time. Simulated impedance spectra were already shown in Figure 3.12, and typically measured impedance spectra are demonstrated in Figure 4.21, whereas resistance and capacitance spectra are not shown because the evaluation of the impedance was sufficient for the interpretation of experiments. These impedance spectra were recorded for each individual experiment and each single well, but they are shown here only for one representative well and experiment, with a comparison of cell-free (Figure 4.21, black curve) and cell-covered conditions (Figure 4.21, red curve). Spectra of cell-free electrodes were always separately measured for pure culture medium before cell seeding, whereas spectra of cell-covered electrodes were always extracted from the first time point of the actual measurement on adherent and confluent cell layers, which were the HTM-N cells in all ECIS experiments.

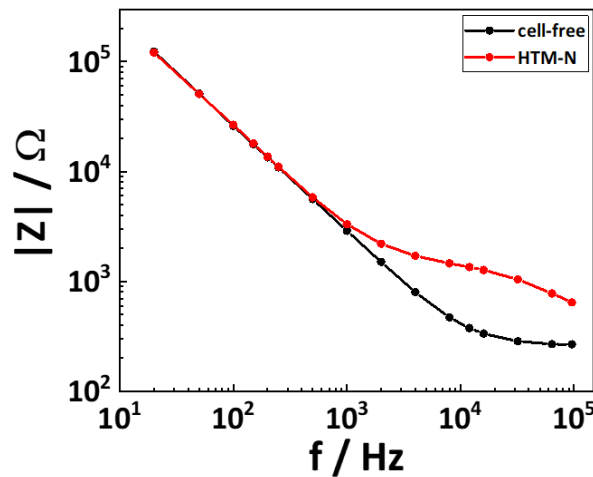


Figure 4.21 Impedance spectra of typical ECIS measurements with an 8W10E array. Data are only shown for one representative WE-CE pair (one well) covered with pure culture medium (cell-free, black) and covered with an adherent and confluent layer of HTM-N cells surrounded by medium (red).

These spectra show close similarities to the simulated ones with the frequency-dependent part dominated by the CPE in the low-frequency region. The frequency-independent horizontal line dominated by R_{bulk} in the high-frequency region is not obtained in the spectrum of cell-covered electrodes and only partially in the spectrum of cell-free

electrodes, because the measurement instrument ECIS® Z0 was not able to go higher in frequency. The cells' influence on the impedance spectrum is observed in between, at frequencies of about 10^3 Hz – 10^5 Hz, with an elevated impedance for the cell-covered electrode. Such spectra were recorded repeatedly over time by the ECIS software. This software also enabled it to extract the impedance at selected frequencies and to monitor the time course for following time-dependent changes of impedance during the measurement. The time resolution of these measurements was 1.8 min – 4.1 min, depending on the number of frequencies and if two arrays were measured in parallel or just one.

Before the first use of the 8W10E arrays, they were treated by so-called pulsing in order to remove impurities that might be present on the electrode surface after their industrial fabrication. This was performed by adding 400 μ L of culture medium into each well and applying a current of 1000 μ A at a frequency of 1500 Hz for 30 s in each well, leading to a decrease in impedance and an increase in capacitance. Impedance spectra of cell-free electrodes of arrays, which were used for the first time, were extracted from the last time point of the pulsing measurement. After that, HTM-N cells were seeded, which reached confluency after 2 d – 3 d of incubation in the incubator Heraeus BB 15 at 37 °C and 5% CO₂ with feeding of the cells on the day before measurement, where the medium (400 μ L) was exchanged with serum-free medium (400 μ L). The measurements were performed in the incubator MCO-170AICUVH at 37 °C, with 5% CO₂ and water vapor saturation. After a stable baseline was obtained with serum-free medium covering the cells (minimum 2 h), the test solutions were added mostly by the half replacement method (–200 μ L serum-free medium, +200 μ L double-concentrated test solution). The cells' reaction to the test solution was observed for at least 24 h. Since eight wells or even 16 wells for two arrays measured in parallel were available, each test solution was used in duplicate for obtaining experimental replicates, which then allowed to investigate four (or eight) different conditions in one experimental run.

The 8W10E arrays were reused several times for experiments, thus, they had to be cleaned thoroughly after usage to re-establish clean electrode surfaces with constant electrical properties. This was achieved by washing each well with PBS[–] and subsequent trypsinization (2.5 g/L trypsin) of the HTM-N cells under incubation at 37 °C for 10 min to completely detach the cells from the well bottom, followed by two further washing steps with PBS[–] and one washing step with distilled water. Re-establishment of sterility was accomplished by treatment with Ar plasma for 30 s before seeding a new cell suspension.

4.8.2 ECIS Measurements in Transwell Format

Transwell ECIS measurements were performed in transwell arrays. Their fabrication is described in section 4.5.1, while three different electrode layouts were investigated (see Figure 4.10). Images of an array were already demonstrated in Figure 4.11 showing the

Plexiglas® 8-well-corpus fixed with silicone glue to the Lexan® plate carrying the electrode structure, yielding wells with a diameter of 16 mm (area of 2 cm²) and a height of 19 mm. Transwell filter inserts with a circular PC membrane with 6.5 mm diameter, a pore density of 10⁸ pores per cm² and 0.4 µm pore diameter were able to be placed into the wells (see Figure 4.11 C). A schematic illustration of the setup is shown in Figure 4.22. These transwell filter inserts were already demonstrated in Figure 4.3 E, which were filled with vitreous body (or agarose gel as a substitute) in a volume of 200 µL, which led to a height of the vitreous body (or agarose gel) in the insert of 6 mm. The extraction of vitreous body from porcine eyes is described in section 4.2.3 and the preparation of agarose gels is described in section 4.2.4. The well bottom with the electrodes was covered by HTM-N cells surrounded by 1 mL of culture medium, into which the transwell filter insert was dipping with a distance between filter membrane and well bottom of 2 mm. The diffusion of fasudil through the vitreous body (or agarose gel) was to be investigated, where it was added in two distinct ways (see further down). After diffusion through the vitreous body (or agarose gel), fasudil can cross the filter membrane, diffuse through the culture medium, and finally reach the HTM-N cells. There it was expected to exert its effect on the cells and lead to morphological changes, which can be followed over time by continuous ECIS measurements.

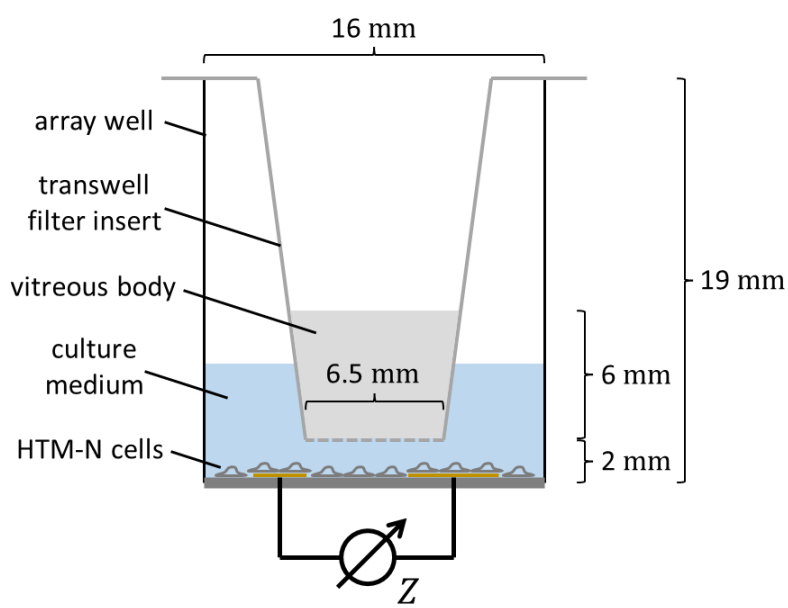


Figure 4.22 Schematic illustration of the transwell setup showing one well of a self-fabricated array with a diameter of 16 mm and a height of 19 mm. Transwell filter inserts exhibited a PC membrane with a diameter of 6.5 mm and were filled with 200 µL (height of 6 mm) of vitreous body freshly extracted from porcine eyes or agarose gel freshly prepared. The transwell filter insert was placed into the well dipping into the culture medium (1 mL) yielding a distance between membrane and well bottom of 2 mm. HTM-N cells grew over the well bottom covering the electrodes. This setup enabled fasudil to diffuse through the vitreous body (or agarose gel), PC membrane, and culture medium and to finally reach the HTM-N cells leading to cellular changes detectable by the ECIS measurement. Drawings of cells and electrodes not to scale.

The performance of the transwell experiments was changed and optimized several times since they were part of an ongoing process of assay development, which was done in cooperation with Dr. Mietzner (University of Regensburg, Germany) and Prof. Dr. Breunig (University of Regensburg, Germany). Two different measurement devices were used for the transwell experiments. The first measurement device was the same as for the regular ECIS measurements: incubator MCO-170AICUVH, measurement instrument ECIS® Zθ, a computer, and a holder. The ECIS software was used for data recording. A current was applied in a frequency range of 20 Hz – 96 kHz with 10 – 14 individually selected frequencies, which led to a time resolution of 0.8 min – 4 min, depending on the number of frequencies and if two arrays were measured in parallel or just one. The impedance of the system was obtained by the determination of its real part (resistance R) and imaginary part (reactance X). The second measurement device was similar to the one used for QCM measurements: incubator New Brunswick Galaxy 48 S, impedance analyzer 1260A, a computer, and a holder. For data recording, a LabVIEW-based ECIS measurement software was used. A sinusoidal driving voltage with a small amplitude of 10 mV (rms) was applied in the frequency range of 10^0 Hz – 10^6 Hz with 61 equally spaced frequencies leading to a time resolution of 4.3 min for two arrays measured in parallel. Here, the impedance of the system was obtained by determination of its magnitude $|Z|$ and phase shift φ between voltage and current. Although the two measurement devices differed in the way of impedance determination and time resolution, influences on the outcome of experiments were not expected. Independent of the measurement device used, spectra of the impedance-based quantities ($|Z|$, R , and C , or $|Z|$ and φ) were recorded over time. Impedance spectra are shown in the results section (see section 7.2) because they have continuously improved during the sensor optimization (see further down).

Since some residues or impurities might remain on the electrode surface of the transwell arrays after their photolithographic fabrication, electrodes were pulsed before use under the same conditions as for the normal 8W10E arrays (1000 μ A, 1500 Hz, 30 s). Incubation with culture medium before pulsing was performed with 1 mL per well and for about 1 h for the first experiments but was extended to 24 h during optimization of the assay. After seeding of the HTM-N cells and incubation for 2 d, the medium was exchanged with 1 mL of medium containing 0.35% FBS, instead of serum-free medium as used in the normal ECIS experiments. About 24 h later, the measurement was started. An additional medium exchange to fresh medium containing 0.35% FBS was introduced right before measurement start as part of the assay optimization process. During the recording of a stable baseline (minimum 2 h) the vitreous body was freshly extracted from porcine eyes. Sometimes, a little bit of water could be removed with a pipet from the vitreous body after centrifugation in a centrifuge 5415 R at 6100 g for 1 min. The application of fasudil to the vitreous body was performed in two different ways. For both ways, the vitreous body was added into micro test tubes by a pipet with a cropped pipet tip in a volume of 200 μ L for each individual transwell filter insert to be filled with vitreous body. Due to the gel-like behavior of the vitreous body, it had to be cut off by scissors. Since this procedure was absolutely not

precise, the exact amount of vitreous body was determined by weighing its mass. The volume was calculated by assuming a water-like density of $10^3 \frac{\text{kg}}{\text{m}^3}$. Thus, the volume of fasudil solution had to be adapted to the exact volume of vitreous body to yield the same final concentration in each well and experiment. For 200 μL of vitreous body (and 1 mL of culture medium), 50 μL of a 625 μM fasudil solution were used, leading to a final fasudil concentration of 25 μM in the wells (total volume: 1250 μL , dilution factor of 25), which was used as the standard concentration in all transwell experiments. The first way of fasudil application was performed by adding the vitreous body into the transwell filter inserts and adding fasudil on top. The second and optimized way of fasudil application was conducted by adding fasudil into the vitreous body in the micro test tubes and mixing with a vortex mixer REAX 1R for about 5 s to yield evenly distributed fasudil in the vitreous body. After that, the mixtures were added to the transwell filter inserts. When an agarose gel was investigated as a substitute for the vitreous body, the gels were freshly prepared during baseline recording. 200 μL of agarose gel were prepared in each transwell filter insert, where 50 μL of the 625 μM fasudil solution were added on top. Directly after adding fasudil on top of the vitreous body or agarose gel, or after adding the vitreous body-fasudil-mixture to the transwell filter inserts, they were placed into the wells of the transwell array during the ECIS measurement. Additionally, in one experiment, one well was used to successively add and remove the transwell filter insert to investigate if placing transwell filter inserts into the array during measurement had an influence on the impedance. For comparison, in most of the transwell experiments, an addition of fasudil directly to the cells without using a transwell filter insert was investigated in a few wells. For this direct addition, the full replacement method was applied in the first few experiments, which was then optimized by using the half replacement method to minimize impairment of the cells. In most of the transwell experiments, *released* fasudil (fasudil released from PLGA microspheres) was used additionally to normal fasudil for simulation of the medical application of fasudil into the human eye by microspheres, where it would be released over time and subsequently diffuse through the vitreous body. The effect of fasudil on the HTM-N cells either added directly or after diffusion through the vitreous body or agarose gel was followed for at least 24 h by continuous ECIS measurements. After a few experiments, the medium in the wells was aspirated and the concentration of fasudil in the medium was determined by HPLC to check for correct and constant fasudil concentration. HPLC measurements were performed as already described in section 4.2.1.

In summary, optimization of the transwell assay comprises the following features: (1) The electrode layout was changed two times, from a simple 1 WE-1 CE-situation (8W1E+ enl. (100 μm) array, see Figure 4.10 A) in the beginning to an idf configuration of WE and CE (8W1000idf enl. (200 μm) array, see Figure 4.10 B), which was further modified (8W1000idf enl. (900 μm) array, see Figure 4.10 C). The electrode area was increased and more distributed over the well surface by these layout changes. (2) The incubation time of the electrodes with medium prior to pulsing was extended from 1 h to 24 h to improve the quality of impedance spectra of cell-free electrodes. (3) Right before measurement start, an

additional medium exchange step to fresh medium containing 0.35% FBS was added to re-establish physiological conditions. (4) For the direct addition of fasudil, the originally applied full replacement method was substituted by the half replacement method, which was expected to disturb the cells less. (5) The way of fasudil application to the vitreous body was changed from a simple on-top application to mixing of both fasudil and vitreous body before adding to the transwell filter inserts to ensure a more uniform diffusion of fasudil through the vitreous body. The respective impacts of all these optimizations are described in section 7.2.

The transwell arrays could also be reused, but only up to maximally three times, because the electrode pattern was sometimes damaged by repeated use since they were less mechanically robust as compared to the commercial ECIS arrays. Their regeneration was performed equally to the normal ECIS arrays. Transwell filter inserts were discarded after usage of agarose gels but were regenerated after usage of vitreous body by repeatedly washing with distilled water, followed by drying in N₂ stream. Sterility was re-established by treatment with Ar plasma (arrays: 30 s, transwell filter inserts: 1 min).

4.8.3 Data Processing and Presentation

As already mentioned above, the magnitude of impedance $|Z|$ was the only parameter used for the evaluation of ECIS experiments, which is, for the sake of convenience, just called impedance. The course of impedance could be followed during the measurement for each single well and each frequency by the ECIS software. After an experiment was finished, the data analysis and graphing program Origin was used to determine the most sensitive frequency. This frequency was typically located in the mid-frequency range between low frequencies (about 400 Hz) for sensing barrier function and high frequencies (about 40 kHz) for sensing cell coverage. Thus, using the most sensitive frequency for evaluation enabled a combined observation of both barrier function and cell coverage. It was determined by considering the impedance spectra of the cell-free and cell-covered electrode (see Figure 4.21) and calculating the ratio $|Z|_{\text{cell-covered}}/|Z|_{\text{cell-free}}$, which is demonstrated exemplary in Figure 4.23 based on the spectra shown in Figure 4.21. The frequency with the maximal value of this ratio was the most sensitive frequency. The higher this value, the more sensitive was the measurement to cellular changes. The most sensitive frequency was figured out for each single experiment, while it was determined to 16 kHz for all the regular ECIS measurements. In the transwell experiments, the most sensitive frequency was not always the same because it was dependent on the electrode layout and the quality of the impedance spectra, and was in the range of 8 kHz – 16 kHz (but mostly 8 kHz).

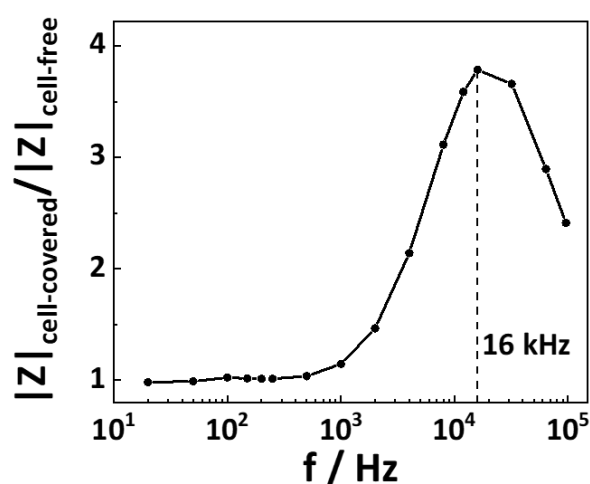


Figure 4.23 Determination of the most sensitive frequency for typical impedance spectra of ECIS measurements with an 8W10E array. Data were calculated from the impedance spectra shown in Figure 4.21 revealing a most sensitive frequency of 16 kHz in this case.

After determination of the most sensitive frequency, the impedance at this specific frequency was plotted as a function of time in Origin with the most sensitive frequency f_{sens} denoted in the index of the impedance: $|Z|_{f_{sens}}$. These time courses were presented normalized to the time point of addition. In contrast to the QCM measurements, here normalization was performed by calculating the ratio instead of the difference, leading to a normalized impedance *norm.* $|Z|_{f_{sens}}$ of 1 at the time point of addition. Although the ratio of magnitudes of complex quantities can actually not be calculated, this is common practice and was done for simplification reasons. To additionally gain information about the biological origin of impedance changes, for one experiment, the parameters α , R_b and C_m and their change over time were calculated by fitting the ECIS model to the raw data with the ECIS software. They were plotted as time courses but without normalization to be able to read the absolute values. Experimental replicates were always guaranteed by the high number of wells of the arrays (16 wells for two parallel arrays). Typically, two experimental replicates of each condition tested were conducted in the experiments by testing eight different conditions and each of them twice. For a few experiments, the number of experimental replicates was three or even four, when it was sufficient to test less than eight different conditions. For the first few transwell experiments, it was not possible to always include experimental replicates because sometimes some wells were leaky due to the self-made fabrication of the arrays, and because only one array was used. Statistical analysis was performed by calculating the arithmetic mean μ and standard deviation σ (see Eq. (74)), while averaging over biological replicates was conducted for regular ECIS measurements but not for the first few transwell experiments due to the varying experimental conditions in the continuous optimization process of the assay. The final optimized transwell experiment was performed three times with constant experimental conditions, while it still occurred sometimes that a few wells were leaky leading to slightly different time resolutions between

single experiments. Thus, averaging over the three biological replicates was only possible by constructing interpolations in advance, which was done by linear interpolations with about 10 000 data points. The number of replicates is given for each experiment in the form: mean $\pm$ SD, $n = x(y)$, with x as the total number of replicates and y as the number of biological replicates. The standard deviation is displayed as error bars into both positive and negative directions. Error bars are given for each single data point, but only up to a total number of 60. If there are more than 60 data points shown in a diagram, there will not be an error bar for each single data point.

4.9 PrestoBlue® Assay

The PrestoBlue® assay, the theoretical background of which is described in section 3.5, was used as a sensing platform for cytotoxicity since it detects mitochondrial activity by the presence of NADH or NADPH after the substance under study has possibly impaired cell metabolism. The cytotoxicity of the three inhibitors $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, bumetanide, and Et_4NCl used in the water flux experiments (description of experiments see section 4.6.3, results see section 8) was investigated by the PrestoBlue® assay, while using HTM-N cells seeded onto a 96-well plate. The outer wells of the 96-well plate were not used due to a non-uniform distribution of light intensity in the measurement instrument, leading to a number of 60 wells available. Thus, ten different conditions were examined with the number of experimental replicates of six for each condition. Regarding the necessity to include a blind control (no cells, only resazurin solution), a negative control (concentration of the test substance of zero), and a positive control (Triton X-100 solution) in the assay, seven different concentrations (six for bumetanide with a DMSO control tested additionally) of the respective test substance were investigated. Since water flux experiments revealed a half maximal inhibitory concentration (IC_{50} value) of $10.8 \mu\text{M}$ for $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (Niemietz & Tyerman, 2002) and this substance was intended in this thesis for a use at a concentration higher than this for an effective inhibition of water flux, the following concentrations were examined in the PrestoBlue® assay: $10 \mu\text{M}$, $25 \mu\text{M}$, $50 \mu\text{M}$, $100 \mu\text{M}$, $200 \mu\text{M}$, $300 \mu\text{M}$, and $400 \mu\text{M}$. For bumetanide, which showed an effective inhibition of RVI processes at a concentration of $10 \mu\text{M}$ (O'Donnell *et al.*, 1995), the following concentrations were examined: $5 \mu\text{M}$, $10 \mu\text{M}$, $20 \mu\text{M}$, $50 \mu\text{M}$, $100 \mu\text{M}$, and $200 \mu\text{M}$. For Et_4NCl , which showed an effective inhibition of RVD processes at a concentration of 7.5 mM (Mitchell *et al.*, 2002), the following concentrations were examined in the PrestoBlue® assay: 2 mM , 5 mM , 7.5 mM , 10 mM , 20 mM , 50 mM , and 100 mM . After the HTM-N cells grew to confluence in the 96-well plate for 2 d of incubation in the incubator Heraeus BB 15 at 37°C , with 5% CO_2 and water vapor saturation, with change to serum-free medium ($200 \mu\text{L}$) on the day in between, the test solutions and the negative control were added by the half replacement method ($-100 \mu\text{L}$ serum-free medium, $+100 \mu\text{L}$ double-concentrated test solution). At the same time, a Triton X-100 solution (0.5% (v/v)) was added as the positive control in the respective wells by the full replacement method. For all three test substances, the time of incubation of

the cells with the test solutions was 2 h, since the water flux experiments took around 2 h. After this incubation time, the test solutions were replaced by 100 μL of a resazurin solution (10 mg/L), which was also added to the wells designated for the blind control. The incubation time with resazurin solution was 30 min, followed by placing the 96-well plate into the fluorescence reader GENios, which applied 10 s of shaking before measurement. The fluorescence of each well was excited at an excitation wavelength λ_{ex} of 532 nm and its intensity was measured at an emission wavelength λ_{em} of 600 nm, while reading fluorescence from the top and bottom. The bottom read was evaluated due to results with smaller deviations. After recording the fluorescence intensities, the cytotoxicity index CI was calculated according to Eq. (71) and plotted versus the concentration of the respective test substance. Errors (ΔCI) were calculated by Gaussian error propagation according to:

$$\Delta CI = \sqrt{\left(-\frac{1}{I_{corr}(neg.ctrl)} \cdot \Delta I_{corr}\right)^2 + \left(\frac{I_{corr}}{I_{corr}(neg.ctrl)^2} \cdot \Delta I_{corr}(neg.ctrl)\right)^2} \quad (75)$$

where these parameters were already introduced by Eq. (71). Errors were plotted as error bars into both positive and negative directions.

4.10 Microscopy

To enable a closer look at the cells under study, two different microscopic techniques were utilized in this thesis: phase contrast microscopy (section 4.10.1) and confocal laser scanning microscopy (CLSM) (section 4.10.2). The latter requires a special preparation of the cells before imaging, which is described in section 4.10.3.

4.10.1 Phase Contrast Microscopy

A phase contrast microscope translates phase changes occurring when light travels through a medium, which are actually invisible by human eye, into amplitude changes visible by human eye (Zernike, 1942), thus, generating additional information about the object under study. This is typically applied to biological specimens like living, unstained cells. In this thesis, an inverted phase contrast microscope Diaphot with 4-fold and 10-fold magnification with an installed camera was used for a variety of purposes: (1) In the weekly process of sub-cultivation (see section 4.1.3), the fraction of cells, which was seeded onto a new culture flask, was adapted to the speed of growth and proliferation of the respective cell type. This was achieved by phase contrast microscopic observation of the cells in the culture flasks each time before passaging and before feeding. This also ensured constant checking of cellular viability. One phase contrast microscopic image of the HTM-N cells was already demonstrated in Figure 4.1. (2) After sub-cultivation and before cells were seeded onto a substrate, the cell number in the cell suspension was estimated by cell counting in a Bürker

chamber using the phase contrast microscope (see section 4.1.4). (3) In the photolithographic fabrication of the transwell arrays (see section 4.5.1), the accuracy of the process regarding exact electrode dimensions was examined by phase contrast microscopy. These images are shown in section 7.2 and evaluated in the context of sensor optimization. (4) Beside the adhesion measurement of HTM-N cells by QCM, the adhesion of these cells was also followed microscopically after seeding in Teflon® vessels (see Figure 4.4). Transparent glass slides were used as growth substrates, which were similar to the quartz disks. Images were taken every 6 h, which are shown in section 6.1 and compared to QCM data.

4.10.2 Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) is a method for the detection of fluorescence with increased lateral and axial resolution. Its principle is on the one hand based on two pinhole apertures, which allow only light from the focal plane to pass and suppress stray light and background fluorescence from out-of-focus planes, and on the other hand is based on horizontal scanning to generate a 2D image (Davidovits & Egger, 1969). It is also possible to generate 3D images by stacking multiple images obtained from focal planes lying above each other. Due to its very high optical resolution, the CLSM is mainly used for observation of cellular structures, as it is also used in this thesis. For most of the measurements performed in this thesis, CLSM observations of the cellular changes occurring upon addition of the respective test substances were performed additionally and complementary. CLSM images were taken for the QCM measurements with CTGF (see section 6.2) and fasudil (see section 6.3), transwell experiments (see section 7), and water flux experiments (see section 8) to help interpret the results. A confocal laser scanning microscope Eclipse 90i with water immersion objective with 60-fold magnification was used for this purpose. Settings were controlled by the software EZ-C1. Because the cellular structures do not show autofluorescence, they had to be stained with fluorescence labels. As already mentioned before, changes of the QCM signal result from changes of viscoelasticity or stiffness, which correlate with changes of the cytoskeleton and cell cortex. In ECIS, the cell shape mainly influences the signal, which is also correlated with the cytoskeleton. The cell cortex consists of filamentous actin, which is also a constituent of the cytoskeleton because actin filaments constitute one of the three big classes of the cytoskeleton, as already explained in section 1.2. Thus, the actin filaments of the cells were stained with fluorescence labels to make them visible in the CLSM. This was achieved by TRITC-phalloidin, where the actin filament binding drug phalloidin was conjugated to the fluorophore TRITC. Additionally, cell nuclei were stained by DAPI for better localization of the cells under the microscope. Both steps are part of the fluorescence staining protocol, which is explained in detail in section 4.10.3. The fluorescence of TRITC was excited with a laser at a wavelength of 543 nm and detected above 650 nm by using a 650-long pass filter. The fluorescence of DAPI was excited with a laser at a wavelength of 408 nm and detected at 450 nm – 535 nm. Laser

intensity amplification (gain) and pinhole size were adjusted individually to the fluorescence brightness in each single experiment but were selected constant during one and the same experiment. These two parameters were also constant when images from two different experiments were compared. Images were taken as average images calculated from eight individual images taken at the same spot, where each image consisted of 1024 x 1024 pixels. The focal plane was chosen close to the substrate to observe actin stress fibers and the cell cortex. In the experiments, many (2 – 8) different conditions (different test substances versus the control, different incubation times) were always investigated. In most of the experiments, two samples of each condition were prepared, and images were taken at 2 – 5 different spots for each sample, resulting in 3 – 10 images for each condition tested. Images taken were processed and evaluated afterwards by the software ImageJ, while one image representative for each condition was selected, demonstrated in the results section, compared to each other, and interpreted.

4.10.3 Cytochemical Fluorescence Staining

Before these fluorescence images were taken as described above, the cells had to be labeled. Images were only taken for HTM-N cells. For this, the HTM-N cells were cultured for 2 d in Petri dishes (diameter of 5.1 cm, area of 20.43 cm²) with several glass slides (diameter of 1.4 cm) deposited on the bottom ensuring confluent cell layers adhered upon these glass slides. After that, on the day of the experiment, each single glass slide was placed on a piece of parafilm in a new Petri dish and the additions of the test substances and controls were performed equally to the experiment to be compared with, regarding concentrations and incubation times. The volume of addition was 100 µL, which completely covered the cell layer and was retained on the glass slide by the non-polar parafilm. When images were taken for the transwell experiment, glass rings with the same dimensions as the wells in the transwell array (diameter of 1.6 cm, height of 1.9 cm) were used for placing the transwell filter inserts. These glass rings were stuck to Lexan® plates with silicone grease, which ensured an easy tear-off before imaging with the water immersion objective. The experimental procedure was the same as in the transwell experiments described in section 4.8.2. When different incubation times were compared, the additions were performed at different time points, so that the staining was started for all samples at the same time. In Table 4.9, the protocol for staining of the HTM-N cells by fluorescence labels is summarized, while 100 µL of each solution were used (200 µL for imaging of the transwell experiment). Since the structure of the actin filaments was to be observed for a certain incubation time of a certain substance, the cells had to be fixed prior to staining. This was achieved by incubation with 40 g/L PFA for 10 min at RT, followed by washing twice with PBS⁺⁺. Because TRITC-phalloidin is hardly membrane permeable, the cells' membranes were permeabilized by 0.2% (v/v) Triton X-100 for 10 min at RT, followed by washing twice with PBS⁺⁺. For actin staining, the incubation with 6 mg/L TRITC-phalloidin was performed for 45 min at 37 °C and protected from light in an incubator with water vapor saturation,

followed by washing three times with PBS⁺⁺. Cell nuclei were stained by incubation with 10 µg/L DAPI for 1 min at RT and protected from light by an aluminum foil, followed by washing three times with PBS⁺⁺. Staining was finished by a second fixation step, which was performed equal to the first one, followed by washing three times with PBS⁺⁺. Cells were completely covered with PBS⁺⁺ prior to imaging.

Table 4.9 Overview of the cytochemical fluorescence staining protocol performed for the HTM-N cells prior to CLSM imaging. The purpose of each step is given with the respective compound used, its concentration, and incubation time, while washing with PBS⁺⁺ was conducted after each step. All steps were performed at RT, except for the actin staining with TRITC-phalloidin, which was done in an incubator at 37 °C with water vapor saturation. This additionally ensured protection from light for this step, which was also necessary for the nuclei staining with DAPI ensured by aluminum foil. The volume of solution used was 100 µL for each step, but in the experiment performed complementary to the transwell experiment, this was 200 µL adapted to the bigger substrate area.

Purpose	Compound	Concentration in PBS ⁺⁺	Incubation time / min
fixation	PFA	40 g/L	10
washing	2 x PBS ⁺⁺		
permeabilization	Triton X-100	0.2% (v/v)	10
washing	2 x PBS ⁺⁺		
staining of actin	TRITC-phalloidin	6 mg/L	45
washing	3 x PBS ⁺⁺		
staining of nuclei	DAPI	10 µg/L	1
washing	3 x PBS ⁺⁺		
post-fixation	PFA	40 g/L	10
washing	3 x PBS ⁺⁺		

5 Assessing Mechanical Parameters of Cells by Using Hydrogels as a Model System

The importance of polymerization reactions is continuously increasing, which leads to an advanced search for novel techniques capable of analyzing and monitoring them. Conventional techniques to follow polymerization reactions are mostly based on the analysis of monomer conversion, for example, by titration of mixture samples (Flory, 1939), infrared spectroscopy (Mijović *et al.*, 1996), ultraviolet spectroscopy (Chai *et al.*, 2006), or nuclear magnetic resonance spectroscopy (Perrier & Haddleton, 2003). Further approaches focus on the analysis of macroradicals during radical polymerization by electron spin resonance spectroscopy (Bresler *et al.*, 1974) or continuous monitoring of reaction products by mass spectrometry (Gruending *et al.*, 2010). But these conventional techniques give only limited information on mechanical properties of the polymer. Viscosimetry-based techniques like the QCM are performing excellently in this field. In the past two decades, the QCM has been used a couple of times to monitor the fabrication of polymers in general (Moya *et al.*, 2005; He *et al.*, 2007) and hydrogels in particular (Åsberg *et al.*, 2005; Criado-Gonzalez *et al.*, 2019). The advantage of the QCM compared to conventional techniques is its capability for real-time monitoring of every type of polymer while providing quantitative information in terms of mass deposition and especially energy dissipation. This energy dissipation is expressed in several different QCM parameters and is closely related to mechanical properties like the Young's modulus and the complex shear modulus of the quartz loading leading to the possibility to establish mathematical correlations between them. In this section, polyacrylamide (PAAm) and polyethylene glycol (PEG) hydrogels, prepared according to sections 4.3.2 and 4.3.3, respectively, were mechanically characterized by compression testing (see section 4.4.1) and rheology (see section 4.4.2) to determine their Young's modulus E (see section 3.3.1) and complex shear modulus G^* (see section 3.3.2), respectively. Results were compared to QCM measurements (see section 4.6) performed during their polymerization. There are three reasons why this was done:

(1) Beside its mass-sensitive nature, the QCM was reported several times as a tool able to reveal mechanical and viscoelastic properties of the load material (Bandey *et al.*, 2000; Johannsmann, 2007; Saitakis & Gizeli, 2012). Thus, it was investigated if this can be confirmed here, too, and it was figured out which QCM parameters are influenced and to what extent. The QCM parameters under study were the minimal impedance $|Z|_{min}$, the load impedance $\Delta|Z|_{Load}$, the half-width-half-maximum of maximal conductance Γ , the frequency of maximal conductance $f(G_{max})$, the real part of the load impedance $Re(\underline{Z}_{Load})$, and the imaginary part of the load impedance $Im(\underline{Z}_{Load})$ (description of these parameters see section 4.6.2). These QCM parameters were correlated with the Young's

modulus and with the complex shear modulus for hydrogels. The synthetic hydrogels PAAm and PEG are perfectly suited for this purpose because their stiffness is easily tunable by varying the concentration of the monomers and their mechanical properties are easy to quantify by determination of the Young's modulus E and complex shear modulus G^* .

(2) As already mentioned in section 1.4, hydrogels and cells show similarities in terms of mechanical and viscoelastic properties, thus, hydrogels can be used as a simplified model for cells with the advantage that they can easily be analyzed by compression testing and rheology. When cells are measured by the QCM, there is no precise model available that is able to correlate the QCM signal change with individual cellular structures. Thus, an empirical approximation was established based on hydrogels modeling the elastic and viscoelastic properties of the cells. The correlations between mechanical properties and QCM parameters were transferred from hydrogels to cells to determine their elastic and viscoelastic properties directly from QCM data and without performing mechanical measurements on the cells like compression testing, atomic force microscopy (AFM), and rheology. In particular, PAAm hydrogels were used to establish correlations between the Young's modulus E and QCM parameters, which were then used to determine the Young's modulus of cells only measured by the QCM (section 5.1). In addition, equations correlating the complex shear modulus G^* with QCM parameters found by Bandey *et al.* (Bandey *et al.*, 2000) were tested for PAAm and PEG hydrogels and applied to cells only measured by the QCM to determine their complex shear modulus (section 5.2). This hydrogel model can maybe help gain additional information on the cellular origin of signal changes when cells are analyzed by QCM readings.

(3) Since it is evident that there is a correlation between QCM parameters and mechanical properties, HTM-N cells were analyzed in this thesis by QCM readings to assess the increased stiffness of the human TM in glaucoma and its decrease in related treatment strategies. But these properties are better described by established parameters like the Young's modulus E than by frequency or impedance changes.

5.1 Correlation of the Young's Modulus with QCM Parameters

The Young's modulus E is a measure to describe elastic properties of a material like its stiffness and is described in detail in section 3.3.1. Whereas the Young's modulus of common elastic and viscoelastic materials is typically measured by compression testing (see section 4.4.1), for cells it is typically obtained by AFM. Additional to topographic scanning of the sample by using a cantilever with a tip as a probe, the AFM allows measuring the forces between probe and sample revealing the sample's Young's modulus. There are many studies published reporting on the determination of the Young's modulus of cells by AFM measurements. Some of them are mentioned further down to compare the results obtained

in this thesis. The advantage of AFM to determine the Young's modulus of single cells and even at specific spots on the plasma membrane turned out to be a disadvantage for the purpose of this thesis because it was about the determination of an average stiffness of the entire cell layer, for example, reflecting the stiffness of the TM when HTM-N cells are analyzed. Thus, a simple and cost-effective approach was established to circumvent expensive AFM measurements and to determine the Young's modulus of cells based on QCM measurements and hydrogels. The hydrogels were used as a model system for the cells, tailored to have similar mechanical and viscoelastic properties. To be more precise, QCM measurements give information on the viscoelastic properties of the load, whereas the Young's modulus describes its elastic properties. Another problem arises from the different directions of distortion in QCM measurements and measurements for the determination of the Young's modulus. While in QCM measurements, the direction of oscillation of the shear wave occurs in horizontal direction, in AFM and compression testing, the material deformation occurs in vertical direction. A further difference between QCM and AFM is their different detection ranges within the cells. The QCM is mainly sensitive to mechanical changes close to the basal membrane, while the AFM mainly measures the apical membrane. Furthermore, the hydrogels used in this study are isotropic materials and cells are not due to their complex architecture. These problems are most probably the causes why no precise correlations between QCM parameters and the Young's modulus have been found by previous studies yet. Nevertheless, in this thesis, certain QCM parameters and the Young's modulus were just tried to be correlated empirically. Whereas both PAAm and PEG hydrogels were used for the correlation of QCM parameters with the complex shear modulus (see section 5.2), in this section, only PAAm hydrogels (prepared according to section 4.3.2) were used for the correlation with the Young's modulus since compression testing was not possible for PEG hydrogels. Both cells and PAAm hydrogels were measured on the QCM, while only the PAAm hydrogels were examined by compression testing to obtain their Young's modulus. Correlations between the Young's modulus and certain QCM parameters were established for the PAAm hydrogels (section 5.1.2), which were then transferred to a variety of cells for calculation of their Young's modulus from QCM data (section 5.1.3). But, at first, a few preliminary tests on PAAm hydrogels regarding temperature, gel layer thickness, and gel swelling in water were performed (section 5.1.1).

5.1.1 Preliminary Tests on Polyacrylamide (PAAm) Hydrogels

Influence of Temperature

The aim was to establish correlations between the Young's modulus E and QCM parameters for PAAm hydrogels and to transfer these correlations to cells only measured by the QCM to determine their Young's modulus. But the determination of the Young's modulus of the PAAm hydrogels by compression testing (see section 4.4.1) could only be performed at RT,

whereas the QCM measurements on cells were conducted at a physiologically relevant temperature of 37 °C. Thus, it was crucial to investigate if the stiffness is influenced by this temperature difference and if yes, which correction factor has to be implemented for compensation. To examine that, the first QCM experiment on PAAm hydrogels included a temperature change from RT to 37 °C while monitoring the stiffness of the PAAm hydrogels after this temperature change (Figure 5.1). The influence of temperature was not investigated during hydrogel polymerization, although a faster polymerization was assumed for higher temperature according to the Arrhenius equation (Arrhenius, 1889), which was also proven in another study (Gelfi & Righetti, 1981*b*). Since the determination of the Young's modulus by compression testing was only possible for PAAm hydrogels after polymerization was completed, the influence of temperature on the stiffness of the PAAm hydrogels was only investigated after their polymerization. The time-resolved nature of the QCM enables a real-time determination of the endpoint of polymerization during the measurement, facilitating an online selection of the time point to apply a temperature change and to analyze its effect on the stiffness of PAAm hydrogels after polymerization. The polymerization time was determined to about 60 min (see further down), and accordingly, the time point of temperature change from RT to 37 °C was performed about 100 min after the addition of the gel mixtures. For monitoring the stiffness of the PAAm hydrogels, many different QCM parameters can be used (see section 5.1.2), but the minimal impedance $|Z|_{min}$ turned out to be a good choice with reliable results. Thus, the change of the minimal impedance $\Delta|Z|_{min}$ was plotted over time with normalization to the time point of gel addition (Figure 5.1 A) and with normalization to the time point of temperature change (Figure 5.1 B). Hence, both parts of Figure 5.1 show one and the same experiment, wherein Figure 5.1 A focuses on the hydrogel polymerization before temperature change and Figure 5.1 B focuses on a potential stiffness change of the PAAm hydrogels after temperature change. This temperature change from RT to 37 °C was performed for half of the measurements (Figure 5.1, red curve), whereas the other half served as a control and remained at RT (Figure 5.1, black curve), both of them shown as mean out of three measurements. The influence of temperature on the stiffness of the PAAm hydrogels was not investigated for all of the used PAAm hydrogel types A – E (see Table 4.8), but instead, only for PAAm gel C due to its average composition and stiffness. The standard hydrogel layer thickness of 2 mm was used.

The first thing, which is conspicuous in Figure 5.1 A and Figure 5.1 B, is the huge degree of signal fluctuations and the relatively high standard deviation at some points of the measurement (for example, at about 75 min for the red curve in Figure 5.1 A). But this is due to the very high sensitivity of the QCM technique and is unproblematic as long as the overall trend of the signal is not distorted. In this specific case at about 75 min, one of the three averaged curves exhibited a few measurement points, which were way too high due to unknown reasons, leading to a few points with enhanced values in the average curve with a high standard deviation. But because the signal decreased again to a plateau value, similar to the black curve's plateau value, this temporary signal increase has been ignored. This is the

reason why averaging was always performed and signal changes with high standard deviations have to be handled with care.

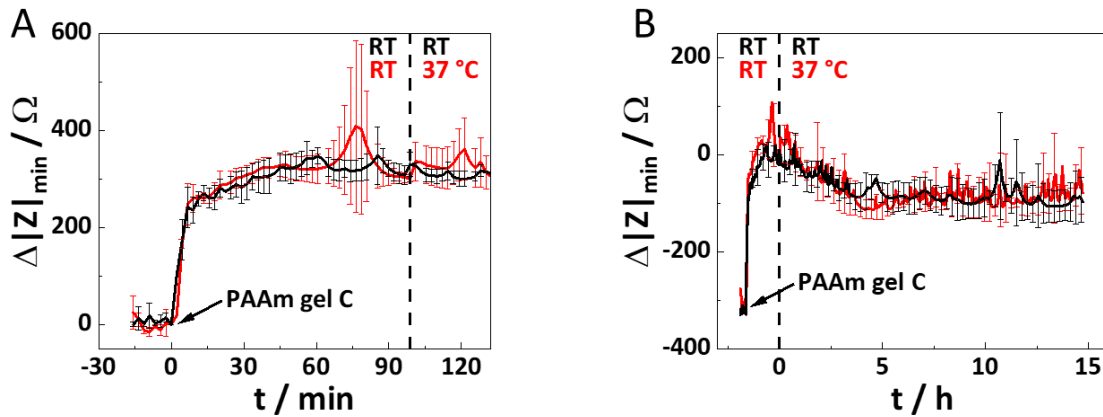


Figure 5.1 Preliminary QCM measurements on PAAm hydrogels regarding the influence of temperature on their stiffness. PAAm gel C was used reflecting an average composition and stiffness with the standard hydrogel layer thickness of 2 mm. The change of the minimal impedance $\Delta|Z|_{min}$ was plotted over time during polymerization at room temperature (RT), followed by a temperature change to 37 °C for the red curve, while the black curve shows data of gels not undergoing this temperature change. Data are presented as mean $\pm$ SD with $n = 3$, while both diagrams show the same data set, but with different normalization and time scales. **(A)** Normalization to the time point of gel addition and focus on the polymerization. The time point of temperature change is at about 100 min. **(B)** Normalization to the time point of temperature change and focus on the behavior of the gels after polymerization.

After baseline recording and addition of the PAAm hydrogel mixtures, the minimal impedance $|Z|_{min}$ showed an increase reflecting a stiffness increase during polymerization (Figure 5.1 A). This stiffness increase during hydrogel polymerization was already detected before by QCM measurements, namely by monitoring resonance frequency and dissipation of hydrogels of a different kind (Nunalee *et al.*, 2006; Criado-Gonzalez *et al.*, 2019). Both the black and red curve in Figure 5.1 A showed very similar polymerization behavior, which was, of course, expected because both curves were subject to the same conditions before temperature change. In detail, the minimal impedance $|Z|_{min}$ showed a fast initial increase of about 250 Ω within the first 10 min, which was most probably due to the instant viscosity increase by replacing the water by the hydrogel mixture, and due to surface polymerization. This was followed by a second but much slower increase of about 70 Ω until plateau values of about 320 Ω were reached at the time of about 60 min after gel addition, which seemed to be due to bulk polymerization. After that, the minimal impedance showed some fluctuations, but it stayed on average at about the same value. Thus, the polymerization of the PAAm hydrogels was completed after about 60 min. A detailed discussion on polymerization time is conducted further down in section 5.1.2. According to the duration of PAAm polymerization of about 60 min determined in Figure 5.1 A, the temperature change from RT to 37 °C for the red curve was performed about 100 min after polymerization start. In Figure 5.1 B, a slow decrease in the minimal impedance $|Z|_{min}$ of about 100 Ω was

observed within the first 5 h after temperature change indicating a stiffness decrease. But this decrease was obtained equally for both the black and red curves, demonstrating that the stiffness decrease was not caused by the temperature change itself, but instead, by some unspecific processes like equilibration of the hydrogels after their polymerization. After this decrease, the minimal impedance exhibited relatively constant values for the next 10 h. Thus, an effect of this temperature change from RT to 37 °C on the minimal impedance of PAAm hydrogels after their polymerization was not observed leading to the conclusion that PAAm gel stiffness was not influenced by the temperature within this specific range. This finding was then utilized for the correlation between hydrogels and cells. For the sake of convenience, all the following QCM measurements on PAAm hydrogels were performed at room temperature, with still having the possibility to correlate to cells, which were measured at 37 °C.

Influence of Hydrogel Layer Thickness

In section 5.2, it was the aim to correlate the complex shear modulus G^* with QCM parameters for hydrogels (PAAm and PEG) and to transfer these correlations to cells only measured by the QCM to determine their complex shear modulus. For this purpose, Eq. (63) and Eq. (64) found by Bandey *et al.* were applied. But for these equations to be applicable, the viscoelastic load on the QCM has to fulfill the semi-infinite condition, thus, the thickness of the hydrogel layer had to be much bigger than the decay length of the shear wave. Hence, the decay length of the shear wave into both PAAm and PEG hydrogels was determined first, which was achieved by using Eq. (32). The dynamic viscosity η'_L of the hydrogels was calculated by $\frac{G''}{\omega}$ according to Eq. (62), while the loss modulus G'' was obtained directly from rheological measurements and $\omega = 2\pi f$ with distortion frequency f of 1 Hz. The difference in frequency of rheological measurements (1 Hz) and QCM measurements (5 MHz) is discussed in section 5.2. The density ρ_L of the hydrogels was calculated by $\frac{m}{V}$, while their mass m was weighed directly after polymerization and the dimensions of the cylindrical hydrogels were measured with a caliper revealing their volume V . For both PAAm gels A – E and PEG gels A – B, the dynamic viscosity η'_L was obtained to be strongly dependent on gel stiffness, with the highest dynamic viscosity for the stiffest gels (PAAm gel A and PEG gel A). Due to the finding that the density ρ_L was more or less independent of gel stiffness for one and the same hydrogel type, the decay length was maximal for the stiffest gels and was calculated for PAAm gel A to 18 μm ($\eta'_L = 5.46 \text{ Pa} \cdot \text{s}$, $\rho_L = 1.05 \cdot 10^3 \frac{\text{kg}}{\text{m}^3}$) and for PEG gel A to 25 μm ($\eta'_L = 5.66 \text{ Pa} \cdot \text{s}$, $\rho_L = 0.58 \cdot 10^3 \frac{\text{kg}}{\text{m}^3}$). Thus, hydrogels of at least 1 mm thickness were used in QCM measurements to ensure fulfilling the semi-infinite condition. To examine, if the hydrogel layer thickness influences their stiffness measured by the QCM within the semi-infinite case, PAAm gels of different thicknesses ranging from 1 mm to 5 mm were used. PAAm gel C was used due to its average composition and stiffness, while the change of the minimal impedance $\Delta|Z|_{\min}$ was measured during polymerization, normalized to the time

point of gel addition (Figure 5.2). The hydrogel was polymerized with a layer thickness of 1 mm (Figure 5.2, black curve), 2 mm (Figure 5.2, red curve), 3 mm (Figure 5.2, green curve), and 5 mm (Figure 5.2, blue curve), presented as mean out of 3 – 4 measurements.

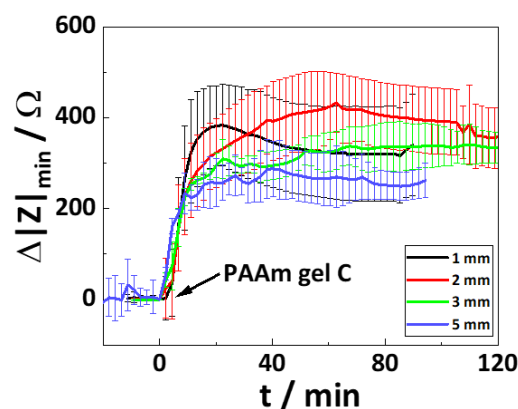


Figure 5.2 Preliminary QCM measurements on PAAm hydrogels regarding the influence of hydrogel layer thickness on their stiffness. PAAm gel C was used reflecting an average composition and stiffness. The change of the minimal impedance $\Delta|Z|_{min}$ was plotted over time during polymerization, while different gel layer thicknesses were investigated: 1 mm (black), 2 mm (red), 3 mm (green), and 5 mm (blue). Data are presented as mean $\pm$ SD with $n = 3 - 4$, with normalization to the time point of gel addition. $T = RT$.

After addition of the PAAm hydrogel mixtures, the minimal impedance $|Z|_{min}$ showed an increase for all the thicknesses investigated (Figure 5.2). But the time course of minimal impedance seemed to be slightly dependent on the hydrogel layer thickness. For the thickness of 1 mm (black curve), the minimal impedance reached a maximum after about 20 min, decreased afterwards and equilibrated finally at a value of $\Delta|Z|_{min}$ of about 320 Ω after 60 – 80 min. The other hydrogel layer thicknesses of 2 mm (red curve), 3 mm (green curve), and 5 mm (blue curve) exhibited a different time course of minimal impedance as compared to the thickness of 1 mm, but a similar time course as compared to each other. Here, a fast initial increase in the minimal impedance within the first 10 min was followed by a slower increase until plateau values were reached after 60 – 80 min. This was similar to the time course of minimal impedance obtained in Figure 5.1 and can be attributed to the distinction between surface polymerization and bulk polymerization. Within these three thicknesses (2 mm, 3 mm, and 5 mm) plateau values differed between 250 Ω and 400 Ω , while a weak trend between plateau value and hydrogel layer thickness was obtained, with the thickest gel exhibiting the lowest plateau value. But these differences were not statistically significant due to overlapping error bars. The different time course of minimal impedance for the thickness of 1 mm indicated faster polymerization kinetics with no clear distinction between surface polymerization and bulk polymerization. Thus, polymerizations of PAAm hydrogels with even smaller thickness can exhibit an even shorter polymerization time, as it was obtained in rheological measurements with the hydrogel layer thickness of 0.5 mm (see Figure 5.7). But, by comparing all four investigated gel thicknesses, overlapping

error bars and no trend in plateau values were observed leading to the conclusion that the hydrogel layer thickness can influence polymerization time, but not gel stiffness in a measurable way. According to this result of the layer thickness being no decisive factor for hydrogel stiffness, a constant thickness of 2 mm was chosen for all the following QCM measurements on PAAm and PEG hydrogels. Consequently, correlations between these QCM measurements with hydrogel layer thickness of 2 mm and rheological measurements with hydrogel layer thickness of 0.5 mm were possible without any problems. Additionally, correlations between QCM measurements and compression testing were easily possible without regarding a potential difference in hydrogel layer thickness, which was then adjusted to 7 mm in compression testing measurements due to better handling of the hydrogels.

Gel Swelling in Water

The determination of the Young's modulus E of PAAm hydrogels by compression testing required gels with completed polymerization. Since these measurements on the hydrogels could sometimes not be performed instantly after their polymerization due to timing reasons, they had to be stored for a certain time. In order not to dry out, the hydrogels were stored in dd-H₂O at RT for this time. But a change of their stiffness by swelling in water was an important issue then because swelling in aqueous solutions has already been reported manifold in the literature for hydrogels in general (Wang *et al.*, 2003; Åsberg *et al.*, 2005; Sadman *et al.*, 2018) and PAAm hydrogels in particular (Boyde, 1976; Tanaka & Fillmore, 1979; Peng *et al.*, 2009). Thus, swelling of PAAm hydrogels in dd-H₂O and a potential associated stiffness decrease was investigated by QCM measurements (Figure 5.3). PAAm gel A was used for these measurements, which was expected to exhibit the greatest potential for swelling due to its highest osmotic gradient relative to dd-H₂O. Stiffness changes mirrored by the change of the minimal impedance $\Delta|Z|_{min}$ (Figure 5.3 A) and mass deposition mirrored by the change of the frequency of maximal conductance $\Delta f(G_{max})$ (Figure 5.3 B) were monitored in real time during PAAm polymerization and subsequent addition of dd-H₂O. The dd-H₂O was added about 2 h after polymerization start, ensuring hydrogels with completed polymerization according to Figure 5.1 and Figure 5.2. Both QCM parameters were extracted from the same measurements and presented as mean out of two individual measurements with normalization to the polymerization start.

As already obtained before in Figure 5.1 and Figure 5.2, the minimal impedance $|Z|_{min}$ showed an increase after addition of the hydrogel mixture, reflecting a stiffness increase during polymerization (Figure 5.3 A). The time course of minimal impedance change during polymerization of PAAm gel A (Figure 5.3 A, zoom-in) seemed to be similar to PAAm gel C obtained in previous measurements (see Figure 5.1 and Figure 5.2), indicating similar polymerization kinetics for PAAm gel A and C. But, the higher monomer and crosslinker concentrations of PAAm gel A compared to PAAm gel C (see Table 4.8) led to the expectation

of a higher stiffness and, accordingly, a bigger increase in minimal impedance. This was confirmed here by the increase of $\Delta|Z|_{min}$ of about 700 Ω for PAAm gel A, which was much greater as compared to the 250 Ω –400 Ω for PAAm gel C observed in previous measurements. This will be analyzed in detail in section 5.1.2 by measuring all five PAAm gel types A – E.

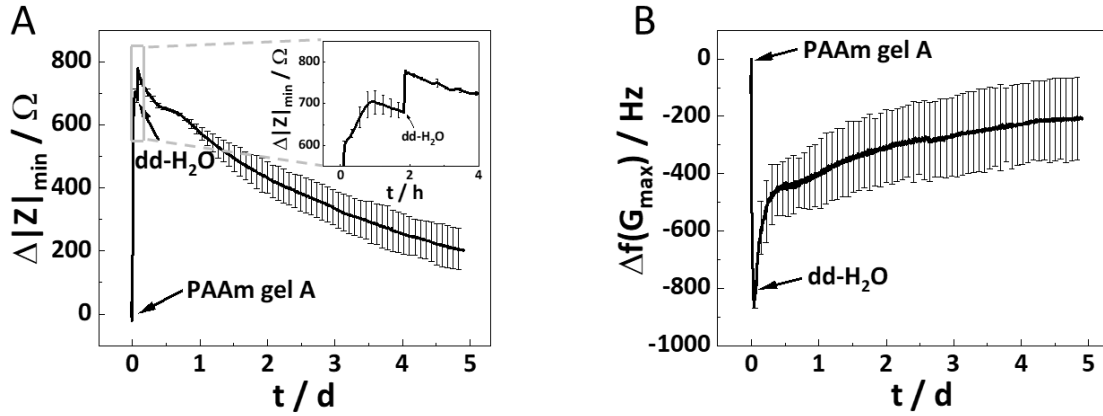


Figure 5.3 Preliminary QCM measurements on PAAm hydrogels regarding the influence of gel swelling in water. PAAm gel A was used due to its highest osmotic gradient relative to dd-H₂O having the greatest potential of swelling. **(A)** The change of the minimal impedance $\Delta|Z|_{min}$, reflecting gel stiffness, with included zoom-in on the first 4 h after polymerization start, and **(B)** the change of the frequency of maximal conductance $\Delta f(G_{max})$, reflecting mass deposition, were plotted over time during initial polymerization (about 2 h) and subsequent addition of dd-H₂O to monitor the swelling behavior of PAAm hydrogels for several days. Both parameters were extracted from the same measurements and data are presented as mean $\pm$ SD with $n = 2$, with normalization to the time point of gel addition. $T = RT$.

An instant increase in the minimal impedance of about 100 Ω was observed upon addition of dd-H₂O (Figure 5.3 A, zoom-in), but this was more like a random signal change by disturbance of the measurement because there is no reasonable correlation between water addition and stiffness increase. But after that, the minimal impedance was observed to decrease gradually by almost 600 Ω within 5 d of contact between PAAm gels and dd-H₂O (Figure 5.3 A), indicating a stiffness decrease by gel swelling in water. A more detailed view on swelling was facilitated by additionally analyzing the frequency of maximal conductance $f(G_{max})$ (Figure 5.3 B). During PAAm polymerization and subsequent addition of dd-H₂O, this QCM parameter showed an inverted signal change as compared to the minimal impedance, since the frequency of maximal conductance was observed to decrease during polymerization indicating a mass increase, and to increase gradually after addition of dd-H₂O indicating a mass decrease. This result was strikingly different to the findings of Wang *et al.*, who reported a decrease in the frequency of maximal conductance $f(G_{max})$ during swelling of poly(*N*-isopropylacrylamide) gels, indicating a mass increase (Wang *et al.*, 2003). Even though these two cases were not perfectly comparable due to slightly different hydrogel types used, these contrary results were not expected. A reason for this difference was then assumed to be the hydrogel layer thickness. Wang *et al.* used very thin hydrogel layers of

50 nm – 250 nm thickness (achieved by spin coating) leading to the so-called finite thickness case because the layer thickness was smaller than the decay length of the shear wave (18 μm , as calculated above in: *Influence of Hydrogel Layer Thickness*). Upon swelling in water, additional mass was detected there by the QCM in the range of the decay length explaining the frequency decrease. Here, the semi-infinite case was valid because the hydrogel layer thickness (2 mm) was much bigger than the decay length leading to the point that no additional mass was detected in the range of the decay length. But instead, hydrogel swelling leads to a density decrease, which explains the detection of less mass in the range of the decay length and the concomitant frequency increase. This, together with the observed decrease in the minimal impedance, confirmed PAAm gel swelling after addition of dd-H₂O over a period of time of 5 d (Figure 5.3). Because no plateau values were reached after this time for both parameters ($|Z|_{min}$ and $f(G_{max})$), the swelling process was maybe even not completed. Since both parameters exhibited signal changes (decrease for $|Z|_{min}$, increase for $f(G_{max})$) beginning immediately after addition of dd-H₂O, the swelling of the hydrogels started instantly without any delay. Another observation was the different time courses of signal change after addition of dd-H₂O for the two parameters under study. The frequency of maximal conductance underwent a fast change within the first 12 h followed by a relatively slow signal change (Figure 5.3 B), whereas the minimal impedance showed a rather constant signal change over the complete time of swelling (Figure 5.3 A). This leads to the conclusion that the mass decrease associated with hydrogel swelling is especially pronounced in the beginning of the swelling process, whereas a decrease in stiffness is observed uniformly during swelling. These results obtained here in this section were then utilized and applied to the practical implementation of PAAm gel storage in water before compression testing. But one further difference between these QCM measurements in Teflon® QCM chambers and immersing the hydrogels in Petri dishes filled with dd-H₂O has to be considered. When a hydrogel polymerization mixture is polymerizing in a Teflon® QCM chamber, the hydrogel is constantly in contact with its substrate, which is the quartz disk, and laterally with the wall of the QCM chamber, unless considerable volume changes or intense gas formation occur, which is both not the case for PAAm hydrogels. This leads to the fact that only the upper face of the hydrogel is able to get in touch with the water added on top, when swelling of the gel is investigated by QCM measurements. In contrast to that, immersing a hydrogel in water in a Petri dish enables the water to enter the gel from all sides, probably leading to an even faster swelling than it was observed in Figure 5.3. A relatively fast swelling of PAAm hydrogels immersed in surrounding water was already demonstrated before in literature, for example when the PAAm gel diameter, which was measured under a microscope, reached a plateau value after about 1 d (Tanaka & Fillmore, 1979). But of course, several other factors like the gel dimensions and temperature are also important for the gel swelling kinetics. As a result of these investigations and considerations, the storage time of the PAAm gels in water before compression testing was reduced as much as possible (< 1 h) to ensure measuring the Young's modulus of gels, that were pretty much freshly prepared and did not undergo swelling to a significant extent.

5.1.2 Establishing Correlations Between the Young's Modulus and Different QCM Parameters for Polyacrylamide (PAAm) Hydrogels

After an influence of temperature and an influence of the hydrogel layer thickness on gel stiffness (as long as the semi-infinite condition is fulfilled) were excluded by preliminary tests (see section 5.1.1), it was the aim to calculate the Young's modulus E for cells from QCM data by correlations between them, which is shown in section 5.1.3. Here, it is demonstrated how these correlations between the Young's modulus and certain QCM parameters were established using PAAm hydrogel data as a type of calibration, which can then be transferred to cells. Four different QCM parameters were selected for correlation with the Young's modulus of PAAm hydrogels. The minimal impedance $|Z|_{min}$ was chosen for this purpose because it was already demonstrated to be robust and highly sensitive to stiffness changes (see section 5.1.1). But to try further ways of correlation, which maybe give even better results for the Young's modulus of cells compared to literature data, two further QCM parameters reflecting the stiffness of the load, were examined, which were the load impedance $\Delta|Z|_{Load}$ and the half-width-half-maximum of maximal conductance Γ . Additionally, the frequency of maximal conductance $f(G_{max})$ as a parameter describing mass deposition was investigated for this correlation. QCM measurements were performed during polymerization of PAAm hydrogels, while extracting these four QCM parameters from the same measurements and presenting them with normalization to the polymerization start (Figure 5.4). $\Delta|Z|_{min}$ is plotted in Figure 5.4 A, $\Delta\Delta|Z|_{Load}$ in Figure 5.4 B, $\Delta\Gamma$ in Figure 5.4 C, and $\Delta f(G_{max})$ in Figure 5.4 D. Five different PAAm hydrogel types with a broad range of different compositions (see Table 4.8) were used in order to obtain a broad range of varying hydrogel stiffness, and accordingly a good distribution of data points to ensure reliable correlations. PAAm gel A (Figure 5.4, black curves), PAAm gel B (Figure 5.4, red curves), PAAm gel C (Figure 5.4, green curves), PAAm gel D (Figure 5.4, blue curves), and PAAm gel E (Figure 5.4, cyan curves) were analyzed by means of the four different QCM parameters. Data are presented as mean out of several individual measurements, while PAAm gel C was the standard gel composition measured several times more often than the other gel types, leading to the number of replicates n varying significantly among the PAAm gel types. Additionally, the number of replicates n differed slightly for each QCM parameter because measurements with huge outliers were eliminated before averaging, which was not equally pronounced for each parameter. According to the duration of polymerization of about 60 min obtained for PAAm gel C (see Figure 5.1 and Figure 5.2) and PAAm gel A (see Figure 5.3), the polymerization of all PAAm hydrogel types was followed for at least 90 min.

Similar to QCM measurements shown before (see Figure 5.1, Figure 5.2, and Figure 5.3 A), here, the minimal impedance $|Z|_{min}$ was also observed to increase after addition of the PAAm hydrogel mixtures demonstrating the stiffness increase during polymerization (Figure 5.4 A). Similarly, an increase was observed for the load impedance $\Delta|Z|_{Load}$ (Figure 5.4 B) and the half-width-half-maximum of maximal conductance Γ (Figure 5.4 C), also proving the

stiffness increase during hydrogel polymerization. A clear dependency of these three QCM parameters on gel type and associated stiffness was observed because decreasing the stiffness in the order from PAAm gel A (black curves), over PAAm gel B (red curves), PAAm gel C (green curves), and PAAm gel D (blue curves) to PAAm gel E (cyan curves) resulted in a stepwise smaller increase in these three parameters. Due to very similar time courses of the QCM parameters minimal impedance $|Z|_{min}$, load impedance $\Delta|Z|_{Load}$, and half-width-half-maximum of maximal conductance Γ , these parameters met the expectation of showing almost equal behavior regarding the stiffness of the load, while the load impedance turned out to be slightly less robust due to some signal fluctuations and a higher standard deviation.

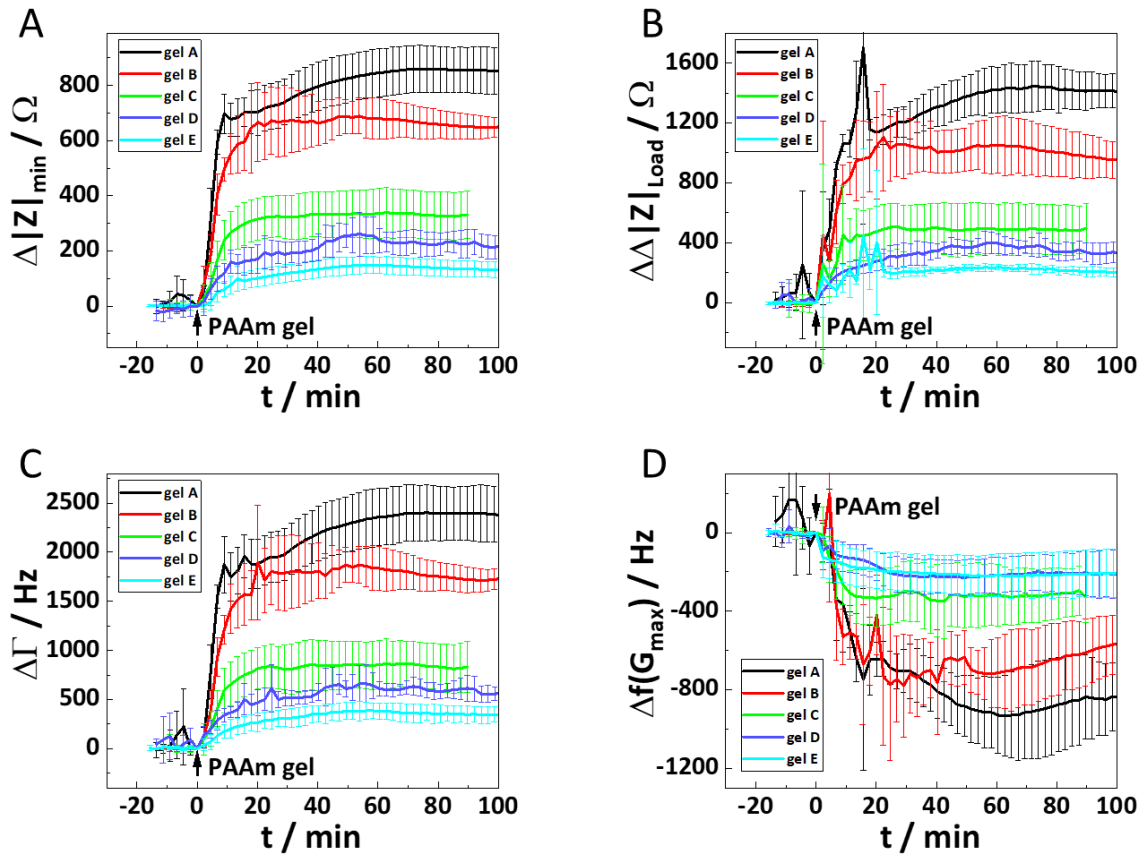


Figure 5.4 QCM measurements during polymerization of PAAm hydrogels for their characterization by means of the change of the minimal impedance $\Delta|Z|_{min}$ (A), the change of the load impedance $\Delta\Delta|Z|_{Load}$ (B), the change of the half-width-half-maximum of maximal conductance $\Delta\Gamma$ (C), and the change of the frequency of maximal conductance $\Delta f(G_{max})$ (D). Five differently composed PAAm hydrogel types were used, PAAm gel A (black), PAAm gel B (red), PAAm gel C (green), PAAm gel D (blue), and PAAm gel E (cyan), with the exact compositions of the gels given in Table 4.8. All the QCM parameters were extracted from the same measurements and were normalized to the time point of gel addition. Data are displayed as mean $\pm$ SD with the number of replicates n varying for each QCM parameter due to a different number of outlier curves eliminated ($\Delta|Z|_{min}$: $n = 4 - 15$, $\Delta\Delta|Z|_{Load}$: $n = 4 - 14$, $\Delta\Gamma$: $n = 3 - 15$, $\Delta f(G_{max})$: $n = 3 - 12$). The range of n was very huge for each parameter because PAAm gel C was measured several times more often than the other gel types. $T = \text{RT}$.

As it was already observed in Figure 5.3, the frequency of maximal conductance $f(G_{max})$ behaved differently (Figure 5.4 D). It decreased during polymerization indicating a mass increase within the detection volume of the decay length. The dependency on gel type was less pronounced due to partially overlapping error bars and PAAm gels D and E exhibiting an almost equal frequency change. With respect to the inverted time course for the frequency of maximal conductance $f(G_{max})$ in comparison to the other investigated QCM parameters, the signal change over time during polymerization seemed to be very similar. In contrast to the faster response of the frequency of maximal conductance $f(G_{max})$ as compared to the minimal impedance $|Z|_{min}$, obtained in the investigation of gel swelling in water (see Figure 5.3), here, the similar time course of signal change for all four investigated QCM parameters indicated that polymerization kinetics is equally expressed in changes of stiffness and mass deposition. But, the two-step time course of signal change during polymerization, which was observed for the minimal impedance in most of the previous measurements and attributed to the distinction between surface polymerization and bulk polymerization, was only observed for PAAm gel A here. As a result, this was no longer regarded as an important feature of PAAm gel polymerization, since it can obviously be masked by averaging over multiple time courses. Examination of polymerization time by the time point of reaching plateau values in Figure 5.4 yielded results that were dependent on the PAAm gel type, but independent of the QCM parameter investigated. A polymerization time of about 70 min was found for PAAm gel A (Figure 5.4, black curves), about 20 min for PAAm gel B (Figure 5.4, red curves), about 30 min for PAAm gel C (Figure 5.4, green curves), and about 60 min for PAAm gel D (Figure 5.4, blue curves) and PAAm gel E (Figure 5.4, cyan curves). This revealed that monomer concentration and associated stiffness did not clearly correlate with the duration of polymerization. The polymerization duration of PAAm hydrogels has been studied several times before in the literature, where many different results can be found. For example, a PAAm hydrogel similar to the PAAm gel A used in this thesis exhibited a polymerization time of about 45 min (McAllister *et al.*, 1963), while a similar polymerization time of 50 min was found for a PAAm hydrogel similar to the PAAm gel B used in this thesis (Peng *et al.*, 2009). On the other hand, a PAAm hydrogel similar to the PAAm gel D used in this thesis showed a duration of polymerization of only 15 min, with an additional influence of crosslinker concentration (Gelfi & Righetti, 1981a). By comparing these literature data to each other, an influence of monomer concentration on the polymerization time of PAAm hydrogels can be assumed, although this has not been investigated yet in one comprehensive study. Additionally, further studies revealed the dependency of the polymerization time of PAAm hydrogels on the temperature (Gelfi & Righetti, 1981b) and on the type of catalyst used (Righetti *et al.*, 1981). With the hydrogel layer thickness, another factor influencing the duration of PAAm polymerization was identified in this thesis before (see above at: *Influence of Hydrogel Layer Thickness*). Thus, regarding all these experimental conditions possibly influencing polymerization time, a statement on the dependency of PAAm polymerization time on monomer concentration can only be made, if a study is performed, where these conditions are kept constant, as it was the case here. Hence, the results regarding polymerization time obtained in Figure 5.4 showed that monomer

concentration can influence the polymerization time of PAAm hydrogels, but there is no clear dependency between both parameters. The crucial aspect of the examination of polymerization time was to obtain hydrogels with completed polymerization, which is achieved after about 70 min for all PAAm hydrogel types since all measurements have reached a plateau value there. More important than polymerization time was the stiffness of the PAAm hydrogels mirrored in the change of the QCM parameters. Although the frequency of maximal conductance $f(G_{max})$ actually reflects mass deposition, a dependency of this parameter on stiffness was also obtained, except for PAAm gel E, which exhibited an almost equal frequency change as compared to PAAm gel D. Thus, this QCM parameter was also used for correlation with the Young's modulus E . To obtain QCM parameters that can be correlated with the Young's modulus of PAAm hydrogels, they had to be extracted at one specific time point from the time-resolved measurements shown in Figure 5.4. The exact selection of the time point was not that crucial because the dependency of the parameters on the monomer concentration was observed over the complete measurement time. In some of the QCM measurements shown before (see Figure 5.1 B and black and red curve in Figure 5.4 D), signal changes were observed after reaching the plateau value indicating equilibration processes of the hydrogels after their polymerization. But this was no problem as long as the QCM parameters and the Young's modulus were taken after the same period of time. Thus, the time point of 90 min after addition of the PAAm gel mixtures was chosen for the QCM parameter extraction and the determination of the Young's modulus. According to the result of the examination of polymerization time, this time point ensured the polymerizations to be definitely completed. These values of the minimal impedance $|Z|_{min}$, load impedance $\Delta|Z|_{Load}$, half-width-half-maximum of maximal conductance Γ , and frequency of maximal conductance $f(G_{max})$ for all the PAAm gel types A – E taken from the QCM measurements (Figure 5.4) after 90 min of polymerization are summarized in Table 5.1. They are given as delta values with respect to the polymerization start reflecting stiffness changes during polymerization. According to the QCM measurements given as mean out of several individual measurements, the values extracted after 90 min of polymerization are presented in the same way with the number of replicates n given for each individual value. Additionally, the Young's modulus E was measured for the PAAm gel types A – E by compression testing according to section 4.4.1 and displayed as mean out of five measurements in Table 5.1, too. The polymerization time of the PAAm gels before compression testing was adjusted to 90 min to ensure perfect comparability between QCM parameters and Young's modulus.

By examining the values of the QCM parameters given in Table 5.1 for a dependency on the PAAm gel type, it was observed that all QCM parameters investigated ($|Z|_{min}$, $\Delta|Z|_{Load}$, Γ , and $f(G_{max})$) showed changes after 90 min of polymerization that were strongly dependent on the monomer concentration with a bigger change of the parameters for higher monomer concentration, confirming the result obtained in Figure 5.4. The only exception was PAAm gels D and E exhibiting an almost equal change of the frequency of maximal conductance

$\Delta f(G_{max})$ (Table 5.1). As expected, a higher monomer concentration of the PAAm hydrogels yielded a higher stiffness expressed by a higher Young's modulus E (Table 5.1).

Table 5.1 The change of the minimal impedance $\Delta|Z|_{min}$, the change of the load impedance $\Delta\Delta|Z|_{Load}$, the change of the half-width-half-maximum of maximal conductance $\Delta\Gamma$, and the change of the frequency of maximal conductance $\Delta f(G_{max})$ extracted from the QCM measurements in Figure 5.4 after 90 min of polymerization of PAAm gels A – E. The mass concentration of AAm is given additionally for each PAAm gel type, while the concentrations of the remaining components can be seen in Table 4.8. Their Young's modulus E is also given, which was measured by compression testing of PAAm gels pre-polymerized for 90 min. Values are displayed as mean $\pm$ SD with the number of replicates n given for each individual value depending on QCM parameter and PAAm gel type, while compression testing was performed five times for each gel type.

PAAm gel type	$\Delta Z _{min} / \Omega$	$\Delta\Delta Z _{Load} / \Omega$	$\Delta\Gamma / \text{Hz}$	$\Delta f(G_{max}) / \text{Hz}$	E / kPa
A 15% (w/v) AAm	860 ± 80 ($n = 4$)	1410 ± 130 ($n = 4$)	2390 ± 270 ($n = 4$)	-840 ± 210 ($n = 4$)	299 ± 14 ($n = 5$)
B 12% (w/v) AAm	650 ± 50 ($n = 6$)	980 ± 150 ($n = 6$)	1720 ± 130 ($n = 6$)	-610 ± 170 ($n = 6$)	86 ± 4 ($n = 5$)
C 7.5% (w/v) AAm	330 ± 90 ($n = 15$)	500 ± 160 ($n = 14$)	830 ± 260 ($n = 15$)	-330 ± 130 ($n = 12$)	21 ± 1 ($n = 5$)
D 5% (w/v) AAm	220 ± 50 ($n = 7$)	340 ± 60 ($n = 4$)	620 ± 130 ($n = 5$)	-200 ± 120 ($n = 3$)	3.1 ± 0.4 ($n = 5$)
E 3% (w/v) AAm	140 ± 30 ($n = 4$)	210 ± 30 ($n = 4$)	350 ± 90 ($n = 3$)	-210 ± 120 ($n = 3$)	0.14 ± 0.06 ($n = 5$)

To validate these results for the Young's modulus of PAAm hydrogels, they were compared to literature data. There is no comprehensive study available analyzing the Young's modulus of PAAm hydrogels in the same monomer concentration range. Instead, the results were compared to literature data taken from different sources measured for individual PAAm hydrogels with similar compositions. Compression testing measurements revealed Young's moduli of 60 kPa for a PAAm hydrogel with 10% (w/v) AAm and 220 kPa for a PAAm hydrogel with 20% (w/v) AAm (Schramm-Baxter *et al.*, 2004). Determination of the Young's modulus by hydrogel bending by gravity yielded a similar result of (60 ± 5) kPa for a PAAm hydrogel with 10% (w/v) AAm (Peng *et al.*, 2009). Measurements based on magnetically triggered movements of microneedles embedded inside the hydrogels resulted in Young's moduli of (0.89 ± 0.10) kPa, (1.41 ± 0.18) kPa, and (5.67 ± 0.81) kPa for PAAm hydrogels with 3% (w/v), 4% (w/v), and 5% (w/v) AAm, respectively (Chippada *et al.*, 2010). Compared to these literature data, the Young's modulus values displayed in Table 5.1 were similar, especially in the mid concentration range. But, the value of (299 ± 14) kPa obtained for PAAm gel A (15% (w/v) AAm) was a bit too high and the value of (0.14 ± 0.06) kPa obtained for PAAm gel E (3% (w/v) AAm) was smaller than reported in literature. But with respect to different experimental conditions and different methods used, the results for the Young's modulus obtained in this thesis were in the same order of magnitude as compared to those

literature data. Summarizing Table 5.1, the monomer concentration of the PAAm hydrogels influenced all four QCM parameters and also the Young's modulus, indicating that there might exist a correlation between QCM parameters and Young's modulus. But the monomer concentration seemed to influence the Young's modulus to a bigger extent than the QCM parameters, leading to the assumption that a potential correlation would not be a linear one. To analyze this correlation in detail, the values for each QCM parameter were plotted graphically versus the respective Young's modulus E . Since this correlation is unknown, different plots and data fits were empirically tested, which is demonstrated exemplary for the minimal impedance $|Z|_{min}$ in Figure S1. In detail, the values for the change of the minimal impedance $\Delta|Z|_{min}$ after 90 min of polymerization of the PAAm gels A – E and the respective values of the Young's modulus were taken from Table 5.1 and plotted against each other, while using a linear scale and logarithmic data fit, a semi-logarithmic scale and sigmoidal data fit, and a double-logarithmic scale and linear data fit. While all of these fits matched the data very good, as can be seen by the R-square values close to 1 (see windows in Figure S1), the logarithmic fit on a linear scale and the sigmoidal fit on a semi-logarithmic scale showed inaccuracy and uncertainty due to a too small number of data points in the high-stiffness region, which was expressed by relatively high standard deviations of the fit parameters for these two data fits. Since the linear fit on a double-logarithmic scale demonstrated a good precision due to very small standard deviations of the fit parameters, this fit was chosen for correlation of all the QCM parameters with the Young's modulus, which additionally provides a very easy calculation. This is demonstrated in Figure 5.5 by plotting the change of the minimal impedance $\Delta|Z|_{min}$ (Figure 5.5 A), the change of the load impedance $\Delta\Delta|Z|_{Load}$ (Figure 5.5 B), the change of the half-width-half-maximum of maximal conductance $\Delta\Gamma$ (Figure 5.5 C), and the change of the frequency of maximal conductance $\Delta f(G_{max})$ (Figure 5.5 D) versus the Young's modulus E in a double-logarithmic manner. The values were taken from Table 5.1, representing PAAm hydrogels after 90 min of polymerization, while $\Delta f(G_{max})$ was plotted as negative change and the data point for PAAm gel E was neglected because its change was almost equal to PAAm gel D. Since all the values in Table 5.1 were given as mean $\pm$ SD, they are presented in the same way in Figure 5.5, leading to error bars into both x- and y-dimension with the same number of replicates n as before. The data points (black symbols) were fitted by linear fits (red lines), which yielded the two fit parameters intercept t and slope m , given with standard deviation for each of the four QCM parameters in the small windows added to the respective graphs.

Since the values of the Young's modulus E were found to be almost equidistant on this logarithmic scale (Figure 5.5), the distribution of stiffnesses of the PAAm hydrogels was selected properly. As already obtained in Table 5.1, PAAm hydrogels after 90 min of polymerization exhibited a bigger change of the QCM parameters for gels with higher Young's modulus E (Figure 5.5). This was observed for all four QCM parameters under study ($|Z|_{min}$, $\Delta|Z|_{Load}$, Γ , and $f(G_{max})$), while the correlation with the Young's modulus seemed to be linear for this double-logarithmic scale.

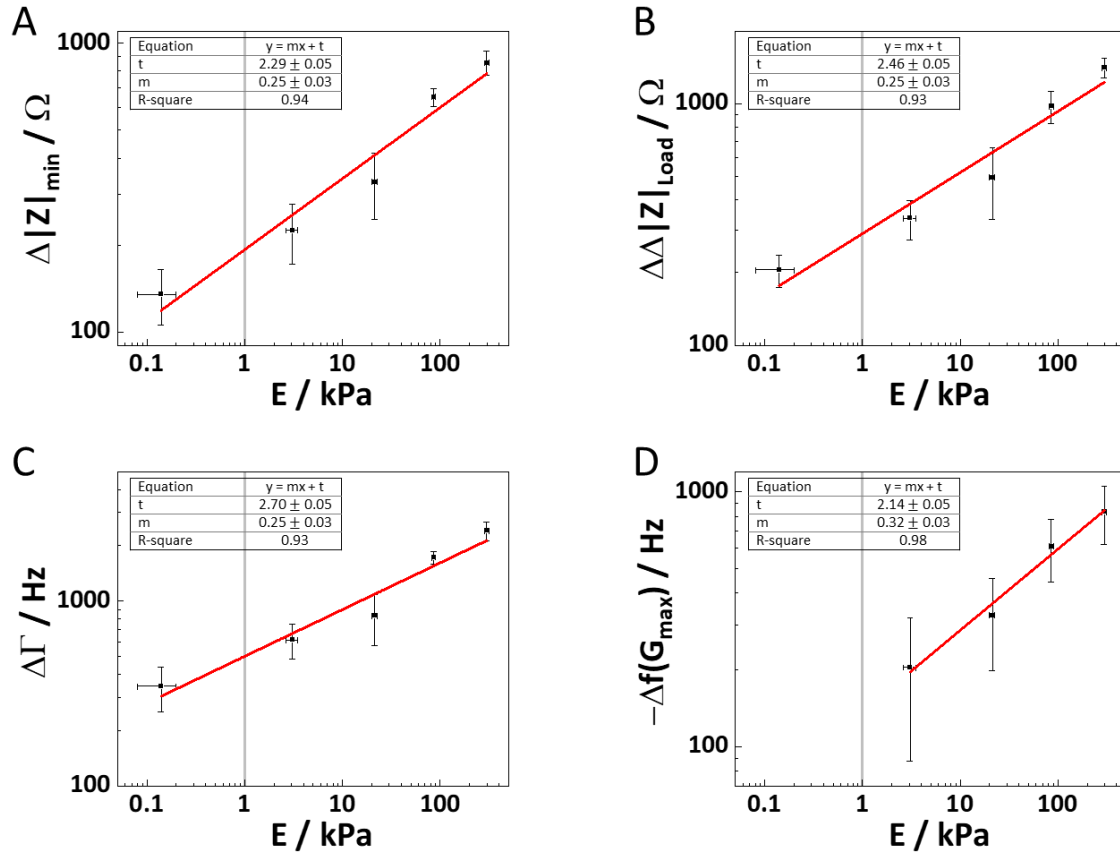


Figure 5.5 Double-logarithmic plots of the change of the minimal impedance $\Delta|Z|_{\min}$ (A), the change of the load impedance $\Delta\Delta|Z|_{\text{Load}}$ (B), the change of the half-width-half-maximum of maximal conductance $\Delta\Gamma$ (C), and the change of the frequency of maximal conductance $\Delta f(G_{\max})$ (plotted as negative change and without the data point for PAAm gel E) (D) versus the Young's modulus E with the data taken from Table 5.1 characterizing PAAm hydrogels A – E after 90 min of polymerization. Data (black) are presented as mean $\pm$ SD with the number of replicates n for the QCM parameters depending on the respective parameter and the PAAm gel type ($\Delta|Z|_{\min}$: $n = 4 - 15$, $\Delta\Delta|Z|_{\text{Load}}$: $n = 4 - 14$, $\Delta\Gamma$: $n = 3 - 15$, $\Delta f(G_{\max})$: $n = 3 - 12$), while $n = 5$ for the Young's modulus. For establishing correlations between QCM parameters and the Young's modulus, linear data fits (red) were applied with the resulting fit parameters (intercept t and slope m) given in the windows added to the graphs. A vertical grey line is added at $E = 1 \text{ kPa}$ to easily read the intercept t . Since both the QCM parameters and the Young's modulus are given on logarithmic scales, they have to be inserted into the equations with their logarithmic values ($y = \log(\text{QCM parameter})$, $x = \log E$).

Thus, linear fitting of the data was performed (Figure 5.5, red lines), although some of the data points were only close or even not intersected by the linear fits within the range of the error bars. But, the R-square values of the fits, which were close to 1, and the very small standard deviations of the fit parameters (intercept and slope) for all the QCM parameters proved that these linear fits on a double-logarithmic scale between QCM parameters and Young's modulus were justified and precise. In the case of the frequency of maximal conductance $f(G_{\max})$ (Figure 5.5 D), the error bars in direction of the QCM parameter were relatively huge, as already obtained in Figure 5.4 and Table 5.1. This made the data fit between $f(G_{\max})$ and the Young's modulus E less precise, although the standard deviations of the fit parameters were as small as for the other QCM parameters. These linear fits with

the fit parameters intercept and slope were then used as correlations between QCM parameters and the Young's modulus. In the following section (section 5.1.3), these correlations were then used to calculate the Young's modulus of cells from QCM data.

5.1.3 Calculation of the Young's Modulus of Cells by Correlations with QCM Parameters Established for Polyacrylamide (PAAm) Hydrogels

It has been demonstrated in the previous section (section 5.1.2), how PAAm hydrogels have been used to establish correlations between certain QCM parameters obtained from time-resolved QCM measurements (minimal impedance $|Z|_{min}$, load impedance $\Delta|Z|_{Load}$, half-width-half-maximum of maximal conductance Γ , and frequency of maximal conductance $f(G_{max})$) and the Young's modulus E obtained from compression testing. Although these correlations were established for PAAm hydrogels, they can easily be transferred to cells according to the abovementioned similarities in terms of stiffness and viscoelasticity and according to the finding that temperature does not influence hydrogel stiffness. Here, these correlations were used to calculate the Young's modulus of different cell lines from QCM parameters obtained from QCM readings, without directly measuring their Young's modulus by methods like compression testing or AFM. No QCM measurement on cells was conducted specifically for this purpose, but instead, the QCM parameters were extracted as average from QCM measurements performed elsewhere in this thesis. One important aspect has to be considered here concerning the reference point of these correlations and QCM data extraction. Since the QCM parameters of the PAAm hydrogels were always used in these correlations as delta values with respect to the polymerization start, the QCM parameters of the cells also have to be taken as delta values relative to a pure liquid loading. Thus, three different situations of QCM measurements have to be distinguished. Typical full-spectrum-mode measurements, where the adhesion of a cell line was followed, enable the extraction of all four investigated QCM parameters as delta values with respect to the cell-free situation, which can be directly used for the calculation of the Young's modulus of the cells. This type of QCM measurement was only performed for HTM-N cells. Although this calculation would be possible at any time point of the measurement revealing the Young's modulus of the HTM-N cells at any time, this was only done for a confluent adhered cell layer. If full-spectrum-mode measurements were performed without recording cell adhesion, as it was the case for NRK cells, the value of the minimal impedance $|Z|_{min}$ had to be taken from a confluent and untreated cell layer and the minimal impedance value of a cell-free control measurement had to be subtracted, before it could be used for calculation of the Young's modulus. Additionally, the load impedance $\Delta|Z|_{Load}$ was used, but without the need to subtract the respective value reflecting the cell-free situation because this is per definition zero. The half-width-half-maximum of maximal conductance Γ and the frequency of maximal conductance $f(G_{max})$ could not be extracted in this case. The third situation of

QCM measurements constitutes the fast-mode, which was described in section 4.6.1 and performed in the investigation of water flux, where MDCK-I and MDCK-II cells were examined. Due to the reduced frequency range, the minimal impedance $|Z|_{min}$ was the only parameter measured, which led to the restriction that the Young's modulus of these cell lines could only be calculated from the minimal impedance. Table 5.2 summarizes the QCM results for different cell lines used in this thesis, with all four QCM parameters ($\Delta|Z|_{min}$, $\Delta\Delta|Z|_{Load}$, $\Delta\Gamma$, and $\Delta f(G_{max})$) extracted for HTM-N cells, $\Delta|Z|_{min}$ and $\Delta\Delta|Z|_{Load}$ for NRK cells, but only $\Delta|Z|_{min}$ for MDCK-I and MDCK-II cells. The values of the QCM parameters were extracted from those time points of QCM experiments reflecting a confluent and untreated cell layer. They are displayed as mean $\pm$ SD out of multiple measurements. Additionally, values for the change of the load impedance $\Delta\Delta|Z|_{Load}$ published by Heitmann *et al.* for MDCK-I cells, MDCK-II cells, 3T3 fibroblasts, porcine brain capillary endothelial cells (PBCEC), and human umbilical vein endothelial cells (HUVEC) are given in Table 5.2, which are marked with asterisks (Heitmann *et al.*, 2007). Both the QCM parameters extracted from measurements performed in this thesis and these literature values for $\Delta\Delta|Z|_{Load}$ were used to calculate the Young's modulus E of the respective cell types by the correlations established before (see section 5.1.2), with the results of these calculations also given in Table 5.2. Different Young's moduli E were calculated for most of the cell types depending on which QCM parameter its calculation was based on. The errors of the values calculated for the Young's modulus were determined by Gaussian error propagation.

All the QCM parameters summarized in Table 5.2 were found within the range of the linear fits established in Figure 5.5, thus, none of the cell lines investigated here exhibited a higher stiffness than PAAm gel A or a lower stiffness than PAAm gel E, except for $\Delta f(G_{max})$, where the value for the HTM-N cells was located beneath the respective range due to the exclusion of PAAm gel E for this correlation. Hence, the range of stiffnesses of the PAAm hydrogels was selected very appropriately for the determination of the Young's modulus of most cell types. By comparing all the errors determined for the different values of the Young's modulus E , the absolute errors were observed to be relatively constant (around 2 kPa) and independent of the value of the Young's modulus (Table 5.2). This led to the finding that cell lines with smaller Young's modulus, like the HTM-N and 3T3 cells, exhibited a higher relative error and, accordingly, a lower precision of the calculation of the Young's modulus by this method. The determination of the Young's modulus E of HTM-N cells yielded almost equal values ((1.0 ± 1.6) kPa, (0.7 ± 1.7) kPa, and (0.7 ± 1.7) kPa) when calculated from $\Delta|Z|_{min}$, $\Delta\Delta|Z|_{Load}$, or $\Delta\Gamma$, respectively, but a smaller one ((0.2 ± 1.8) kPa) was calculated based on $\Delta f(G_{max})$ (Table 5.2). This confirmed the result from section 5.1.2, where the correlation between the change of the frequency of maximal conductance $\Delta f(G_{max})$ and the Young's modulus E was found to be less precise. But with respect to the high errors for the Young's modulus of HTM-N cells, as determined from the applied correlations, this difference was not really significant.

Table 5.2 Summary of the change of the minimal impedance $\Delta|Z|_{min}$, the change of the load impedance $\Delta\Delta|Z|_{Load}$, the change of the half-width-half-maximum of maximal conductance $\Delta\Gamma$, and the change of the frequency of maximal conductance $\Delta f(G_{max})$ extracted from QCM measurements (37 °C) on HTM-N, NRK, MDCK-I, and MDCK-II cells reflecting confluent and untreated cell layers. The values are displayed as mean $\pm$ SD with the number of replicates n given for each individual value. For MDCK-I and MDCK-II cells, and additionally for 3T3 fibroblasts, PBCEC, and HUVEC, values for $\Delta\Delta|Z|_{Load}$ are given as they were published by Heitmann *et al.* (Heitmann *et al.*, 2007; marked with asterisks). Based on all these QCM data (measured in this thesis or taken from literature), the Young's modulus E of these cell types was calculated by using the correlations between QCM parameters and Young's modulus established in section 5.1.2, while the results are also given here depending on the QCM parameter used for calculation. Gaussian error propagation was used for the errors of the Young's modulus. For comparison, literature data of the Young's modulus E (measured by AFM) are given in the last row, while no literature values are available for MDCK-I and HTM-N cells (HTM-N cells are compared to a value reported for primary HTM cells instead). The corresponding references are given in the text.

Parameter	HTM-N	NRK	MDCK-I	MDCK-II	3T3	PBCEC	HUVEC
$\Delta Z _{min} / \Omega$	191 $\pm$ 9 ($n = 4(2)$)	352 $\pm$ 3 ($n = 4(2)$)	525 $\pm$ 26 ($n = 6(2)$)	362 $\pm$ 44 ($n = 19(4)$)	–	–	–
$\Delta\Delta Z _{Load} / \Omega$	264 $\pm$ 17 ($n = 4(2)$)	528 $\pm$ 10 ($n = 4(2)$)	550 $\pm$ 35*	425 $\pm$ 26*	247 $\pm$ 40*	380 $\pm$ 23*	435 $\pm$ 65*
$\Delta\Gamma / \text{Hz}$	464 $\pm$ 18 ($n = 4(2)$)	–	–	–	–	–	–
$\Delta f(G_{max}) / \text{Hz}$	–85 $\pm$ 13 ($n = 4(2)$)	–	–	–	–	–	–
E / kPa (by $\Delta Z _{min}$)	1.0 $\pm$ 1.6	11.5 $\pm$ 1.7	58.6 $\pm$ 2.0	12.9 $\pm$ 2.1	–	–	–
E / kPa (by $\Delta\Delta Z _{Load}$)	0.7 $\pm$ 1.7	10.7 $\pm$ 1.8	12.5 $\pm$ 1.9	4.5 $\pm$ 1.8	0.5 $\pm$ 2.2	2.9 $\pm$ 1.8	4.9 $\pm$ 2.2
E / kPa (by $\Delta\Gamma$)	0.7 $\pm$ 1.7	–	–	–	–	–	–
E / kPa (by $\Delta f(G_{max})$)	0.2 $\pm$ 1.8	–	–	–	–	–	–
E / kPa (literature)	1.6 $\pm$ 0.2	16 $\pm$ 11	–	2 $\pm$ 1 6.2 $\pm$ 1.2 12 $\pm$ 4	1.0 $\pm$ 0.4	5.1 $\pm$ 1.9	0.39 $\pm$ 0.07 1.00 $\pm$ 0.24 3.23 $\pm$ 0.54

For NRK cells, the two values of the Young's modulus E calculated from $\Delta|Z|_{min}$ and $\Delta\Delta|Z|_{Load}$ also fitted very well to each other ((11.5 $\pm$ 1.7) kPa compared to (10.7 $\pm$ 1.8) kPa) (Table 5.2). For MDCK-I cells, as well as for MDCK-II cells, the Young's moduli E calculated from $\Delta|Z|_{min}$ and $\Delta\Delta|Z|_{Load}$, respectively, differed significantly ((58.6 $\pm$ 2.0) kPa compared to (12.5 $\pm$ 1.9) kPa, and (12.9 $\pm$ 2.1) kPa compared to (4.5 $\pm$ 1.8) kPa) (Table 5.2), probably due to the fact that the two QCM parameters were extracted for these cell lines from two different data sources (measured in this thesis versus Heitmann *et al.*), although experimental conditions were the same. To validate this approach for determination of the Young's modulus, the values calculated for a variety of cell types

and summarized in Table 5.2 were compared to literature data reported for these cell types, all of them obtained by AFM, except for MDCK-I, because there is no value reported in the literature yet. Since also no literature value of the Young's modulus is available for HTM-N cells, the result for this cell type was compared to primary HTM cells, which were expected to be quite similar in terms of stiffness and, accordingly, the Young's modulus. A Young's modulus of (1.6 ± 0.2) kPa was published for primary HTM cells (McKee *et al.*, 2011), where the Young's moduli of HTM-N cells calculated here from the different QCM parameters $((0.2 - 1.0)$ kPa) (Table 5.2) were not far away with respect to the errors. The Young's moduli calculated for NRK cells $((10.7 - 11.5)$ kPa) (Table 5.2) were also relatively close to the literature value of (16 ± 11) kPa reported from AFM measurements (Kagiwada *et al.*, 2010). For MDCK-II cells, three clearly distinct values were reported, (2 ± 1) kPa (Nehls *et al.*, 2019), (6.2 ± 1.2) kPa (Steltenkamp *et al.*, 2006), and (12 ± 4) kPa (Rabinovich *et al.*, 2005), where both the values calculated here $((4.5 \pm 1.8)$ kPa) and (12.9 ± 2.1) kPa) (Table 5.2) fitted very good in this range. When these three references reporting on the Young's modulus of MDCK-II cells are compared to each other regarding technical details of the AFM measurements, the different results can probably be attributed to different diameters of the AFM tips used, with a sharper AFM tip yielding a higher Young's modulus. For 3T3 cells $((0.5 \pm 2.2)$ kPa) (Table 5.2) and PBCEC $((2.9 \pm 1.8)$ kPa) (Table 5.2), the calculated Young's moduli were only slightly different to literature values $((1.0 \pm 0.4)$ kPa (Park *et al.*, 2005); (5.1 ± 1.9) kPa (Schrot *et al.*, 2005)), respectively. With (0.39 ± 0.07) kPa, (1.00 ± 0.24) kPa, and (3.23 ± 0.54) kPa, HUVEC were reported in one single study to exhibit different Young's moduli depending on the AFM tip size (Vargas-Pinto *et al.*, 2013), confirming the strong dependency of the Young's modulus on the tip size when measured by AFM, as it was already mentioned above for MDCK-II cells. The Young's modulus calculated in this thesis for HUVEC $((4.9 \pm 2.2)$ kPa) (Table 5.2) fitted quite well to the literature value measured with the sharpest AFM tip $((3.23 \pm 0.54)$ kPa).

Taken all together, a method was developed based on QCM measurements and hydrogels to calculate the Young's modulus of cells without performing elaborate and invasive deformation measurements on the cells. Correlations between the Young's modulus measured by compression testing and several QCM parameters can easily be established for hydrogels, where PAAm hydrogels have shown to be well suited. These correlations can be transferred to cells, which only have to be analyzed by the QCM, enabling a calculation of their Young's modulus from QCM data. Despite the problems described before regarding the difference between the elastic nature of the Young's modulus and the viscoelastic nature of QCM parameters, the different directions of distortion, the different detection ranges (basal versus apical membrane), and the difference between isotropic and anisotropic materials, this empirical method facilitated the calculation of the Young's modulus for a variety of cells, which fitted quite well to literature data obtained by AFM measurements. When the Young's modulus is to be calculated from more than one QCM parameter, care must be taken due to the finding that Young's moduli calculated from QCM parameters obtained from different data sources can vary significantly, as it was observed for MDCK-I and MDCK-II cells. But this

problem is not unique to QCM, it is a general problem of all measurements on living cells and can be overcome by using QCM parameters extracted from one and the same measurement. Although this model describes most of the presented cell types quite precisely compared to literature data, there might be cell types that can possibly not be described accurately by this approach. Furthermore, the Young's modulus can still depend on confluency (Nehls *et al.*, 2019) and cellular senescence (Morgan *et al.*, 2015), which can both not be avoided by the AFM and also not by using this QCM-based approach. Due to the dependency of the Young's modulus on the AFM tip size, values obtained by AFM measurements have to be handled with care because information on the tip size is not always given. Taking this into account, the approach demonstrated here enables a cost-effective and powerful alternative to AFM for the determination of the Young's modulus of any cell type by measuring their behavior on the QCM and establishing a correlation between QCM parameters and Young's modulus for hydrogels. At the same time, the disadvantage of the dependency on the tip size in AFM measurements is avoided. Another advantage of this QCM-approach is the integral signal readout, which provides a holistic average of the cell layer and is not influenced by differences between single cells or even differences within a single cell's plasma membrane. The dependency of the Young's modulus on substrate stiffness typically obtained in AFM measurements on very thin cells (Vichare *et al.*, 2014) is also avoided by this approach because the QCM result is only influenced by the stiffness of the load, and not by the rigidly behaving quartz disk. In contrast to AFM, the QCM's non-invasive nature generates the possibility to further analyze the cells, because they are not destroyed or even influenced by the measurement. Another advantage over AFM is the possible future application in organ-on-a-chip devices, where the QCM is probably the only technique allowing mechanical sensing. In addition, for both AFM and QCM, the Young's modulus can depend on cell size altered, for example, by osmotic pressure (Steltenkamp *et al.*, 2006), which can be utilized as an advantage in this QCM-based approach due to the time-resolved measurements. Real-time information on the stiffness of the cells can be gained by the QCM, when volume changes are triggered osmotically, as it is utilized in this thesis in section 8.

5.2 Correlation of the Complex Shear Modulus with QCM Parameters

Since cells behave like viscoelastic bodies, the determination of their Young's modulus, which describes elastic properties, is not enough to fully characterize their mechanical behavior. With the complex shear modulus G^* , which is described in detail in section 3.3.2, viscoelastic properties of a material can be characterized comprehensively. The complex shear modulus of cells is typically obtained by AFM-based microrheology (Rother *et al.*, 2014), optorheology (Wottawah *et al.*, 2005), or optical tweezers (Ayala *et al.*, 2016). As already mentioned in section 3.3.2, the complex shear modulus G^* is a complex quantity,

thus, at least two of the following four quantities are needed to fully describe the material's viscoelastic behavior: the magnitude of the complex shear modulus $|G^*|$, the storage modulus G' , the loss modulus G'' , and the phase shift φ . Mostly, the storage modulus G' is used to describe elastic properties of the material under observation. This is typically used in combination with either the loss modulus G'' , directly describing viscous properties of the material, or with the magnitude of the complex shear modulus $|G^*|$, where viscous properties are then represented by the difference between both quantities (in the complex context, see Eq. (60)). The complex shear modulus has been reported several times to be dependent on the oscillatory distortion frequency. Within the range of measurement frequency of (0.02 – 0.3) Hz, BALB/3T3 cells and SV-T2 cells were found to exhibit a higher storage modulus G' ((20 – 70) Pa) for increasing frequency of the optorheology measurements, with the loss modulus G'' ((10 – 40) Pa) showing a complicated behavior with no clear dependency on the frequency (Wottawah *et al.*, 2005). In a higher frequency range of (1 – 35) Hz, another study using optical tweezers demonstrated a linear dependency on the frequency for both the storage modulus G' ((30 – 140) Pa) and the loss modulus G'' ((20 – 80) Pa) of cortical astrocytes, cortical neurons, and NIH3T3 fibroblasts (Ayala *et al.*, 2016). When using AFM-based microrheology with even higher frequencies in the range of (5 – 100) Hz, a similar dependency on frequency was observed for MCF-10A cells, MCF-7 cells, and MDA-MB-231 cells, where the loss modulus G'' ((0.3 – 6) kPa) showed an even stronger dependency than the storage modulus G' ((0.6 – 3) kPa) (Rother *et al.*, 2014). Beside this constantly observed proportionality between complex shear modulus G^* and frequency within one single study, comparing these literature data to each other reveals the same dependency of G^* on the frequency, although different cells and different measurement techniques were used. This frequency dependency of the complex shear modulus was also demonstrated for dough in the low-frequency region (0.01 Hz – 400 kHz) by rheological and ultrasonic shear wave measurements (Leroy *et al.*, 2010) and for the polymer poly(3-hexylthiophene) in the high-frequency region ((10 – 110) MHz) by QCM measurements (Brown *et al.*, 2000). Due to this frequency dependency, values of the complex shear modulus always have to be considered with respect to the oscillatory distortion frequency of the measurement. But this also requires the search for measurement techniques, which allow for determination of the complex shear modulus in new frequency ranges. With the QCM as a method sensitive to viscoelastic changes of the load, this technique was tested here for determination of the complex shear modulus of cells providing data recording in the high-frequency region (5 MHz in fundamental resonance). But, since a direct extraction of the complex shear modulus from QCM measurements is not possible, equations correlating the complex shear modulus G^* with QCM parameters reported by Bandey *et al.* were used, which were already mentioned before expressed by Eq. (63) and Eq. (64) (Bandey *et al.*, 2000). These equations were transferred into Eq. (65) and Eq. (66) to calculate the magnitude of the complex shear modulus $|G^*|$ and the storage modulus G' out of the QCM parameters real part of the load impedance $Re(\underline{Z}_{Load})$ and imaginary part of the load impedance $Im(\underline{Z}_{Load})$. Fulfilling the condition of a semi-infinite viscoelastic material was already ensured before by calculation of the decay length of the

shear wave and by using sufficiently thick hydrogel layers (see section 5.1.1, *Influence of Hydrogel Layer Thickness*). In the following, these equations were tested for PAAm and PEG hydrogels by measuring them on the QCM, calculating $|G^*|$ and G' from $Re(\underline{Z}_{Load})$ and $Im(\underline{Z}_{Load})$, and comparing the results with values obtained from rheological measurements (section 5.2.1). Due to the frequency dependency of the complex shear modulus and due to the different measurement frequencies of QCM (5 MHz, see section 4.6.1) and rheology (1 Hz, see section 4.4.2), the quality and accuracy of these equations can actually not be evaluated, but instead, it can be investigated how these results fit into the frequency dependency of the complex shear modulus often obtained in literature. After that, these equations were applied to cells for calculation of their complex shear modulus from QCM data in the high-frequency region (section 5.2.2).

5.2.1 Using Hydrogels to Examine an Existing Correlation Between the Complex Shear Modulus and QCM Parameters

Before the complex shear modulus G^* of cells was calculated from QCM parameters using equations found by Bandey *et al.* (Bandey *et al.*, 2000) (see section 5.2.2), here, these equations were tested for PAAm and PEG hydrogels prepared according to sections 4.3.2 and 4.3.3, respectively. In detail, the magnitude of the complex shear modulus $|G^*|$ and the storage modulus G' were calculated from the QCM parameters real part of the load impedance $Re(\underline{Z}_{Load})$ and imaginary part of the load impedance $Im(\underline{Z}_{Load})$ by Eq. (65) and Eq. (66) and compared to values of $|G^*|$ and G' obtained from rheological measurements (performed according to section 4.4.2). For PAAm gels, these two QCM parameters were extracted from the measurements shown in Figure 5.4, thus, they were recorded during polymerization of the same five PAAm hydrogel types as used for the correlation between the Young's modulus and QCM parameters (PAAm gels A – E, for exact compositions, see Table 4.8). Additionally, $Re(\underline{Z}_{Load})$ and $Im(\underline{Z}_{Load})$ were extracted from QCM measurements during polymerization of two differently composed PEG hydrogels, PEG gel A with macromonomer concentrations of 5% (w/v) and PEG gel B with macromonomer concentrations of 3% (w/v) (see section 4.3.3). Further attempts on monitoring the polymerization of a 2% (w/v) PEG gel by the QCM failed due to an incomplete polymerization, and 7% (w/v) and 10% (w/v) PEG gels exhibited a too fast polymerization with too many N₂ bubbles emerging leading to contact loss to the quartz substrate. Thus, these equations were tested for five different PAAm gels and two different PEG gels. As already mentioned before in section 4.6.2, the two QCM parameters were used for these equations as delta values ($\Delta Re(\underline{Z}_{Load})$, $\Delta Im(\underline{Z}_{Load})$) with normalization to the polymerization start. Figure 5.6 demonstrates the change of the real part of the load impedance $\Delta Re(\underline{Z}_{Load})$ (Figure 5.6 A and C) and the change of the imaginary part of the load impedance $\Delta Im(\underline{Z}_{Load})$ (Figure 5.6 B and D) during polymerization of PAAm gels A – E

(Figure 5.6 A and B) and PEG gels A – B (Figure 5.6 C and D). Data are presented as mean out of several individual measurements, while the number of replicates n varied significantly among the PAAm gel types due to PAAm gel C measured several times more often, and $n = 4 - 5$ for the PEG gels. While the polymerization of all PAAm hydrogel types was followed for at least 90 min, the PEG gels were found to polymerize faster, which enabled a shorter measurement time of 60 min for PEG gel B and only 30 min for PEG gel A.

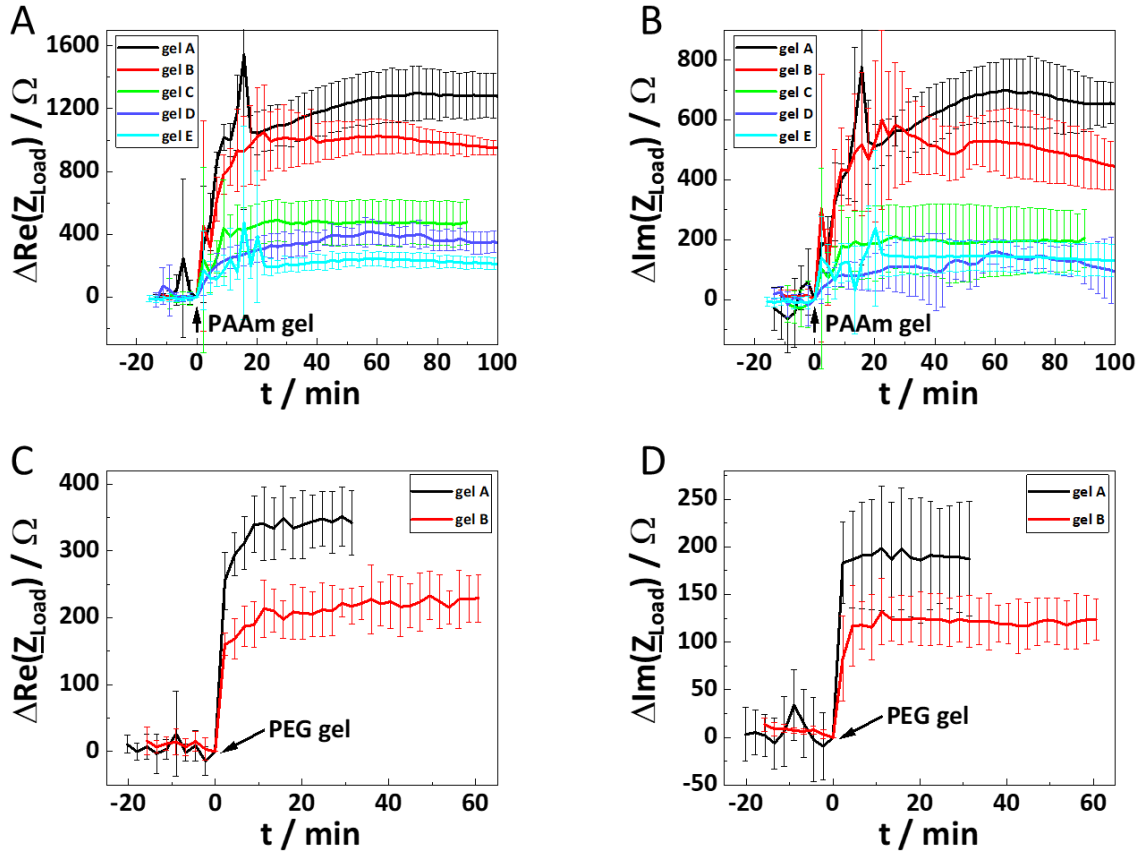


Figure 5.6 QCM measurements at 5 MHz during polymerization of PAAm (A, B) and PEG (C, D) hydrogels for their characterization by means of the change of the real part of the load impedance $\Delta \text{Re}(Z_{\text{Load}})$ (A, C) and the change of the imaginary part of the load impedance $\Delta \text{Im}(Z_{\text{Load}})$ (B, D). For PAAm hydrogels, five differently composed gel types were used, PAAm gel A (black), PAAm gel B (red), PAAm gel C (green), PAAm gel D (blue), and PAAm gel E (cyan), with the exact compositions of the gels given in Table 4.8, while two different PEG hydrogels were used, PEG gel A (black) and PEG gel B (red), with the exact compositions given in section 4.3.3. The data for PAAm hydrogels were extracted from the measurements already shown in Figure 5.4. Data were normalized to the time point of gel addition and are displayed as mean $\pm$ SD with the number of replicates n varying depending on the gel type (PAAm: $n = 4 - 14$, PEG: $n = 4 - 5$). $T = \text{RT}$ for PAAm, but $T = 37^\circ \text{C}$ for PEG.

For the change of the real part of the load impedance $\Delta \text{Re}(Z_{\text{Load}})$ of PAAm gels (Figure 5.6 A), the time courses for the different PAAm hydrogel types looked almost identical to the ones for the change of the load impedance $\Delta \Delta |Z|_{\text{Load}}$ itself, as observed before in Figure 5.4 B. For all PAAm hydrogel types, an increase during polymerization was followed by

flattening of the curves with plateau values reached not later than 70 min after polymerization start. Despite partially overlapping error bars and several signal fluctuations, a good dependency of $\Delta Re(\underline{Z}_{Load})$ on gel composition and associated stiffness was observed with the highest signal response for the stiffest gel. The change of the imaginary part of the load impedance $\Delta Im(\underline{Z}_{Load})$ of PAAm gels (Figure 5.6 B) exhibited similar behavior in terms of signal increase and reaching of plateau values. A dependency on gel stiffness was also disclosed but less pronounced due to bigger error bars, which were overlapping to a bigger extent as compared to $\Delta Re(\underline{Z}_{Load})$, and due to PAAm gels D and E exhibiting an almost equal signal change. Similar to PAAm hydrogels, also PEG hydrogels showed an increase in $\Delta Re(\underline{Z}_{Load})$ (Figure 5.6 C) and $\Delta Im(\underline{Z}_{Load})$ (Figure 5.6 D) during their polymerization reflecting a stiffness increase. But a faster polymerization was observed for the PEG gels as expressed by the plateau values reached after about 10 min for all PEG gel types. Since a higher temperature can reduce polymerization time (Gelfi & Righetti, 1981b), this shorter polymerization time for the PEG gels can probably be attributed to the elevated temperature of 37 °C during polymerization, although different hydrogel types were used. As expected, PEG gel A exhibited a higher signal change for both parameters than PEG gel B, which was less significant for $\Delta Im(\underline{Z}_{Load})$ because error bars were partially overlapping. Accordingly, the dependency of $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$ on gel stiffness was observed for both PAAm and PEG hydrogels. Parameter extraction from these QCM measurements and calculation of the magnitude of the complex shear modulus $|G^*|$ and the storage modulus G' is described further down. First, rheological measurements (see section 4.4.2) on PAAm and PEG hydrogels are demonstrated, which facilitated the direct extraction of values of $|G^*|$ and G' of the hydrogels for later comparison. These rheological measurements were performed with the same PAAm gels A – E (Figure 5.7 A and B) and PEG gels A – B (Figure 5.7 C and D) as already used before in Figure 5.6, revealing the magnitude of the complex shear modulus $|G^*|$ (Figure 5.7 A and C) and the storage modulus G' (Figure 5.7 B and D) during polymerization of the hydrogels. Problems regarding the different temperatures during the QCM measurements (RT) and rheological measurements (37 °C) of PAAm hydrogels were not expected since an influence of the temperature on gel stiffness was already excluded before (see section 5.1.1, *Influence of Temperature*). Each of the five different PAAm gels was measured five times and each of the two different PEG gels was measured three times. Due to slightly different start points of the measurements, normalization and averaging were not possible, but instead, the data are shown for each individual measurement. Measurements were terminated when $|G^*|$ and G' were obtained to be constant mirroring gels with completed polymerization, leading especially for the PAAm gels to shorter measurement times as compared to the QCM measurements.

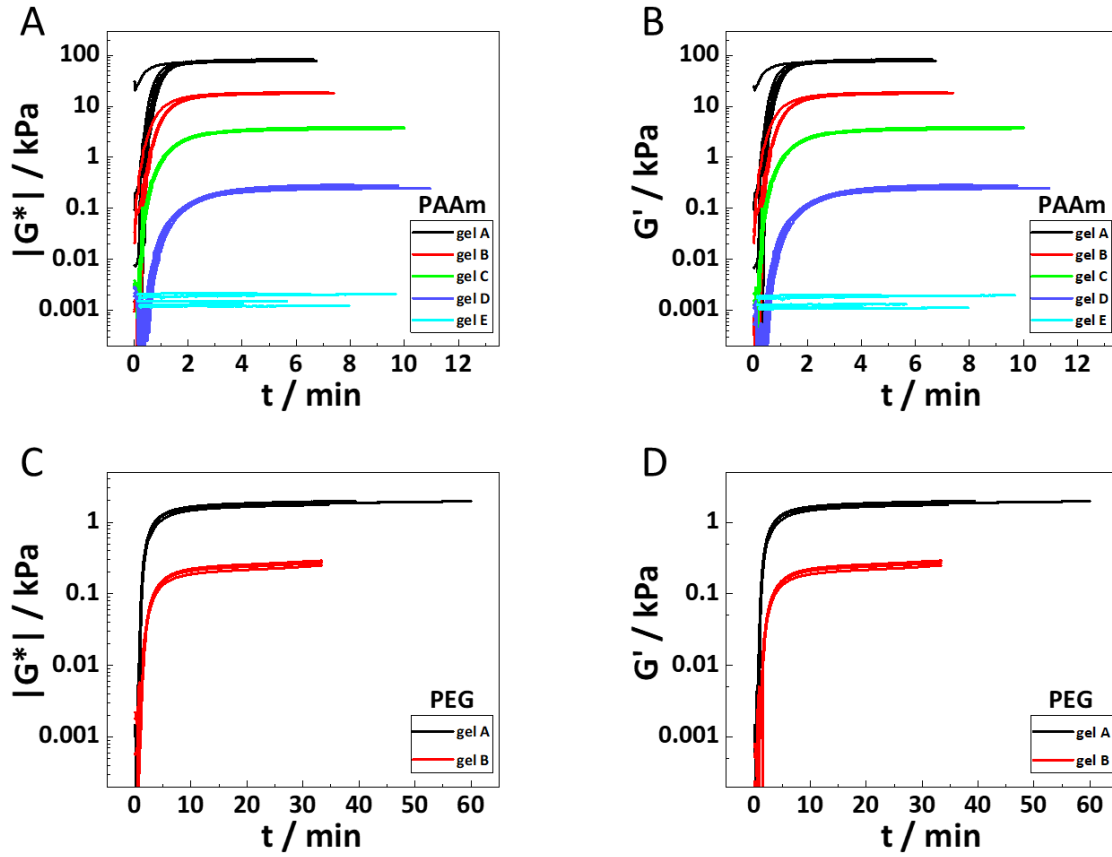


Figure 5.7 Time-resolved rheological measurements at 1 Hz during polymerization of PAAm (A, B) and PEG (C, D) hydrogels for their characterization by means of the magnitude of the complex shear modulus $|G^*|$ (A, C) and the storage modulus G' (B, D). With the five differently composed PAAm hydrogels, PAAm gel A (black), PAAm gel B (red), PAAm gel C (green), PAAm gel D (blue), and PAAm gel E (cyan), and the two different PEG hydrogels, PEG gel A (black) and PEG gel B (red), the same compositions were used as before in Figure 5.6. These measurements were performed five times for each of the five different PAAm gels and three times for each of the two different PEG gels, while they could not be normalized and averaged due to the slightly different start points of the measurements. $T = 37^\circ\text{C}$.

For all the PAAm and PEG gel types investigated rheologically in Figure 5.7, individual measurements on the same gel composition yielded results for both the magnitude of the complex shear modulus $|G^*|$ and the storage modulus G' that exhibited almost no variation, demonstrating the good precision of this method. Additionally, the results for $|G^*|$ and G' seemed to be identical to each other (more detailed further down in Table 5.3), which would indicate predominantly elastic behavior for all the hydrogel types. Similar to all the QCM measurements shown before, here, $|G^*|$ and G' were also observed to continuously increase during the hydrogel polymerization process until plateau values were reached indicating the end of the polymerization, with the exception of PAAm gel E showing constant values of $|G^*|$ and G' over the complete measurement time, because this gel type was pre-polymerized for 20 min – 30 min due to its very low stiffness. As already obtained in Figure 5.6 for the QCM parameters $Re(Z_{Load})$ and $Im(Z_{Load})$, here, $|G^*|$ and G' were also observed to be dependent on gel stiffness. A comparison to Table 5.1 revealed higher $|G^*|$ and G' for PAAm

gels with higher Young's modulus E (the stiffer ones). Although an in-depth analysis of polymerization time is not possible here due to the different start points of the measurements, PEG gels seemed to exhibit a similar polymerization time of about 10 min in QCM measurements (Figure 5.6 C and D) and rheological measurements (Figure 5.7 C and D), probably due to the same temperature. The difference in hydrogel layer thickness of 2 mm in QCM measurements and 0.5 mm in rheological measurements seemed to not influence the polymerization time of PEG gels, although such an influence was found to be possible (see section 5.1.1, *Influence of Hydrogel Layer Thickness*). But for PAAm gels, the polymerization time seemed to be indirectly proportional to gel stiffness (Figure 5.7 A and B), since plateau values were reached later for softer gels. Additionally, significantly shorter polymerization times of PAAm hydrogels were observed here by the rheological measurements as compared to the QCM measurements (Figure 5.6 A and B), which can be attributed to the higher temperature and/or to the thinner hydrogels.

It was the aim now to calculate the magnitude of the complex shear modulus $|G^*|$ and the storage modulus G' from the change of the real part of the load impedance $\Delta Re(\underline{Z}_{Load})$ and the change of the imaginary part of the load impedance $\Delta Im(\underline{Z}_{Load})$ for the PAAm and PEG hydrogels by using Eq. (65) and Eq. (66) and to compare the results to values of $|G^*|$ and G' obtained from rheological measurements. Although this would be possible for multiple time points of the time-resolved measurements, this was only done for gels with completed polymerization, which is mirrored in the measurements by their plateau values. Thus, values of $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$ had to be extracted from the QCM measurements in Figure 5.6, and values of $|G^*|$ and G' had to be extracted from the rheological measurements in Figure 5.7. Since the rheological measurements exhibited non-uniform start points, the parameter extraction could not be performed at one fixed time point for all measurements, but instead, the time point was selected individually. The selection of this time point was not that crucial as long as the respective parameter has reached a plateau value there. The extraction of $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$ for PAAm gels was conducted equally to the extraction of all the other QCM parameters as performed before for the correlation with the Young's modulus, which was done after 90 min of polymerization. This is justified here because these two parameters also exhibited plateau values at this time. $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$ for PEG gels and $|G^*|$ and G' for both PAAm and PEG gels were extracted from the respective end of the measurement, which were also characterized by plateau values. Because $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$ are two associated parameters, the calculation of $|G^*|$ and G' by Eq. (65) and Eq. (66) could not be performed from average values of the two QCM parameters, thus, for each single hydrogel measured, they were extracted individually and used to calculate $|G^*|$ and G' of this hydrogel and averaged afterwards. In Table 5.3, these QCM parameters are not given with all the individual values for reasons of clarity, but instead, they are summarized as mean $\pm$ SD with the number of replicates n , although these average values were actually not used for calculation. The density ρ_L , which was needed for this calculation, was found to be more or less independent of gel stiffness with an average

value of $1.05 \cdot 10^3 \frac{\text{kg}}{\text{m}^3}$ for PAAm gels and $0.58 \cdot 10^3 \frac{\text{kg}}{\text{m}^3}$ for PEG gels. The values of $|G^*|$ and G' derived by this calculation were averaged afterwards and also given in Table 5.3 as mean $\pm$ SD for each gel composition. For comparison of the calculated results of $|G^*|$ and G' , these parameters were extracted from the rheological measurements, also individually for each single hydrogel measured and averaged afterwards, which are then also given in Table 5.3 as mean $\pm$ SD with the number of replicates n .

Table 5.3 Summary of the change of the real part of the load impedance $\Delta Re(Z_{Load})$ and the change of the imaginary part of the load impedance $\Delta Im(Z_{Load})$ for PAAm gels A – E and PEG gels A – B, calculation of the magnitude of the complex shear modulus $|G^*|$ and the storage modulus G' from these two QCM parameters by Eq. (65) and Eq. (66), and comparison to values obtained from rheological measurements. The mass concentration of AAm is given additionally for each PAAm gel type (exact compositions in Table 4.8), while the mass concentration of macromonomers (MM) is given for each PEG gel type (exact compositions in section 4.3.3). $\Delta Re(Z_{Load})$ and $\Delta Im(Z_{Load})$ were extracted from the QCM measurements at 5 MHz in Figure 5.6, while for PAAm gels, this was performed after 90 min of polymerization, whereas the values for PEG gels were extracted from the respective end of the measurement. The QCM parameters are given here as mean $\pm$ SD with the number of replicates n , although the calculation of $|G^*|$ and G' was not performed from the average values, but instead, from individually extracted values. The density ρ_L was determined to $1.05 \cdot 10^3 \frac{\text{kg}}{\text{m}^3}$ for PAAm gels and $0.58 \cdot 10^3 \frac{\text{kg}}{\text{m}^3}$ for PEG gels. $|G^*|$ and G' were averaged after calculation and given as mean $\pm$ SD. The values for $|G^*|$ and G' measured by rheology at 1 Hz were extracted from the rheological measurements in Figure 5.7 from the respective end of the measurement, averaged, and given as mean $\pm$ SD.

Gel type	$\Delta Re(Z_{Load})$ / Ω	$\Delta Im(Z_{Load})$ / Ω	$ G^* $ / kPa (calc. from QCM data)	G' / kPa (calc. from QCM data)	$ G^* $ / kPa (measured by rheology)	G' / kPa (measured by rheology)
PAAm gel A 15% (w/v) AAm	1280 $\pm$ 150 ($n = 4$)	660 $\pm$ 90 ($n = 4$)	180 $\pm$ 40	110 $\pm$ 30	80 $\pm$ 3 ($n = 5$)	80 $\pm$ 3 ($n = 5$)
PAAm gel B 12% (w/v) AAm	970 $\pm$ 70 ($n = 6$)	470 $\pm$ 100 ($n = 6$)	100 $\pm$ 20	60 $\pm$ 10	18.4 $\pm$ 0.3 ($n = 5$)	18.4 $\pm$ 0.3 ($n = 5$)
PAAm gel C 7.5% (w/v) AAm	480 $\pm$ 140 ($n = 14$)	200 $\pm$ 100 ($n = 14$)	30 $\pm$ 20	20 $\pm$ 10	3.8 $\pm$ 0.1 ($n = 5$)	3.8 $\pm$ 0.1 ($n = 5$)
PAAm gel D 5% (w/v) AAm	360 $\pm$ 60 ($n = 4$)	120 $\pm$ 70 ($n = 4$)	13 $\pm$ 5	10 $\pm$ 3	0.28 $\pm$ 0.02 ($n = 5$)	0.28 $\pm$ 0.02 ($n = 5$)
PAAm gel E 3% (w/v) AAm	220 $\pm$ 40 ($n = 4$)	130 $\pm$ 60 ($n = 4$)	6 $\pm$ 3	2 $\pm$ 1	0.0016 $\pm$ 0.0004 ($n = 5$)	0.0015 $\pm$ 0.0004 ($n = 5$)
PEG gel A 5% (w/v) MM	350 $\pm$ 40 ($n = 5$)	190 $\pm$ 60 ($n = 5$)	26 $\pm$ 8	14 $\pm$ 4	1.9 $\pm$ 0.1 ($n = 3$)	1.9 $\pm$ 0.1 ($n = 3$)
PEG gel B 3% (w/v) MM	230 $\pm$ 40 ($n = 4$)	120 $\pm$ 20 ($n = 4$)	11 $\pm$ 3	6 $\pm$ 3	0.27 $\pm$ 0.02 ($n = 3$)	0.27 $\pm$ 0.02 ($n = 3$)

For both PAAm and PEG hydrogels, Table 5.3 showed that the change of the real part of the load impedance $\Delta Re(Z_{Load})$ and the change of the imaginary part of the load impedance

$\Delta Im(\underline{Z}_{Load})$ measured by QCM and the magnitude of the complex shear modulus $|G^*|$ and the storage modulus G' measured by rheology exhibited plateau values that were strongly dependent on gel composition and associated stiffness, which confirmed the findings from Figure 5.6 and Figure 5.7. The only exception was PAAm gels D and E exhibiting an almost equal change of the imaginary part of the load impedance $\Delta Im(\underline{Z}_{Load})$ (Table 5.3). The small standard deviations of $|G^*|$ and G' obtained by rheological measurements mirrored the very low variation of results observed before in Figure 5.7. As it was also observed before, the values of $|G^*|$ and G' measured by rheology were identical to each other with only one slight difference for PAAm gel E (Table 5.3), indicating predominantly or even purely elastic behavior. Due to the results that the parameters $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$ as well as the parameters $|G^*|$ and G' increased with increasing gel stiffness, a correlation between these QCM parameters and these rheological parameters was obvious, which was claimed by Bandey *et al.* (Bandey *et al.*, 2000) and tested here subsequently by calculation of $|G^*|$ and G' by Eq. (65) and Eq. (66). These values of $|G^*|$ and G' calculated from QCM data also showed a strong dependency on gel stiffness for both PAAm and PEG hydrogels (Table 5.3). When the results of $|G^*|$ and G' , either calculated from $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$ or measured by rheology, are compared to each other, first, it was striking that all hydrogels exhibited almost purely elastic behavior for low frequency (1 Hz, rheology) because $|G^*|$ and G' were almost identical there, but more or less balanced elastic and viscous properties for high frequency (5 MHz, QCM) due to values of G' lying between 1/3 and 3/4 of $|G^*|$ (Table 5.3). This result of hydrogels behaving predominantly elastically for low distortion frequency has already been reported in the literature for PAAm hydrogels (Foegeding *et al.*, 1994; Wang & Ugaz, 2006). Additionally, this finding that the proportion of the viscous part increases in contrast to the elastic part with increasing frequency was also demonstrated before for dough (Leroy *et al.*, 2010), poly(3-hexylthiophene) (Brown *et al.*, 2000), and different cell lines (Rother *et al.*, 2014). To validate the results for the complex shear modulus obtained here by rheological measurements in the low-frequency region, $|G^*|$ or G' were compared to literature data obtained from PAAm and PEG hydrogels with similar compositions. For a PEG hydrogel with the exact same composition as PEG gel A, a value of $|G^*|$ of (1.8 ± 0.1) kPa was observed in rheological measurements with a distortion frequency of 1 Hz (Ziegler *et al.*, 2021), where the value of (1.9 ± 0.1) kPa measured in this thesis fitted very good (Table 5.3). The comparison of the complex shear modulus measured rheologically for the PAAm hydrogels to literature data is summarized in Figure 5.8 by means of the storage modulus G' .

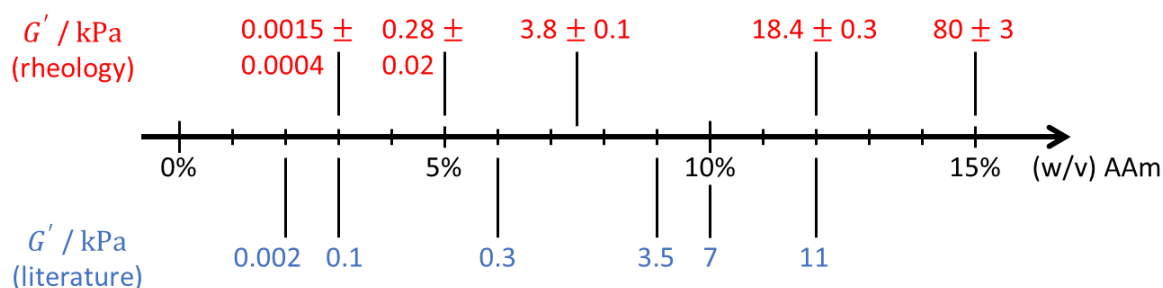


Figure 5.8 Comparison of the storage modulus G' measured by rheology for PAAm gels A – E (red) to literature data (blue) on a number line displaying the different AAm contents of the hydrogels. The values measured for the PAAm gels A – E were taken from Table 5.3. The corresponding references of the literature data are given in the text.

Rheological measurements with a distortion frequency of 0.2 Hz on differently composed PAAm gels revealed a value of G' of about 11 kPa for a PAAm gel with about 12% (w/v) AAm, which was similar to PAAm gel B, a value of G' of about 3.5 kPa for a PAAm gel with about 9% (w/v) AAm, a gel with stiffness lying between PAAm gel B and C, and a value of G' of about 0.3 kPa for a PAAm gel with about 6% (w/v) AAm, a gel with stiffness lying between PAAm gel C and D (Wang & Ugaz, 2006; Figure 5.8). In another study using rheological measurements with a distortion frequency of 0.05 Hz, a value of G' of about 7 kPa was obtained for a PAAm gel with 10% (w/v) AAm, a gel with stiffness lying between PAAm gel B and C (Foegeding *et al.*, 1994; Figure 5.8). Microscopic observations of thermal fluctuations of particles embedded inside the hydrogels at a frequency of 1 Hz yielded a value of G' of about 0.1 kPa for a PAAm gel with 3% (w/v) AAm, which was equivalent to PAAm gel E, and a value of G' of about 0.002 kPa for a PAAm gel with 2% (w/v) AAm (Gittes *et al.*, 1997; Figure 5.8). Compared to these literature data, the values of the storage modulus G' measured for PAAm gels A – E were in a similar order of magnitude (Figure 5.8). Thus, for both PAAm and PEG hydrogels, the values of $|G^*|$ and G' measured by rheology and displayed in Table 5.3 fitted quite well to literature.

Since $|G^*|$ and G' for such hydrogels have never been measured for high frequencies, these values obtained here by the calculation from $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$, which were measured by the QCM at 5 MHz, were not able to be compared with literature data, but these values were evaluated with respect to the frequency dependency of the complex shear modulus. All the values of $|G^*|$ and G' calculated from QCM data were bigger than the respective values measured by rheology, as can also be seen in Table 5.3. This difference was equally pronounced for PAAm and PEG hydrogels and obtained to be bigger for softer hydrogels (Table 5.3). But, due to the frequency dependency of the complex shear modulus regularly reported in literature, it was expected that the values of $|G^*|$ and G' calculated from QCM data at 5 MHz were bigger than $|G^*|$ and G' measured by rheological measurements at 1 Hz. Although the quality of these equations from Bandey *et al.* could not exactly be assessed due to missing literature values of $|G^*|$ and G' for these hydrogels in the

high-frequency region, these results obtained here generally fitted quite well to the frequency dependency of the complex shear modulus. Thus, in the following section, these equations were then applied to cells for the determination of their complex shear modulus from QCM data in the high-frequency region (5 MHz).

5.2.2 Calculation of the Complex Shear Modulus of Cells by an Existing Correlation with QCM Parameters

After the equations from Bandey *et al.* between the complex shear modulus and QCM parameters, expressed by Eq. (65) and Eq. (66) and valid for semi-infinite viscoelastic materials, were successfully tested for PAAm and PEG hydrogels, this was applied to cells to only perform QCM measurements on the cells and to calculate their complex shear modulus from QCM parameters. This enabled the determination of the complex shear modulus of cells in the high-frequency region (5 MHz). In detail, by using these two equations, the magnitude of the complex shear modulus $|G^*|$ and the storage modulus G' were calculated from the change of the real part of the load impedance $\Delta Re(\underline{Z}_{Load})$ and the change of the imaginary part of the load impedance $\Delta Im(\underline{Z}_{Load})$ obtained from regular QCM measurements on the cells. The semi-infinite condition and, accordingly, the applicability of these equations to cells was evaluated by comparing the decay length of the shear wave into cells as the quartz loading to the average epithelial cell height. The decay length of the shear wave into cells was estimated before to be similar to that in water of about 250 nm (see section 3.2.1, *The QCM beyond the Sauerbrey Limitations*). The exact decay length can be calculated for each single cell line after $|G^*|$ and G' have been determined, which is demonstrated further down in this section. The epithelial cell height was determined in literature to about 6 μm (Breitenstein *et al.*, 2007) or 7 μm (Van Driessche *et al.*, 1993). Thus, the average epithelial cell height is much bigger than the decay length of the shear wave, ensuring the semi-infinite condition. But these calculations could only be performed for those cell lines, which were analyzed by QCM measurements in the full-spectrum-mode revealing their change of the load impedance $\Delta\Delta|Z|_{Load}$, from which $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$ could be extracted then. Thus, out of all the cell lines analyzed in this thesis, the calculation of $|G^*|$ and G' was only possible for HTM-N cells and NRK cells. Although values of $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$ for several other cell lines were reported in the literature (for example, in Heitmann *et al.*, 2007), these average values could not be used for the calculation of $|G^*|$ and G' because only the calculation from individual values of the two associated parameters $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$ was reasonable. Thus, values of $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$ were extracted from real-time QCM measurements on HTM-N cells and NRK cells performed elsewhere in this thesis, while they were extracted from those time points of the measurements reflecting confluent and untreated cell layers in reference to cell-free conditions. In Table 5.4, they are displayed as mean $\pm$ SD out of multiple measurements, although $|G^*|$ and G' were calculated from individually extracted values.

$|G^*|$ and G' were calculated by Eq. (65) and Eq. (66), while for both cell types, the density ρ_L was set as an average value for mammalian cells to $1.05 \cdot 10^3 \frac{\text{kg}}{\text{m}^3}$ (Wegener *et al.*, 1998). The calculated values of $|G^*|$ and G' were averaged afterwards and also given as mean $\pm$ SD in Table 5.4.

Table 5.4 The change of the real part of the load impedance $\Delta Re(\underline{Z}_{Load})$ and the change of the imaginary part of the load impedance $\Delta Im(\underline{Z}_{Load})$ extracted from QCM measurements (37 °C) at 5 MHz on HTM-N cells and NRK cells reflecting confluent and untreated cell layers. Based on these QCM data, the magnitude of the complex shear modulus $|G^*|$ and the storage modulus G' were calculated by Eq. (65) and Eq. (66), while using an average density ρ_L of $1.05 \cdot 10^3 \frac{\text{kg}}{\text{m}^3}$ for both cell types (Wegener *et al.*, 1998). The QCM parameters are given here as mean $\pm$ SD with the number of replicates n , although the calculation of $|G^*|$ and G' was not performed from the average values, but instead, from individually extracted values. $|G^*|$ and G' were averaged after calculation and given as mean $\pm$ SD.

Parameter	HTM-N	NRK
$\Delta Re(\underline{Z}_{Load}) / \Omega$	280 ± 10 ($n = 4(2)$)	510 ± 10 ($n = 4(2)$)
$\Delta Im(\underline{Z}_{Load}) / \Omega$	50 ± 10 ($n = 4(2)$)	130 ± 10 ($n = 4(2)$)
$ G^* / \text{kPa}$ (calc. from QCM data)	7.0 ± 0.6	24.6 ± 0.9
G' / kPa (calc. from QCM data)	6.4 ± 0.7	21.7 ± 0.9

The average values of the change of the real part of the load impedance $\Delta Re(\underline{Z}_{Load})$ of the two cell types (Table 5.4) were found to be similar to the values of certain hydrogels measured before (see Table 5.3). $\Delta Re(\underline{Z}_{Load})$ of HTM-N cells ($(280 \pm 10) \Omega$) was located between the values of PAAm gel D ($(360 \pm 60) \Omega$) and PAAm gel E ($(220 \pm 40) \Omega$) or between the values of PEG gel A ($(350 \pm 40) \Omega$) and PEG gel B ($(230 \pm 40) \Omega$), while $\Delta Re(\underline{Z}_{Load})$ of NRK cells ($(510 \pm 10) \Omega$) was found to be very similar to the value of PAAm gel C ($(500 \pm 100) \Omega$). Thus, testing the equations, correlating the complex shear modulus with QCM parameters, for the PAAm and PEG hydrogels was quite representative for these cell types due to similar viscoelastic properties, indicating that the calculation of the magnitude of the complex shear modulus $|G^*|$ and the storage modulus G' of the cells should also yield reasonable results. The values of the change of the imaginary part of the load impedance $\Delta Im(\underline{Z}_{Load})$ were found to be smaller (with respect to $\Delta Re(\underline{Z}_{Load})$) for the cells as compared to the respective values of the hydrogels (Table 5.4 and Table 5.3). The standard deviations of $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$ were observed to be smaller for these cell types as compared to the hydrogels, which also led to smaller standard deviations of $|G^*|$ and G' . For NRK cells, higher values of $|G^*|$ and G' were calculated compared to HTM-N cells (24.6 kPa compared to 7.0 kPa, and 21.7 kPa compared to 6.4 kPa), which was expected

since higher values of $\Delta Re(\underline{Z}_{Load})$ and $\Delta Im(\underline{Z}_{Load})$ were observed for NRK cells (Table 5.4). This was in agreement with the previous finding that materials with higher Young's modulus E exhibit also a higher complex shear modulus G^* (compare to Table 5.2). For both HTM-N and NRK cells, G' was found to be almost as high as $|G^*|$ (Table 5.4), indicating prevailing elastic characteristics. These values of $|G^*|$ and G' for HTM-N and NRK cells obtained here by the calculation from QCM data in the high-frequency region (5 MHz) were then compared to literature data. Since no literature data of the complex shear modulus for cells are available for this high frequency, they were just roughly compared to available values, which is only the case for frequencies lower than 100 Hz, while regarding the frequency dependency of the complex shear modulus. No values of $|G^*|$ and G' for HTM-N and NRK cells are available in literature, thus, they were compared to values for different cell lines. As already mentioned before in the discussion of the frequency dependency of the complex shear modulus in section 5.2, BALB/3T3 cells and SV-T2 cells exhibited values of G' of (20 – 70) Pa for frequencies of (0.02 – 0.3) Hz (Wottawah *et al.*, 2005), cortical astrocytes, cortical neurons, and NIH3T3 fibroblasts exhibited values of G' of (30 – 140) Pa for frequencies of (1 – 35) Hz (Ayala *et al.*, 2016), and MCF-10A cells, MCF-7 cells, and MDA-MB-231 cells exhibited values of G' of (0.6 – 3) kPa for frequencies of (5 – 100) Hz (Rother *et al.*, 2014). With the significantly higher values of G' obtained here for HTM-N cells ((6.4 $\pm$ 0.7) kPa) and NRK cells ((21.7 $\pm$ 0.9) kPa) for the frequency of 5 MHz, they fitted to the frequency dependency of the complex shear modulus.

Using the values of $|G^*|$ and G' determined for HTM-N and NRK cells (Table 5.4), the decay length of the shear wave δ into these cell types was calculated, which has never been performed before. This calculation was done according to Eq. (32), while the dynamic viscosity η' was determined by $\frac{G''}{\omega}$ according to Eq. (62), and G'' was calculated out of $|G^*|$ and G' according to Eq. (60). The frequency used for this calculation was 5 MHz. Same as for the calculation of $|G^*|$ and G' , the average cell density of $1.05 \cdot 10^3 \frac{\text{kg}}{\text{m}^3}$ was used. The errors of the values calculated for the decay length were determined by Gaussian error propagation. This calculation resulted in a decay length δ of (70 $\pm$ 30) nm for HTM-N cells and (150 $\pm$ 20) nm for NRK cells, which were both smaller than the 250 nm for water. Compared to the much bigger average epithelial cell heights of about 6 μm (Breitenstein *et al.*, 2007) or 7 μm (Van Driessche *et al.*, 1993), the assumption of fulfilling the semi-infinite condition was justified. Since cell-surface distances are in the range from 10 nm (Braun & Fromherz, 1997) to 70 nm (Lanni *et al.*, 1985), mechanical sensing by the QCM is indeed restricted close to the basal membrane.

Taken all together, a method was developed to obtain the complex shear modulus G^* , especially the magnitude of the complex shear modulus $|G^*|$ and the storage modulus G' , for cells and hydrogels, which is based on QCM measurements and equations found by Bandey *et al.*, as expressed by Eq. (65) and Eq. (66), without performing elaborate and invasive deformation measurements. The most important issue is the different measurement frequencies of QCM measurements (MHz-region) and rheological

measurements that serve as gold standard (Hz-region). But, considering this difference in frequency not as a disadvantage just because of its low comparability, but instead, as an opportunity to find complex shear moduli in a new frequency region, this approach enables a way to obtain complex shear moduli in the MHz-region, which is hardly accessible by other techniques. Results for PAAm and PEG hydrogels as well as for cells were in a reasonable order of magnitude as predicted by the frequency dependency of the complex shear modulus. Similar to rheological measurements, the QCM offers several useful advantages like its time-resolved and non-invasive nature with integral signal readout, which makes the QCM perfectly suited for determination of the complex shear modulus of soft viscoelastic materials like cells or hydrogels. Additionally, the decay length of the shear wave can be determined for the cell line under study. Another advantage of this QCM-approach is the possibility to simultaneously determine the Young's modulus E of cells when correlations with QCM parameters are established for hydrogels before, as it was demonstrated in section 5.1.

6 Investigating Cellular Changes Involved in the Pathogenesis and Treatment of Primary Open-Angle Glaucoma

As described in detail in section 1.1.2, one hallmark in the pathogenesis of primary open-angle glaucoma (POAG) are cytoskeletal changes in trabecular meshwork (TM) cells, which lead to an increased outflow resistance and, accordingly, to an increased intraocular pressure (IOP). To study these cytoskeletal changes, immortalized human trabecular meshwork (HTM-N) cells were used as a model for the TM cells of a human eye. Most commonly, primary HTM cells are used for this purpose, although HTM-N cells were demonstrated not to differ from primary HTM cells regarding the expression of cytoskeletal and extracellular matrix proteins and intracellular signal transduction systems (Pang *et al.*, 1994). The similar behavior between both types of HTM cells was also confirmed in a study investigating the effect of a Rho-associated protein kinase (ROCK) inhibitor on cell proliferation (Chen *et al.*, 2020), whereas slight differences regarding the response to drugs potentially inducing cytoskeletal changes were obtained elsewhere (Vollmer *et al.*, 2020). In this thesis, HTM-N cells were preferred to primary HTM cells due to two critical advantages for cell culture. They can be passaged more often and used in culture for an extended period of time due to their immortalization, and additionally, they are growing faster (Pang *et al.*, 1994). Cytoskeletal changes may trigger two distinct major phenotypic changes, viscoelastic changes and/or morphological changes. When a cytoskeleton influencing drug only induces one of those phenotypic changes, the use of different methods may be required to ensure that cytoskeletal changes are detected anyway. On the other hand, when cytoskeletal changes lead to both of those phenotypic changes, this offers the opportunity to confirm results by using different methods of detection. Thus, for all experiments performed or substances analyzed in this chapter, at least two independent techniques of detection were used to characterize the associated phenotypic changes. For recording viscoelastic changes of the cells, the QCM technique has proven to be perfectly suited. In the present chapter, it was always complemented by ECIS and/or microscopic techniques (phase contrast microscopy, CLSM) to detect morphological changes. First, the adhesion behavior of HTM-N cells was analyzed by QCM readings and by phase contrast microscopy (section 6.1). The connective tissue growth factor (CTGF), which may play a critical role in the pathogenesis of POAG, was studied regarding its effect on HTM-N cells, examined by the QCM and by CLSM imaging (section 6.2). As described in detail in section 1.1.3, although the ROCK inhibitor fasudil has not yet been approved for the treatment of POAG, it has been demonstrated several times to exhibit positive effects for the treatment of this disease. Thus, the HTM-N cells' response to fasudil was investigated by QCM, ECIS, and CLSM imaging (section 6.3).

6.1 Monitoring the Adhesion of HTM-N Cells

Before investigating the effects of CTGF and fasudil on the HTM-N cells, first, the suitability of these cells for impedimetric and microscopic analysis was tested. Since an adherent cell layer is required for these techniques, that also has to be confluent for ECIS, the adhesion behavior of the HTM-N cells was investigated on quartz disks by QCM measurements and on glass slides by phase contrast microscopy. Cell adhesion is a combined process of cell attachment and spreading, which were both tried to be observed experimentally for the HTM-N cells. Microgravimetric monitoring of HTM-N cell adhesion was started immediately after seeding the cells into the QCM chambers and letting them settle down for 10 min in the laminar flow hood. The HTM-N cells were seeded in an areal cell density that was expected to lead to a confluent cell layer after attachment and spreading and without proliferation ($200\,000\text{ cells/cm}^2$). The change of the minimal impedance $\Delta|Z|_{\min}$ and the change of the load impedance $\Delta\Delta|Z|_{\text{Load}}$ over time are displayed in Figure 6.1 A and B, respectively, while both QCM parameters were normalized to the measurement start and averaged (mean $\pm$ SD with $n = 4(2)$). The first 3 h of the measurements are highlighted in the small inserts. Non-normalized and non-averaged time courses of the minimal impedance $|Z|_{\min}$ and the load impedance $\Delta|Z|_{\text{Load}}$ are depicted in Figure S2. Additionally, the change of the half-width-half-maximum of maximal conductance $\Delta\Gamma$ and the change of the frequency of maximal conductance $\Delta f(G_{\max})$ extracted from the same measurements are shown in Figure S3.

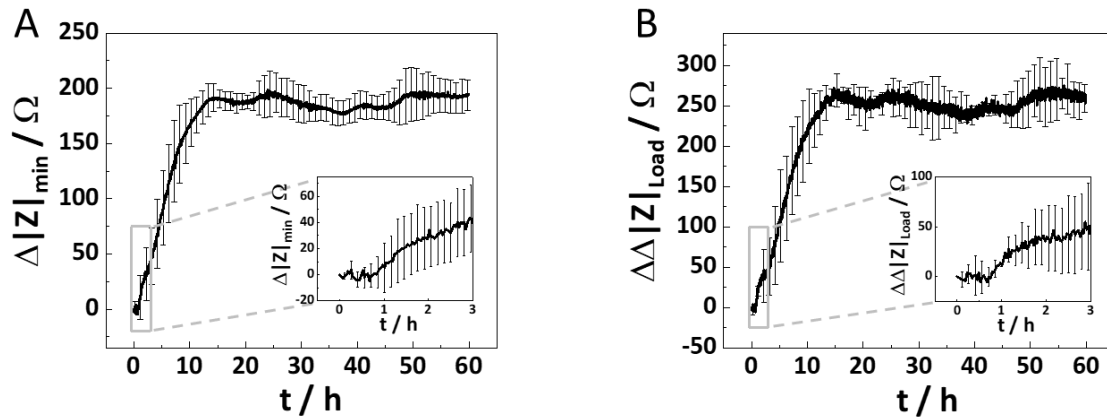


Figure 6.1 The change of the minimal impedance $\Delta|Z|_{\min}$ (A) and the change of the load impedance $\Delta\Delta|Z|_{\text{Load}}$ (B) extracted from QCM measurements during HTM-N cell adhesion (seeded confluent). Measurements were started directly after addition of the cell suspension (including the 10 min storage in the laminar flow hood for settling down of cells), while the included inserts zoom on the first 3 h. Both QCM parameters were extracted from the same measurements and were normalized to the measurement start. Data are displayed as mean $\pm$ SD out of four total replicates with two biological replicates ($n = 4(2)$). Non-normalized and non-averaged data are presented in Figure S2. Corresponding data for the change of the half-width-half-maximum of maximal conductance $\Delta\Gamma$ and the change of the frequency of maximal conductance $\Delta f(G_{\max})$ are shown in Figure S3. $T = 37\text{ }^{\circ}\text{C}$.

The time courses of both the change of the minimal impedance $\Delta|Z|_{min}$ (Figure 6.1 A) and the change of the load impedance $\Delta\Delta|Z|_{Load}$ (Figure 6.1 B) during adhesion of HTM-N cells showed close similarities to each other, which was already observed for hydrogels before (see Figure 5.4). Non-normalized and non-averaged data revealed start values for the minimal impedance $|Z|_{min}$ of about 450 Ω and start values for the load impedance $\Delta|Z|_{Load}$ close to 0 Ω (see Figure S2), which did not differ from the values experienced over multiple measurements for a cell-free control with pure medium or water as the quartz loading. This proved that the simple presence of suspended cells in the medium and even cell settling by sedimentation do not induce additional damping of the resonator's shear oscillation. Although it is unknown how close the cells approach the surface by sedimentation, and whether it is within the decay length of the shear wave or not, the finding that the impedance did not change during this process was reasonable because the cells do not yet exhibit anchoring points to the surface. The small inserts in Figure 6.1 showed a relatively constant signal of $\Delta|Z|_{min}$ and $\Delta\Delta|Z|_{Load}$ for about the first 1 h before an increase was observed, which demonstrated that signal recording was started not too late to detect the complete adhesion process, and settling down of cells is probably continuously occurring until this time point. After that, $\Delta|Z|_{min}$ and $\Delta\Delta|Z|_{Load}$ were observed to continuously increase for about 15 h reflecting an increasing amount of viscoelastic loading. When the cells are coming closer to the surface, they start interacting with the sensor surface and anchor to it by integrin-mediated formation of focal contacts and hemidesmosomes. Since completely adhered cells exhibit cell-surface distances (10 nm (Braun & Fromherz, 1997) to 70 nm (Lanni *et al.*, 1985)) that are smaller than the corresponding decay length of the shear wave (calculated to (70 ± 30) nm for HTM-N cells (see section 5.2.2)) and exhibit strong cell-substrate contacts, the viscoelastic cell bodies oscillate with the resonator and contribute to the damping of its oscillation. Thus, the increase in $\Delta|Z|_{min}$ and $\Delta\Delta|Z|_{Load}$ mirrored the formation of contacts between the quartz surface and a gradually increasing number of cell bodies confirming a successful attachment of the HTM-N cells onto the quartz disk. But additional to cell attachment, also cell spreading can cause such an increase in minimal impedance and load impedance because it goes along with an increasing contact area and, accordingly, with a formation of further cell-substrate contacts. But discrimination between attachment and spreading of the cells is not possible in these QCM measurements due to the integral signal readout. This increase in $\Delta|Z|_{min}$ and $\Delta\Delta|Z|_{Load}$ stopped after about 15 h of adhesion with values of $\Delta|Z|_{min}$ of almost 200 Ω and $\Delta\Delta|Z|_{Load}$ of about 250 Ω , followed by some further signal fluctuations without significant deviation from these values obtained after 15 h. These fluctuations can also be observed in the individual time courses in Figure S2 and were attributed to cell motility. The relatively constant signal after the initial increase demonstrated that no cell proliferation occurred after adhesion since the quartz surface seemed to be completely covered with cells then. This confirmed the expectation of reaching a confluent cell layer after attachment and spreading, which is completed after about 15 h. Thus, these values of $\Delta|Z|_{min}$ and $\Delta\Delta|Z|_{Load}$ obtained after 15 h of HTM-N cell adhesion were regarded as plateau values representative for a confluent and adherent layer of HTM-N cells, which were for example used in section 5.1.3 to calculate their Young's

modulus E . The finding that the QCM signal after 60 h was still at the level of the value after 15 h demonstrated the HTM-N cells to be a stable and robust cell line for phenotypic assays along this time window. Similar time courses of $\Delta|Z|_{min}$, as obtained here for the HTM-N cells, were already observed for NRK cells and MDCK-II cells by combined ECIS-QCM measurements (Oberleitner, 2018). Significantly higher values of $\Delta|Z|_{min}$ were observed there, but this is actually not comparable due to the significantly higher base values of $|Z|_{min}$ due to the use of specially designed double-quartz-disks. With respect to that and according to the only slightly longer period of time needed to reach confluency, the HTM-N cells showed an adhesion behavior similar to that of established epithelial cell lines.

To confirm and complement these observations from the QCM measurements, the adhesion of HTM-N cells was monitored additionally by phase contrast microscopic imaging. This was performed on glass slides embedded in specially designed Teflon® vessels (see Figure 4.4) mimicking the QCM chambers in terms of size, which were also used for the polymerization of PAAm hydrogels prior to compression testing. Experimental conditions were the same as for the QCM measurements (areal cell density of 200 000 cells/cm² and included time of 10 min for settling down of cells). In Figure 6.2, these images are shown when taken immediately (Figure 6.2 A), after 6 h (Figure 6.2 B), 12 h (Figure 6.2 C), 18 h (Figure 6.2 D), 24 h (Figure 6.2 E), 30 h (Figure 6.2 F), 36 h (Figure 6.2 G), 42 h (Figure 6.2 H), 48 h (Figure 6.2 I), 54 h (Figure 6.2 J), 60 h (Figure 6.2 K), and 66 h (Figure 6.2 L) after seeding the cell suspension. Two experimental replicates were performed, from which each image was selected individually showing a spot of the cell layer representative for the respective time point.

The HTM-N cells were observed to exhibit a spherical shape directly after cell seeding (Figure 6.2 A), which was obtained to be stepwise reduced after 6 h (Figure 6.2 B) and 12 h (Figure 6.2 C) and completely vanished after 18 h (Figure 6.2 D). The cells started to form clusters, became bigger, and gradually deviated from their initially spherical shape. Additionally, the HTM-N cells exhibited relatively big cell distances with many cell-free spaces in the beginning (Figure 6.2 A), which was observed to be stepwise reduced until the surface was completely covered with cells after 30 h (Figure 6.2 F) and also attributed to the increasing cell size. This increase in cell size indicated flattening of the cells by attachment and spreading with an increasing contact area to the substrate, which was in good agreement with the increase in $\Delta|Z|_{min}$ and $\Delta\Delta|Z|_{Load}$ obtained in Figure 6.1, although the time point of reaching confluency was observed to be later here (30 h compared to the 15 h obtained in QCM).

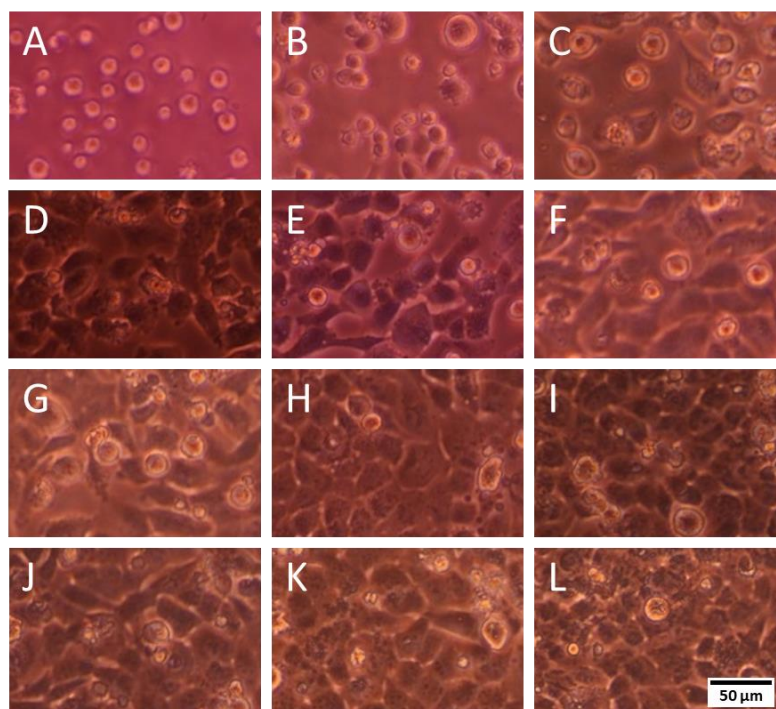


Figure 6.2 Phase contrast microscopic images of confluent seeded HTM-N cells during their adhesion on glass slides embedded in Teflon® vessels (see Figure 4.4). The images were taken directly after addition of the cell suspension (including the 10 min storage in the laminar flow hood for settling down of cells), thus, corresponding to a time of 0 h in the QCM measurement (Figure 6.1) (A), and 6 h (B), 12 h (C), 18 h (D), 24 h (E), 30 h (F), 36 h (G), 42 h (H), 48 h (I), 54 h (J), 60 h (K), and 66 h (L) after seeding the cell suspension. Typical images were selected from two experimental replicates, while representative spots are shown. $T = 37\text{ }^{\circ}\text{C}$.

Beyond that, in the time from 30 h (Figure 6.2 F) to 66 h (Figure 6.2 L), the cells were observed to undergo no significant changes regarding cell shape or confluency, but they became more compact over time, which was attributed to a certain extent of proliferation. The reason why this proliferation could not be observed in the QCM measurements was probably the fact that the confluency did not change in this period of time leading to a constant strength and number of cell-substrate contacts. Furthermore, in the image for 48 h after cell seeding (Figure 6.2 I) and subsequent images, residues of dead cells were visible indicating that the cells lose viability after 2 d of cultivation without addition of fresh cell culture medium.

In total, adhesion of HTM-N cells could be successfully demonstrated in QCM measurements by the increase in $\Delta|Z|_{min}$ and $\Delta\Delta|Z|_{Load}$ and in phase contrast microscopic imaging by the change in cell shape and size. This also proved that surface coating for improving cell adhesion was not necessary. For subsequent experiments with these cells, they were seeded with the same areal cell density as used here in these adhesion studies ($200\,000\text{ cells/cm}^2$) and cultivated for 2 d (3 d for the transwell experiments (see section 7)) to obtain a confluent cell layer. To avoid a potential loss in cell viability, cells were fed 24 h before measurement start and measurements never took longer than 48 h.

6.2 Studying the Effect of CTGF on HTM-N Cells

Connective tissue growth factor (CTGF) is a protein that is assumed to play a major role in the pathogenesis of POAG, as described in detail in section 1.1.2. It was demonstrated to induce many different changes also found in glaucomatous eyes, like an increase in the number of stress fibers, an increase in cellular contractility, an increase in IOP, and a reduction of the number of axons in the visual nerve (Junglas *et al.*, 2012). Since a connection between POAG and stiffening of cells in the TM was discovered (Last *et al.*, 2011; Wang *et al.*, 2018), a correlation between increased levels of CTGF and this TM cell stiffening was hypothesized, but has never been definitely confirmed yet. Thus, this was analyzed here by CLSM imaging and QCM studies. CLSM imaging was performed as a first indicator of cytoskeletal changes that may be relevant for a stiffness increase, which should be confirmed by QCM readings known to be sensitive to stiffness changes, which was already demonstrated before for hydrogels (see section 5.1). TM cells exhibit several different extracellular binding molecules for CTGF (Junglas *et al.*, 2009), which can also be assumed for HTM-N cells, enabling the testing of the effect of artificially added CTGF on the cells. CTGF was used in a standard mass concentration of 50 $\mu\text{g}/\text{L}$, since this concentration was already demonstrated to induce significant cytoskeletal changes 24 h after treatment (Junglas *et al.*, 2012). A confluent HTM-N cell layer was treated with 50 $\mu\text{g}/\text{L}$ CTGF or a respective control solution, and CLSM images of the actin cytoskeleton were taken 24 h after treatment. Cells were treated according to section 4.10.3 and images were taken according to section 4.10.2. Images were selected from two experimental replicates, showing a spot of the cell layer that is representative for the respective condition (Figure 6.3).

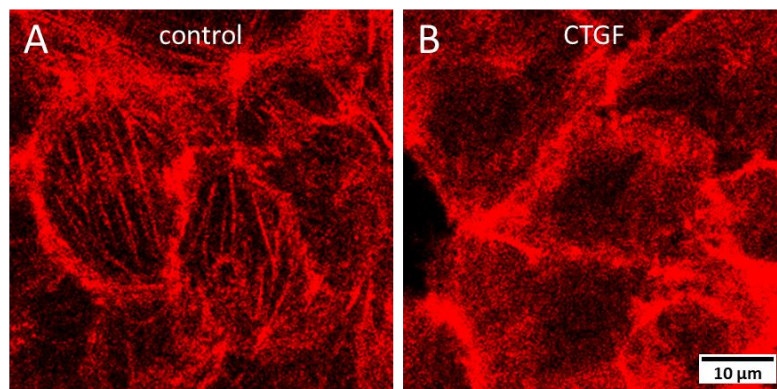


Figure 6.3 Confocal laser scanning microscopic images of confluent HTM-N cell layers with TRITC-phalloidin labeling of actin filaments, treated with a control solution (**A**) or treated with 50 $\mu\text{g}/\text{L}$ CTGF (**B**). Images were taken 24 h after start of exposure and were chosen from two experimental replicates, while representative spots were selected. The focal plane was chosen close to the substrate to observe stress fibers and the cell cortex. $T = 37^\circ\text{C}$.

Many thin and long fibers were observed in the CLSM image for the control (Figure 6.3 A), which demonstrated the presence of stress fibers in HTM-N cells under control conditions.

No similar structures were observed in the CLSM image after CTGF treatment (Figure 6.3 B) indicating that CTGF may lead to a degradation of stress fibers. In both images, the cell cortex was clearly visible by the presence of actin in ring-shaped structures, which was observed to be stronger pronounced after CTGF treatment (Figure 6.3 B), indicating a strengthening of the cell cortex by CTGF. A significant alteration in cell size was not observed. Both stress fibers and cell cortex are important structures affecting cell stiffness. Since the CTGF treatment led to a degradation of stress fibers indicating a stiffness decrease, and simultaneously led to a strengthening of the cell cortex indicating a stiffness increase, a conclusion about the behavior of cell stiffness was not possible here. These findings were contrary to studies on primary HTM cells, which were obtained to become larger and to exhibit stronger pronounced and longer stress fibers after CTGF treatment, which was expected to correlate with a stiffness increase (Junglas *et al.*, 2012). These differences may be attributed to differences between primary HTM cells and HTM-N cells. Although different microscopic observations of the cytoskeleton were made after CTGF stimulation, both the results obtained in this thesis and by Junglas *et al.* indicated that the stiffness of the cells may be changed after treatment with CTGF. Thus, QCM measurements were conducted to analyze cell stiffness more precisely. The change of the minimal impedance $\Delta|Z|_{min}$ already known to reflect stiffness of the quartz loading (see section 5.1) was recorded until about 1 d after treatment of HTM-N cells with 50 $\mu\text{g/L}$ CTGF, in comparison to the addition of a control solution (Figure 6.4). Data were normalized to the time point of addition and averaged over three (CTGF, Figure 6.4, red curve) or five (control, Figure 6.4, black curve) replicates.

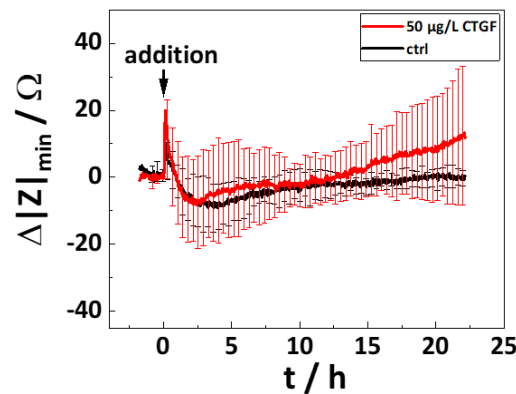


Figure 6.4 The change of the minimal impedance $\Delta|Z|_{min}$ during QCM measurements analyzing the influence of 50 $\mu\text{g/L}$ CTGF (red) on confluent HTM-N cells for about 1 d in contrast to control conditions (ctrl, black). Data were normalized to the time point of addition and are displayed as mean $\pm$ SD out of several measurements (CTGF: $n = 3(3)$, ctrl: $n = 5(5)$). $T = 37^\circ\text{C}$.

Directly after stimulation of the HTM-N cells with the 50 $\mu\text{g/L}$ CTGF test solution an instant increase in the minimal impedance of about 20 Ω was observed (Figure 6.4, red curve). But the change of the minimal impedance $\Delta|Z|_{min}$ returned to baseline levels after only 1 h and

the same behavior was obtained for control conditions (Figure 6.4, black curve), which both demonstrated that this initial increase was just a liquid handling peak reflecting a slight impairment of the cells by liquid additions. These addition peaks were regularly observed in QCM measurements investigating cellular responses to certain substances all over this thesis and were considered a normal cellular reaction as long as $\Delta|Z|_{min}$ was smaller than $30\ \Omega$ and the signal change recovered back to baseline levels within 2 h. After the addition peak, as it was expected for control conditions, the HTM-N cells exhibited no significant deviation of $\Delta|Z|_{min}$ from baseline levels with values between $-10\ \Omega$ and $0\ \Omega$ (Figure 6.4, black curve). A very similar behavior of $\Delta|Z|_{min}$ was obtained after CTGF stimulation within 1 d of monitoring (Figure 6.4, red curve). The signal increase of about $10\ \Omega$ reached at the end of the measurement was very small and not significant with respect to the error bars. Thus, this QCM measurement indicated that no stiffness changes occurred after treatment of the HTM-N cells with $50\ \mu\text{g/L}$ CTGF. But, since $\Delta|Z|_{min}$ exhibited a slightly ascending trend in the end of the measurement reporting on the cellular reaction to CTGF for about 1 d (Figure 6.4, red curve), this was performed again with an extension of the time of data recording from about 1 d to about 2 d, which is demonstrated in Figure 6.5. Additionally, to ensure that a potential effect of CTGF on cell stiffness is not obstructed by a possible binding of CTGF to the plastic walls of the micro test tubes and the Teflon® QCM chambers, BSA was used as a blocker to prevent this binding. It was used in a mass concentration of $5\ \text{g/L}$ and present in all the solutions added to the HTM-N cells. Moreover, to test if CTGF maybe only influences HTM-N cell stiffness in a higher concentration, once it was added double concentrated ($100\ \mu\text{g/L}$). Data for the change of the minimal impedance $\Delta|Z|_{min}$ were normalized to the time point of addition and averaged for $50\ \mu\text{g/L}$ CTGF with additional $5\ \text{g/L}$ BSA (Figure 6.5, red curve) and control conditions (Figure 6.5, black curve), whereas $100\ \mu\text{g/L}$ CTGF with additional $5\ \text{g/L}$ BSA (Figure 6.5, green curve) was only tested once.

After adding test and control solutions, addition peaks were observed in Figure 6.5, as already observed in Figure 6.4. Again, control conditions did not show any influence on the change of the minimal impedance $\Delta|Z|_{min}$ because it exhibited values between $-10\ \Omega$ and $0\ \Omega$ over the complete measurement time of about 2 d (Figure 6.5, black curve). This also demonstrated that $5\ \text{g/L}$ BSA did not induce a stiffness change of HTM-N cells, as it was expected. After addition of $50\ \mu\text{g/L}$ CTGF in presence of $5\ \text{g/L}$ BSA $\Delta|Z|_{min}$ showed a slight decrease with values that were constantly below the values of the control and a final value of about $-20\ \Omega$ (Figure 6.5, red curve). But, these differences in $\Delta|Z|_{min}$ were very small and especially the differences in the final values were not significant with respect to the error bars. The HTM-N cells' response to $100\ \mu\text{g/L}$ CTGF in presence of $5\ \text{g/L}$ BSA (Figure 6.5, green curve) was very similar to the one of normal CTGF concentration. A slight decrease in $\Delta|Z|_{min}$ was observed with a final value of about $-30\ \Omega$, which could not be regarded as significant because it was also very small and measured only once.

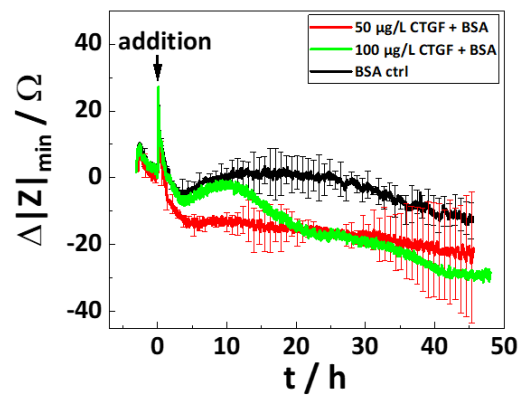


Figure 6.5 The change of the minimal impedance $\Delta|Z|_{\min}$ during QCM measurements analyzing the influence of 50 $\mu\text{g/L}$ CTGF + 5 g/L BSA (red) and 100 $\mu\text{g/L}$ CTGF + 5 g/L BSA (green) on confluent HTM-N cells for about 2 d in contrast to control conditions (black). Data were normalized to the time point of addition and are displayed as mean $\pm$ SD for the red and black curves (50 $\mu\text{g/L}$ CTGF: $n = 2(2)$, BSA ctrl: $n = 3(3)$), while data for the green curve could not be averaged because this was only measured once. $T = 37^\circ\text{C}$.

Thus, an influence of CTGF on HTM-N cell stiffness was not observed in these QCM measurements (Figure 6.4 and Figure 6.5), neither after 1 d of monitoring nor after 2 d, neither for 50 $\mu\text{g/L}$ CTGF nor for 100 $\mu\text{g/L}$ CTGF, and neither without BSA nor in presence of 5 g/L BSA as a blocker. These results fitted actually not too bad to the CLSM images taken for HTM-N cells 24 h after treatment with 50 $\mu\text{g/L}$ CTGF (see Figure 6.3), although cytoskeletal changes were observed there. Following hypothesis was proposed: The stiffness decrease indicated by the degradation of stress fibers seemed to be compensated by the stiffness increase indicated by the strengthening of the cell cortex resulting in no significant net change of cell stiffness. Since no significant change of the QCM signal was observed at all, and also no temporary increase or decrease, this indicated a more or less simultaneous occurrence of both processes of degradation of stress fibers and strengthening of the cell cortex. Another reason may be that the cytoskeletal changes obtained by CLSM imaging were not big enough to be detected with significance in QCM readings. This could maybe be resolved by future studies on taking CLSM images at multiple time points after addition of CTGF. As already mentioned above, these results were contrary to those by Junglas *et al.*, where a strengthening of stress fibers was obtained and an increase in cell stiffness was predicted to happen after CTGF treatment of primary HTM cells (Junglas *et al.*, 2012). These differences can probably be attributed to a big extent to a different behavior of HTM-N cells and primary HTM cells, which has been discussed controversially in the literature. Major differences between these two cell types were confirmed (Vollmer *et al.*, 2020), but also excluded (Pang *et al.*, 1994; Chen *et al.*, 2020) in previous studies. Future studies on performing QCM measurements with primary HTM cells can help resolve these discrepancies regarding the influence of CTGF on the stiffness of TM cells.

6.3 Investigating the Relaxation Effect of Fasudil on HTM-N Cells

After the discovery of fasudil as an efficient inhibitor of Rho-associated protein kinase (ROCK) (Asano *et al.*, 1998) it was found to lower IOP, the most important risk factor of POAG, in rabbits (Sugiyama *et al.*, 2011) and in humans (Pakravan *et al.*, 2017). It exhibits several further positive effects for the treatment of this disease (increase in blood flow in the visual nerve head, prevention of retinal ganglion cell death), as already described in detail in section 1.1.3. Nevertheless, fasudil has not yet been approved for the treatment of POAG, whereas other ROCK inhibitors like ripasudil and netarsudil have already been. Positive effects of ROCK inhibitors on the decrease in IOP are considered to be the result of an increase in aqueous humor outflow capacity through the TM correlated with a destabilization and depolymerization of actin fibers in TM cells. But only a few studies have been published, that demonstrated a correlation between fasudil treatment and degradation of actin fibers by using fluorescence imaging (Lau *et al.*, 2011; Li *et al.*, 2018). Thus, a comprehensive investigation of the relaxation effect of fasudil on the actin cytoskeleton of HTM-N cells was performed here, which was evaluated by QCM and ECIS measurements to assess viscoelastic and morphological changes, known to be closely correlated with alterations of the actin cytoskeleton, and confirmed by CLSM imaging. Since the TM tissue was shown to express ROCKs intracellularly (Fukiage *et al.*, 2001), the HTM-N cells are also expected to express these intracellular kinases. According to the membrane-permeable behavior of fasudil, its effect on the HTM-N cells can be studied by adding solutions of fasudil extracellularly to these cells. For comparison, these investigations on fasudil were accompanied by studies on cytochalasin D, known as an actin filament binding drug and inhibitor of their polymerization, so it induces a decomposition of the actin cytoskeleton over time (Casella *et al.*, 1981). Cytochalasin D was also demonstrated to have a potential application for the treatment of POAG by increasing aqueous humor outflow capacity (Johnson, 1997). Additional to the possibility of studying morphological changes of the cells, ECIS also enabled a fast screening of a variety of concentrations of the drugs under investigation by its high throughput, which was performed first of all. HTM-N cells seeded confluent in 8W10E arrays were treated with different concentrations of fasudil or cytochalasin D and their impedance was recorded at multiple frequencies in an extended frequency range (500 Hz – 96 kHz) by ECIS measurements (Figure 6.6). The concentration range of the drugs was oriented on studies in the literature, which found positive cell responses for fasudil concentrations of (12.5 – 50) μM (Li *et al.*, 2018) and 100 μM (Lau *et al.*, 2011), and a half-maximal inhibition of actin polymerization by 0.5 μM cytochalasin D (Casella *et al.*, 1981). Thus, fasudil was used in seven different concentrations in the range of (1 – 200) μM (Figure 6.6 A), and cytochalasin D was used in three different concentrations in the range of (0.05 – 5) μM including the reported IC_{50} value (Figure 6.6 B), both in comparison to the addition of a control solution. The most sensitive frequency was determined to 16 kHz, thus, the impedance was plotted for this specific frequency and

normalized to the time point of addition. Consequently, the normalized impedance at 16 kHz $norm. |Z|_{16\text{ kHz}}$ is displayed in Figure 6.6, which was averaged over four wells from two biological replicates for each fasudil concentration and over two wells from one experiment for each cytochalasin D concentration.

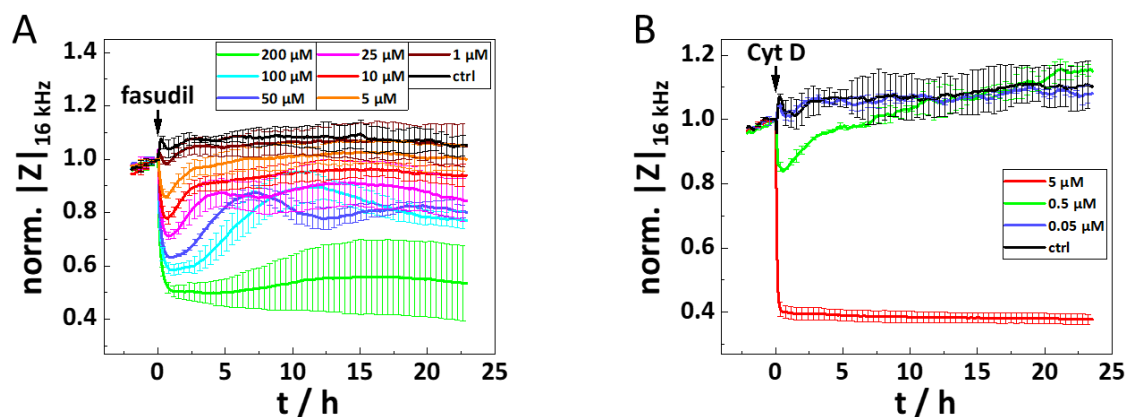


Figure 6.6 Normalized impedance at 16 kHz $norm. |Z|_{16\text{ kHz}}$ during ECIS measurements analyzing the influence of different concentrations of fasudil (A) and cytochalasin D (Cyt D) (B) on HTM-N cells seeded confluent in 8W10E arrays. Fasudil concentrations were 200 μM (A, green), 100 μM (A, cyan), 50 μM (A, blue), 25 μM (A, purple), 10 μM (A, red), 5 μM (A, orange), 1 μM (A, brown) in contrast to control conditions (A, black), and cytochalasin D concentrations were 5 μM (B, red), 0.5 μM (B, green), and 0.05 μM (B, blue) in contrast to control conditions (B, black). Data were normalized (ratio) to the time point of addition and are displayed as mean $\pm$ SD out of four wells from two biological replicates for fasudil ($n = 4(2)$) and out of two wells from one measurement for cytochalasin D ($n = 2(1)$). Data of $norm. |Z|_{16\text{ kHz}}$ are displayed down to a value of 0.27 representing cell-free conditions. $T = 37^\circ\text{C}$.

Calculations of the normalized impedance at 16 kHz $norm. |Z|_{16\text{ kHz}}$ from spectra of cell-free electrodes recorded in advance revealed a value of 0.27 for cell-free conditions, which was the reason why the plots of $norm. |Z|_{16\text{ kHz}}$ in Figure 6.6 A and B started at this value. As expected, control measurements for both fasudil and cytochalasin D (Figure 6.6, both black curves) exhibited a relatively constant impedance over the complete measurement time of about 1 d. No significant signal change was also observed for the respective lowest concentrations of both substances, for 1 μM fasudil (Figure 6.6 A, brown curve) and 0.05 μM cytochalasin D (Figure 6.6 B, blue curve). The addition of higher fasudil and cytochalasin D concentrations to the HTM-N cells led to instant and significant changes of $norm. |Z|_{16\text{ kHz}}$ reflecting major morphological changes, where the signal decrease indicated cell contraction as the current can cross the cell layer more easily after treatment. This was in agreement with the expectation that both drugs induce a degradation of actin filaments, leading to a certain degree of contact loss to neighboring cells and the growth substrate. To gain information about the exact biological origin of these impedance changes, modeling of the ECIS data is needed, which was performed for a similar experiment further down. The time courses of $norm. |Z|_{16\text{ kHz}}$ for fasudil concentrations of 5 μM (Figure 6.6 A, orange curve), 10 μM (Figure 6.6 A, red curve), 25 μM (Figure 6.6 A, purple curve), 50 μM (Figure 6.6 A, blue

curve), and 100 μM (Figure 6.6 A, cyan curve) and for 0.5 μM cytochalasin D (Figure 6.6 B, green curve) exhibited a minimum at about 1 h after addition. This was followed by a partial recovery of the signal indicating an equilibration process of the cells, which was more pronounced for lower concentrations. Both fasudil and cytochalasin D induced similar impedance changes, especially 5 μM fasudil (Figure 6.6 A, orange curve) and 0.5 μM cytochalasin D (Figure 6.6 B, green curve), indicating similar effects of both substances on the HTM-N cells. The highest fasudil concentration of 200 μM (Figure 6.6 A, green curve) triggered a huge decrease in $\text{norm. } |Z|_{16 \text{ kHz}}$ with almost no recovery, while the relatively huge error bars demonstrated that some experienced a small extent of recovery and some did not. These data suggested a strong and irreversible impairment of cellular viability. Similarly, 5 μM cytochalasin D (Figure 6.6 B, red curve) caused a huge signal decrease almost down to the level of a cell-free electrode ($\text{norm. } |Z|_{16 \text{ kHz}}$ of about 0.4 in comparison to 0.27 for cell-free conditions) without subsequent recovery and without signal fluctuations, which indicated that the HTM-N cells were killed due to the complete destruction of their actin cytoskeleton by cytochalasin D. In total, for both fasudil and cytochalasin D, a good concentration dependency of the decrease in $\text{norm. } |Z|_{16 \text{ kHz}}$ was obtained, with a more pronounced minimum (smaller value) for higher concentrations. The concentration dependency also seemed to be expressed in the extent of recovery after reaching the minimum. Thus, this concentration dependency was evaluated for fasudil in detail by the minimum reached after about 1 h and, additionally, by the area under the curve. The minimum was not taken from the average curves, but instead, extracted from each individual time course and averaged afterwards. The area was determined by integration from 0 h to 20 h and down to a value of $\text{norm. } |Z|_{16 \text{ kHz}}$ of 0.27 (leading to a theoretical area of 0 for cell-free conditions), which is shown for one typical time course in Figure S4. The area was determined individually for each single time course, averaged afterwards, and normalized to a value of 1 for control conditions (Figure 6.6 A, black curve). With this, the area mirrored both the minimum and the extent of recovery. The results of the normalized area are, together with the minimum, depicted in Figure 6.7.

As already observed in Figure 6.6 A, the detailed analysis of the minimum of $\text{norm. } |Z|_{16 \text{ kHz}}$ also revealed a very good concentration dependency, with a smaller minimum for higher fasudil concentrations (Figure 6.7 A). A concentration dependency was also observed for the area under the curve with higher concentrations of fasudil leading to a smaller area (Figure 6.7 B), reflecting a smaller extent of recovery. But this was of less significance than for the minimum since error bars were partially overlapping and due to the exception of 100 μM fasudil exhibiting an area similar to the one for 50 μM fasudil, which resulted from the overlapping time courses during recovery for these two fasudil concentrations (see Figure 6.6 A, blue and cyan curves). IC_{50} values could not be extracted since the data points did not follow a sigmoidal behavior.

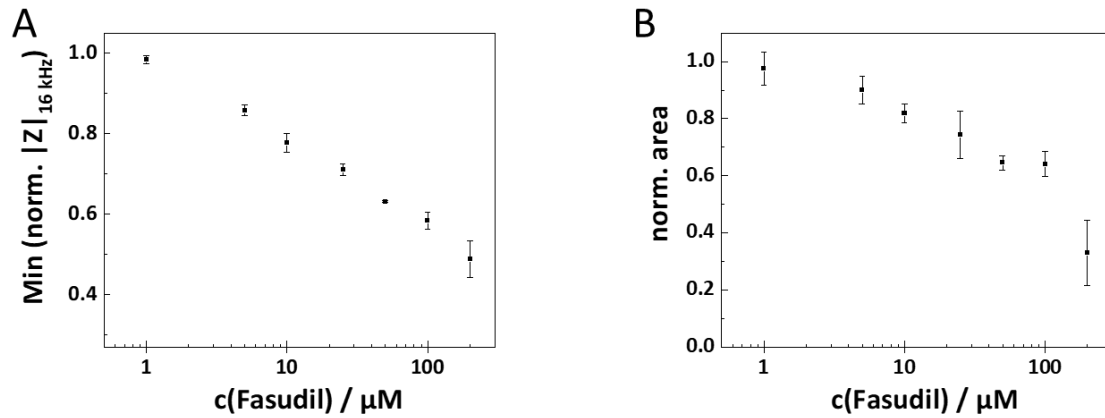


Figure 6.7 Semi-logarithmic evaluation of the minimum of the normalized impedance at 16 kHz $\text{norm. } |Z|_{16 \text{ kHz}}$ (A) and the normalized area under the curve (B) extracted from ECIS measurements shown in Figure 6.6 A on fasudil treatment of HTM-N cells seeded confluent in 8W10E arrays. The area was determined as demonstrated in Figure S4, by integration from 0 h to 20 h and between the curves and a value of $\text{norm. } |Z|_{16 \text{ kHz}}$ of 0.27. The area was normalized to a value of 1 for control conditions. Both minimum and area were determined individually for each single time course and averaged afterwards. Data are displayed as mean $\pm$ SD out of four wells from two biological replicates ($n = 4(2)$).

The stimulation of the HTM-N cells with 25 μM fasudil caused a medium cell reaction, as expressed in minimum and area, which was the reason why this was used as the standard concentration for further ECIS experiments and especially the transwell experiments (see section 7). Since ECIS measurements are typically more sensitive than QCM measurements, sensing for viscoelastic changes upon fasudil stimulation was performed with a higher concentration. Because 200 μM fasudil was found to exert a strong and irreversible impairment of the cells, a fasudil concentration of 100 μM seemed to be appropriate for QCM measurements.

In the following, such QCM measurements were performed to examine the viscoelastic response of the HTM-N cells to a fasudil treatment. The change of the minimal impedance $\Delta|Z|_{\min}$ was recorded for about 1 d after treatment of the cells with 100 μM fasudil in comparison to the addition of a control solution (Figure 6.8 A). For comparison to a complete degradation of the actin cytoskeleton (see Figure 6.6 B, red curve), the viscoelastic cell response was also monitored after addition of 5 μM cytochalasin D in comparison to control conditions (Figure 6.8 B). Data were normalized to the time point of addition and averaged over four (100 μM fasudil and its corresponding control, Figure 6.8 A), three (5 μM cytochalasin D, Figure 6.8 B, green curve) or two (control for 5 μM cytochalasin D, Figure 6.8 B, black curve) replicates.

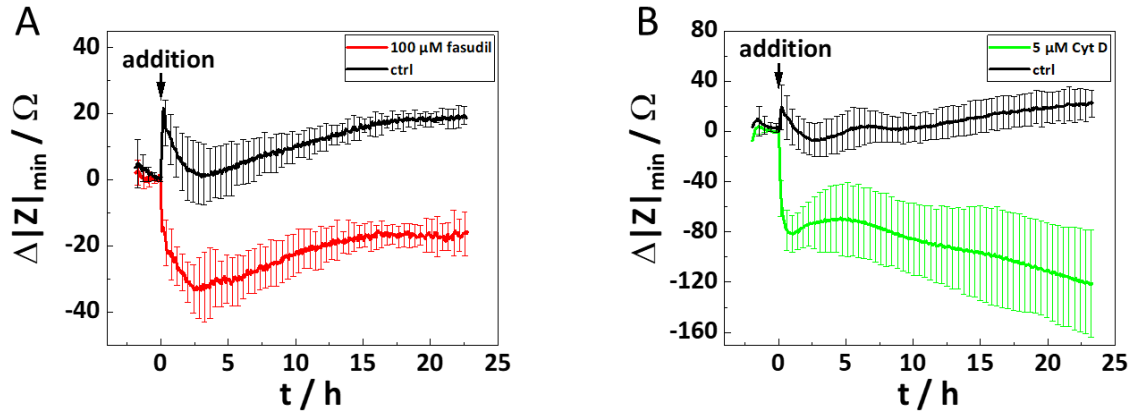


Figure 6.8 The change of the minimal impedance $\Delta|Z|_{\min}$ during QCM measurements analyzing the influence of 100 μM fasudil (**A**) (red) and 5 μM cytochalasin D (**B**) (green) on confluent HTM-N cells in comparison to control conditions (black). Data were normalized to the time point of addition and are displayed as mean $\pm$ SD out of several measurements (A: both fasudil and ctrl: $n = 4(4)$, B: Cyt D: $n = 3(3)$, B: ctrl: $n = 2(2)$). $T = 37^\circ\text{C}$.

After exhibiting addition peaks, control measurements for both fasudil and cytochalasin D showed only a slight increase in the minimal impedance with final values of $\Delta|Z|_{\min}$ of only 20 Ω reached after about 1 d of monitoring, proving a relatively constant signal for both controls (Figure 6.8, both black curves). The treatment of the HTM-N cells with 100 μM fasudil caused an instant decrease in $\Delta|Z|_{\min}$ with a minimum at about -35Ω reached after about 3 h, followed by a slight signal recovery to about -20Ω (Figure 6.8 A, red curve). Although this total decrease in the minimal impedance of only 20 Ω was quite low, this was still a significant decrease in contrast to control conditions because the control measurement slightly increased meanwhile. Thus, the signal recovery was not completely back to baseline levels indicating that cellular changes were permanent to a certain extent. The decrease in the minimal impedance correlated with a decrease in cell stiffness, which was in agreement with the proposed degradation of actin filaments by fasudil, since actin filaments contribute to cell stability and stiffness. Osmolality measurements (see section 4.2.2) revealed a value of (341 ± 4) mosmol/kg ($n = 2$) for 100 μM fasudil in serum-free HTM-N medium and a value of (340 ± 4) mosmol/kg ($n = 2$) for pure serum-free HTM-N medium. Thus, a contribution of water flux to the minimal impedance was excluded, since no significant osmolality differences between the fasudil solution and the medium surrounding the cells were measured. Viscoelastic changes of the cells by fasudil seemed to be triggered later than morphological changes since the minimum of $\Delta|Z|_{\min}$ in QCM measurements was obtained after about 3 h, whereas the minimum of $\text{norm.}|Z|_{16\text{ kHz}}$ in ECIS measurements was reached after about 1 h (see Figure 6.6 A). The value of $\Delta|Z|_{\min}$ for cell-free conditions would be at about -200Ω because this was the difference in $\Delta|Z|_{\min}$ between a confluent HTM-N cell layer and a cell-free quartz disk observed in adhesion studies (see Figure 6.1 A). Compared to that, this decrease of $\Delta|Z|_{\min}$ of 35 Ω at the minimum observed after treatment with 100 μM fasudil was relatively low (18%).

Stimulating the HTM-N cells with 5 μM cytochalasin D led, as expected, also to a decrease in the minimal impedance (Figure 6.8 B, green curve). But, in contrast to the ECIS measurement, where a huge signal decrease almost down to the level of cell-free conditions was observed (see Figure 6.6 B, red curve), here, a minimum of $\Delta|Z|_{\min}$ of about $-80\ \Omega$ was obtained and a short phase of recovery was followed by a further decrease in $\Delta|Z|_{\min}$ to a final value of about $-120\ \Omega$ after 1 d. As expected, 5 μM cytochalasin D caused a bigger signal decrease than 100 μM fasudil, but the complete degradation of the actin cytoskeleton, as obtained in ECIS measurements, was not observed in these QCM readings. Since the cellular reactions to both fasudil and cytochalasin D were observed bigger in ECIS than in QCM, this indicated the higher sensitivity of ECIS and/or that these two substances influenced the morphology of the HTM-N cells to a bigger extent than their stiffness. Both the results from ECIS (see Figure 6.6) and QCM measurements (Figure 6.8) were in accordance with a degradation of actin filaments, which is reported for both fasudil and cytochalasin D.

Thus, in order to confirm these changes of the actin cytoskeleton, CLSM imaging of actin filaments was performed, which were stained by TRITC-phalloidin labeling in HTM-N cells. CLSM images were taken for a confluent HTM-N cell layer at different time points after stimulation with 100 μM fasudil (Figure 6.9). The image for the time point of 0 h was taken for an untreated cell layer and represents control conditions, while actin cytoskeletal changes were followed over time by taking images in intervals of 1 h until the time point of 3 h after treatment, which was the time point of the minimum of $\Delta|Z|_{\min}$ observed in QCM measurements (Figure 6.8 A, red curve). Images were selected as representatives for the respective condition from three images taken each. For a more detailed view, 4-fold zoom-ins of these images are shown further down (see Figure 6.10). The brightness of the images depicted in Figure 6.9 was evaluated by the software ImageJ with averaging over the three images taken each and normalization to a value of 1 for the image at 0 h (Figure 6.9 E).

By comparing the CLSM images for different time points after treatment of the HTM-N cells with 100 μM fasudil (Figure 6.9 A – D), a stepwise decrease in total brightness over time was suggested. This was confirmed by the evaluation of the brightness, which revealed a reduction of the normalized brightness to about 0.8 after 1 h, to about 0.5 after 2 h, and to about 0.25 after 3 h (Figure 6.9 E). Although changes of fluorescence intensity have to be handled with care, this huge brightness decrease by a factor of 4 within 3 h was substantial and correlated with a significantly smaller extent of staining of actin filaments after fasudil treatment, which occurred gradually over time. This indicated a degradation of actin filaments over time down to its monomeric form (globular actin), which can no longer be bound and stained by TRITC-phalloidin explaining the brightness decrease. A similar brightness decrease was observed in a study on investigating the effect of 100 μM fasudil on the actin cytoskeleton of mouse brain astrocytes, which was explained and confirmed by a reducing amount of filamentous actin and an increasing amount of globular actin, according to a degradation of actin filaments (Lau *et al.*, 2011).

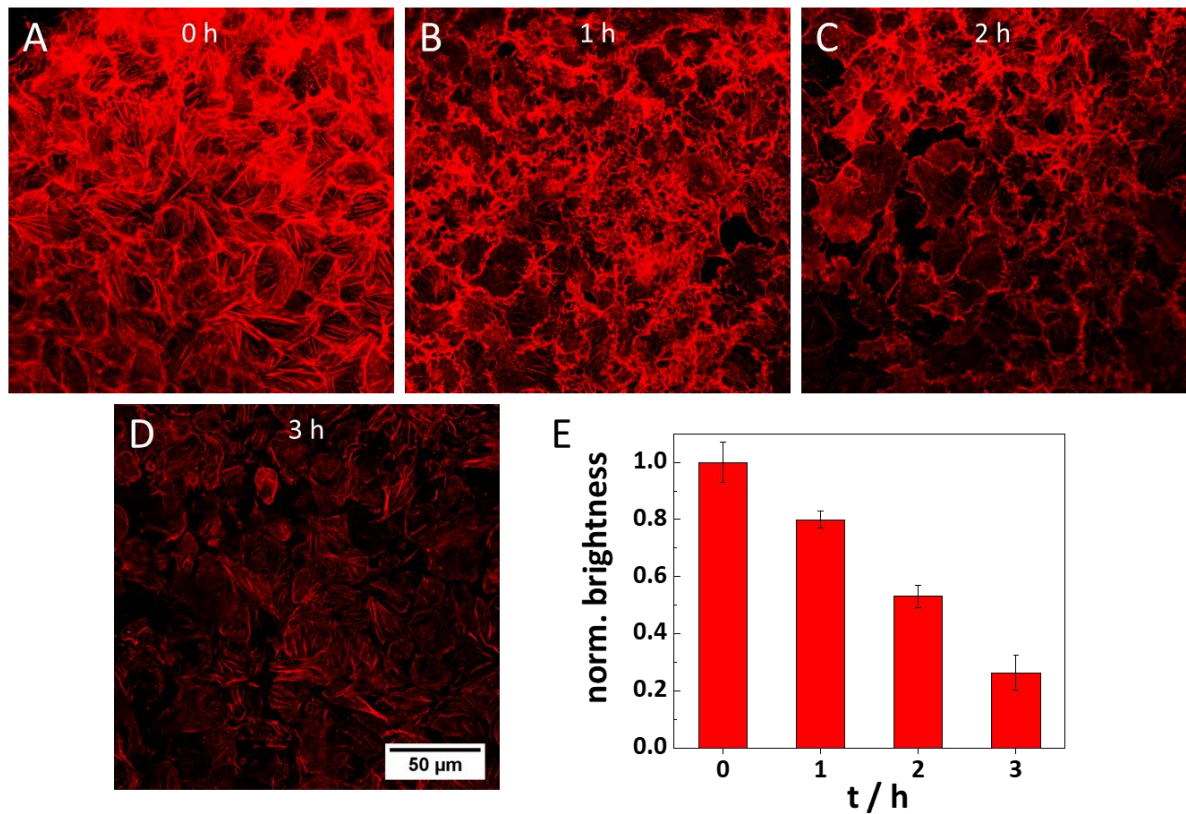


Figure 6.9 Confocal laser scanning microscopic images of confluent HTM-N cell layers with TRITC-phalloidin labeling of actin filaments, untreated (0 h) (A) and taken at 1 h (B), 2 h (C), and 3 h (D) after treatment with 100 μ M fasudil. Their total brightness was evaluated, which was normalized to 1 for the image at 0 h and averaged over three images each ($n = 3(1)$) (E). Three images were taken for each condition, but only one image representative for the respective condition is demonstrated here, while the focal plane was chosen close to the substrate. 4-fold zoom-ins of these images are demonstrated in Figure 6.10. $T = 37^\circ\text{C}$.

Structural changes within the actin cytoskeleton of the HTM-N cells upon fasudil treatment were analyzed better by looking at zoom-ins of the CLSM images. Thus, the same images as already demonstrated in Figure 6.9 are shown again in Figure 6.10, while zooming in with a factor of 4 on representative spots of the cell layer. According to the above-obtained decrease in brightness over time, the image for 3 h after fasudil treatment was brightened by a factor of 1.6 to improve the visibility of the actin filaments (Figure 6.10 D). Additionally, a CLSM image corresponding to a time of 3 h after treatment of the HTM-N cells with 5 μ M cytochalasin D is shown, which was also selected from several images taken as being representative for these conditions and with the same zoom and without brightening (Figure 6.10 E).

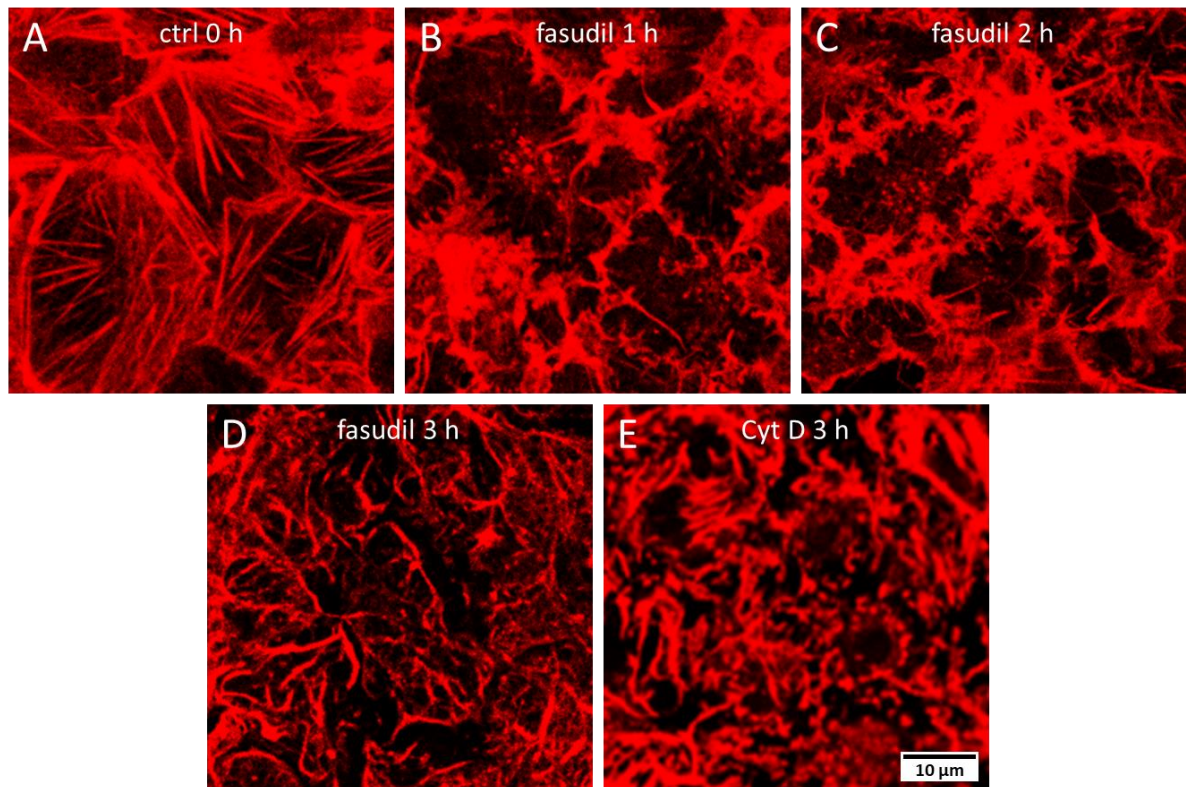


Figure 6.10 Confocal laser scanning microscopic images of confluent HTM-N cell layers with TRITC-phalloidin labeling of actin filaments (focal plane close to the substrate), untreated (0 h) (**A**) and taken at 1 h (**B**), 2 h (**C**), and 3 h (**D**) after treatment with 100 μ M fasudil or 3 h after treatment with 5 μ M cytochalasin D (**E**). Images A – D are the same as already demonstrated in Figure 6.9 with 4-fold zoom-in on representative spots, while image D was brightened by a factor of 1.6. Image E was also selected as representative out of several images and zoomed in by a factor of 4 without brightening. $T = 37^\circ\text{C}$.

As already obtained above in the investigation of the effect of CTGF (see Figure 6.3 A), HTM-N cells were observed to exhibit strongly pronounced stress fibers under control conditions (Figure 6.10 A). These thin and long fibers were not visible in the images after treatment with 100 μ M fasudil (Figure 6.10 B – D), indicating a degradation of stress fibers by fasudil, which was already observed after 1 h. Additionally, in both the images for 1 h and 2 h after fasudil addition, the cell cortex appeared frizzy and diffuse (Figure 6.10 B and C), which indicated a beginning degradation of the cell cortex. This was observed to be further progressed after 3 h with a partial fragmentation since less actin was present at the cell borders and shorter pieces of filaments were observed (Figure 6.10 D). Treatment of HTM-N cells with 5 μ M cytochalasin D for 3 h caused similar changes of the actin cytoskeleton, which was, however, stronger pronounced than for 100 μ M fasudil because even shorter filament pieces were observed and cell borders were no longer clearly visible (Figure 6.10 E). Although this degradation of actin filaments seemed to be stronger pronounced for 5 μ M cytochalasin D, a decrease in brightness, as obtained for fasudil, was not observed for cytochalasin D. This indicated that filaments were not degraded completely down to their monomeric form as triggered by fasudil, but instead, a depolymerization to shorter pieces of

actin filaments occurred by cytochalasin D. This fitted the expectation from literature for cytochalasin D as an inhibitor of actin polymerization (Casella *et al.*, 1981). CLSM images for fasudil proved that it induced a decomposition of actin filaments, as demonstrated by the decrease in brightness and by the degradation of stress fibers and cell cortex. This matched studies previously reported for fasudil, where it was demonstrated to reduce and disassemble bundles of actin filaments in human urethral scar fibroblasts (Li *et al.*, 2018) and in mouse brain astrocytes (Lau *et al.*, 2011). In total, changes of the actin cytoskeleton by fasudil and cytochalasin D were detected by CLSM imaging, confirming ECIS and QCM results and literature data. Quantitative measurement techniques like ECIS and QCM revealed a bigger influence on cell morphology and stiffness for 5 μ M cytochalasin D as compared to 100 μ M fasudil, and a bigger influence on cell morphology than on stiffness for both substances.

Although the effect of CTGF on TM cells could not be finally resolved in the previous section (section 6.2), it was used in the following to study the effect of fasudil on HTM-N cells, which were pre-stimulated by CTGF, since an application of fasudil is intended for glaucomatous eyes instead of healthy ones. Glaucomatous eyes were simulated by treatment of HTM-N cells with 50 μ g/L CTGF (1. addition), which were subsequently treated with 100 μ M fasudil 1 d later (2. addition). The change of the minimal impedance $\Delta|Z|_{min}$ was recorded by QCM measurements during these stimulations of the cells (Figure 6.11, red curve), while a control measurement was performed for the CTGF stimulation. For this CTGF control, HTM-N cells were pre-stimulated with a control solution (1. addition) and subsequently treated with fasudil (2. addition) (Figure 6.11, black curve). Data were normalized to the time point of fasudil addition and averaged over eight total replicates from six biological replicates.

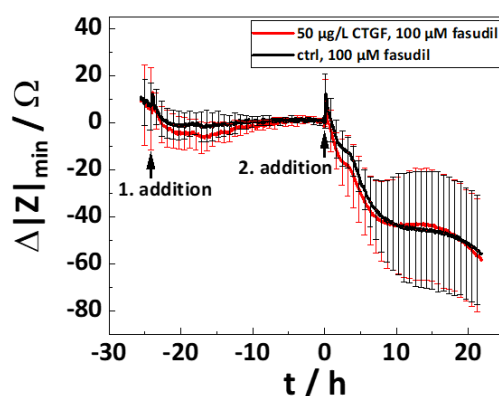


Figure 6.11 The change of the minimal impedance $\Delta|Z|_{min}$ during QCM measurements analyzing the influence of 100 μ M fasudil (2. addition) on confluent HTM-N cells, which were pre-stimulated 1 d before by 50 μ g/L CTGF (1. addition) (red) in contrast to a control measurement, where 100 μ M fasudil (2. addition) was tested on the cells, which were pre-stimulated 1 d before by a control solution (1. addition) (black). Data were normalized to the time point of fasudil addition and are displayed as mean $\pm$ SD out of eight total replicates from six biological replicates ($n = 8(6)$). $T = 37^\circ\text{C}$.

As already discussed for Figure 6.4 and Figure 6.5, here again, the HTM-N cells did not respond with a significant stiffness change to a CTGF treatment (1. addition), as expressed by the relatively constant minimal impedance (Figure 6.11, red curve). The subsequent treatment with 100 μ M fasudil (2. addition) induced a decrease in $\Delta|Z|_{min}$ for both with (Figure 6.11, red curve) and without (Figure 6.11, black curve) pre-stimulation with 50 μ g/L CTGF. This demonstrated that CTGF did also not show an effect on the HTM-N cell reaction to fasudil if the cells are pre-stimulated with it. But compared to Figure 6.8 A, where a minimum of $\Delta|Z|_{min}$ at about -35Ω followed by a slight signal recovery was obtained after fasudil treatment without pre-stimulation, here, a stronger pronounced decrease of the minimal impedance was observed with a plateau at about -45Ω followed by some further signal decrease. Since this was also observed for the fasudil treatment with pre-stimulation with the control solution, the HTM-N cells seemed to be more susceptible after additional 24 h of cultivation on the quartz disk. But fasudil was also demonstrated to induce a stiffness decrease of HTM-N cells pre-stimulated with 50 μ g/L CTGF.

This experiment was additionally monitored by ECIS to examine the effect of fasudil on the morphology of the HTM-N cells pre-stimulated with 50 μ g/L CTGF (Figure 6.12). The concentration of fasudil was 25 μ M, as defined before as the standard concentration in ECIS experiments. Thus, HTM-N cells seeded confluent in 8W10E arrays were treated with 25 μ M fasudil (2. addition) 1 d after stimulation with 50 μ g/L CTGF or a control solution (1. addition), while the impedance was recorded at multiple frequencies in an extended frequency range (20 Hz – 96 kHz). The most sensitive frequency was determined to 16 kHz, thus, the impedance was plotted for this specific frequency and normalized to the time point of fasudil addition. Consequently, the normalized impedance at 16 kHz $norm. |Z|_{16 \text{ kHz}}$ is displayed in Figure 6.12, which was averaged over two wells from one measurement.

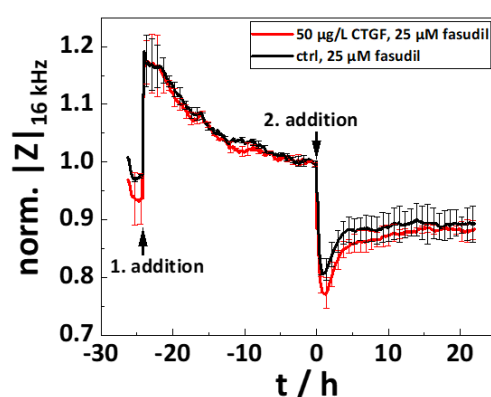


Figure 6.12 Normalized impedance at 16 kHz $norm. |Z|_{16 \text{ kHz}}$ during ECIS measurements analyzing the influence of 25 μ M fasudil (2. addition) on HTM-N cells seeded confluent in 8W10E arrays, which were pre-stimulated 1 d before by 50 μ g/L CTGF (1. addition) (red) in contrast to a control measurement, where 25 μ M fasudil (2. addition) was tested on the cells, which were pre-stimulated 1 d before by a control solution (1. addition) (black). Data were normalized to the time point of fasudil addition and are displayed as mean $\pm$ SD out of two wells from one measurement ($n = 2(1)$). $T = 37^\circ \text{C}$.

An instant increase in $norm. |Z|_{16\text{ kHz}}$ was observed after treatment of the HTM-N cells with 50 $\mu\text{g/L}$ CTGF (1. addition) (Figure 6.12, red curve), but this was obtained to be equally pronounced after addition of the control solution (Figure 6.12, black curve). Thus, this signal change after the pre-stimulation was not a result of the CTGF treatment, but instead, a result of the solution exchange itself, which was performed here by the full replacement method leading to a significant impairment of the cells. But this cell impairment by the first addition was no problem since a signal recovery back to start values was observed before the fasudil addition (2. addition) was performed. This subsequent treatment of the HTM-N cells with 25 μM fasudil led to an instant decrease in $norm. |Z|_{16\text{ kHz}}$ with a minimum at about 0.8 reached after about 1 h followed by a slight signal recovery, which was very similar for both with and without CTGF pre-stimulation. Thus, the cell reaction to the fasudil treatment was very similar to the one observed before (see Figure 6.6 A, purple curve) and independent of a previous CTGF stimulation. Accordingly, an influence of CTGF was also not observed here, neither a direct influence on HTM-N cell morphology nor an influence on a subsequent fasudil treatment. But fasudil was observed to influence the cell morphology by a degradation of the actin cytoskeleton of HTM-N cells with and without CTGF pre-stimulation.

This ECIS measurement in Figure 6.12 was then used to identify the biological origin of impedance changes upon fasudil treatment by fitting the ECIS model to the raw spectra, as introduced in section 3.4. This enabled the extraction of the resistance beneath the cells α (Figure 6.13 A), the paracellular resistance R_b (Figure 6.13 B), and the membrane capacitance C_m (Figure 6.13 C), and their change over time. With this, it was analyzed how these three parameters change after stimulating the HTM-N cells with 50 $\mu\text{g/L}$ CTGF (1. addition) and subsequently treating with 25 μM fasudil (2. addition), which can help reveal the cellular origin of the observed impedance changes. Same as for Figure 6.12, these three parameters were also averaged over two wells from one measurement, but the data were not normalized to also observe the absolute values.

For both the parameters α (Figure 6.13 A) and R_b (Figure 6.13 B), the time courses were observed to be almost identical to the ones obtained for $norm. |Z|_{16\text{ kHz}}$ in Figure 6.12. A signal increase followed by a recovery back to start values was induced by 50 $\mu\text{g/L}$ CTGF or the control solution (1. addition), and a signal decrease with a minimum after about 1 h followed by a slight signal recovery was triggered by the subsequent treatment with 25 μM fasudil (2. addition) independently of a CTGF pre-stimulation. Upon fasudil addition, the resistance beneath the cells α decreased from about $4.5 \sqrt{\Omega} \cdot \text{cm}$ to about $3.6 \sqrt{\Omega} \cdot \text{cm}$ at the minimum and the paracellular resistance R_b decreased from about $3.3 \Omega \cdot \text{cm}^2$ to about $2.3 \Omega \cdot \text{cm}^2$ at the minimum.

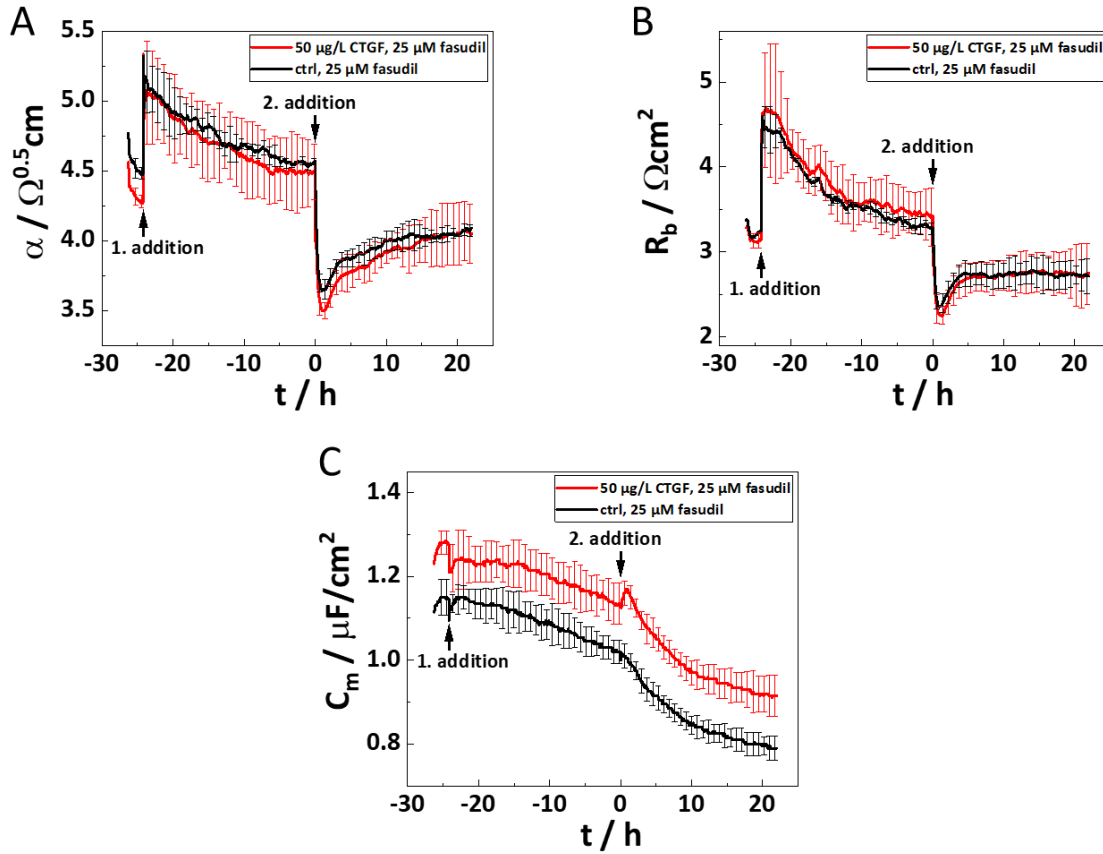


Figure 6.13 The cell-related parameters α (A), R_b (B), and C_m (C), extracted by fitting the ECIS model to the raw spectra, for the ECIS experiment shown in Figure 6.12. HTM-N cells seeded confluent in 8W10E arrays were stimulated by 25 μM fasudil (2. addition) 1 d after treatment with 50 $\mu\text{g/L}$ CTGF (red) or a control solution (black) (1. addition). Data were not normalized to read the absolute values of the parameters, and are displayed as mean $\pm$ SD out of two wells from one measurement ($n = 2(1)$). $T = 37^\circ\text{C}$.

Although the absolute values of the membrane capacitance C_m were a bit different to each other for cells with (Figure 6.13 C, red curve) and without (Figure 6.13 C, black curve) pre-stimulation with 50 $\mu\text{g/L}$ CTGF, the change over time was very similar, but clearly different to α and R_b . Upon fasudil addition, the membrane capacitance C_m did not exhibit a minimum, but instead, slowly and continuously decreased, from about $1.1 \frac{\mu\text{F}}{\text{cm}^2}$ to about $0.9 \frac{\mu\text{F}}{\text{cm}^2}$ with CTGF pre-stimulation (Figure 6.13 C, red curve) and from about $1.0 \frac{\mu\text{F}}{\text{cm}^2}$ to about $0.8 \frac{\mu\text{F}}{\text{cm}^2}$ without CTGF pre-stimulation (Figure 6.13 C, black curve). Such low values of C_m smaller than $1 \frac{\mu\text{F}}{\text{cm}^2}$ are unusual and can be caused by an inaccurate determination of the electrode area or when more than two plasma membranes lie above each other (like in a series circuit of capacitors). Since the electrode area of the commercially purchased ECIS arrays was considered as reliably constant, the rather small values of C_m were probably the result of cell protrusions partially lying above each other. The influence of fasudil on C_m was not as pronounced as for α and R_b because C_m already showed a slight downwards drift before fasudil addition. Thus, a significant signal change upon fasudil treatment of HTM-N

cells was observed for all three parameters, even though a bit different for C_m . In total, each parameter experienced a signal decrease to about 0.7 – 0.8 of the start values, which was in good agreement with the minimum of $norm. |Z|_{16\text{ kHz}}$ of about 0.8 observed in Figure 6.12. Since C_m already showed a slight downwards drift before fasudil addition, impedance changes after fasudil stimulation were attributed to similar parts of cellular changes beneath the cells (α) and in the paracellular clefts (R_b), but to a smaller part in the membranes (C_m). The signal change of all three parameters after CTGF stimulation was not specific for CTGF since it did not differ from the control, leading to the conclusion that an effect of CTGF was also not detected here. When the absolute values of these three parameters for HTM-N cells are compared to epithelial (MDCK-I: $\alpha \approx 120 \sqrt{\Omega} \cdot \text{cm}$, $R_b \approx 3000 \Omega \cdot \text{cm}^2$, $C_m \approx 1.9 \frac{\mu\text{F}}{\text{cm}^2}$; MDCK-II: $\alpha \approx 16 \sqrt{\Omega} \cdot \text{cm}$, $R_b \approx 90 \Omega \cdot \text{cm}^2$, $C_m \approx 3 \frac{\mu\text{F}}{\text{cm}^2}$) and epithelial-like (NRK: $\alpha \approx 8 \sqrt{\Omega} \cdot \text{cm}$, $R_b \approx 9 \Omega \cdot \text{cm}^2$, $C_m \approx 1.2 \frac{\mu\text{F}}{\text{cm}^2}$) cell lines (Reiss & Wegener, 2015), much smaller values were observed here for HTM-N cells. Especially the big differences in R_b values are reasonable because TM cells have to be leaky to allow a strongly pronounced aqueous humor outflow.

Taken all together, fasudil was found to trigger a degradation of actin filaments in HTM-N cells, as indicated by the stiffness decrease observed in QCM measurements and the morphological changes observed in ECIS measurements, and confirmed by CLSM imaging. This was obtained equally in cells pre-stimulated with CTGF, simulating the treatment of glaucomatous eyes. This relaxation of the actin cytoskeleton and concomitant reduction of TM stiffness correlates with a reduction of outflow resistance in the TM, leading to an increase in aqueous humor outflow and consequently to a reduction of IOP, which has already been reported in literature studies (Sugiyama *et al.*, 2011; Pakravan *et al.*, 2017). These results may help better understand the effect of fasudil and possibly contribute to find new ROCK inhibitors for glaucoma treatment. The techniques of QCM and ECIS can be used to accelerate the screening of drug candidates and to give an additional insight into their viscoelastic and morphological effects on the cells. Similar to other ROCK inhibitors, the easiest way of application of fasudil is via injection into the vitreous body, which induces a subsequent diffusion through the vitreous body. Thus, the following section (section 7) focuses on the diffusion of fasudil through the vitreous body before it gets in contact with the HTM-N cells, which is performed by a new transwell assay.

7 Development of a Transwell Assay to Monitor the Diffusion of Fasudil through the Vitreous Body and its Subsequent Cellular Response

In the previous section (section 6), the ROCK inhibitor fasudil was proven to induce a degradation of actin filaments in HTM-N cells, which is supposed to correlate with a reduction of intraocular pressure (IOP) in glaucomatous eyes. Application of ROCK inhibitors is typically performed by injection into the vitreous body, which triggers a diffusion through the vitreous body into the posterior chamber. From there, they are transported with the flow of aqueous humor through the anterior chamber to finally reach the target tissue, the trabecular meshwork (TM). Thus, this diffusion process through the vitreous body was analyzed in detail in this section with fasudil as the diffusing molecule. This biological situation of fasudil diffusing through the vitreous body before acting on TM cells was simulated in a transwell filter setup, which was developed in this thesis in cooperation with Dr. Mietzner and Prof. Dr. Breunig. HTM-N cells were used as a model for the TM cells of a human eye and porcine vitreous body was used as a model for the human one, the extraction of which is described in section 4.2.3. To detect the cellular response to the fasudil treatment as well as the retardation of fasudil by its diffusion through the vitreous body, ECIS as a non-invasive and time-resolved technique was regarded to be perfectly suited for this purpose. For this, special ECIS electrode arrays were used, which were fabricated according to section 4.5.1. They were designed to hold transwell filter inserts for the vitreous body above the cells under study. This setup, which is demonstrated in detail in Figure 4.22, enabled fasudil to diffuse through the vitreous body in the transwell filter inserts, through the filter membrane and the culture medium to finally reach the HTM-N cells sitting on the electrodes leading to cellular changes detectable by the ECIS measurement. In section 7.1, the vitreous body was first characterized regarding its viscoelastic properties by QCM measurements and regarding its properties as a diffusion medium by calculations and diffusion tests with a trypan blue solution (described in section 4.2.5). The transwell assay was optimized several times in the course of the assay development, as already described in section 4.8.2, and the impacts of these optimizations on ECIS spectra and experiment outcome are demonstrated in section 7.2. The final result of this assay development process is shown in section 7.3, which has already been published by Mietzner *et al.* (Mietzner *et al.*, 2020), and includes, for comparison, a direct addition of fasudil to the HTM-N cells without diffusion through the vitreous body and application of fasudil previously released from PLGA microspheres. Some further tests and modifications of the transwell assay are also demonstrated in this section, which includes, as already described in section 4.8.2, an investigation of the effect of placing transwell filter inserts into

the wells, the determination of the final fasudil concentration by HPLC, and the usage of agarose gels as a substitute for the vitreous body (preparation described in section 4.2.4). For comparison of the ECIS results, CLSM imaging of this transwell experiment (described in section 4.10.3) was performed and also demonstrated in section 7.3.

7.1 Characterization of the Vitreous Body

Before the vitreous body was used for diffusion assays in the transwell setup, it was first characterized regarding its viscoelastic properties by QCM measurements. The minimal impedance $|Z|_{min}$ was recorded, while the vitreous body was added to blank quartz disks (Figure 7.1, black curve) or added to quartz disks preloaded with water and exchanged with it (Figure 7.1, red curve). Averaging was performed over two replicates but without normalization.

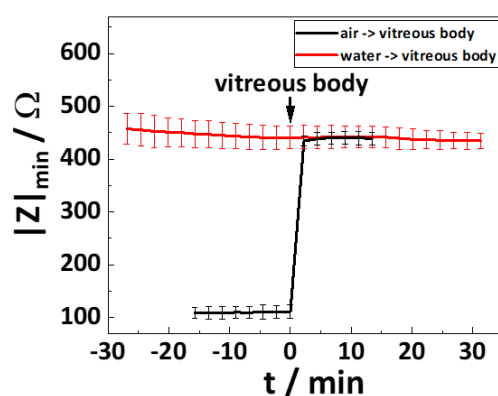


Figure 7.1 The minimal impedance $|Z|_{min}$ during QCM measurements analyzing the viscoelastic properties of the vitreous body added on blank resonators (black) or exchanged for water (red). Data were not normalized and are displayed as mean $\pm$ SD out of two replicates ($n = 2$). $T = 37^\circ\text{C}$.

Before addition of the vitreous body, the blank quartz resonators exhibited a minimal impedance $|Z|_{min}$ of about $100\ \Omega$ (Figure 7.1, black curve), a value obtained regularly for unloaded conditions. Upon addition of the vitreous body, $|Z|_{min}$ immediately increased to a constant value of about $450\ \Omega$, a typical value for cell-free conditions with pure medium or water as the quartz loading (as, for example, obtained in Figure S2). When the QCM measurement was performed with water as the initial quartz loading, values of $|Z|_{min}$ of about $450\ \Omega$ were observed in the beginning, which experienced no change at all upon exchanging the load from water to vitreous body (Figure 7.1, red curve). As expected, final values of $|Z|_{min}$ for the vitreous body were the same for both measurements, irrespective of the initial loading. But, the minimal impedance for the vitreous body was observed to be equal to that of water. As already observed before in Figure 6.1 by the initially unchanged minimal impedance after cell seeding and sedimentation, which was attributed to not yet

existing anchoring points between cells and quartz surface, leading to no additional damping of the resonator's shear oscillation, this was also expected here because the vitreous body can also not anchor to the quartz surface. But the vitreous body, by its gel-like nature, was expected to exhibit a higher viscosity than water or minor viscoelastic properties that would suggest an increase in the minimal impedance. With $1.6 \cdot 10^{-3} \text{ Pa} \cdot \text{s}$, a slightly increased dynamic viscosity of the vitreous body in comparison to that of water ($10^{-3} \text{ Pa} \cdot \text{s}$) was also reported elsewhere (Murphy *et al.*, 2016). But, these results indicated that the viscoelastic properties of the vitreous body were very similar to those of water, at least in the range of the decay length, which is determined to about 250 nm in water (see section 3.2.1, *The QCM beyond the Sauerbrey Limitations*). According to the gel-like nature of the vitreous body, which gives reason to expect an increased viscosity, these results obtained here suggested an inhomogeneous distribution of higher viscous parts and lower viscous parts in the vitreous body, with lower viscous parts (some kind of water film) present within the range of the decay length. These results fitted quite well to literature, where the structure of the vitreous body is described by a dilute network of collagen fibrils in water surrounded by hyaluronic acid (Murphy *et al.*, 2016).

Diffusion dynamics of fasudil in the vitreous body were analyzed in the following by a calculation of the average duration of diffusion by Eq. (72) and Eq. (73) and subsequently by using a trypan blue solution as a colored substitute for fasudil for visualization of the diffusion. When fasudil is applied on top of the vitreous body, the concentration gradient between added fasudil solution and vitreous body triggers the diffusion of fasudil molecules downwards into and through the vitreous body. The diffusion coefficient D of fasudil in the vitreous body was calculated by Eq. (73), while 310.15 K (37 °C) was used as the temperature and $1.6 \cdot 10^{-3} \text{ Pa} \cdot \text{s}$ was used as the dynamic viscosity of the vitreous body (Murphy *et al.*, 2016). Fasudil was assumed to behave like a spherical particle. Its radius was calculated to $2.37 \cdot 10^{-10} \text{ m}$ out of the previously published surface area of $70.7 \text{ \AA}^2$ (National Center for Biotechnology Information, 2022). Thus, this calculation of the diffusion coefficient D of fasudil in the vitreous body yielded a value of about $5.99 \cdot 10^{-6} \frac{\text{cm}^2}{\text{s}}$. Applying this result to the random walk model (see Eq. (72)) yielded an average duration of diffusion of fasudil across the vitreous body, which was always prepared in a height of 6 mm, of about 167 min (2.8 h). In comparison to the calculated radius of fasudil of $2.37 \cdot 10^{-10} \text{ m}$, the filter membrane with 6.5 mm diameter, pore density of 10^8 pores per cm^2 , and $0.4 \text{ }\mu\text{m}$ pore diameter does absolutely not constitute a diffusion barrier for fasudil, which can therefore easily cross the filter membrane. After its diffusion through the vitreous body, fasudil has to traverse the 2 mm of medium between filter membrane and well bottom to reach the cells, which is also achieved by diffusion. Using similar calculations by Eq. (72) and Eq. (73) with the dynamic viscosity of water of $10^{-3} \text{ Pa} \cdot \text{s}$ and the distance of 2 mm yielded a diffusion coefficient D of fasudil in the medium of about $9.58 \cdot 10^{-6} \frac{\text{cm}^2}{\text{s}}$ and, accordingly, an average duration of diffusion of about 12 min. Thus, when fasudil is applied on top of the vitreous body, these calculations suggested an average total time of diffusion of about 3 h. Not all

fasudil molecules will reach the cells at the same time, but instead, some of them will reach the cells earlier than others and some will never, with an average time of 3 h, as estimated by these calculations. When fasudil application is performed by pre-mixing with the vitreous body, as it was done for the purpose of sensor optimization (see section 7.2), fasudil molecules from the lower layers of the vitreous body only have to diffuse through the medium, reaching the cells after about 12 min according to the calculations from above. These fasudil molecules leaving the vitreous body trigger the development of a concentration gradient within the vitreous body leading to the diffusion of further molecules of fasudil from layers of the vitreous body more distant to the filter membrane. Those fasudil molecules will then also diffuse through the medium and reach the cells after some delay, up to a delay of about 3 h for fasudil molecules starting from the uppermost layers of the vitreous body. Thus, this pre-mixing application of fasudil was expected to lead to less retardation of fasudil in the vitreous body and to show an earlier onset of the fasudil effect on the cells after about 12 min. For both cases of fasudil application, the diffusion process was expected to last until the fasudil concentration is evenly distributed over vitreous body and medium. These calculations were performed according to the random walk model, which describes the random movement of particles and does not consider a concentration gradient, and the flux of molecules increases with the concentration gradient. Thus, the diffusion of fasudil through the vitreous body in the transwell experiments was expected to be a bit faster than suggested by these calculations. For the direct addition of fasudil to the medium without previous diffusion through the vitreous body (performed additionally to compare to regular additions), no time delay of the fasudil effect was expected because the addition of fasudil by a pipet into the medium causes a convection. This immediately mixes the added fasudil solution with the medium to lead to a constant fasudil concentration in the well within seconds. It is possible that some extra convection, which can for example be caused by placing the transwell filter insert into the well, also contributes to the transport of fasudil through the vitreous body and the medium, leading to a reduction of the time for fasudil to reach the cells. Additionally, the different liquid heights of vitreous body and surrounding medium, as can be seen in Figure 4.22, causes a hydrodynamic pressure difference and subsequent water transport, which probably increases the fasudil transport. Nevertheless, these calculations were a first indicator to roughly estimate the duration of diffusion of fasudil through the vitreous body.

Additionally, experimental diffusion tests were performed with a trypan blue solution instead of fasudil. Since these tests were just conducted for the purpose of a rough estimation of the duration of diffusion, the drawback of the differences in molar mass and water solubility between these two substances was compensated by the fact that trypan blue is a colored molecule enabling a visualization of the diffusion by taking a series of images. These experiments were not performed for the simulation of the diffusion of fasudil in the 2 mm of medium, but only in the 6 mm of vitreous body. Since agarose gels were used as a substitute for the vitreous body in one transwell experiment, the diffusion of the trypan blue solution was also followed in agarose gels, while different concentrations of agarose

(1.5% (w/v), 1% (w/v), and 0.5% (w/v)) were tested. Both vitreous body and agarose gels were added to transwell filter inserts in a volume of 200 μL providing a height of the diffusion medium of 6 mm. One single drop (10 μL – 20 μL) of the trypan blue solution (0.5 g/L) was added on top and its diffusion through the vitreous body or agarose gels was observed by taking several images until this blue dye seemed to be completely distributed. The corresponding images are shown in Figure 7.2.

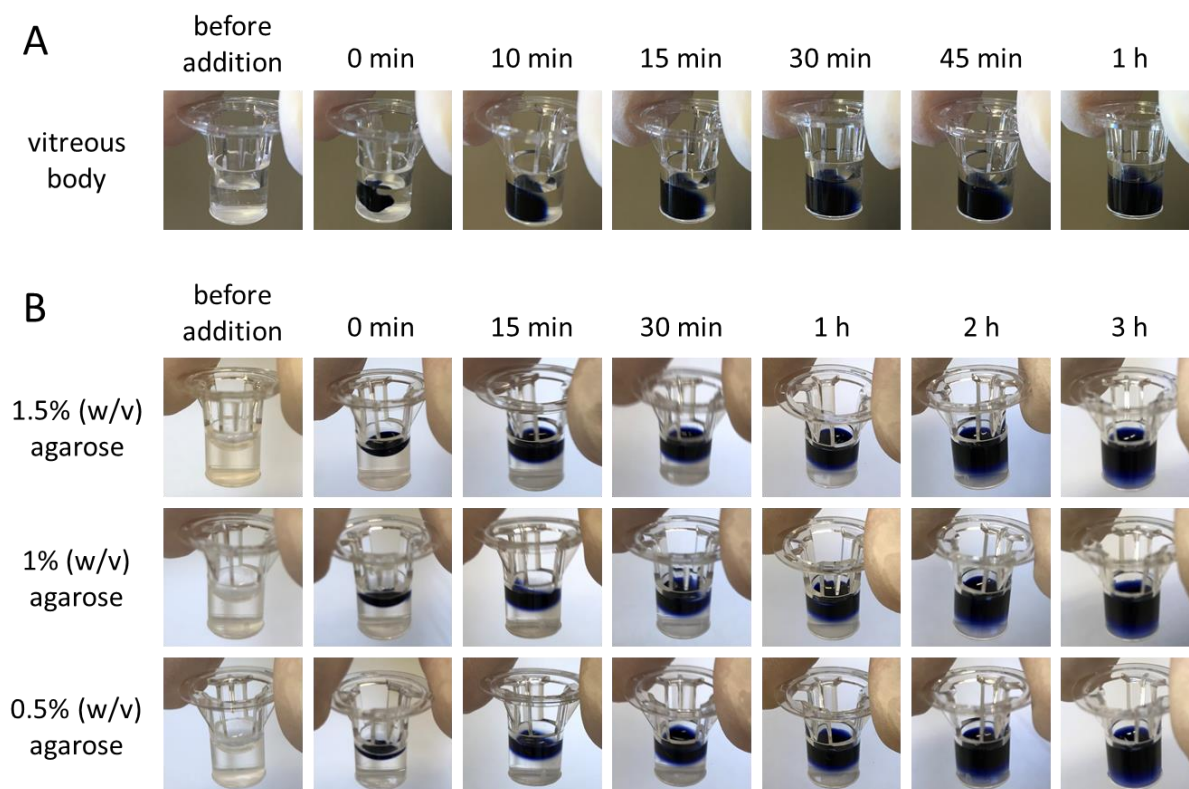


Figure 7.2 Images demonstrating the diffusion of a trypan blue solution through the vitreous body (A) or agarose gels (B) in transwell filter inserts. With 1.5% (w/v), 1% (w/v), and 0.5% (w/v), three different concentrations of agarose were tested in the gels, while both vitreous body and agarose gels were used in a volume of 200 μL (6 mm height) and one single drop (10 μL – 20 μL) of the trypan blue solution (0.5 g/L) was added on top. Images were taken at several time points within 1 h of observation for the vitreous body and within 3 h of observation for agarose gels. The images for 0 min were taken directly after addition of the trypan blue solution. $T = 37^\circ\text{C}$.

By comparing the images taken directly after addition (0 min) of the trypan blue solution, one crucial difference between vitreous body (Figure 7.2 A) and agarose gels (Figure 7.2 B) was observed. The drop of trypan blue solution immediately penetrated into the vitreous body, whereas it stayed at the surface of the agarose gels. Thus, for the vitreous body, this diffusion test can hardly be compared to the calculations from above, but it mirrored the results from Figure 7.1, where a discontinuity in the viscosity of the vitreous body was found. In these diffusion tests, the trypan blue solution obviously penetrated easily through regions of lower viscosity, which can also be assumed for fasudil. For both vitreous body and agarose gels, a diffusion of the trypan blue solution was observed by the gradual spreading of the

blue dye over time. No differences in diffusion behavior were observed regarding the different concentrations of agarose. The trypan blue solution was observed to be completely distributed over the vitreous body after about 1 h (Figure 7.2 A) and over agarose gels after about 3 h (Figure 7.2 B). The shorter duration of diffusion in the vitreous body was reasonable because the trypan blue solution was able to start its diffusion in the center of the vitreous body leading to shorter distances to travel in comparison to agarose gels, where the diffusion occurred over the complete distance of 6 mm. Thus, the durations of diffusion of the trypan blue solution in the vitreous body or agarose gels can hardly be compared to each other. Additional to a reduction of the duration for a complete distribution of the dye, the instant penetration of the trypan blue solution into the vitreous body also significantly reduced the time to reach the filter membrane to less than 10 min, as observed in the respective image in Figure 7.2 A. Since this time point represents the start point of diffusion through the medium in the transwell experiments, which takes about 12 min as calculated before, a total duration of diffusion of less than 22 min was expected for the on-top application of fasudil onto the vitreous body. But, since this initial penetration into the vitreous body, as observed in Figure 7.2 A for the trypan blue solution, can vary drastically depending on the exact distribution of material properties within the vitreous body, the duration of diffusion can also vary significantly between single experiments. This was the reason for the development of the second and optimized application strategy of fasudil, where it was mixed with the vitreous body in advance, which is discussed in detail in section 7.2.5. This was expected to be better because it avoids inhomogeneities within the vitreous body, leading to a constant migration time of fasudil, with the first fasudil molecules reaching the cells after about 12 min as calculated before. For agarose gels, the trypan blue solution reached the filter membrane after about 2 – 3 h (Figure 7.2 B), suggesting a total duration of diffusion of fasudil through agarose and culture medium of about 3 h. This was very close to the calculated value of about 3 h for a diffusion of fasudil through vitreous body and medium, indicating that these diffusion tests fitted very well to the calculations from above in case of agarose.

7.2 Assay Optimization

The transwell assay was developed in this thesis to monitor the diffusion of fasudil through the vitreous body and to detect its subsequent effect on the HTM-N cells. This assay development process comprised five variations in experimental procedure for optimization of the results. These five optimizations were already described in section 4.8.2 and comprise the electrode layout (section 7.2.1), the pre-incubation time of the electrodes with medium prior to cell seeding (section 7.2.2), an additional medium exchange right before measurement start (section 7.2.3), the method of direct additions of fasudil (section 7.2.4), and the way of fasudil application to the vitreous body (section 7.2.5). The respective influences on ECIS spectra or time course data are discussed in the following.

7.2.1 Electrode Layout

The first optimization of the transwell assay concerned the electrode layout. Three different electrode layouts were used, which were fabricated photolithographically as described in section 4.5.1 and already demonstrated in Figure 4.10. The first electrode layout used was the 8W1E+ enl. (100 μm) array with small circular WEs and CE with 400 μm diameter each and supply lines of 100 μm width adjacent to the electrodes leading to a total electrode area in each well of $3.31 \cdot 10^{-3} \text{ cm}^2$ (see Figure 4.10 A). It was obtained before in diffusion assays in Figure 7.2 A by the example of the trypan blue solution instantly penetrating, that the vitreous body can exhibit regions of lower viscosity enabling a faster diffusion of fasudil at certain spots and potentially triggering a locally limited cell reaction. Thus, the initially used electrode layout with its small electrode area may not detect the cell response in each individual case. Hence, the electrode layout was modified for the purpose of an increase in electrode area and a broader distribution of the electrode pattern over the well surface. This was achieved by the development of two electrode layouts with an inter-digitated finger configuration, the 8W1000idf enl. (200 μm) array with total electrode area in each well of $1.26 \cdot 10^{-1} \text{ cm}^2$ (see Figure 4.10 B) and the even further improved 8W1000idf enl. (900 μm) array with total electrode area in each well of $1.84 \cdot 10^{-1} \text{ cm}^2$ (see Figure 4.10 C). The inkjet printer, which printed the designed electrode layouts with black inkjet dye onto transparent inkjet films yielding the masks used for the UV illumination step, as well as the complete photolithographic fabrication process of the sensors were expected to have limited precision. Thus, these masks (Figure 7.3 A – C) and the ready-to-use Lexan® plates (Figure 7.3 D – F) for the three different electrode layouts were investigated by phase contrast microscopy. For each condition, two images were taken showing critical parts of the electrode layout in one well, with one of them demonstrated in Figure 7.3 as a representative for the respective electrode type.

The phase contrast microscopic images revealed that the inkjet film masks of the three different electrode layouts exhibited very frayed borders of the dye-coated regions (Figure 7.3 A – C), but the borders of the gold-coated regions on the Lexan® plates were smooth and accurate (Figure 7.3 D – F). For each image taken, electrode dimensions were measured, while this concerned the electrode diameter and the supply line width adjacent to the electrodes for the 8W1E+ enl. (100 μm) array (Figure 7.3 A and D), whereas the electrode finger width, the electrode finger distance, and the supply line width adjacent to the electrodes were measured for the two inter-digitated electrode structures of the 8W1000idf enl. (200 μm) array (Figure 7.3 B and E) and the 8W1000idf enl. (900 μm) array (Figure 7.3 C and F). For both masks and Lexan® plates, these electrode dimensions were measured at two individual spots in each image yielding four values (two images, two spots) for each dimension, which were averaged and summarized in Table 7.1 in comparison to the intended electrode dimensions.

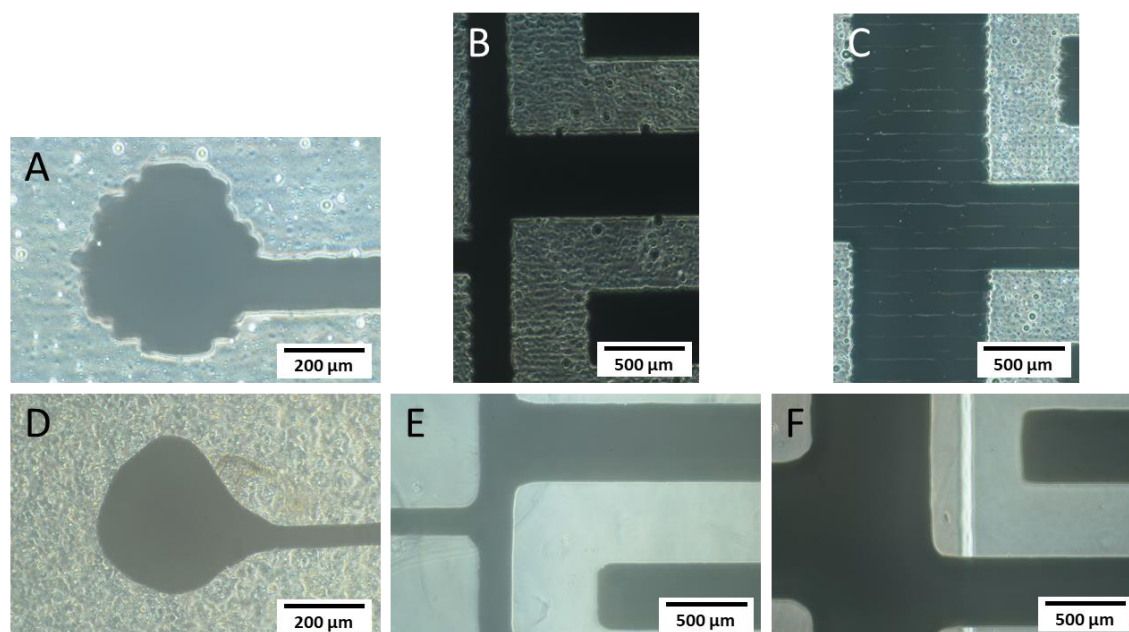


Figure 7.3 Phase contrast microscopic images of the inkjet film masks used for the sensor fabrication (**A, B, C**) and the ready-to-use Lexan® plates (**D, E, F**) for the three different electrode layouts of the 8W1E+ enl. (100 μm) array (**A, D**), the 8W1000idf enl. (200 μm) array (**B, E**), and the 8W1000idf enl. (900 μm) array (**C, F**). Representative images show the electrode layout in one single well. While dark areas on the masks are those covered with black inkjet dye preventing the UV light from destabilizing the photopolymer in the UV illumination step of the sensor fabrication process, dark areas on the Lexan® plates make up the sputtered gold electrode layout.

Table 7.1 Summary of electrode diameter and supply line width adjacent to the electrodes for the 8W1E+ enl. (100 μm) array and electrode finger width, electrode finger distance, and supply line width adjacent to the electrodes for the 8W1000idf enl. (200 μm) array and the 8W1000idf enl. (900 μm) array. Dimensions were measured for the respective masks and Lexan® plates in all phase contrast microscopic images taken (not only those shown in Figure 7.3) at two individual spots. These electrode dimensions are displayed as mean $\pm$ SD over four values in comparison to the intended electrode dimensions.

Array type	Dimension	Intended / μm	Mask / μm	Lexan® plate / μm
8W1E+ enl. (100 μm)	electrode diameter	400	440 ± 40	350 ± 40
	supply line width	100	150 ± 20	70 ± 20
8W1000idf enl. (200 μm)	electrode finger width	500	490 ± 30	450 ± 40
	electrode finger distance	500	460 ± 10	510 ± 10
	supply line width	200	220 ± 20	180 ± 20
8W1000idf enl. (900 μm)	electrode finger width	500	540 ± 10	440 ± 10
	electrode finger distance	500	410 ± 60	500 ± 40
	supply line width	900	870 ± 50	760 ± 60

Comparing the electrode dimensions for the masks to intended values given in Table 7.1 revealed slight differences between both for all three array types, which was attributed to

the inaccuracy of the inkjet printer. By comparing the electrode dimensions for the masks to the Lexan® plates it was recognized that electrode diameter, electrode finger width, and supply line width adjacent to the electrodes were smaller and electrode finger distance was bigger for the Lexan® plates (Table 7.1), proving that electrode dimensions have reduced during the photolithographic fabrication process. This was obviously the result of the frayed borders of the dye-coated regions on the masks (as obtained in Figure 7.3 A – C) allowing additional UV light to destabilize the photopolymer during UV illumination through the mask, and leading to additional photopolymer removed by NaOH and additional gold etched by KI/I₂ in the subsequent steps of the fabrication process. This explained that gold-coated regions were smaller and uncoated regions (like electrode finger distance) were bigger on the Lexan® plates than initially printed on the masks with black inkjet dye. But, final gold electrode dimensions on the Lexan® plates were still very close to intended dimensions for all three array types, with the biggest difference for the supply line width of the 8W1000idf enl. (900 µm) array (760 ± 60 µm compared to 900 µm) (Table 7.1), which did not matter because the supply lines were completely covered by photopolymer for this array type (see Figure 4.10 C).

Beside this phase contrast microscopic evaluation of electrode dimensions, in the following, the three different electrode layouts were compared regarding their impedance spectra measured in ECIS experiments. Only spectra of cell-free electrodes were analyzed, whereas spectra of cell-covered electrodes are shown as part of the next optimization further down (see section 7.2.2). For each of the three different array types (8W1E+ enl. (100 µm), 8W1000idf enl. (200 µm), and 8W1000idf enl. (900 µm)), impedance spectra of cell-free electrodes were taken from one typical measurement and are displayed in Figure 7.4 A for well 1, which was the well on the top left (arrangement of wells can be viewed in Figure 4.10). Since the eight different wells exhibited significantly different lengths of the supply lines, the dependency of the impedance spectra of cell-free electrodes on the well position was analyzed additionally in Figure 7.4 B for the 8W1000idf enl. (900 µm) array.

In Figure 7.4 A, a stepwise shift of the impedance spectrum of cell-free electrodes to lower impedance values was observed when switching the electrode layout from the 8W1E+ enl. (100 µm) array (Figure 7.4 A, black curve) to the 8W1000idf enl. (200 µm) array (Figure 7.4 A, red curve) and finally to the 8W1000idf enl. (900 µm) array (Figure 7.4 A, green curve). This impedance decrease was observed in both the low (10^1 Hz – 10^3 Hz) and high-frequency regions (10^4 Hz – 10^5 Hz). In the low-frequency region, the stepwise shift of the frequency-dependent capacitive part of the spectrum can be correlated with the total electrode area, which was one big difference between the three electrode layouts. As already mentioned before, the total electrode area stepwise increased from $3.31 \cdot 10^{-3}$ cm² to $1.26 \cdot 10^{-1}$ cm² and finally to $1.84 \cdot 10^{-1}$ cm² for the 8W1E+ enl. (100 µm) array, the 8W1000idf enl. (200 µm) array and the 8W1000idf enl. (900 µm) array, respectively. According to Eq. (67) and Eq. (68), a higher electrode area leads to a higher capacitance and subsequently to a smaller impedance in the capacitive part of the spectrum, explaining the stepwise impedance decrease in the low-frequency region when increasing the total

electrode area by changing the electrode layouts. In the high-frequency region, this stepwise decrease in impedance can be explained by the constriction resistance and the supply line resistance, which are both not considered in the theoretical ECIS model, where the resistance of the electrolyte R_{bulk} is the only impedance contribution to the frequency-independent resistive part of the spectrum at high frequencies. The constriction resistance increases with decreasing electrode area, leading to a higher impedance in the high-frequency region for the small electrode area of the 8W1E+ enl. (100 μm) array (Figure 7.4 A, black curve). Additionally, if the supply lines get sufficiently thin, going along with a very high resistance, this serial resistance contribution dominates the high-frequency region then. Since the supply line width adjacent to the electrodes increased in the order from the 8W1E+ enl. (100 μm) array to the 8W1000idf enl. (200 μm) array and finally to the 8W1000idf enl. (900 μm) array, as already indicated in their denotations, this explained the decrease in impedance in that order in the high-frequency region.

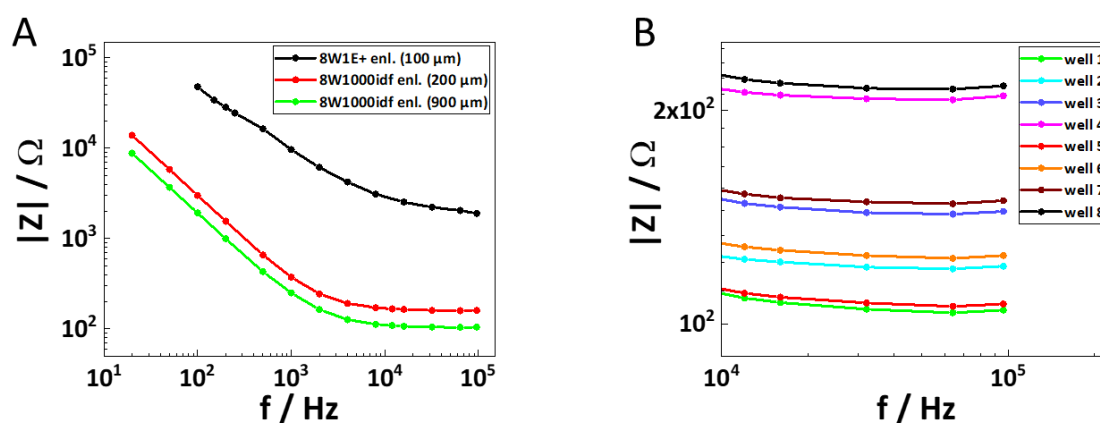


Figure 7.4 Impedance spectra of cell-free electrodes of typical ECIS measurements during optimization of the electrode layout of the transwell assay. **(A)** Impedance spectra of cell-free electrodes for the three different electrode layouts of the 8W1E+ enl. (100 μm) array (black), 8W1000idf enl. (200 μm) array (red), and the 8W1000idf enl. (900 μm) array (green), while data are shown only for well 1. **(B)** Dependency of the impedance spectra of cell-free electrodes on the well number chosen from well 1 (green), well 2 (cyan), well 3 (blue), well 4 (purple), well 5 (red), well 6 (orange), well 7 (brown), and well 8 (black), while data are shown only for the 8W1000idf enl. (900 μm) array with focus on the high-frequency region above 10 kHz. $T = 37^\circ\text{C}$.

Detailed analysis of the impedance spectra in Figure 7.4 A yielded an impedance decrease from 48 k Ω to 3 k Ω at 10^2 Hz when the array type is switched from the 8W1E+ enl. (100 μm) array (Figure 7.4 A, black curve) to the 8W1000idf enl. (200 μm) array (Figure 7.4 A, red curve), which corresponds to an increase in electrode area by a factor of about 40. Further increasing the electrode area by a factor of about 1.5 by switching from the 8W1000idf enl. (200 μm) array to the 8W1000idf enl. (900 μm) array (Figure 7.4 A, green curve) led to an impedance decrease from 3 k Ω to 2 k Ω at 10^2 Hz. Since the values of the electrode area were calculated from the intended electrode dimensions and small differences between actual and intended electrode dimensions were obtained before in Table 7.1, the given values of electrode area can only roughly be used for the explanation of

the exact impedance decrease. Nevertheless, these shifts in impedance correlated very well with the respective increase in electrode area and mirror the indirect proportionality between electrode area and impedance given by Eq. (67) and Eq. (68). The electrode area of $3.31 \cdot 10^{-3} \text{ cm}^2$ of the 8W1E+ enl. (100 μm) array was in the same order of magnitude (although slightly smaller) as the electrode area of $5 \cdot 10^{-3} \text{ cm}^2$ used for the simulations in Figure 3.12 and the electrode area of $4.9 \cdot 10^{-3} \text{ cm}^2$ of conventional 8W10E ECIS arrays, from which one measured spectrum was shown in Figure 4.21. Thus, impedance values of cell-free electrodes in the low-frequency region were compared to each other. The impedance value of 48 k Ω at 10^2 Hz for the 8W1E+ enl. (100 μm) array (Figure 7.4 A, black curve) was slightly higher than the 28 k Ω at 10^2 Hz obtained in the simulations (see Figure 3.12) and the 26 k Ω at 10^2 Hz measured for conventional 8W10E ECIS arrays (see Figure 4.21), which correlated well with the slightly smaller electrode area. When the shape of the impedance spectra of cell-free electrodes in Figure 7.4 A is compared to those in Figure 3.12 and Figure 4.21, the spectra for the 8W1000idf enl. (200 μm) array (Figure 7.4 A, red curve) and the 8W1000idf enl. (900 μm) array (Figure 7.4 A, green curve) were found to be very similar to simulated ones and those measured for conventional 8W10E ECIS arrays. The 8W1E+ enl. (100 μm) array (Figure 7.4 A, black curve) exhibited an atypical shape of the spectrum with a small shoulder at $5 \cdot 10^2$ Hz and a hardly obtainable horizontal part of the spectrum at high frequencies. In Figure 7.4 B, the impedance spectra of cell-free electrodes in the high-frequency region of the 8W1000idf enl. (900 μm) array were observed to be dependent on the respective well chosen for data recording. Pairs of spectra with similar impedance were identified with stepwise impedance increases between these pairs. The impedance was observed smallest for wells 1 (Figure 7.4 B, green curve) and 5 (Figure 7.4 B, red curve), a bit higher for wells 2 (Figure 7.4 B, cyan curve) and 6 (Figure 7.4 B, orange curve), still higher for wells 3 (Figure 7.4 B, blue curve) and 7 (Figure 7.4 B, brown curve), and highest for wells 4 (Figure 7.4 B, purple curve) and 8 (Figure 7.4 B, black curve). This observation was in accordance with the length of the supply line from the working electrodes in the respective well to the contact pad, which was shortest for wells 1 and 5, and stepwise increased for wells 2 and 6, further increased for wells 3 and 7, and was maximal for wells 4 and 8, as can be seen in Figure 4.10. The longer the supply line, the higher was the impedance in the high-frequency region, which can be explained by a higher supply line resistance. Similar to above, where the supply line resistance was decreased by an increase in the supply line width adjacent to the electrodes, here, a decreased supply line resistance was observed for wells with shorter supply lines, both of them leading to a smaller impedance in the high-frequency region. Although the two wells of each pair (wells 1 and 5, wells 2 and 6, wells 3 and 7, wells 4 and 8) exhibited a similar impedance, a closer look revealed slightly higher values for the wells located on the right side of the array (wells 5 – 8), which may be attributed to a non-uniform gold sputtering process. Because these differences in impedance obtained for the different wells were actually not huge ((105 – 215) Ω at 10^5 Hz), this had no consequences for the experimental procedure and the measurement.

In total, for all three electrode layouts of the transwell assay tested, impedance spectra of cell-free electrodes were obtained, which were similar in shape to those from simulations and measurements with conventional 8W10E ECIS arrays, while the two electrode layouts with inter-digitated finger configurations were considered to be better. The electrode area was increased two times, which was clearly identifiable in the spectra. The disadvantage of the loss in sensitivity, which is associated with the increase in electrode area, is compensated by the gain of representativity since a higher number of cells is monitored. Thus, the 8W1000idf enl. (900 μm) array was chosen for all the transwell experiments shown in section 7.3 because this was the electrode layout with the biggest electrode area. Since the width of the supply lines adjacent to the electrodes was identified as an important factor influencing the quality of the spectra and this parameter was highest for the 8W1000idf enl. (900 μm) array, this also favored the selection of this array type. But, due to the relatively broad supply lines, which would significantly contribute to the impedance signal, they were completely covered with photopolymer for insulation in the end of the photolithographic fabrication process.

7.2.2 Pre-Incubation Time of the Electrodes

The second issue for optimization of the transwell assay was the pre-incubation time of the electrodes with culture medium before cell seeding. As already mentioned in section 4.8, for each individual experiment, impedance spectra of cell-covered electrodes were always extracted from the first time point of the actual measurement reflecting electrodes covered with an adherent and confluent layer of HTM-N cells ensured by starting the measurement three days after cell seeding. Before cell seeding, impedance spectra of cell-free electrodes were recorded separately (for reused arrays) or extracted from the last time point of the pulsing measurement (for arrays used for the first time), both of them performed in pure culture medium. For the first few transwell experiments, the pre-incubation time of the electrodes with culture medium was about 1 h, which yielded impedance spectra of cell-free electrodes as displayed representatively for one typical transwell experiment and one well in Figure 7.5 A. The corresponding impedance spectra of electrodes covered with HTM-N cells recorded later on are shown additionally. Since these impedance spectra looked quite unusual in the low-frequency region, as discussed in detail further down, this was attempted to be improved by extending the pre-incubation time of the electrodes with medium from about 1 h to about 24 h, which yielded impedance spectra (cell-free and covered with HTM-N cells) as displayed representatively in Figure 7.5 B. For both cases, spectra for the 8W1000idf enl. (900 μm) array were recorded and discussed.

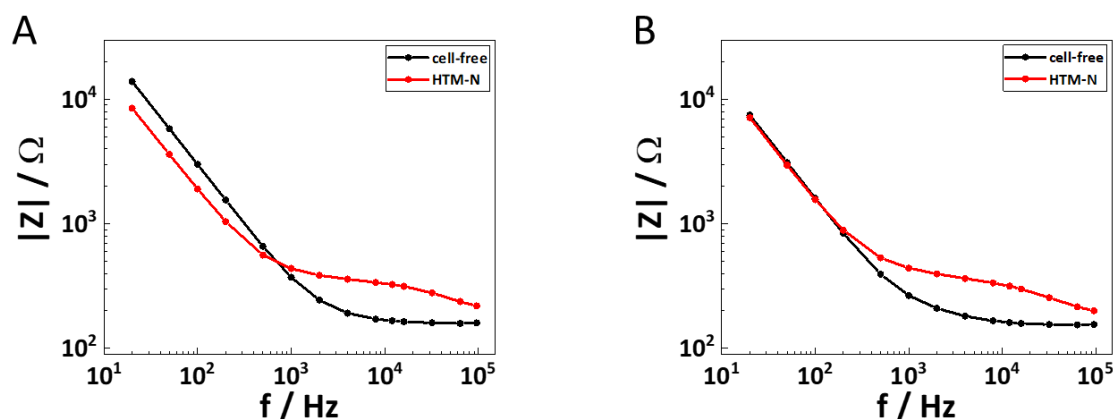


Figure 7.5 Typical impedance spectra of 8W1000idf enl. (900 μm) electrodes for about 1 h (**A**) or about 24 h (**B**) of pre-incubation of the electrodes with culture medium prior to the measurement of the spectra of cell-free electrodes. Data are only shown for one representative well covered with pure culture medium (cell-free, black) and covered with an adherent and confluent layer of HTM-N cells (red). $T = 37^\circ\text{C}$.

For both the pre-incubation times of the 8W1000idf enl. (900 μm) array with culture medium of about 1 h (Figure 7.5 A) and about 24 h (Figure 7.5 B), in the mid and high-frequency region, the spectra of HTM-N cell-covered electrodes (Figure 7.5, red curves) exhibited higher impedance values than the respective spectra of cell-free electrodes (Figure 7.5, black curves) with a similar shape of the spectra as already measured for conventional 8W10E ECIS arrays (see Figure 4.21). The influence of the HTM-N cells was observed by their additional impedance contribution leading to an elevated impedance in this region. Overlapping impedance spectra for both cell-free and cell-covered electrodes in the high-frequency region, as it was obtained in simulations in Figure 3.12 above 10^5 Hz, was not observed here because 10^5 Hz was the maximum of the measurement frequency. In the low-frequency region, spectra of cell-covered electrodes (Figure 7.5, red curves) were observed to exhibit lower impedance values than the spectra of cell-free electrodes (Figure 7.5, black curves), which was strongly pronounced for 1 h of pre-incubation (Figure 7.5 A) and still present, but only minimally, for 24 h of pre-incubation (Figure 7.5 B). This was unusual and a clear discrepancy to the ECIS model, where impedance spectra of both cell-free and cell-covered electrodes overlap in the low-frequency region, as can be seen in simulations (see Figure 3.12) and was also observed in measurements with conventional 8W10E ECIS arrays (see Figure 4.21). Since this strange behavior of the impedance spectra was observed in the frequency-dependent capacitive part in the low-frequency region, this must be the result of a change of the electrode capacitance over time between the time points of measuring the spectra of cell-free and cell-covered electrodes. This decrease in impedance for longer incubation times correlates with an increase in capacitance and is most likely explained by cysteine molecules from the medium covering the gold electrodes over time. This also explained that the difference between the spectra of cell-free and cell-covered electrodes became smaller for an increase in the pre-incubation time with culture medium to about 24 h (Figure 7.5 B) because then it was enough time for the increase in capacitance to

happen also for the spectra of cell-free electrodes, associated with a decrease in impedance. This process of increase in capacitance by incubation with medium was not avoidable for the spectra of cell-covered electrodes due to the long incubation time. Since the spectra of cell-covered electrodes still looked quite normal, this process of increase in capacitance was not considered a disadvantage, but instead, a possibility to align the spectra of cell-free electrodes to the cell-covered ones by increasing the pre-incubation time with culture medium before measuring cell-free electrodes. This increase in pre-incubation time from about 1 h to about 24 h led to a significant improvement of impedance spectra, which, nevertheless, still exhibited slightly smaller impedance in the low-frequency region for cell-covered electrodes (Figure 7.5 B). But the impedance spectra were similar to simulated ones (see Figure 3.12) and to those measured for conventional 8W10E ECIS arrays (see Figure 4.21) and, thus, good enough for a precise determination of the most sensitive frequency. Accordingly, for all the transwell experiments shown in section 7.3, the transwell arrays were incubated with culture medium for about 24 h before spectra of cell-free electrodes were measured.

7.2.3 Additional Medium Exchange

As already described in section 4.8.2, on day two of the three days of incubation between seeding of the HTM-N cells into the transwell arrays and measurement start, the culture medium was exchanged with medium containing 0.35% FBS to simulate the physiological situation in the human eye. Within these 24 h before measurement start, the cells had enough time to accustom to the new conditions, but this small amount of FBS was assumed to be completely consumed by the cells within this time. When fasudil and control solutions, which both contained 0.35% FBS, were added during the assay, an influence on the cells was possible only caused by the different content of FBS. To avoid that, an additional medium exchange to fresh medium containing 0.35% FBS was introduced right before measurement start as part of the optimization of the transwell assay to provide the cells with a fresh amount of FBS and to re-establish physiological conditions. Thus, the cells had a time of 2 h (during measurement of the baseline) for equilibrating to this addition of FBS before fasudil and control solutions were added, which was expected to minimize the unspecific reaction of the cells. Figure 7.6 demonstrates the effect of this implementation of an additional medium exchange by showing typical time courses of the normalized impedance $norm. |Z|$ for additions of control solutions from different transwell experiments, while six of them were taken from experiments without this additional medium exchange and six of them from such experiments with additional medium exchange. For data selection, no discrimination was made if the addition was directly or through the vitreous body or agarose gels. Although the data were selected from different experiments with different most sensitive frequencies (8 kHz, 10 kHz, 12 kHz, or 16 kHz), for a better comparison, all the time courses are displayed in one single diagram for the normalized impedance at variable

frequency $\text{norm. } |Z|_{X \text{ kHz}}$ (with $X = 8, 10, 12, 16$), so that any detailed comparison of the normalized impedance has to be handled with care.

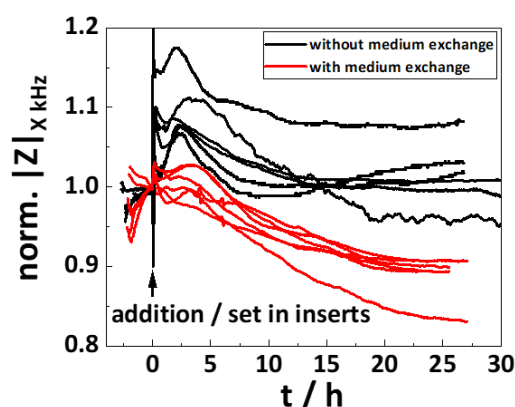


Figure 7.6 Comparison of time courses of the normalized impedance at variable frequency $\text{norm. } |Z|_{X \text{ kHz}}$ ($X = 8, 10, 12, 16$) taken from different transwell experiments with electrodes confluent covered with HTM-N cells for analyzing the influence of an additional medium exchange to fresh medium containing 0.35% FBS right before measurement start. Twelve typical time courses are shown, six without (black) and six with (red) this additional medium exchange. In every case, only the control solution ($0 \mu\text{M}$ fasudil) was added to the cell layer, disregarding the way of addition. Data were normalized to the time point of addition and are displayed without averaging. $T = 37^\circ\text{C}$.

Despite the reduced comparability due to different most sensitive frequencies, a clear difference was observed between time courses of the normalized impedance extracted from experiments without (Figure 7.6, black curves) and with (Figure 7.6, red curves) an additional medium exchange to fresh medium containing 0.35% FBS right before measurement start. Before this additional medium exchange was implemented, the normalized impedance $\text{norm. } |Z|_{X \text{ kHz}}$ increased to 1.07 – 1.17 within about 2 h after addition of control solutions, followed by a signal decrease back to about baseline levels (Figure 7.6, black curves). In contrast to that, when the amount of FBS was refreshed before measurement start, no significant signal change was observed upon addition of control solutions, followed by a signal decrease to about 0.9 till the end of the measurements (Figure 7.6, red curves). Additionally, the implementation of this additional medium exchange led to less variation between individual time courses. These results demonstrated that the HTM-N cells were sensitive to the FBS content and needed some time to equilibrate to the altered conditions. Without previous medium exchange, the FBS content was changed by the additions of control solutions during the measurement leading to a significant signal change. But when the medium was exchanged before measurement start, giving the cells sufficient time to accustom to the altered FBS content (during measurement of the baseline), this avoided the signal change upon addition of control solutions. Thus, the implementation of this additional medium exchange before measurement start for refreshing the FBS content had a positive effect on experiment outcome and was therefore applied to all the transwell experiments shown in section 7.3.

7.2.4 Method of Direct Additions

As already mentioned in section 4.8.2, in most of the transwell experiments, an addition of fasudil directly to the cells without using a transwell filter insert was investigated in parallel for comparison to the diffusion through the vitreous body. This direct addition was initially performed by the full replacement method, which was substituted by the half replacement method to minimize impairment of the HTM-N cells as the next issue for optimization of the transwell assay. The influence of the change of this addition strategy is demonstrated in Figure 7.7, where three typical time courses of the normalized impedance at variable frequency $norm. |Z|_{X \text{ kHz}}$ (with $X = 8, 10, 12$) are depicted for both the full replacement method and the half replacement method. Only direct additions of control solutions are shown extracted from different transwell experiments with different most sensitive frequencies (8 kHz, 10 kHz, or 12 kHz). The focus of the diagram is the behavior directly after the additions.

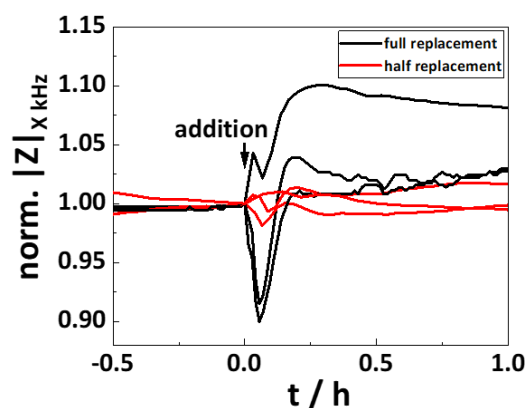


Figure 7.7 Comparison of time courses of the normalized impedance at variable frequency $norm. |Z|_{X \text{ kHz}}$ ($X = 8, 10, 12$) taken from different transwell experiments with electrodes confluent covered with HTM-N cells for analyzing the influence of the method of direct additions with focus on the first hour after the additions. Six typical time courses are shown, three for the full replacement method (black) and three for the half replacement method (red), while only direct additions of control solutions were analyzed. Data were normalized to the time point of addition and are displayed without averaging. $T = 37^\circ \text{C}$.

Direct additions of control solutions performed by the full replacement method caused a significant signal change directly after addition, as evident from the three respective time courses in Figure 7.7, while two of them exhibited an instant decrease in $norm. |Z|_{X \text{ kHz}}$ to about 0.9 and one showed an instant increase to about 1.05 followed by a further increase to about 1.1 reached after about 15 min (Figure 7.7, black curves). In contrast to that, when the half replacement method was applied, direct additions of control solutions induced much smaller signal changes directly after addition (Figure 7.7, red curves). This proved that the HTM-N cells were significantly impaired by liquid additions using the full replacement method, which was strongly reduced for the half replacement method. Thus, for all the

transwell experiments shown in section 7.3, the half replacement method was applied to direct additions of fasudil and control solutions.

7.2.5 Way of Fasudil Application

The last issue for optimization of the transwell assay was the way how fasudil was applied to the vitreous body. As already described in section 4.8.2, in initial experiments, the fasudil solution was added on top of the vitreous body in the transwell filter inserts. Since inhomogeneities within the material properties of the vitreous body were described earlier in section 7.1, this on-top application of fasudil was expected to lead to diffusion times varying significantly between single experiments. More constant diffusion times of fasudil were expected when fasudil and vitreous body were mixed prior to the assay, leading to a uniform distribution of fasudil within the vitreous body. This pre-mixing application of fasudil is compared to the on-top application in Figure 7.8 by showing three typical time courses each, extracted from different transwell experiments with different most sensitive frequencies (8 kHz, 16 kHz). The time courses show the behavior of the normalized impedance at variable frequency $norm. |Z|_{X \text{ kHz}}$ (with $X = 8, 16$) with focus on the first 10 h (Figure 7.8 A) or 2 h (Figure 7.8 B) after the transwell filter inserts were placed above the HTM-N cell-covered electrodes. Fasudil solutions were always applied to yield a final concentration of 25 μM in the wells.

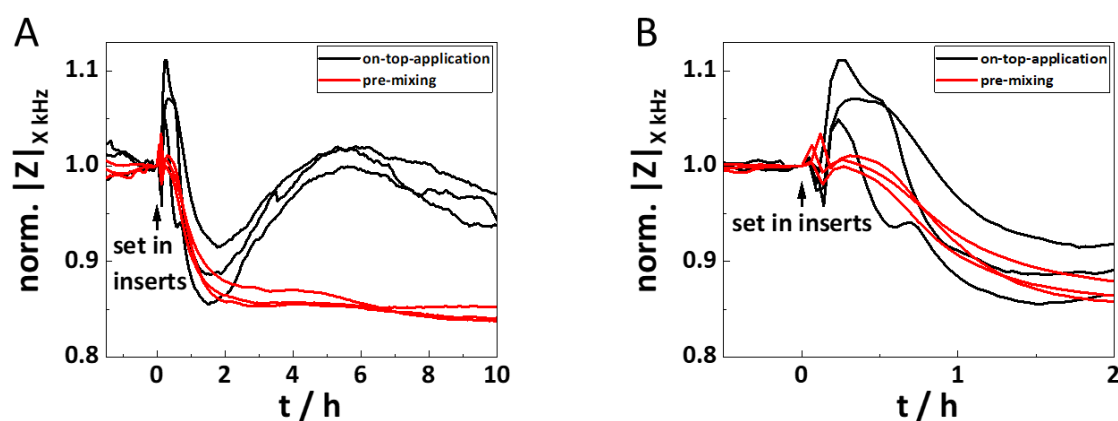


Figure 7.8 Comparison of time courses of the normalized impedance at variable frequency $norm. |Z|_{X \text{ kHz}}$ ($X = 8, 16$) taken from different transwell experiments with electrodes confluent covered with HTM-N cells for analyzing the influence of the way of fasudil application to the vitreous body (final fasudil concentration of 25 μM). Both diagrams show the same data with different time scales focusing on the first 10 h (A) or the first 2 h (B) after the transwell filter inserts were placed into the arrays. Six typical time courses are shown, three for the on-top application of fasudil onto the vitreous body (black) and three for the application of fasudil by pre-mixing with the vitreous body (red). Data were normalized to the time point of addition of the transwell filter inserts and are displayed without averaging. $T = 37^\circ\text{C}$.

For the on-top application of fasudil onto the vitreous body, the time courses of $norm. |Z|_{X\text{ kHz}}$ exhibited minima at about 1.5 h after addition of the transwell filter inserts followed by a signal recovery (Figure 7.8 A, black curves). This was very similar to direct additions of fasudil in normal ECIS experiments (see Figure 6.6 A), where the stimulation of the HTM-N cells with multiple fasudil molecules at the same time led to overshoot reactions with subsequent signal recovery. Thus, this similarity in the time courses also suggested a simultaneous stimulation of the HTM-N cells by a huge amount of fasudil when it was applied on top of the vitreous body and can be explained by fasudil reaching the cells almost all at once by an easy and fast penetration of the vitreous body by sneak pathways. But there was also one clear difference between the time courses of the normalized impedance for the on-top application and the direct additions. Whereas direct additions of fasudil always led to an instant signal decrease (see Figure 6.6 A), after on-top application, the normalized impedance first experienced some signal fluctuations caused by the additions of the transwell filter inserts, followed by a signal decrease starting with some time delay (Figure 7.8 B, black curves). Since the cell reaction to a fasudil treatment was expected to happen instantly after the fasudil solution gets in touch with the cells, as it was obtained in direct additions (see Figure 6.6 A), the time point of the onset of signal decrease is regarded as the time point when the first fasudil molecules reach the cells. This was observed about 15 min – 30 min after addition of the transwell filter inserts (Figure 7.8 B, black curves), which obviously resulted from the time fasudil needed to diffuse out of the vitreous body and across the medium to the cells on the electrodes. Thus, these results fitted quite well to the duration of diffusion of less than 22 min, as estimated in section 7.1 by diffusion tests and calculations. The huge variation of diffusion times between single experiments, as expected for the on-top application of fasudil as a result of inhomogeneities within the vitreous body, was also proven here.

When fasudil and vitreous body were mixed prior to the assay (Figure 7.8, red curves), time courses of $norm. |Z|_{X\text{ kHz}}$ were observed, which were significantly different to those of the on-top application (Figure 7.8, black curves). After some signal fluctuations due to the additions of the transwell filter inserts (Figure 7.8 B, red curves), this pre-mixing application of fasudil led to a slow and sustained signal decrease without signal recovery (Figure 7.8 A, red curves), which was caused by the gradual diffusion of fasudil without any transient overshoot, but instead, with a longer lasting effect of fasudil on the HTM-N cells. The onset of signal decrease was observed about 15 min after addition of the transwell filter inserts (Figure 7.8 B, red curves), which fitted very well to the 12 min of diffusion of fasudil through the culture medium, as calculated in section 7.1. There was less variation in the time delays of signal decrease between single experiments for the pre-mixing application of fasudil, which proved the more reproducible diffusion profile for this type of application. Additionally, there was also less variation in the maximal cell response for the pre-mixing application of fasudil. The maximal cell response for the on-top application, reflected by the value of $norm. |Z|_{X\text{ kHz}}$ at the minimum, varied between 0.85 and 0.91 (Figure 7.8, black curves), whereas the pre-mixing application yielded values of $norm. |Z|_{X\text{ kHz}}$ of about 0.85

with less variation between single experiments (Figure 7.8, red curves). As a consequence of these results, the pre-mixing of fasudil with vitreous body was used for all the transwell experiments shown in section 7.3, when the diffusion through the vitreous body was analyzed. When agarose gels were used as the diffusion medium, fasudil was added by the on-top application because mixing with agarose gels was not possible due to their relatively stiff nature.

7.3 Studying the Diffusion of Fasudil through the Vitreous Body and its Subsequent Effect on HTM-N Cells Using a Transwell Approach

The transwell assay was developed to simulate the biological situation of fasudil diffusing through the vitreous body and subsequently exerting its relaxation effect on TM cells by degradation of actin filaments to finally lower IOP for the treatment of glaucoma. After the transwell assay has been developed, including self-fabrication of arrays (see section 4.5.1), characterization of the vitreous body (see section 7.1), and optimization of the assay (see section 7.2), the final transwell experiment is demonstrated here (Figure 7.9). All the optimizations described and discussed in section 7.2 were applied here. Thus, 8W1000idf enl. (900 μm) arrays were used, the electrodes were previously incubated with culture medium for about 24 h, an additional medium exchange was performed right before measurement start, the half replacement method was applied for direct additions, and fasudil and control solutions were previously mixed with vitreous body for their diffusion. This final transwell experiment was performed three times with constant experimental conditions and has already been published by Mietzner *et al.* (Mietzner *et al.*, 2020). After its diffusion through the vitreous body in the transwell filter inserts and the culture medium in the wells, fasudil stimulated the HTM-N cells adherently grown on the bottom electrodes, while both the retardation by diffusion and cellular changes were monitored by ECIS measurements. For all three biological replicates, the most sensitive frequency was 8 kHz and, thus, the normalized impedance at 8 kHz $norm. |Z|_{8\text{ kHz}}$ was plotted and averaged over these three biological replicates. Experimental replicates were always included by parallelly testing the same conditions in 2 – 3 wells, leading to a total number of replicates of 6 – 9. Beside the treatment with normal free fasudil (Figure 7.9, red and blue curves), *released* fasudil was used additionally (Figure 7.9, orange and cyan curves). It was initially encapsulated in PLGA microspheres and released over time as described in section 4.2.1. This was done to test if fasudil is still biologically active after encapsulation and release from PLGA microspheres, which can be used as an intravitreally injectable depot formulation for a prolonged drug release (Mietzner *et al.*, 2020). For comparison to control conditions, control solutions for *released* fasudil were used (Figure 7.9, black and brown curves). Application of solutions was performed by pre-mixing with vitreous body (Figure 7.9, blue, cyan, and brown

curves), which was compared to direct additions (Figure 7.9, red, orange, and black curves). For both free and *released* fasudil and for both application strategies, solutions were added to yield final fasudil concentrations in the wells of 25 μM . This transwell experiment is shown with focus on the first 15 h (Figure 7.9 A) or the first 2 h (Figure 7.9 B) after the additions (placing of transwell filter inserts into the wells or direct additions).

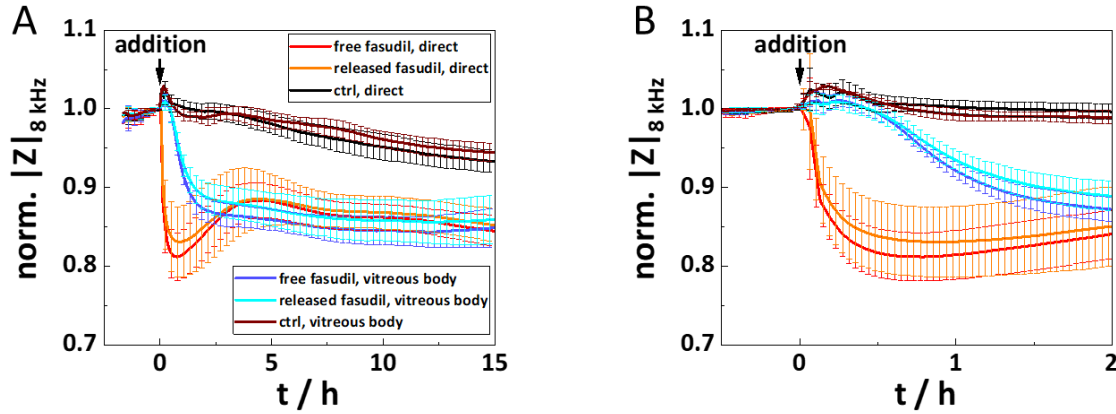


Figure 7.9 Normalized impedance at 8 kHz $norm. |Z|_{8 \text{ kHz}}$ during transwell experiments analyzing the diffusion of fasudil (final concentration of 25 μM) through the vitreous body and its subsequent influence on HTM-N cells seeded confluent in 8W1000idf enl. (900 μm) transwell arrays. Both diagrams show the same data with different time scales focusing on the first 15 h (A) or the first 2 h (B) after addition of solutions or transwell filter inserts. Solutions were added directly to the cells (red, orange, black) or mixed with vitreous body in the transwell filter inserts for a previous diffusion (blue, cyan, brown), while normal free fasudil (red, blue) and *released* fasudil (orange, cyan) were compared in contrast to control conditions (black, brown). Data were normalized to the time point of addition and are displayed as mean $\pm$ SD out of several wells from three biological replicates (free fasudil: $n = 7 - 9(3)$, *released* fasudil: $n = 8(3)$, ctrl: $n = 6(3)$). $T = 37^\circ\text{C}$.

When control solutions were investigated in the transwell experiment, the normalized impedance at 8 kHz $norm. |Z|_{8 \text{ kHz}}$ experienced a decrease over time, but this was only down to values of about 0.95 after 15 h (Figure 7.9 A, black and brown curves), proving that no unspecific cell reactions occurred. No significant difference was observed between a direct addition of the control solution (Figure 7.9 A, black curve) and a previous diffusion of the control solution through vitreous body and culture medium (Figure 7.9 A, brown curve). As expected, direct additions of fasudil to the HTM-N cells led to an instant and huge signal decrease with a minimum after about 1 h due to its relaxation effect, mirroring an instant and strong cell reaction (overshoot reaction) due to an instant concentration increase (Figure 7.9, red and orange curves). This was followed by a signal recovery, as obtained similarly in normal ECIS experiments (see Figure 6.6 A) and for the on-top application of fasudil onto the vitreous body (see Figure 7.8 A). When fasudil was applied by pre-mixing with the vitreous body inducing its diffusion within the vitreous body and through the culture medium, a slow and sustained decrease in $norm. |Z|_{8 \text{ kHz}}$ without signal recovery was observed caused by the gradual diffusion of fasudil and slowly increasing concentration of fasudil in the wells (Figure 7.9, blue and cyan curves). Additionally, this diffusion of fasudil

caused a time delay of signal decrease in comparison to direct additions. As already obtained in Figure 7.8 B, here again, the onset of signal decrease was observed about 15 min after addition of the transwell filter inserts (Figure 7.9 B, blue and cyan curves). This fitted very well to the duration of diffusion of fasudil through the culture medium of 12 min, as calculated in section 7.1. The maximal diffusion time, which is the time for fasudil molecules starting the diffusion from the uppermost layers of the vitreous body until reaching the HTM-N cells, is expressed by the time point where the time courses of $norm. |Z|_{8\text{ kHz}}$ for the two different application strategies of fasudil reach the same level. This was observed at about 3 h after the additions (Figure 7.9 A), which perfectly fitted to the value of 3 h, as calculated in section 7.1. For the direct additions of fasudil (Figure 7.9, red and orange curves) as well as for the application of fasudil via the vitreous body (Figure 7.9, blue and cyan curves), no significant difference between *released* fasudil and free fasudil was observed, proving the continued biological activity of fasudil after its encapsulation in PLGA microspheres and subsequent release. Although different time courses of $norm. |Z|_{8\text{ kHz}}$ were observed for the different application strategies of fasudil, final values after 15 h were almost the same (about 0.85) (Figure 7.9 A, red, orange, blue, and cyan curves), proving similar final concentrations and similar total effects of fasudil on the HTM-N cells irrespective of the way of application. Thus, this transwell experiment demonstrated a successful diffusion of fasudil within the vitreous body and through the culture medium with a subsequent total cellular response similar to that of a direct supply, with an additional time delay. These results suggested that fasudil and other ROCK inhibitors can, after previous intravitreal injection and diffusion through the vitreous body, elicit relaxation effects to the TM, potentially leading to a reduction of IOP in glaucomatous eyes. Additionally, the results for *released* fasudil indicated that intravitreally injectable PLGA microspheres containing and releasing fasudil for a prolonged time without loss in biological activity can improve glaucoma therapy, as it was also reported by Mietzner *et al.* (Mietzner *et al.*, 2020).

To finally evaluate the development of this transwell assay, it was compared to existing systems that are able to follow the diffusion of fasudil to the trabecular meshwork. Commonly, such experiments are performed by eye perfusion studies. Originally, an anterior cavity of an eyeball was fixed and perfused with a medium containing the test solution under study via a cannula (Johnson & Tschumper, 1987). This original test system did not yet include the vitreous body for diffusion and the evaluation was only possible by microscopic observations of TM sections after perfusion. But, based on that, more comprehensive organ-on-a-chip devices were developed using the complete eyeball including the vitreous body, where, for example, drug distribution was evaluated by perfusing fluorescent microparticles and performing fluorescence imaging after cryo-sectioning (Chan *et al.*, 2022). In comparison to that, the transwell assay developed in this thesis is not that close to the in-vivo physiological situation, but the diffusion of fasudil and its cellular effect can be followed in real time due to the time-resolved measurement. Thus, this transwell assay complements existing devices to analyze the diffusion of fasudil and other drug candidates to treat POAG.

In the following, some further complementary tests and variations of the transwell assay are demonstrated, which include a test if placing transwell filter inserts into the wells shows a significant influence on the ECIS measurement, HPLC measurements for checking the final fasudil concentration in the wells, the use of agarose gels as a substitute for the vitreous body, and CLSM imaging of the HTM-N cells.

For testing the effect of placing transwell filter inserts into the wells during measurement, one additional transwell experiment was performed, where one well was used to successively add and remove the transwell filter insert (Figure 7.10). In the remaining wells, direct additions of fasudil (Figure 7.10, red curve) and control solution (Figure 7.10, black curve) and pre-mixing application of fasudil (Figure 7.10, blue curve) and control solution (Figure 7.10, brown curve) were performed with final fasudil concentrations in the wells of 25 μM . But, in this one specific well, nothing was added at 0 h, but a transwell filter insert (filled with vitreous body and control solution) was added at about 4 h, which was removed and immediately added again at about 6 h (Figure 7.10, green curve). The effect of this successive addition of the transwell filter insert was investigated by analyzing the time courses of the normalized impedance at 8 kHz $norm. |Z|_{8 \text{ kHz}}$ recorded by continuous ECIS measurements in this specific well and all the other wells.

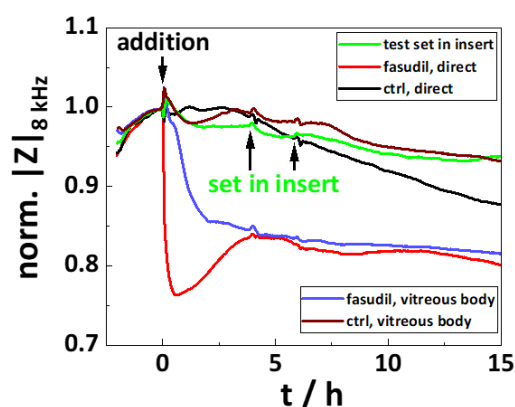


Figure 7.10 Normalized impedance at 8 kHz $norm. |Z|_{8 \text{ kHz}}$ during an additional transwell experiment analyzing the influence of placing transwell filter inserts into the wells during the ECIS measurement. HTM-N cells seeded confluent in 8W1000idf enl. (900 μm) transwell arrays were treated directly with fasudil (red) or a control solution (black) or were treated after previous diffusion through the vitreous body (application by pre-mixing) with fasudil (blue) or a control solution (brown), while the final fasudil concentration in the wells was adjusted to 25 μM . In one well, the transwell filter insert (filled with vitreous body and control solution) was not added at 0 h, but instead, at about 4 h, which was removed and immediately added again at about 6 h (green). Data were normalized to the time point of addition (0 h). $T = 37^\circ\text{C}$.

When the time courses of $norm. |Z|_{8 \text{ kHz}}$ of the direct additions and the applications by pre-mixing with the vitreous body (Figure 7.10, red, black, blue, and brown curves) are compared to the respective time courses in Figure 7.9, very close similarities were observed. Although no addition was performed at 0 h in the test well (Figure 7.10, green curve), a signal

change was observed similar to the addition peak in the other wells, where solutions were added directly or transwell filter inserts were placed into the wells. Moreover, the addition of the transwell filter insert in this test well at 4 h and 6 h induced slight signal peaks, which were observable in all wells. These small signal changes can be caused by a slight movement of the array or by temperature changes associated with an opening of the incubator during additions and are typical for ECIS measurements due to the very high sensitivity of this technique. But these signal changes can be ignored because they were very small and followed by an instant recovery. Thus, placing transwell filter inserts into the wells during the transwell experiment did not significantly influence the impedance signal.

To check for correct and constant final fasudil concentration in the wells, after the ECIS measurement, the culture medium surrounding the HTM-N cells was taken out of the wells and analyzed by HPLC measurements (see section 4.2.1). This was performed for two individual transwell experiments (two biological replicates), where the pre-mixing application of fasudil with the vitreous body was applied. This type of fasudil application was compared to direct additions, while for both application strategies, several wells were analyzed and each of them with triplicate HPLC determinations, leading to a total number of replicates of 9 for direct additions and 12 for the pre-mixing with vitreous body. HPLC results are displayed as mean $\pm$ SD out of all these replicates in Table 7.2.

Table 7.2 Determination of the final fasudil concentration c in the wells of the transwell array by HPLC. While fasudil solutions were always added to yield final concentrations in the wells of 25 μM , the actual fasudil concentrations in the wells were determined after the ECIS measurement for direct additions and pre-mixing with vitreous body. HPLC results are given as mean $\pm$ SD with averaging over two biological replicates, several wells, and triplicate HPLC determinations, leading to a total number of replicates of 9 – 12.

Application strategy	c (fasudil) / μM
direct	21.4 ± 1.1 ($n = 9(2)$)
pre-mixing with vitreous body	18.2 ± 0.6 ($n = 12(2)$)

Since the standard deviations of the final fasudil concentration determined by HPLC were very small (1.1 μM and 0.6 μM) (Table 7.2), this proved a good precision of the HPLC based quantification as well as a constant fasudil concentration over experimental and biological replicates. Compared to the intended final fasudil concentration in the wells of 25 μM , the value determined by HPLC after direct additions of fasudil of (21.4 ± 1.1) μM was a bit smaller (Table 7.2). Reasons for this deviation could be a partial uptake of fasudil by the HTM-N cells, binding of fasudil to plastic walls and to BSA, or due to the inaccuracy of pipetting steps during preparation and addition of the fasudil solutions. The final fasudil concentration after the pre-mixing application with vitreous body was determined by HPLC measurements to (18.2 ± 0.6) μM , which was 15% smaller than compared to direct

additions (Table 7.2). This proved that, even after the 24 h of measurement, fasudil was not completely distributed by diffusion over culture medium and vitreous body with more fasudil molecules stuck within the vitreous body leading to a smaller fasudil concentration in the wells. But because both values of the final fasudil concentration determined by HPLC in the wells of the transwell array after the two different application strategies were not really far away from each other, this was in agreement with the finding from above that both application strategies exhibited similar final impedance values in the ECIS measurements (see Figure 7.9 A).

As a substitute for the vitreous body, agarose gels were used in one transwell experiment, which is demonstrated in Figure 7.11. The agarose gels were prepared according to section 4.2.4 and added to the transwell filter inserts as the diffusion medium for fasudil. As already mentioned before, solutions (fasudil and control) were added by the on-top application onto the agarose gels for a previous diffusion, while agarose was used at a concentration of 1.5% (w/v) (Figure 7.11, blue (fasudil) and brown (control) curves), 1% (w/v) (Figure 7.11, cyan (fasudil) and orange (control) curves) and 0.5% (w/v) (Figure 7.11, green (fasudil) and purple (control) curves). This was compared to direct additions to the HTM-N cells (Figure 7.11, red (fasudil) and black (control) curves). Again, either added directly or via an on-top application onto agarose gels, a fasudil solution was added to yield a final concentration of 25 μ M. The remaining experimental conditions (including volumes and dimensions) were the same as for the final transwell experiment with vitreous body demonstrated in Figure 7.9. The most sensitive frequency of the ECIS measurement was 10 kHz and, thus, the normalized impedance at 10 kHz $norm. |Z|_{10\text{ kHz}}$ was plotted. For most conditions, averaging was performed over two experimental replicates (two wells), whereas the addition of the control solution onto the 1.5% (w/v) agarose gel (Figure 7.11, brown curve) and the 1% (w/v) agarose gel (Figure 7.11, orange curve) were only measured once. This transwell experiment is shown with focus on the first 15 h (Figure 7.11 A) or the first 5 h (Figure 7.11 B) after the addition of solutions or transwell filter inserts.

Although slight differences in the reactions of the HTM-N cells to control stimulations, either added directly (Figure 7.11 A, black curve) or via an on-top application onto agarose gels (Figure 7.11 A, brown, orange, and purple curves), were observed, all of them showed a slight decrease in the normalized impedance at 10 kHz over time with values of 0.9 – 0.95 obtained after 15 h, proving no significant unspecific cell reactions. As expected, the direct addition of fasudil to the cells caused a time course of $norm. |Z|_{10\text{ kHz}}$ with minimum and signal recovery (Figure 7.11 A, red curve), as observed similarly several times before (see Figure 6.6 A, Figure 7.8 A, and Figure 7.9 A).

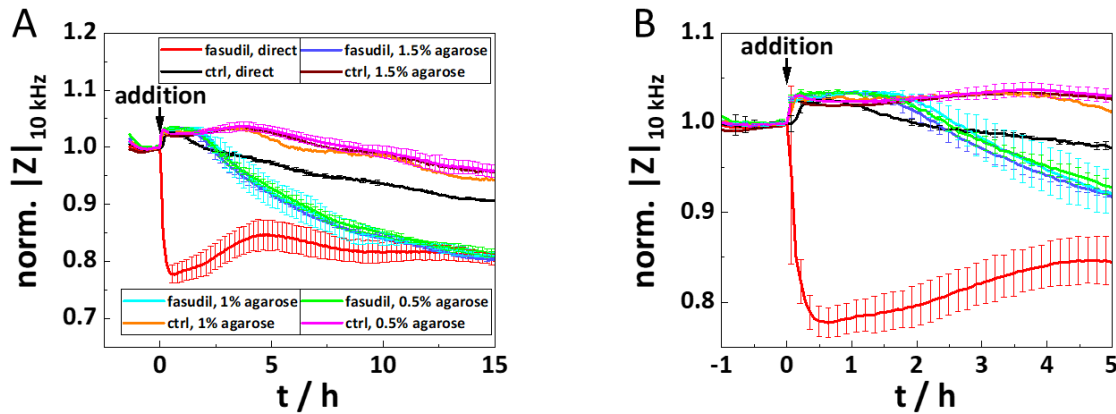


Figure 7.11 Normalized impedance at 10 kHz $norm. |Z|_{10 \text{ kHz}}$ during transwell experiments analyzing the diffusion of fasudil (final concentration of 25 μM) through agarose gels and its subsequent influence on HTM-N cells seeded confluent in 8W1000idf enl. (900 μm) transwell arrays. Both diagrams show the same data with different time scales focusing on the first 15 h (A) or the first 5 h (B) after addition of solutions or transwell filter inserts. Direct additions (fasudil: red, control: black) were compared to a previous diffusion by on-top application onto a 1.5% (w/v) agarose gel (fasudil: blue, control: brown), a 1% (w/v) agarose gel (fasudil: cyan, control: orange), and a 0.5% (w/v) agarose gel (fasudil: green, control: purple). Data were normalized to the time point of addition and are displayed as mean $\pm$ SD out of two wells ($n = 2(1)$), while the control measurements for the 1.5% (w/v) agarose gel (brown) and the 1% (w/v) agarose gel (orange) were only performed in one single well, thus, not allowing averaging. $T = 37^\circ\text{C}$.

When fasudil was applied through agarose gels, it triggered a slow and sustained signal decrease without recovery (Figure 7.11 A, blue, cyan, and green curves). There was no measurable influence of the agarose concentration, as it was already obtained in diffusion tests demonstrated in section 7.1. These time courses of $norm. |Z|_{10 \text{ kHz}}$ were similar with respect to their shape (but not with respect to their time scales) as obtained for the diffusion of fasudil through the vitreous body (see Figure 7.9 A) and attributed to the slowly increasing concentration of fasudil in the wells by the gradual diffusion through the agarose gels without causing an overshoot reaction. But in contrast to the diffusion through the vitreous body, fasudil exhibited a longer maximal diffusion time across the agarose gels, since it took about 13 h for these time courses to reach the level of the direct addition (Figure 7.11 A), which was obtained at about 3 h for the vitreous body (see Figure 7.9 A). This longer period of time needed for fasudil to be completely distributed over agarose gel and medium mirrored the reduced mass transport through agarose gels as compared to the vitreous body, but this was also partially caused by the different application strategies of fasudil. Whereas the pre-mixing application of fasudil and vitreous body triggered an instant diffusion start through the culture medium, the on-top application onto agarose gels required a previous diffusion of fasudil through the complete agarose gel before entering the medium, leading to a longer time for a complete distribution of fasudil. Additionally, the onset of signal decrease was also later for agarose gels, which was observed after about 1.7 h (Figure 7.11 B, blue, cyan, and green curves), compared to the 15 min observed for vitreous body in Figure 7.9 B. This difference was, of course, also induced by the different

application strategies of fasudil. Significant differences were obtained when these 1.7 h of diffusion time are compared with diffusion tests and calculations, where a total duration of diffusion of fasudil through agarose and culture medium of about 3 h was suggested (see section 7.1). Reasons for this deviation could be the concentration gradient of fasudil within the agarose gel and medium, which was not regarded for the calculations, a possible convection induced by addition of the transwell filter inserts, or the hydrodynamic pressure difference between the different liquid heights of agarose and surrounding medium. All these possible reasons increase the flux of fasudil molecules through the agarose gels, inducing that an effective fasudil concentration is achieved earlier in the wells. Although a longer duration of diffusion of fasudil was obtained in agarose gels, this experiment demonstrated that agarose gels can be used as a good model when vitreous body is not available. This also suggests the usage of any kind of hydrogel to be tested in this transwell assay as a substitute for the vitreous body.

Morphological changes of the HTM-N cells caused by fasudil treatment during this transwell experiment were recorded by ECIS measurements so far. For gaining additional structural information about the actin cytoskeleton, ECIS was complemented by actin staining and subsequent CLSM imaging in the following. CLSM images of the cells were taken in a glass ring setup with the same dimensions as the wells of the transwell arrays, as described in detail in section 4.10.3. Images were taken for different time points (0 h, 1 h, and 4 h) during a transwell experiment with diffusion of fasudil (final concentration of 25 μM) through vitreous body with pre-mixing of both, which is again compared to direct additions. According to the ECIS measurement in Figure 7.9, after 1 h, the normalized impedance was at the minimum for direct additions and has arrived about half of the cell response for fasudil diffusing through the vitreous body, while after 4 h, both additions were down at about the same level of impedance decrease. Images for the time point of 0 h were taken for an untreated cell layer and represent control conditions. For each condition, images were taken at three different spots with one image shown as representative for the respective condition in Figure 7.12.

As already observed before (see Figure 6.3 and Figure 6.10), HTM-N cells exhibit strongly pronounced stress fibers under control conditions (Figure 7.12 A). 1 h (Figure 7.12 B) and 4 h (Figure 7.12 C) after direct addition of fasudil, the stress fibers were no longer present indicating a degradation of stress fibers by fasudil treatment, and the cell cortex appeared frizzy and diffuse, which indicated a beginning degradation of the cell cortex. This was observed similarly before (see Figure 6.10), where a higher fasudil concentration was used (100 μM), which led to a stronger and more pronounced degradation of the cell cortex after already 3 h.

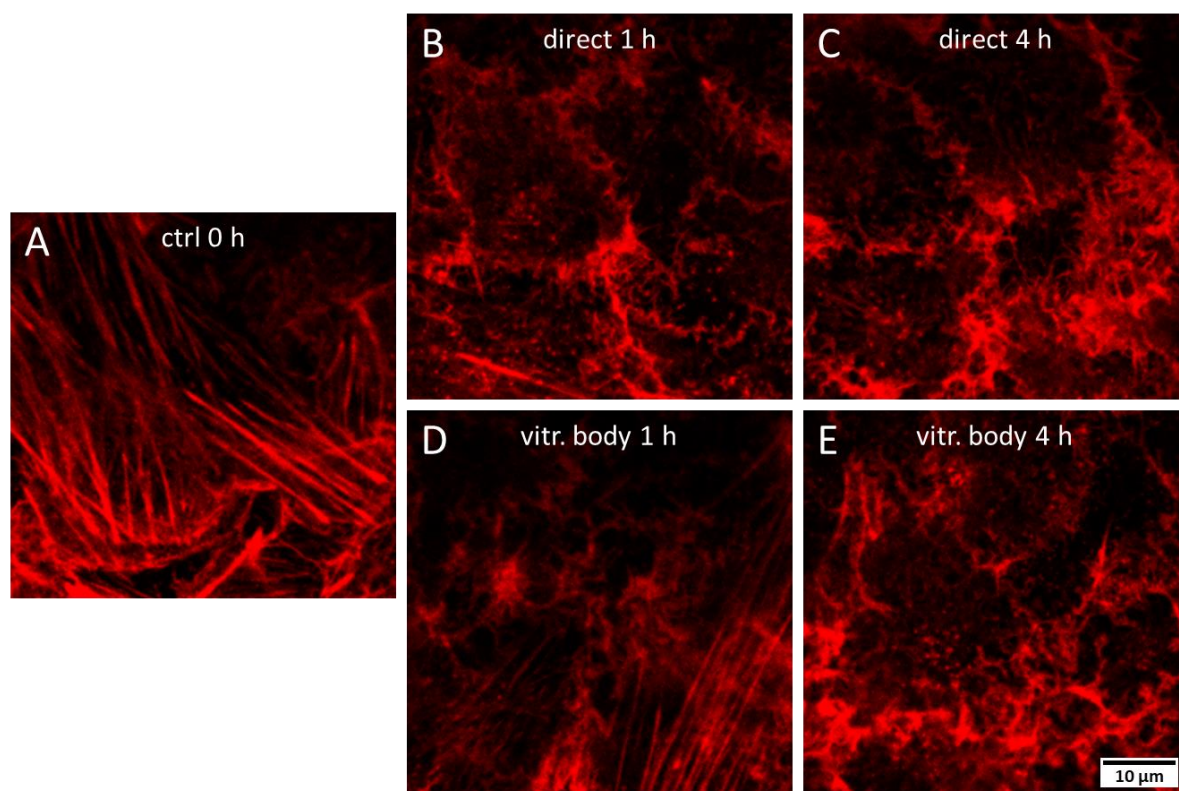


Figure 7.12 Confocal laser scanning microscopic images of confluent HTM-N cell layers with TRITC-phalloidin labeling of actin filaments (focal plane close to the substrate) taken during a transwell experiment with 25 μM fasudil treatment through vitreous body or after direct application. Cells were imaged for control conditions (A), 1 h (B), and 4 h (C) after direct addition of fasudil and 1 h (D) and 4 h (E) after addition of transwell filter inserts filled with a vitreous body-fasudil-mix. Three images were taken for each condition, but only one image representative for the respective condition is demonstrated here. $T = 37^\circ\text{C}$.

The degradation of stress fibers and cell cortex was observed similarly after 4 h of diffusion of fasudil within the vitreous body and through the culture medium (Figure 7.12 E). This was expected because the normalized impedance in Figure 7.9 revealed an equal cell reaction to fasudil after this time for both additions. But after 1 h of diffusion of fasudil, some kind of intermediate cell reaction was observed, where both linear and frizzy structures were obtained (Figure 7.12 D). This indicated that some stress fibers were still intact, but the degradation of stress fibers and cell cortex has already started. This was also in accordance with the ECIS measurement (see Figure 7.9), where the normalized impedance suggested about half of the cell response for this type of fasudil application as compared to a direct addition of fasudil for this time of 1 h after addition. As already mentioned before, these cytoskeletal changes fitted to the obtained impedance decrease upon fasudil stimulation since the current can cross the cell layer more easily when actin filaments are degraded, accompanied by a certain degree of contact loss to neighboring cells and the growth substrate. The observations fitted to literature studies on the fasudil effect in human urethral scar fibroblasts (Li *et al.*, 2018) and in mouse brain astrocytes (Lau *et al.*, 2011). Thus, these CLSM investigations for the transwell experiment revealed actin cytoskeletal

changes of the HTM-N cells upon fasudil treatment, which were observed similarly with and without previous diffusion within vitreous body and through culture medium. The only difference is a time delay of the cell reaction obtained with previous diffusion. ECIS and CLSM results matched very well.

Taken all together, the physiological situation of a fasudil diffusion through the vitreous body and subsequent effect on TM cells was simulated by a transwell assay, which was developed in this thesis. This assay is able to analyze the response of HTM-N cells to a fasudil treatment after its previous diffusion through the vitreous body in transwell filter inserts, with a comparison to direct additions in the same setup. Arrays were self-fabricated with modification of the electrode layout and several further optimizations of the experimental procedure. Continuous ECIS measurements were able to record the cellular response to fasudil as well as the retardation by diffusion, which fitted quite well to calculations and diffusion tests. Morphological changes of the HTM-N cells during this transwell experiment proved the relaxation effect of fasudil even after previous diffusion, which was additionally confirmed by CLSM imaging. Moreover, tests on agarose gels recommended their usage as a substitute for the vitreous body although their higher stiffness is accompanied by a reduced mass transport. The successful performance of this transwell experiment also suggests screening different diffusing substances, for example, other ROCK inhibitors, to test their diffusion through the vitreous body and their potential influence on the cells. Different cell types can also be cultured on the electrodes, for example, primary HTM cells for a supplementary investigation of the fasudil effect. Other ocular diseases with intended treatment via intravitreal injection can also be investigated with this transwell assay by culturing the respective cell type in the wells of the transwell array and adding drug candidates to the vitreous body. Instead of the vitreous body, different biomaterials used as drug carriers can also be added to the transwell filter inserts to test a prolonged drug release by measuring the cell response over time.

8 Microgravimetric Sensing of Osmotically Induced Transmembrane Water Flux

As it was already discussed in detail in section 1.3, there is a correlation between glaucoma and water flux. On the one hand, AQP inhibition in the ciliary body can reduce aqueous humor production and secretion, and consequently, can reduce IOP (Zhang *et al.*, 2002). On the other hand, evidence was found for a correlation between TM cell shrinking by water outflux and an increase in TM permeability to aqueous humor outflow and a correlated decrease in IOP (O'Donnell *et al.*, 1995). Thus, studying water flux and its inhibition can help find new treatment strategies for glaucoma. Regarding the general discrimination between transepithelial and transmembrane water flux, the latter is way more important in this thesis since the applied measurement technique of QCM largely prevents a water flux across the entire cell layer by adhesion of the cells onto an impermeable quartz disk. A transepithelial water flux, with an additional water flux between cell bodies and the narrow liquid compartment beneath the cells (across the basal membrane), associated with an alteration of the cell-substrate distance, may occur and contribute to the overall QCM response for epithelial cell lines with strong barrier function (Wegener *et al.*, 2000c). The QCM, as a method shown to be sensitive to stiffness changes, is able to detect this transmembrane water flux because this is directly associated with cell volume changes and, thus, stiffness changes. As already described in section 4.6.3 and demonstrated schematically in Figure 4.17, the water flux is triggered osmotically, where extracellular hypertonic conditions lead to water outflux, associated with cell shrinking and stiffening. Extracellular hypotonic conditions lead to water influx, associated with cell swelling and softening. Solutions of varying osmolality were prepared by adding different amounts of sucrose, as described in detail in section 4.2.1, and their osmolalities were determined by cryoscopy (see section 4.2.2). Osmolality differences $\Delta\pi$ (to the respective isotonic solution) were already given in Table 4.6 and are summarized again in Table 8.1 with discrimination between full replacement method and half replacement method.

Since the cell response to osmolality changes typically happens on a relatively fast time scale (within a few minutes), the QCM was operated in the fast-mode enabling a time resolution of 16.5 s, as already explained in section 4.6.1. The disadvantage of this fast-mode was that the minimal impedance $|Z|_{min}$ was the only parameter that could be extracted from the measurements, which, however, was shown before to be a reliable parameter for stiffness (see section 5.1). Therefore, $|Z|_{min}$ was expected to mirror the volume changes associated with water flux. This parameter was expected to increase upon hypertonically induced cell shrinking and to decrease upon hypotonically induced cell swelling.

Table 8.1 Summary of final osmolality differences $\Delta\pi$ of hypotonic, isotonic, and hypertonic sucrose solutions used for water flux experiments with discrimination between full replacement method and half replacement method, which were already given in Table 4.6. The solutions were prepared as described in section 4.2.1 and their osmolalities were determined as described in section 4.2.2. Data are given as average over all solutions prepared with two individual samples each. $\Delta\pi$ of the 800 mM sucrose solution for the half replacement method was not determined because this solution was only added by the full replacement method.

Solution	$\Delta\pi$ / mosmol/kg (full replacement)	$\Delta\pi$ / mosmol/kg (half replacement)
hypo 1	−194	−103
hypo 2	−130	−66
iso	0	0
PBS ⁺⁺ /Glc	0	0
20 mM	+24	+16
100 mM	+108	+57
200 mM	+246	+116
300 mM	+350	+170
400 mM	+484	+233
800 mM	+1102	-

Based on the time courses of $\Delta|Z|_{min}$, quantitative evaluation of certain aspects of water flux was performed by determination of a variety of parameters with distinct interpretations, which are described in the following. The maximum of the change of the minimal impedance $Max(\Delta|Z|_{min})$ (for hypertonic stimulations) and the minimum of the change of the minimal impedance $Min(\Delta|Z|_{min})$ (for hypotonic stimulations) reflect the total amount of water flux occurring after stimulation. The velocity of water flux is described by the speed of signal increase or decrease and can be mathematically determined by fitting the time course of $\Delta|Z|_{min}$. Among many different fit functions, the curve shape of signal increase or decrease was matched best by an exponential fit with the following equation:

$$\Delta|Z|_{min} = A_1 \cdot e^{\frac{t}{\tau}} + y_0 \quad (76)$$

with the time t and the three fit parameters A_1 , y_0 , and the time constant τ . The factors A_1 and y_0 determine the exact position and orientation of this exponential fit function, while the time constant τ describes how fast it increases or decreases. The determination of the time constant τ is demonstrated for typical water flux measurements in Figure S5, while both hypertonic and hypotonic stimulations were fitted by this exponential fit function described by Eq. (76). The data for Figure S5 were selected from water flux experiments with clearly obtainable signal regeneration, demonstrating that the speed of regeneration can also be determined by exponential fitting with Eq. (76). The time constant is then called time constant of regeneration τ_{reg} and describes its velocity. If this signal regeneration is the

consequence of RVC (RVI or RVD) processes, this will also be investigated in this chapter. Both time constants were negative for all the water flux experiments, thus, their magnitude was used for a better comparison, where a smaller time constant reflects a faster water flux. The excellent quality of the fits was expressed by the small standard deviations of the fit parameters and the R-square values close to 1 (see windows in Figure S5). The last parameter of evaluation was the area under the curve reflecting the extent of total water flux including signal regeneration. This parameter was determined similarly as already demonstrated for ECIS measurements on fasudil stimulations (see Figure S4). While the area for hypertonic stimulations was indeed determined under the curve, it was determined above the curve for hypotonic stimulations, so that the extent of water flux is directly proportional to the area in both cases. The limits of integration are given for each individual experiment, but were kept constant within an experiment or when two experiments were compared. When replicates were included in an experiment, these parameters were determined for each individual time course and averaged afterwards.

In section 8.1.1, the method of addition was first analyzed by comparison of half replacement method and full replacement method, and the influence of viscosity differences on the total cell response was investigated. Different compounds were examined in this thesis reported to elicit inhibitory effects on certain aspects of water flux, including subsequent RVC processes, summarized as water flux inhibitors. These were the AQP inhibitor hydrogen tetrachloroaurate trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) (Niemietz & Tyerman, 2002), the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport inhibitor bumetanide (O'Donnell *et al.*, 1995), and the K^+ channel inhibitor tetraethylammonium chloride (Et_4NCl) (Mitchell *et al.*, 2002). Before these water flux inhibitors were applied in QCM-based water flux experiments, their cytotoxicity was first assessed by the PrestoBlue[®] assay (section 8.1.2) and their influence on the solutions' osmolality was measured (section 8.1.3). By comparing MDCK-I cells and MDCK-I-AQP1 cells and using $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ as an inhibitor, the recombinant expression of aquaporin-1 (AQP1) and its inhibition were investigated (section 8.2.1). Among these experiments on AQP1 and its inhibition, double-quartz-disks (see section 4.7) were tested in addition to normal quartz disks. In section 8.2.2, the full range of water flux experiments was performed with HTM-N cells, which naturally express AQP1 (Liton *et al.*, 2006). Beside the conventional addition strategy of one addition per cell layer (individual addition), the osmolality was also stepwise increased/decreased by consecutive additions to the same cell layer (serial addition). The inhibition of this water flux in HTM-N cells, including subsequent RVC processes, was then analyzed by the use of the three water flux inhibitors $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, bumetanide, and Et_4NCl (section 8.2.3). Finally, QCM results for these water flux experiments were compared to cytoskeletal changes observed by CLSM imaging (section 8.2.4).

8.1 Preliminary Experiments

8.1.1 Investigating the Method of Additions and the Influence of Viscosity

Although the half replacement method was applied in almost all cell-based experiments in this thesis due to its minimized cell impairment, which was also demonstrated in Figure 7.7, the full replacement method is regarded as a meaningful alternative in water flux experiments, since the solutions' osmolalities are known precisely during this addition strategy and higher osmotic gradients can be achieved. Thus, these two methods of addition were compared to each other in a preliminary water flux experiment on hypertonic stimulations of MDCK-II cells (Figure 8.1 A and B). Hypertonicity with varying osmotic gradient was induced by addition of sucrose solutions with varying osmolality: 20 mM (Figure 8.1 A and B, green curves), 100 mM (Figure 8.1 A and B, cyan curves), 200 mM (Figure 8.1 A and B, blue curves), 300 mM (Figure 8.1 A and B, purple curves), and 400 mM (Figure 8.1 A and B, red curves), in comparison to an addition of the isotonic PBS⁺⁺/Glc solution (Figure 8.1 B, black curve). The osmolalities of these solutions were already given in Table 8.1. The water flux and associated cell volume change was monitored by recording the change of the minimal impedance $\Delta|Z|_{min}$ extracted from fast-mode QCM measurements. Normalization was performed for the time point of addition (0 min). Since the applied sucrose solutions showed rather high solute concentrations, their enhanced viscosity was assumed to contribute significantly to the composite QCM response. To detect the influence of viscosity alone without inducing a water flux, a cell-free control experiment was conducted, where the initially present PBS⁺⁺/Glc solution was exchanged with the 400 mM sucrose solution, which was done by the full replacement method (Figure 8.1 C, black curve) and by the half replacement method (Figure 8.1 C, red curve). For the sake of completeness, water flux experiments on MDCK-II cells were additionally performed with hypotonic stimulations, while the two different hypotonic solutions hypo 2 (Figure 8.1 D, green curve) and hypo 1 (Figure 8.1 D, red curve) were added in comparison to an addition of the isotonic solution (iso) (Figure 8.1 D, black curve). The osmolalities of these solutions can also be seen in Table 8.1. These hypotonic additions were, however, only conducted by the half replacement method. Replicates were only included for hypo 2 and hypo 1 solutions, where averaging was performed over two experimental replicates.

Control measurements for both hypertonic (PBS⁺⁺/Glc, Figure 8.1 B, black curve) and hypotonic stimulations (iso, Figure 8.1 D, black curve), where isotonic buffer was exchanged by isotonic buffer, showed no significant change of $\Delta|Z|_{min}$ demonstrating that signal changes were not generated by the liquid handling itself. As expected, hypertonic stimulations induced an increase of $\Delta|Z|_{min}$, as can be seen in Figure 8.1 A and B, according to a water outflux, cell shrinking and stiffening. Hypotonic stimulations induced a decrease of $\Delta|Z|_{min}$, as can be seen in Figure 8.1 D, according to a water influx, cell swelling and softening. The higher the osmolality difference $\Delta\pi$ to the corresponding isotonic solution,

the bigger was the change of the minimal impedance $\Delta|Z|_{\min}$, which was observed for both hypertonic (Figure 8.1 A and B) and hypotonic stimulations (Figure 8.1 D). This correlation between the magnitude of the osmotic gradient and the extent of water flux has already been reported before (McCarty & O'Neil, 1992).

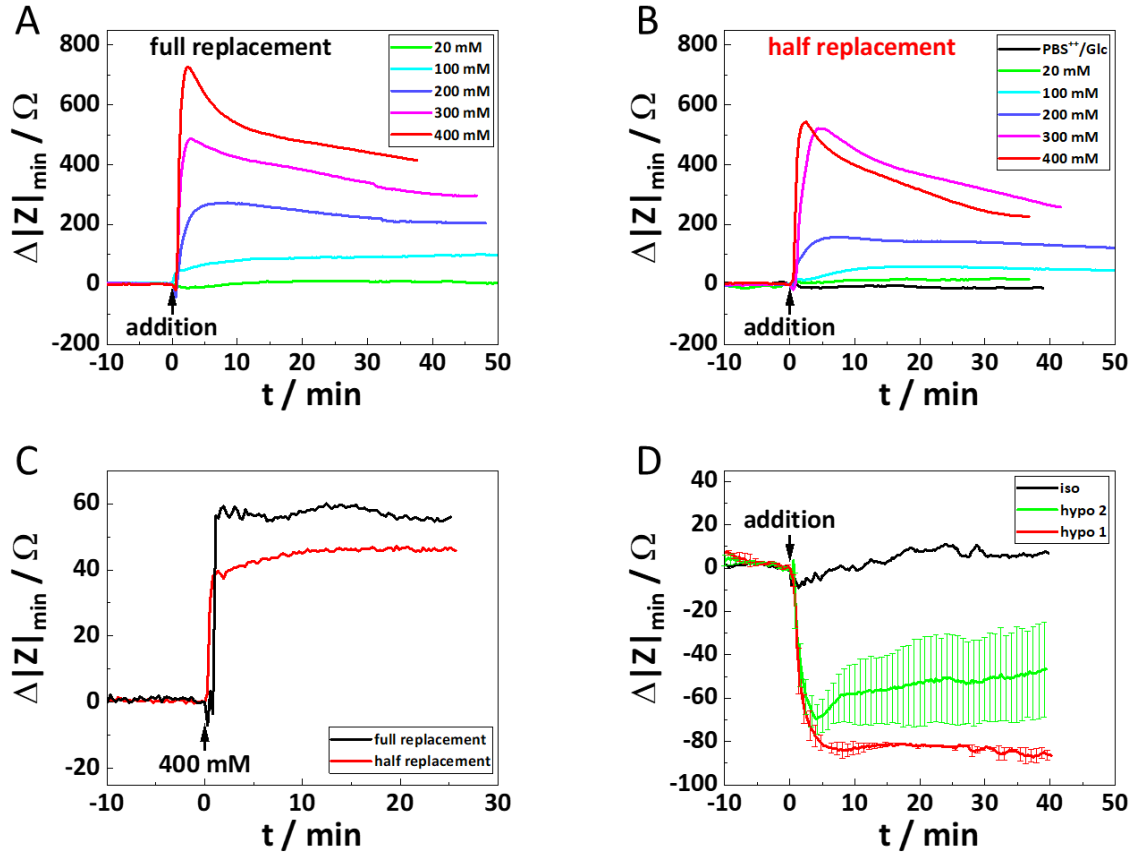


Figure 8.1 The change of the minimal impedance $\Delta|Z|_{\min}$ during QCM measurements (fast-mode) analyzing the method of additions and the influence of viscosity in water flux experiments. MDCK-II cells were stimulated hypertonically by the full replacement method (A) or by the half replacement method (B), while the cells were exposed to different solutions of varying osmolality achieved by different concentrations of sucrose: 20 mM (green), 100 mM (cyan), 200 mM (blue), 300 mM (purple), and 400 mM (red) in comparison to the isotonic PBS⁺⁺/Glc solution (black) (for the osmolalities of these solutions see Table 8.1). (C) The 400 mM sucrose solution was added to cell-free quartz disks, either by the full replacement method (black) or by the half replacement method (red), to investigate the contribution of viscosity differences. (D) MDCK-II cells were stimulated hypotonically by the half replacement method, while the two different hypotonic solutions hypo 2 (green) and hypo 1 (red) were used in comparison to the isotonic solution (iso, black) (osmolalities see Table 8.1). All solutions were added at time point 0 min and data were normalized to that time point. Reproducibility was only tested for hypo 2 and hypo 1, where the data are displayed as mean $\pm$ SD out of two replicates ($n = 2(1)$). $T = 37^\circ\text{C}$.

Analyzing the shape of the curves revealed similarities but also significant differences between hypertonic and hypotonic stimulations. The signal change started immediately after addition and continuously increased (for hypertonic) or decreased (for hypotonic) until maxima (for hypertonic) or minima (for hypotonic) were reached after about 5 min on

average (Figure 8.1 A, B, and D). After reaching a maximum of $\Delta|Z|_{min}$, hypertonic stimulations were followed by a signal regeneration, which was especially pronounced for the 300 mM and 400 mM sucrose solutions (Figure 8.1 A and B, purple and red curves), indicating an RVI process. In contrast, after reaching a minimum of $\Delta|Z|_{min}$ for hypotonic stimulations, the signal stayed at about this level without significant regeneration (Figure 8.1 D), indicating that there is no RVD process in MDCK-II cells. This was contrary to certain literature studies, where MDCK cells exhibited RVD processes (Rothstein & Mack, 1991; Banderali & Roy, 1992). A more detailed investigation of RVC processes is demonstrated further down (see sections 8.2.1 and 8.2.3). When full replacement method and half replacement method are compared to each other, the MDCK-II cells were observed to respond with a higher increase in the minimal impedance for the full replacement method for all investigated hypertonic solutions (Figure 8.1 A and B), except for the 300 mM sucrose solution inducing a similar cell response for both addition strategies (Figure 8.1 A and B, purple curves). This observation was reasonable because the osmolality difference $\Delta\pi$ was higher when solutions were added by the full replacement method (Table 8.1). Upon addition, none of the methods induced a significant impairment of the cells, which would be expressed by a temporary signal drop or huge outliers. The 300 mM and 400 mM sucrose solutions triggered very similar cell responses for the half replacement method (Figure 8.1 B, purple and red curves), indicating a possible saturation for these hypertonic values of $\Delta\pi$ (+170 mosmol/kg and +233 mosmol/kg, Table 8.1). But since it was demonstrated before that the shrinking behavior of MDCK-II cells is by far not saturated at these osmolalities (Steltenkamp *et al.*, 2006), here, the half replacement method exhibited a limited dependency on the osmotic gradient. Thus, the full replacement method appeared to be the better addition strategy for water flux experiments. A more detailed comparison of both methods is facilitated by determination of a variety of parameters (see further down in Figure 8.2). Analyzing the impact of viscosity differences in Figure 8.1 C revealed time courses of $\Delta|Z|_{min}$ that were significantly different to those obtained with MDCK-II cells adhered on the quartz resonators. Adding the 400 mM sucrose solution onto cell-free quartz disks induced a signal increase that was also immediate, but the maximal signal change was also reached immediately leading to these step-like time courses typical for cell-free investigations (Figure 8.1 C). As expected due to the higher osmotic gradient for the full replacement method, the increase in $\Delta|Z|_{min}$ of 60 Ω for this application strategy (Figure 8.1 C, black curve) was higher than the 45 Ω observed for the half replacement method (Figure 8.1 C, red curve). Compared to the increase in $\Delta|Z|_{min}$ induced by water flux after addition of the same solution (400 mM) to adherent MDCK-II cell layers, where a maximal increase of 730 Ω for the full replacement method (Figure 8.1 A, red curve) and 540 Ω for the half replacement method (Figure 8.1 B, red curve) was obtained, the influence of viscosity accounts for only 8% of the total signal change. Thus, the solutions' viscosity differences can be ignored for the investigation of water flux. In the following, these water flux experiments on MDCK-II cells (Figure 8.1 A, B, and D) were evaluated quantitatively by determination of the parameters $Max(\Delta|Z|_{min})$ (for hypertonic), $Min(\Delta|Z|_{min})$ (for hypotonic), summarized as $Max/Min(\Delta|Z|_{min})$ (Figure 8.2 A), $|\tau|$ (Figure 8.2 B), the area under the curve (Figure

8.2 C), and $|\tau_{reg}|$ (Figure 8.2 D). This enabled a better comparison of hypertonic stimulations by the full replacement method (Figure 8.2, black curves), hypertonic stimulations by the half replacement method (Figure 8.2, red curves), and hypotonic stimulations by the half replacement method (Figure 8.2, green curves), while for the latter averaging was performed over two experimental replicates. All these parameters were plotted versus the osmolality difference $\Delta\pi$, which is given in Table 8.1. Determination of the area was performed by integration from 0 min to 20 min, while this was done for hypertonic stimulations down to a value of 0 Ω and for hypotonic stimulations up to a value of 0 Ω . Although the area was positive in all cases, it was marked with a negative sign for hypotonic stimulations to indicate a decrease of the minimal impedance. Since for hypotonic stimulations, a regeneration was not obtained at all, and for hypertonic stimulations, it was strongly pronounced only for the 300 mM and 400 mM sucrose solutions, $|\tau_{reg}|$ was only determined for these two solutions.

Whereas the reproducibility of hypotonic stimulations was very good for the hypo 1 solution, but not that good for the hypo 2 solution, as demonstrated by the error bars in Figure 8.1 D, the corresponding evaluation parameters exhibited an excellent reproducibility due to very small error bars (Figure 8.2 A, B, and C, green curves). As already indicated in Figure 8.1, the osmolality difference $\Delta\pi$ correlated well with the cell response for both hypertonic and hypotonic stimulations, as especially observed here by $Max/Min(\Delta|Z|_{min})$ representing the maximal water flux (Figure 8.2 A) and the area representing the total water flux including regeneration (Figure 8.2 C). This confirmed the correlation between the magnitude of the osmotic gradient and the extent of water flux. For hypertonic stimulations, the full replacement method gave better results because $Max(\Delta|Z|_{min})$ and the area correlated almost linearly with $\Delta\pi$ (Figure 8.2 A and C, black curves), whereas this linearity was not really obtained for the half replacement method (Figure 8.2 A and C, red curves). A similar result was already reported in literature, where a linear correlation between the motional resistance and the osmolality was observed for similar QCM-based water flux measurements (Wegener *et al.*, 2000c). For both hypertonic and hypotonic stimulations, time constants $|\tau|$ and $|\tau_{reg}|$ were smaller for higher osmolality difference $\Delta\pi$ with some kind of indirect proportionality (Figure 8.2 B and D). This indicated that a higher osmotic gradient induced, in addition to a bigger extent of water flux, also a faster water flux. The values of $|\tau_{reg}|$ were significantly higher than those of $|\tau|$ (with respect to the same osmolality) (Figure 8.2 B and D) demonstrating that the water flux occurring during regeneration was much slower than the initially triggered water flux. Compared to the time constant $|\tau|$ of (1.45 ± 0.12) min published for SPR measurements during a +70 mosmol/kg osmotic stimulation of MDCK-II cells (Robelek & Wegener, 2010), hypertonic stimulations with similar osmolality performed in this thesis resulted in values for $|\tau|$ in the range of (1.6 – 5.8) min (Figure 8.2 B). Since the time constants observed in this thesis were similar or at least in a similar order of magnitude as those reported for SPR, a measurement technique insensitive to cell mechanics, the QCM results can be considered reliable.

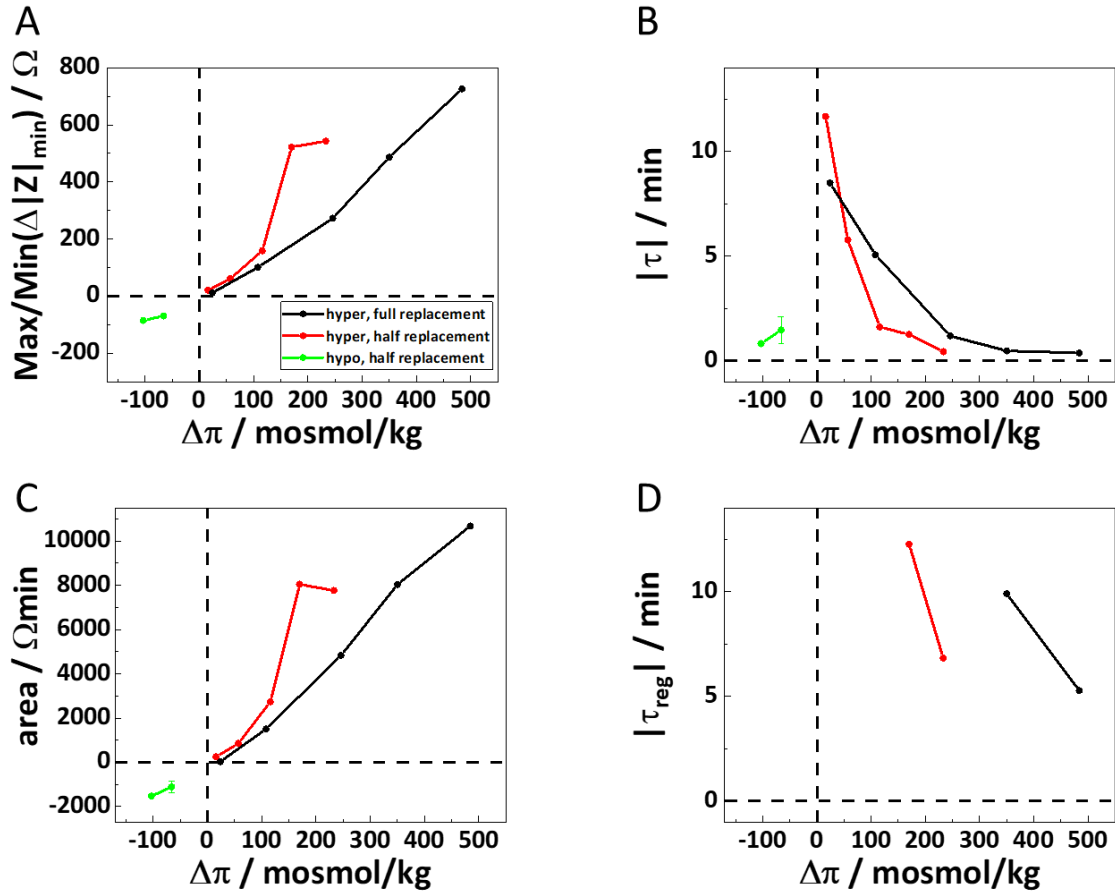


Figure 8.2 Quantitative evaluation of hypertonically and hypotonically induced water flux in MDCK-II cells in terms of a variety of parameters extracted from the QCM measurements (fast-mode) in Figure 8.1. Both the maximum of the change of the minimal impedance $\text{Max}(\Delta|Z|_{\min})$ (for hypertonic stimulations) and the minimum of the change of the minimal impedance $\text{Min}(\Delta|Z|_{\min})$ (for hypotonic stimulations), summarized as $\text{Max/Min}(\Delta|Z|_{\min})$ (A), the magnitude of the time constant $|\tau|$ (B), the area under the curve (C), and the magnitude of the time constant of regeneration $|\tau_{\text{reg}}|$ (D) were plotted versus the osmolality difference $\Delta\pi$. Hypertonic stimulations by the full replacement method (black), hypertonic stimulations by the half replacement method (red), and hypotonic stimulations by the half replacement method (green) were compared, while data for the latter are displayed as mean $\pm$ SD out of two replicates ($n = 2(1)$). For determination of the area, the integration was performed between 0 min and 20 min, with 0 Ω as the lower integration limit in y-direction for hypertonic stimulations and the upper integration limit in y-direction for hypotonic stimulations. The area for hypotonic stimulations was marked with a negative sign to indicate a decrease of the minimal impedance. $|\tau_{\text{reg}}|$ could only be determined for the 300 mM and 400 mM sucrose solutions.

In total, these preliminary results were unambiguous and promising, demonstrating an osmolality dependency of water flux and an advantage of the full replacement method over the half replacement method. According to that, the full replacement method was used for all remaining water flux experiments. But because most of these measurements were performed without replicates, the results have to be handled with care since the reproducibility has only been proven for hypotonic stimulations.

8.1.2 Screening the Cytotoxicity of Water Flux Inhibitors by the PrestoBlue® Assay

Before the water flux inhibitors $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, bumetanide, and Et_4NCl were applied in QCM-based water flux experiments, their cytotoxicity was first tested by the PrestoBlue® assay. The range of concentrations and the experimental procedure were described in section 4.9. Since these water flux inhibitors were mostly applied to HTM-N cells, these cells were used here adherently grown on the well bottom of a 96-well plate. Cytotoxicity was assessed after 2 h of incubation with these water flux inhibitors. Because the stock solution of bumetanide was prepared in DMSO, a DMSO control was included in the PrestoBlue® assay for bumetanide, while Triton X-100 served as the positive control for all water flux inhibitors. Figure 8.3 demonstrates the resulting cytotoxicity index CI plotted versus the respective concentrations of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (Figure 8.3 A), bumetanide (Figure 8.3 B), and Et_4NCl (Figure 8.3 C). Averaging was performed over six experimental replicates (six wells) from one measurement, while errors were calculated by Gaussian error propagation.

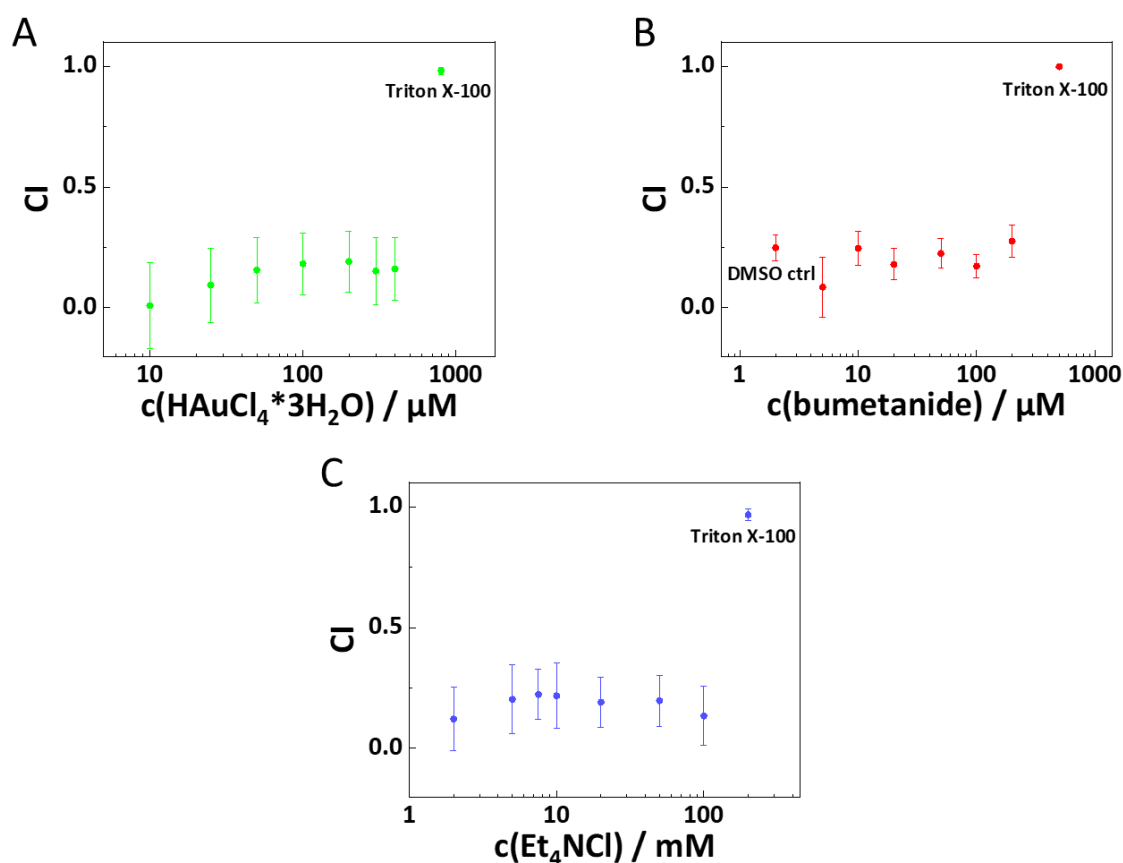


Figure 8.3 Cytotoxicity screening of water flux inhibitors by the PrestoBlue® assay applied to HTM-N cells. The cytotoxicity index CI determined for $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (green) (A), bumetanide (red) (B), and Et_4NCl (blue) (C) was plotted versus the respective concentration, while a DMSO control was included for bumetanide. The cells were incubated with these test substances for 2 h and Triton X-100 served as the positive control. The data are displayed as mean $\pm$ error (with the errors calculated by Gaussian error propagation) out of six experimental replicates from one measurement ($n = 6(1)$). $T = 37^\circ\text{C}$.

The positive control Triton X-100 exhibited a *CI* value of almost 1 for all three water flux inhibitors (Figure 8.3 A, B, and C) proving the functionality of the PrestoBlue® assays performed. For the water flux inhibitors themselves, no concentration dependent cytotoxicity was observed with *CI* values between 0 and 0.25 (Figure 8.3 A, B, and C), which was also true for the DMSO control for bumetanide (Figure 8.3 B). Thus, none of the water flux inhibitors $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, bumetanide, and Et_4NCl exhibited a significant cytotoxic influence on mitochondrial activity of the HTM-N cells. These results were then used to select the standard concentrations of the inhibitors. Due to the finding that none of them exerts a considerable cytotoxicity, they could be applied in any concentration within the investigated concentration range. For $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 50 μM was chosen as the standard concentration, which lies above the reported IC_{50} value of 10.8 μM (Niemietz & Tyerman, 2002). For bumetanide, a standard concentration of 10 μM was selected because an effective inhibition of RVI processes was already reported for this concentration (O'Donnell *et al.*, 1995). For Et_4NCl , 7.5 mM was chosen as the standard concentration because at this concentration Et_4NCl was demonstrated before in literature to show an effective inhibition of RVD processes (Mitchell *et al.*, 2002).

8.1.3 Examining the Influence of Water Flux Inhibitors on the Osmolality

The water flux inhibitors $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, bumetanide, and Et_4NCl were tested for a potential influence on the osmolality of hypertonic and hypotonic solutions. Since they were mostly used in the 200 mM sucrose solution and the hypo 1 solution, the osmolalities of these two solutions were measured in presence and absence of water flux inhibitors. Osmolality measurements were performed according to section 4.2.2. Additionally, the osmolalities of the respective isotonic solutions ($\text{PBS}^{++}/\text{Glc}$ for hyper, *iso* for hypo) were measured in presence and absence of water flux inhibitors to calculate the osmolality difference $\Delta\pi$ for each individual inhibitor, which was then compared to control conditions without inhibitor. The water flux inhibitors were used in their standard concentrations as selected in the previous section (section 8.1.2) (50 μM $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 10 μM bumetanide, 7.5 mM Et_4NCl). Results are displayed in Figure 8.4, where the osmolality differences $\Delta\pi$ of the 200 mM sucrose solution (Figure 8.4 A) and the hypo 1 solution (Figure 8.4 B) were plotted versus the inhibitor present in the solutions. Averaging was performed over two individually measured samples.

The osmolality difference $\Delta\pi$ of the 200 mM sucrose solution in presence of 10 μM bumetanide was very similar to the one without inhibitor, whereas the presence of 50 μM $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ or 7.5 mM Et_4NCl induced slightly smaller values than without inhibitor (Figure 8.4 A). But these differences were very small and absolutely not significant. The same was observed for the hypo 1 solution (Figure 8.4 B).

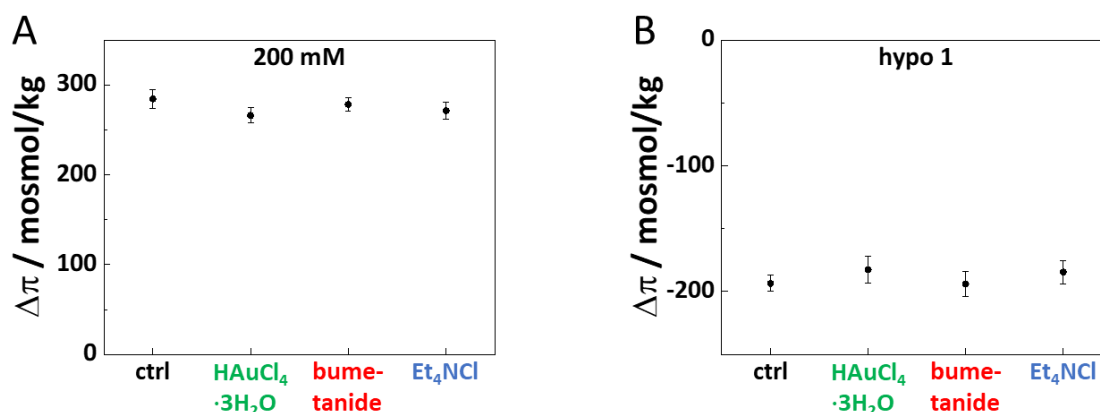


Figure 8.4 Examination of the water flux inhibitors' influence on osmolality. The influence of 50 μ M HAuCl₄·3H₂O, 10 μ M bumetanide, or 7.5 mM Et₄NCl on the osmolality difference $\Delta\pi$ of the 200 mM sucrose solution (A) and the hypo 1 solution (B) was analyzed with respect to the corresponding isotonic solution in comparison to control conditions without inhibitor (ctrl). Osmolality measurements were performed according to section 4.2.2. The data are displayed as mean $\pm$ SD out of two replicates ($n = 2$).

Thus, at the concentrations used, none of the water flux inhibitors had a significant influence on the osmolality difference $\Delta\pi$ of the investigated hypertonic and hypotonic solutions. Due to the rather low concentrations of HAuCl₄·3H₂O and bumetanide, this result was expected for these two inhibitors. But these results demonstrated that Et₄NCl, with its rather high concentration, also showed no influence on the solutions' osmolality difference. Accordingly, the water flux inhibitors HAuCl₄·3H₂O, bumetanide, and Et₄NCl can be applied in QCM-based water flux measurements without the need to consider an influence on cytotoxicity (see section 8.1.2) or osmolality.

8.2 Using QCM to Detect Water Flux and its Inhibition

8.2.1 Investigating the Influence of a Recombinant AQP1 Expression on Water Flux and its Inhibition

As discussed in section 1.3, aquaporins (AQPs) and especially aquaporin-1 (AQP1) can enhance water flux across the plasma membrane, which can be inhibited by AQP inhibitors, like HAuCl₄·3H₂O. In detail, as it was already stated by Urban, the presence of AQP1 is not expected to increase the total amount of water flux, but is expected to accelerate water flux, although it could not be verified there (Urban, 2019). The AQP inhibitor HAuCl₄·3H₂O was shown to decrease the speed of water flux (Niemietz & Tyerman, 2002). The influence of a recombinant expression of AQP1 on water flux and its inhibition by HAuCl₄·3H₂O was investigated in this thesis by fast-mode QCM measurements. These water flux experiments

are displayed in Figure 8.5, where MDCK-I cells (Figure 8.5 A and B) were compared to MDCK-I-AQP1 cells (Figure 8.5 C and D), which recombinantly express AQP1, and MDCK-I-AQP1 cells in presence of 50 μM $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (Figure 8.5 E and F). Beside QCM measurements with normal quartz disks (Figure 8.5 A, C, and E), double-quartz-disks were tested here (Figure 8.5 B, D, and F), which parallelly generate two QCM signals of the same cell layer (see section 4.7), with a discrimination between coated (Figure 8.5 B, D, and F, ●) and uncoated side (Figure 8.5 B, D, and F, ○). Only hypertonic stimulations were investigated here, by sucrose solutions with varying osmolality: 20 mM (Figure 8.5, green curves), 100 mM (Figure 8.5, cyan curves), 200 mM (Figure 8.5, blue curves), 300 mM (Figure 8.5, purple curves), and 400 mM (Figure 8.5, red curves), in comparison to an addition of the isotonic $\text{PBS}^{++}/\text{Glc}$ solution (Figure 8.5, black curves) (osmolalities see Table 8.1). The water flux was monitored by the change of the minimal impedance $\Delta|Z|_{\min}$, normalized to the time point of addition (0 min).

For all the conditions investigated, control additions with the $\text{PBS}^{++}/\text{Glc}$ solution induced no significant signal change (Figure 8.5, black curves). As already observed for MDCK-II cells in Figure 8.1 A and B, the minimal impedance of MDCK-I cells increased upon hypertonic stimulations with a clearly identifiable dependency on the osmotic gradient (Figure 8.5 A). This was observed similarly for MDCK-I-AQP1 cells (Figure 8.5 C) and in presence of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (MDCK-I-AQP1 + $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, Figure 8.5 E). At a first glance, only slight differences were observed between these three cases, where the MDCK-I-AQP1 cells in presence of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ exhibited a slightly smaller increase in $\Delta|Z|_{\min}$ for the 300 mM and 400 mM sucrose solutions (Figure 8.5 E, purple and red curves). In comparison to normal quartz disks, using double-quartz-disks led to very similar time courses of $\Delta|Z|_{\min}$, although slightly higher values were observed there (Figure 8.5 B, D, and F), which was strongest pronounced for the 400 mM sucrose solution (Figure 8.5 B, D, and F, red curves). For most of the stimulations, the coated side (Figure 8.5 B, D, and F, ●) exhibited slightly higher values of $\Delta|Z|_{\min}$ than the uncoated one (Figure 8.5 B, D, and F, ○). This was induced by higher baseline minimal impedance values for the coated side (data not shown) and an associated higher sensitivity. This was already described by Oberleitner (Oberleitner, 2018) and was attributed to a better adhesion of the cells onto the photopolymer coating. Similar to normal quartz disks, the double-quartz-disks resulted in the smallest increase in $\Delta|Z|_{\min}$ for the MDCK-I-AQP1 cells in presence of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (Figure 8.5 F). Maxima of $\Delta|Z|_{\min}$ seemed to be reached earliest for MDCK-I-AQP1 cells (Figure 8.5 C and D), which would fit the expectation of AQP1 accelerating water flux, but this was evaluated in detail further down by the time constant. After reaching maxima of $\Delta|Z|_{\min}$, the presented hypertonic stimulations, especially the 300 mM and 400 mM sucrose solutions, led to a signal regeneration (Figure 8.5, purple and red curves) indicating an RVI process, which was already observed similarly for MDCK-II cells (see Figure 8.1 A and B).

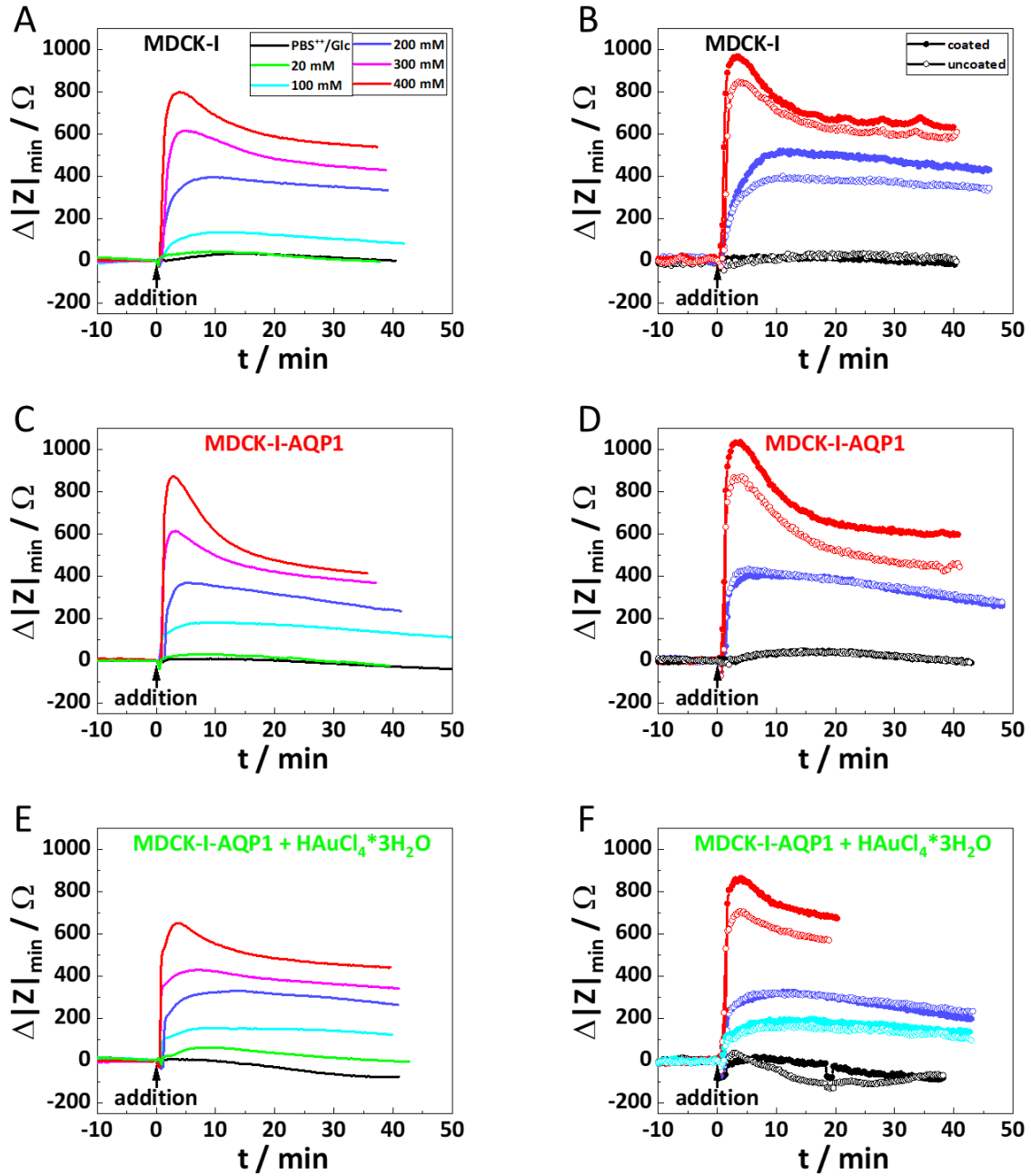


Figure 8.5 The change of the minimal impedance $\Delta|Z|_{min}$ during QCM measurements (fast-mode) analyzing the differences in water flux in MDCK-I cells (**A, B**), MDCK-I-AQP1 cells (**C, D**), and MDCK-I-AQP1 cells with 50 μM of the AQP inhibitor $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (**E, F**). Additional to normal quartz disks (**A, C, E**), double-quartz-disks were used (**B, D, F**), providing a coated ($\bullet$) and uncoated side ($\circ$). The water flux was triggered by hypertonic solutions of varying osmolality: 20 mM (green), 100 mM (cyan), 200 mM (blue), 300 mM (purple), and 400 mM (red) in comparison to the isotonic $\text{PBS}^{++}/\text{Glc}$ solution (black) (osmolalities see Table 8.1). All solutions were added at time point 0 min and data were normalized to that time point. $T = 37^\circ\text{C}$.

For a more detailed analysis of these water flux experiments, both the measurements with normal quartz disks (Figure 8.5 A, C, and E) and double-quartz-disks (Figure 8.5 B, D, and F) were evaluated quantitatively by determination of several parameters plotted in Figure 8.6 and Figure 8.7, respectively. The parameters $\text{Max}(\Delta|Z|_{min})$ (Figure 8.6 A and Figure 8.7 A),

$|\tau|$ (Figure 8.6 B and Figure 8.7 B), the area under the curve (Figure 8.6 C and Figure 8.7 C), and $|\tau_{reg}|$ (Figure 8.6 D and Figure 8.7 D) were plotted versus the osmolality difference $\Delta\pi$. This facilitated an easy comparison of MDCK-I cells (Figure 8.6 and Figure 8.7, black curves), MDCK-I-AQP1 cells (Figure 8.6 and Figure 8.7, red curves), and MDCK-I-AQP1 cells in presence of 50 μM $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (Figure 8.6 and Figure 8.7, green curves). Determination of the area was performed by integration from 0 min to 20 min and down to a value of 0 Ω . For normal quartz disks, $|\tau_{reg}|$ was determined for the 300 mM and 400 mM sucrose solutions, whereas for double-quartz-disks, this was only possible for the 400 mM sucrose solution. Since the time courses of $\Delta|Z|_{min}$ for the coated and uncoated side were not significantly different, the parameters were averaged over these two individual cell responses for double-quartz-disks.

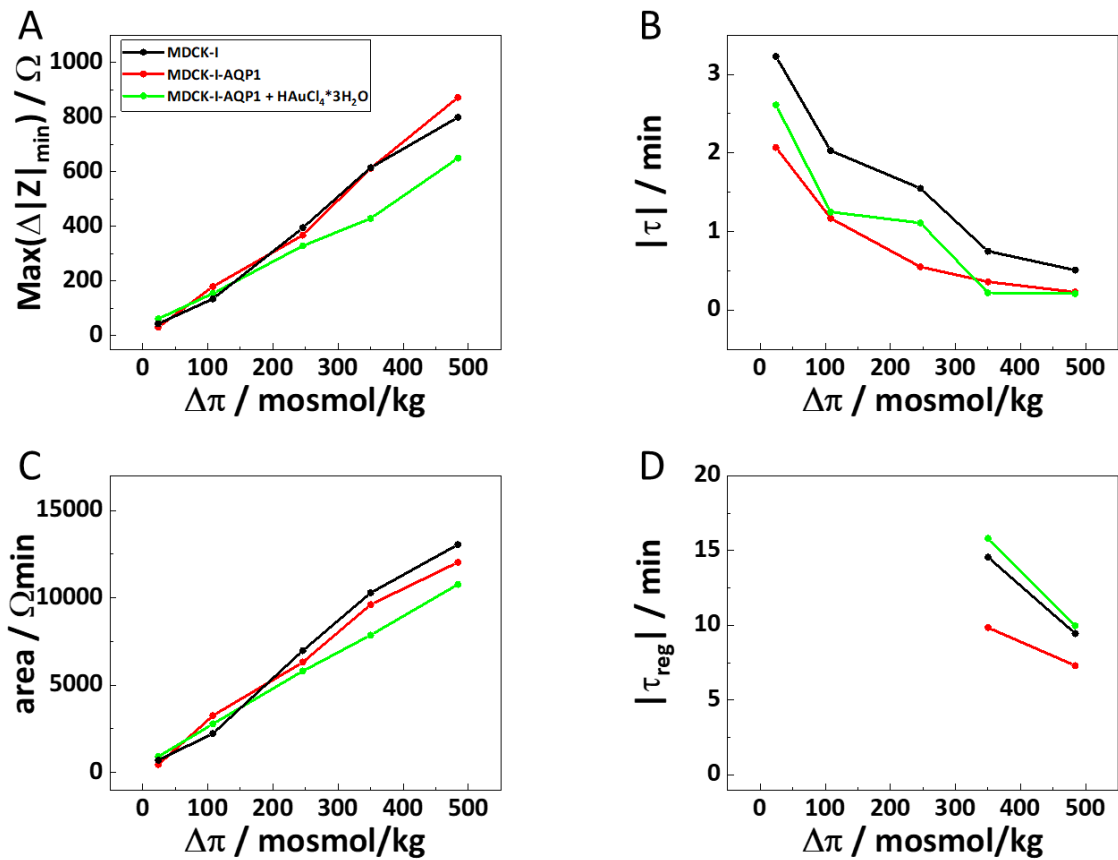


Figure 8.6 Quantitative evaluation of the QCM-based water flux experiments (fast-mode) with **normal quartz disks** demonstrated in Figure 8.5 A, C, and E, in terms of the parameters maximum of the change of the minimal impedance $\text{Max}(\Delta|Z|_{min})$ (A), magnitude of the time constant $|\tau|$ (B), area under the curve (C), and magnitude of the time constant of regeneration $|\tau_{reg}|$ (D) plotted versus the osmolality difference $\Delta\pi$ of the hypertonic solutions. MDCK-I cells (black) were compared to MDCK-I-AQP1 cells (red) and MDCK-I-AQP1 cells with 50 μM of the AQP inhibitor $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (green). For determination of the area, the integration was performed between 0 min and 20 min and down to a value of 0 Ω . $|\tau_{reg}|$ was only determined for the 300 mM and 400 mM sucrose solutions.

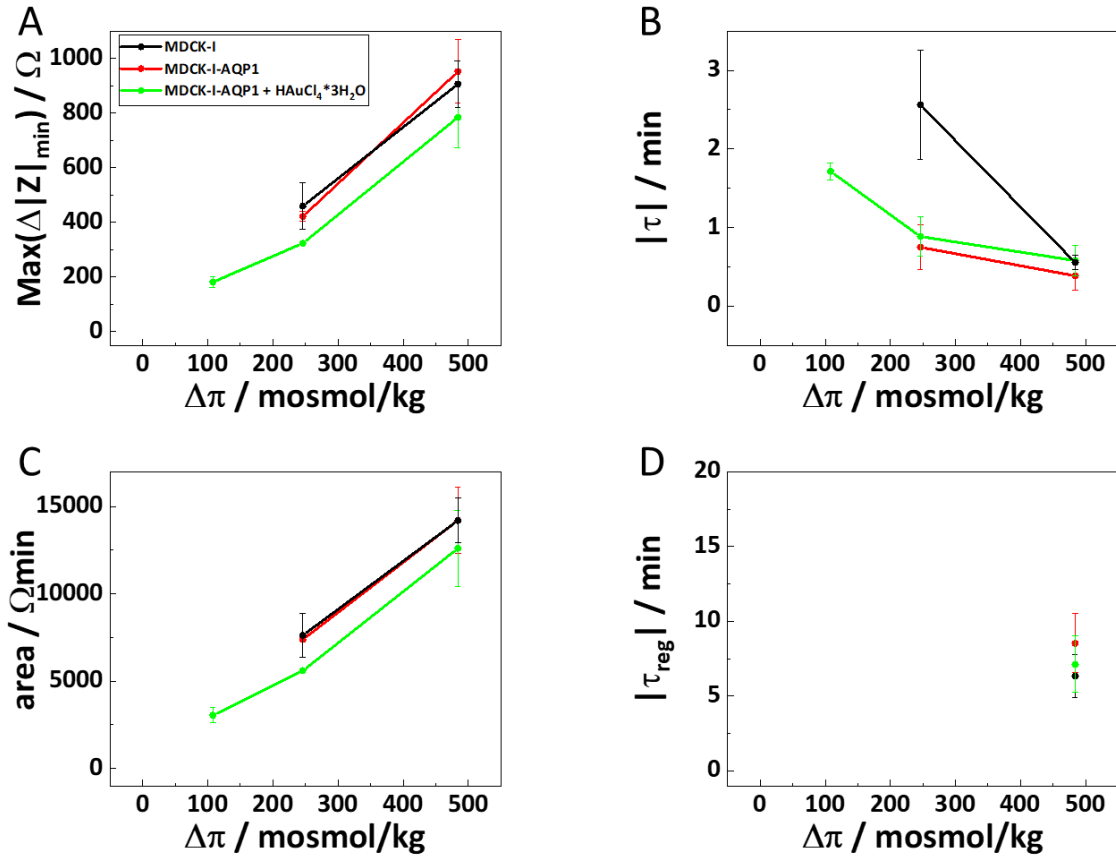


Figure 8.7 Quantitative evaluation of the QCM-based water flux experiments (fast-mode) with **double-quartz-disks** demonstrated in Figure 8.5 B, D, and F, in terms of the parameters maximum of the change of the minimal impedance $\text{Max}(\Delta|Z|_{\min})$ (A), magnitude of the time constant $|\tau|$ (B), area under the curve (C), and magnitude of the time constant of regeneration $|\tau_{\text{reg}}|$ (D) plotted versus the osmolality difference $\Delta\pi$ of the hypertonic solutions. MDCK-I cells (black) were compared to MDCK-I-AQP1 cells (red) and MDCK-I-AQP1 cells with 50 μM of the AQP inhibitor $\text{HgCl}_2 \cdot 3\text{H}_2\text{O}$ (green). For determination of the area, the integration was performed between 0 min and 20 min and down to a value of 0 Ω . $|\tau_{\text{reg}}|$ was only determined for the 400 mM sucrose solution. The data are displayed as mean $\pm$ SD out of the two individual cell responses from the coated and uncoated sides of the quartz disks.

As already obtained for MDCK-II cells in Figure 8.2 A and C, $\text{Max}(\Delta|Z|_{\min})$ and the area correlated almost linearly with the osmolality difference $\Delta\pi$ for all three investigated cases (MDCK-I, MDCK-I-AQP1, and MDCK-I-AQP1 + $\text{HgCl}_2 \cdot 3\text{H}_2\text{O}$), as can especially be observed for the normal quartz disks (Figure 8.6 A and C), but was also indicated by double-quartz-disks (Figure 8.7 A and C). The time constants $|\tau|$ and $|\tau_{\text{reg}}|$ were smaller for higher osmolality difference $\Delta\pi$ (Figure 8.6 B and D and Figure 8.7 B), with the exception of $|\tau_{\text{reg}}|$ for double-quartz-disks, where no statement was possible about the dependency on $\Delta\pi$ since there were only data points for the 400 mM sucrose solution (Figure 8.7 D). Some kind of indirect proportionality between $|\tau|$ and $\Delta\pi$ was observed for normal quartz disks (Figure 8.6 B), which was also very similar to MDCK-II cells (see Figure 8.2 B), whereas the number of data points for $|\tau_{\text{reg}}|$ for normal quartz disks (Figure 8.6 D) and for $|\tau|$ for double-quartz-disks (Figure 8.7 B) was too small for a similar statement. When the influence of AQP1 was

examined, MDCK-I cells and MDCK-I-AQP1 cells were observed to exhibit very similar values of $Max(\Delta|Z|_{min})$ and the area, which was obtained for both normal quartz disks (Figure 8.6 A and C, black and red curves) and double-quartz-disks (Figure 8.7 A and C, black and red curves). Smaller values of $|\tau|$ were observed for MDCK-I-AQP1 cells in comparison to MDCK-I cells, as obtained for normal quartz disks (Figure 8.6 B, black and red curves) and double-quartz-disks (Figure 8.7 B, black and red curves), proving the expected acceleration of water flux by the presence of AQP1. Whereas no significant differences between the three cases were observed for $|\tau_{reg}|$ for double-quartz-disks (Figure 8.7 D), $|\tau_{reg}|$ for normal quartz disks was observed to be smaller for MDCK-I-AQP1 cells in comparison to MDCK-I cells (Figure 8.6 D, black and red curves), showing that AQP1 also accelerates the water flux during regeneration. Since AQPs are also involved in RVC processes, this is a first hint that the observed regeneration after hypertonic stimulations is maybe an RVI process. Investigating the influence of the AQP inhibitor $HAuCl_4 \cdot 3H_2O$ by comparing MDCK-I-AQP1 cells in presence of $HAuCl_4 \cdot 3H_2O$ (Figure 8.6 and Figure 8.7, green curves) with the other two cases revealed results for $Max(\Delta|Z|_{min})$ that were already indicated in Figure 8.5. Smaller values of $Max(\Delta|Z|_{min})$ for the 300 mM and 400 mM sucrose solutions for normal quartz disks (Figure 8.6 A) and also smaller values of $Max(\Delta|Z|_{min})$ for double-quartz-disks (Figure 8.7 A) were observed when both AQP1 and $HAuCl_4 \cdot 3H_2O$ were present. The results for the area were very similar to that (Figure 8.6 C and Figure 8.7 C). This indicated that the speed of water flux may be somehow correlated with the total amount of water flux. A possible explanation could be that for low water flux rates, the starting regeneration process partially compensates the total amount of water flux. The values of the time constant $|\tau|$ for MDCK-I-AQP1 cells in presence of $HAuCl_4 \cdot 3H_2O$ were observed to be located between the other two cases for normal quartz disks (Figure 8.6 B), which was also obtained but less significantly for double-quartz-disks (Figure 8.7 B), indicating that the speed of water flux was reduced by $HAuCl_4 \cdot 3H_2O$ but not completely back to the original speed without AQP1. The speed of regeneration seemed to be completely reset to the original speed without AQP1 since the values of $|\tau_{reg}|$ for MDCK-I-AQP1 cells in presence of $HAuCl_4 \cdot 3H_2O$ were close to or even slightly higher than those of MDCK-I cells (Figure 8.6 D). This also indicated that an RVI process is possibly responsible for the observed signal regeneration. A more detailed statement, if RVC processes are mainly responsible for these signal regenerations, would require a more sophisticated analysis by using specific inhibitors, as it will be performed for HTM-N cells in section 8.2.3.

Taken together, expectations were met since the total amount of water flux was mainly unaffected by AQP1 and $HAuCl_4 \cdot 3H_2O$, whereas a successful acceleration of water flux by AQP1 and partial inhibition of this acceleration by $HAuCl_4 \cdot 3H_2O$ was demonstrated here. But repetitive future studies have to be made to verify these results and to check their reproducibility. Double-quartz-disks were presented as a promising alternative to normal quartz disks because they gave very similar results, although they were more delicate and more prone to errors. Additionally, double-quartz-disks already provide some kind of replicates by the two parallelly recorded signals (coated and uncoated), which, however,

does not enhance the throughput since only one chamber with a double-quartz-disk can be measured at the same time when two chambers with normal quartz disks are measured. But the advantage of double-quartz-disks is facilitated by averaging over two responses of the same cell layer, and an additional ECIS measurement would be possible, as has been demonstrated before (Oberleitner, 2018).

8.2.2 Basic Water Flux Experiments with HTM-N Cells

After water flux experiments were successfully performed for MDCK cells (MDCK-II in section 8.1.1, MDCK-I in section 8.2.1), the water flux was also examined in HTM-N cells, which were already used before for the investigation of glaucoma-related stiffness changes (see sections 6 and 7). Although slightly downregulated in comparison to primary HTM cells, AQP1 is also expressed in HTM-N cells (Liton *et al.*, 2006), which is expected to facilitate a fast water flux. Experiments were performed equally to those demonstrated before for MDCK cells, while the water flux was triggered by hypertonic and hypotonic sucrose solutions. A new addition protocol was established, named serial addition, during which the osmolality was stepwise increased/decreased by consecutive additions to the same cell layer. This protocol was compared to the conventional individual addition, where each cell layer was stimulated only once. The successful application of the serial addition protocol with its advantage of enhancing throughput was already reported for hypertonic stimulations of MDCK-I cells (Wegener *et al.*, 2000c). The results of the water flux experiments on HTM-N cells are displayed in Figure 8.8, while individual hypertonic additions (Figure 8.8 A), serial hypertonic additions (Figure 8.8 B), and serial hypotonic additions (Figure 8.8 C) were performed. Hypertonic conditions with varying osmolality were induced by the same sucrose solutions as already used before: 20 mM (Figure 8.8 A, green curve), 100 mM (Figure 8.8 A, cyan curve), 200 mM (Figure 8.8 A, blue curve), 300 mM (Figure 8.8 A, purple curve), and 400 mM (Figure 8.8 A, red curve) in comparison to an addition of the isotonic PBS⁺⁺/Glc solution (Figure 8.8 A, black curve). For individual hypertonic additions, an 800 mM sucrose solution was used additionally (Figure 8.8 A, orange curve). For serial hypertonic additions, the osmolality of the medium surrounding the cells was stepwise increased by consecutive additions of these sucrose solutions (without 800 mM) in time intervals of 20 min (Figure 8.8 B). Serial hypotonic additions were conducted by addition of hypo 2 and hypo 1 solutions in a time interval of 20 min (Figure 8.8 C). The osmolalities of all these solutions are presented in Table 8.1. The water flux was monitored by the change of the minimal impedance $\Delta|Z|_{min}$, normalized to the time point of the first addition (0 min). Averaging was performed over several replicates (QCM chambers), while serial additions ($n = 4 - 6(1)$) allowed a higher number of replicates than individual additions ($n = 2(2)$).

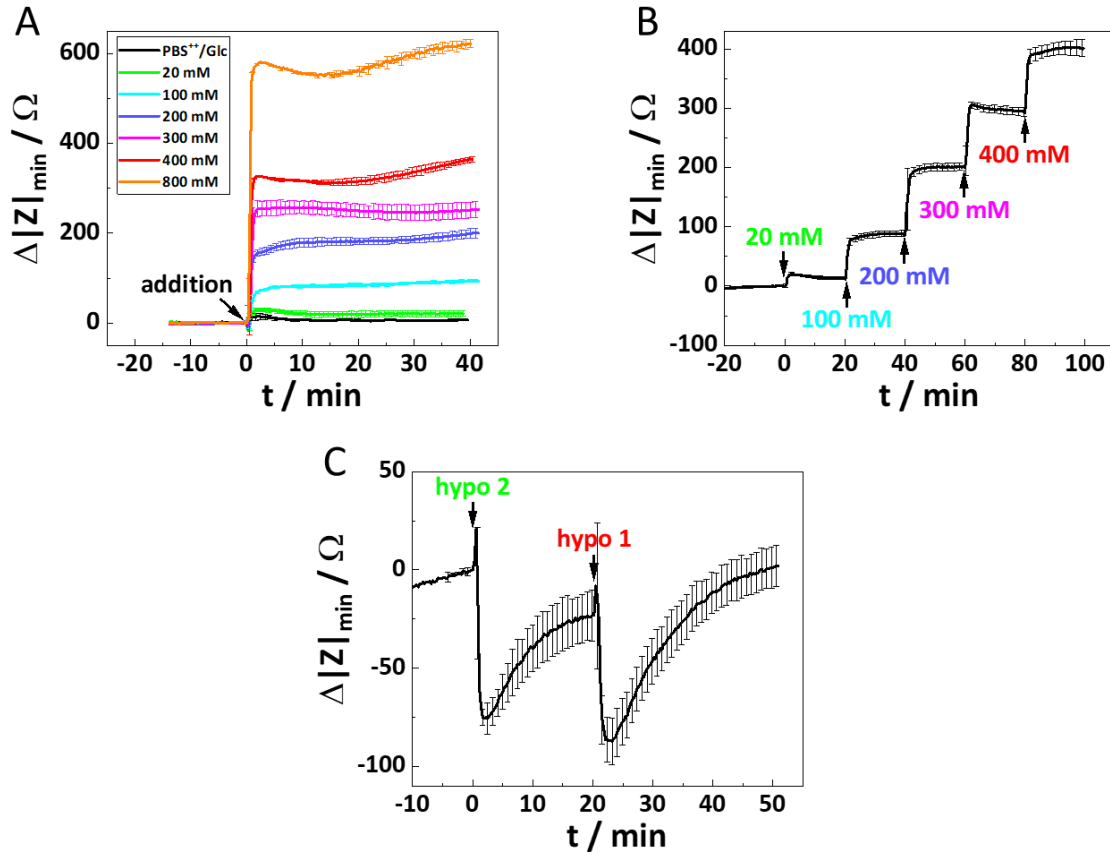


Figure 8.8 The change of the minimal impedance $\Delta|Z|_{\min}$ during QCM measurements (fast-mode) analyzing water flux in HTM-N cells. **(A)** The water flux was triggered hypertonically by individual additions at time point 0 min using different sucrose solutions of varying osmolality: 20 mM (green), 100 mM (cyan), 200 mM (blue), 300 mM (purple), 400 mM (red), and 800 mM (orange) in comparison to the isotonic PBS⁺⁺/Glc solution (black) (osmolalities see Table 8.1). **(B)** The water flux was triggered hypertonically by serial additions, where the same sucrose solutions (but without the 800 mM) were added consecutively to one cell layer in time intervals of 20 min. **(C)** The water flux was triggered hypotonically by serial additions of hypo 2 and hypo 1 solutions in a time interval of 20 min (osmolalities see Table 8.1). Data were normalized to the time point of the first addition (0 min) and are displayed as mean $\pm$ SD over several replicates (A: $n = 2(2)$, B: $n = 6(1)$, C: $n = 4(1)$). $T = 37^\circ\text{C}$.

As expected, the control stimulation of HTM-N cells with the PBS⁺⁺/Glc solution led to no significant change of $\Delta|Z|_{\min}$ (Figure 8.8 A, black curve). As already obtained for MDCK-II cells in Figure 8.1 and MDCK-I cells in Figure 8.5, the minimal impedance was observed to increase upon hypertonic additions, which was strongly dependent on the osmolality (Figure 8.8 A). The stepwise osmolality increase by serial additions of hypertonic solutions induced a stepwise increase in $\Delta|Z|_{\min}$ (Figure 8.8 B), which showed time courses that were similar to those published before for MDCK-I cells (Wegener *et al.*, 2000c), although the resistance of the load R_L was monitored there. The serial addition protocol was also successfully applied to hypotonic stimulations. A stepwise decrease in the minimal impedance was observed after consecutive additions of the hypo 2 and hypo 1 solutions, where the solution with the bigger osmolality difference (hypo 1) induced the smaller minimum (Figure 8.8 C). Thus, the

cell response was also osmolality-dependent. But the time courses of $\Delta|Z|_{min}$ upon hypertonic and hypotonic stimulations of HTM-N cells were different to those obtained for MDCK cells. After reaching minima of $\Delta|Z|_{min}$ for hypotonic stimulations, an almost full signal regeneration was observed for HTM-N cells (Figure 8.8 C), whereas $\Delta|Z|_{min}$ reached a rather constant level without regeneration for hypertonic stimulations (Figure 8.8 A). This was exactly reversed for MDCK cells, where $\Delta|Z|_{min}$ partly regenerated after hypertonic (see Figure 8.1 A and B and Figure 8.5), but not after hypotonic stimulations (see Figure 8.1 D). It is discussed in detail in section 8.2.3 if RVC processes are mainly responsible for these signal regenerations. According to these observations, performing serial additions for hypertonic stimulations was justified because the minimal impedance did not significantly change directly before the additions (Figure 8.8 B). This was a bit different for hypotonic stimulations because $\Delta|Z|_{min}$ was still in a recovery phase when the hypo 1 solution was added (Figure 8.8 C). Nevertheless, this was not problematic since the determination of $|\tau_{reg}|$ was still possible for the hypo 2 solution. However, especially the stimulations with the 400 mM and 800 mM sucrose solutions indicated a long-term signal change, which could not be recorded completely within the 40 min of measurement time (Figure 8.8 A, red and orange curves). Thus, all further experiments were performed with an extended measurement time to detect a possible regeneration process for hypertonic stimulations of HTM-N cells. While the minimal impedance exhibited a standard deviation, that was still quite good for hypotonic stimulations (Figure 8.8 C), the extremely small error bars for hypertonic stimulations for both individual additions (Figure 8.8 A) and serial additions (Figure 8.8 B) indicated a very good reproducibility of these experiments. This was especially true for the serial additions, where six experimental replicates were conducted. The quantitative evaluation of these water flux experiments was performed by determination of the parameters $Max/Min(\Delta|Z|_{min})$ (Figure 8.9 A), $|\tau|$ (Figure 8.9 B), the area under the curve (Figure 8.9 C), and $|\tau_{reg}|$ (Figure 8.9 D), all of them plotted versus the osmolality difference $\Delta\pi$. Individual hypertonic stimulations (Figure 8.9, black curves), serial hypertonic stimulations (Figure 8.9, red curves), and serial hypotonic stimulations (Figure 8.9, green curves) were compared. The determination of the area was only performed for individual additions and by integration from 0 min to 20 min and down to a value of 0 Ω . Since a regeneration was not observed for hypertonic stimulations, $|\tau_{reg}|$ was only determined for hypotonic stimulations.

As already obtained before for MDCK-II cells (see Figure 8.2 A and C) and MDCK-I cells (see Figure 8.6 A and C), approximately linear correlations were observed for HTM-N cells between $Max/Min(\Delta|Z|_{min})$ and $\Delta\pi$ (Figure 8.9 A) and between the area and $\Delta\pi$ (Figure 8.9 C). A comparison between the individual and serial addition protocol of hypertonic solutions yielded similar, but even slightly higher values of $Max(\Delta|Z|_{min})$ for the serial addition of the 300 mM and 400 mM sucrose solutions (Figure 8.9 A, black and red curves). This indicated that a hyposensibilization of the cells, typically obtained for serial additions of drugs (Stolwijk *et al.*, 2019), did not take place for these water flux experiments.

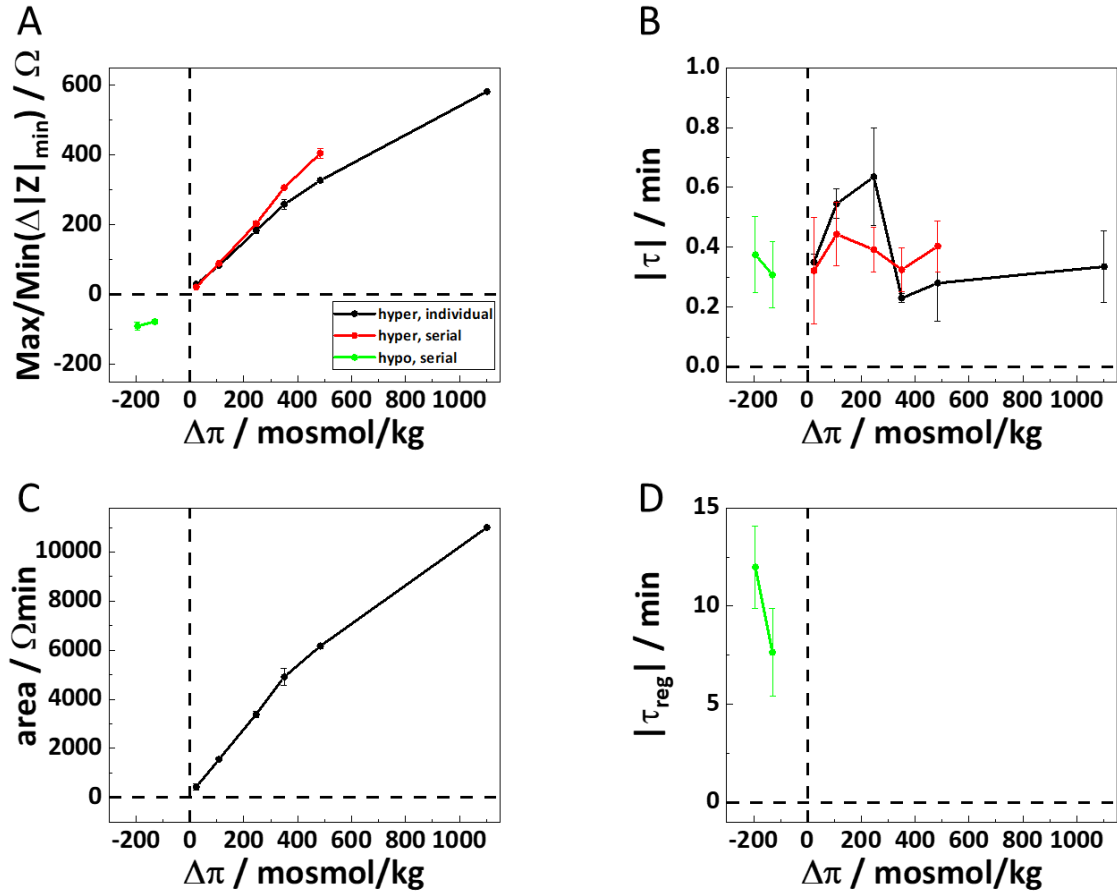


Figure 8.9 Quantitative evaluation of the QCM-based water flux experiments (fast-mode) on HTM-N cells demonstrated in Figure 8.8, in terms of the parameters maximum/minimum of the change of the minimal impedance $\text{Max/Min}(\Delta|Z|_{\min})$ (A), magnitude of the time constant $|\tau|$ (B), area under the curve (C), and magnitude of the time constant of regeneration $|\tau_{\text{reg}}|$ (D) plotted versus the osmolality difference $\Delta\pi$ of the hypertonic and hypotonic solutions. Individual hypertonic stimulations (black), serial hypertonic stimulations (red), and serial hypotonic stimulations (green) were compared. The area was only determined for individual additions, while the integration was performed between 0 min and 20 min and down to a value of 0 Ω . $|\tau_{\text{reg}}|$ could only be determined for hypotonic stimulations. Data are displayed as mean $\pm$ SD with a varying number of replicates, depending on the experiment the parameters were extracted from (A and B: $n = 2 - 6(1 - 2)$, C: $n = 2(2)$, D: $n = 4(1)$).

When these results of $\text{Max}(\Delta|Z|_{\min})$ for hypertonic stimulations of HTM-N cells are compared to those obtained for MDCK-II cells and MDCK-I cells, significantly lower values were observed here for HTM-N cells. For example, the 400 mM sucrose solution induced a $\text{Max}(\Delta|Z|_{\min})$ of about 330 Ω in individual stimulations (Figure 8.9 A, black curve) and about 400 Ω in serial stimulations (Figure 8.9 A, red curve) of HTM-N cells, whereas the same solution induced a $\text{Max}(\Delta|Z|_{\min})$ of about 730 Ω for MDCK-II cells (see Figure 8.2 A, black curve) and about 800 Ω for MDCK-I cells (see Figure 8.6 A, black curve). It is not possible that these huge differences in the cell responses to the treatment with hypertonic solutions correlate with the presence of AQP1 since it was demonstrated in the previous section (section 8.2.1) that the total amount of water flux was mainly unaffected by AQP1. Instead,

these differences were assumed to result from the different types of cell lines influencing the total amount of water flux. MDCK cells (type I and II) are kidney epithelial cells with strong barrier function, which leads to the possibility that a water flux across the basal membrane, associated with an alteration of the cell-substrate distance, can contribute to the QCM signal since this was already demonstrated before for MDCK-I cells (Wegener *et al.*, 2000c). This was obviously observed in this thesis for MDCK-II cells and MDCK-I cells with a significant contribution to the overall QCM response, leading to these higher values of $Max(\Delta|Z|_{min})$ in comparison to those of HTM-N cells. HTM-N cells exhibit a very weak barrier function enabling an easy diffusion of sucrose through the paracellular clefts. Thus, the water flux only occurred across the apical membrane without additional alteration of the cell-substrate distance. The values of $Min(\Delta|Z|_{min})$ after hypotonic stimulations observed here for HTM-N cells (Figure 8.9 A, green curve) were very similar to those obtained for MDCK-II cells (see Figure 8.2 A, green curve), indicating that hypotonic stimulations did not significantly influence the liquid compartment beneath the cells and, accordingly, the cell-substrate distance. The time constant $|\tau|$ did not show an osmolality-dependent trend with unexpectedly big error bars and values fluctuating between 0.2 min and 0.6 min for all three investigated cases (Figure 8.9 B). Compared to that, in MDCK cells (type I and II), where an indirect proportionality between $|\tau|$ and $\Delta\pi$ was observed, only the 300 mM and 400 mM sucrose solutions induced similarly small values of $|\tau|$ (see Figure 8.2 B, black curve, and Figure 8.6 B, black curve), proving a very fast water flux in HTM-N cells for each solution added. This fitted to the fact that HTM-N cells express AQP1 facilitating such a fast water flux, that it was unaffected by the osmolality. The time constant of regeneration $|\tau_{reg}|$ after hypotonic stimulations exhibited a smaller value for a smaller osmolality difference $\Delta\pi$, which was, however, not significant due to relatively big error bars (Figure 8.9 D, green curve). Nevertheless, the values were in the same order of magnitude as obtained for hypertonic stimulations of MDCK cells, but with a contrary osmolality dependency (see Figure 8.2 D, black curve, and Figure 8.6 D, black curve). The reason for this was maybe the serial addition, where the hypo 1 solution was added during the regeneration phase after stimulation with the hypo 2 solution (Figure 8.8 C), leading to an interrupted regeneration and distorted results of $|\tau_{reg}|$ for the hypo 2 solution. This also showed that the serial addition, although it can significantly enhance the throughput, as demonstrated by Figure 8.8 B, has to be applied with care. Serial additions with short time intervals should only be applied if the cells do not undergo regeneration processes. But if a regeneration occurs, this should be completed before the next addition, which, when the regeneration takes too long, maybe even cancels the advantage of the serial addition and the individual addition becomes the preferred addition protocol because an untreated cell layer is used there for each addition. Disregarding the addition protocol applied, osmotically (hypertonic and hypotonic) triggered water flux was successfully demonstrated in HTM-N cells, which was occurring very fast, most probably due to the presence of AQP1.

8.2.3 Analyzing the Inhibition of Certain Aspects of Water Flux

Since evidence was found in the previous section (section 8.2.2) for the presence of AQP1 in HTM-N cells due to a very fast transmembrane water flux, and the AQP inhibitor $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was demonstrated to decrease the speed of water flux (Niemietz & Tyerman, 2002), which was confirmed for MDCK-I cells in section 8.2.1, $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was also investigated for a potential AQP1 inhibition in HTM-N cells. Additionally, the regeneration processes observed after initial water flux were investigated in detail by studying the influence of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ and further more selective ion channel inhibitors. Bumetanide, which was identified as an effective $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport inhibitor able to inhibit RVI processes (O'Donnell *et al.*, 1995), and Et_4NCl , which is known as a K^+ channel inhibitor and was reported to inhibit RVD processes (Mitchell *et al.*, 2002), were examined for their influence on the regeneration processes to resolve a potential contribution of RVC processes. Beyond that, Et_4NCl was also examined for a potential AQP1 inhibition, since this has been controversially discussed in literature (Detmers *et al.*, 2006; Müller *et al.*, 2008; Yang *et al.*, 2006; Søggaard & Zeuthen, 2008). Thus, QCM-based water flux experiments in presence of these inhibitors are presented in Figure 8.10. One hypertonic stimulation (200 mM, Figure 8.10 A) and one hypotonic stimulation (hypo 1, Figure 8.10 B) were investigated (osmolalities see Table 8.1). These stimulations in presence of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (Figure 8.10, green curves), bumetanide (Figure 8.10, red curves), and Et_4NCl (Figure 8.10, blue curves) were compared to control conditions without inhibitor (Figure 8.10, black curves), while their standard concentrations were used as selected in section 8.1.2 (50 μM $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 10 μM bumetanide, 7.5 mM Et_4NCl). It was already demonstrated in previous sections that these water flux inhibitors exhibit no significant influence on cytotoxicity (see section 8.1.2) or osmolality (see section 8.1.3). Since especially the regeneration processes were examined, additions were only conducted by the individual addition protocol. The water flux was monitored by the change of the minimal impedance $\Delta|Z|_{\min}$, normalized to the time point of addition (0 min). Averaging was performed over several experimental and biological replicates.

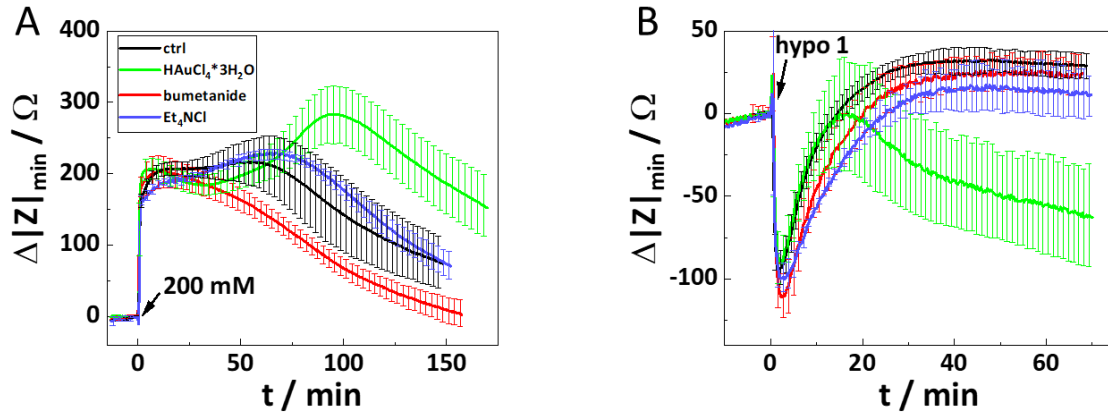


Figure 8.10 The change of the minimal impedance $\Delta|Z|_{\min}$ during QCM measurements (fast-mode) analyzing the inhibition of water flux in HTM-N cells. The water flux was triggered hypertonically by addition of the 200 mM sucrose solution (A) or hypotonically by addition of the hypo 1 solution (B), while the presence of different inhibitors was tested: 50 μM $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (green), 10 μM bumetanide (red), and 7.5 mM Et_4NCl (blue) in comparison to control conditions without inhibitor (black). All solutions were added at time point 0 min and data were normalized to that time point and are displayed as mean $\pm$ SD out of several experimental and biological replicates (A: $n = 4 - 6(1 - 3)$, B: $n = 4 - 5(1 - 4)$). $T = 37^\circ\text{C}$.

When the time courses of $\Delta|Z|_{\min}$ for stimulations of water flux without inhibitor (Figure 8.10, black curves) are compared to the basic water flux experiments in Figure 8.8, hypotonic stimulations were observed to be almost identical with their minimum after about 2 min and very fast regeneration (Figure 8.10 B, black curve, and Figure 8.8 C), whereas a crucial difference was obtained for hypertonic stimulations (Figure 8.10 A, black curve, and Figure 8.8 A, blue curve). As it was already indicated by the higher osmolar sucrose solutions in Figure 8.8 A, an extended measurement time led to a further signal change. This was expressed here for the 200 mM sucrose solution by some kind of two-step-mechanism, with a second maximum of $\Delta|Z|_{\min}$ at about 60 min after the first maximum at about 10 min, followed by a regeneration process. Although HTM-N cells were now observed to perform a regeneration also after hypertonic stimulations, compared to the very fast regeneration after hypotonic stimulations this was very delayed and slow, as will be confirmed by determination of $|\tau_{\text{reg}}|$ further down in this section. The presence of inhibitors appeared to have no significant influence on the (first) maximum after hypertonic stimulation (Figure 8.10 A) and on the minimum after hypotonic stimulation (Figure 8.10 B), neither on the time point nor on the minimal impedance value. The inhibitors' influence on the speed of water flux will be examined by determination of $|\tau|$ further down in this section. Whereas the time course of $\Delta|Z|_{\min}$ after hypertonic stimulation was relatively unaffected by Et_4NCl (Figure 8.10 A, blue curve), the other two inhibitors showed a significant influence. The presence of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ led to an even more delayed regeneration with a more pronounced two-step-mechanism (Figure 8.10 A, green curve), whereas bumetanide led to an earlier regeneration (Figure 8.10 A, red curve). After hypotonic stimulation, slightly delayed regenerations (but not significantly) were observed for bumetanide (Figure 8.10 B, red curve) and Et_4NCl (Figure

8.10 B, blue curve), whereas the presence of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ led to a significant inhibition of the regeneration, where the minimal impedance started again to decrease (Figure 8.10 B, green curve). These experiments were also evaluated quantitatively by determination of the parameters $\text{Max}(\Delta|Z|_{\min})$ (for hypertonic) (Figure 8.11 A), $\text{Min}(\Delta|Z|_{\min})$ (for hypotonic) (Figure 8.12 A), $|\tau|$ (Figure 8.11 B and Figure 8.12 B), the area under the curve (Figure 8.11 C and Figure 8.12 C), and $|\tau_{\text{reg}}|$ (Figure 8.11 D and Figure 8.12 D), all of them plotted versus the inhibitor applied. The parameter $\text{Max}(\Delta|Z|_{\min})$ was taken from the local maximum at about 10 min to describe the total amount of initial water flux. To cover the total regeneration processes by the area, the integration was performed from 0 min to 150 min and down to a value of -15Ω for hypertonic stimulations and from 0 min to 60 min and up to a value of 45Ω for hypotonic stimulations.

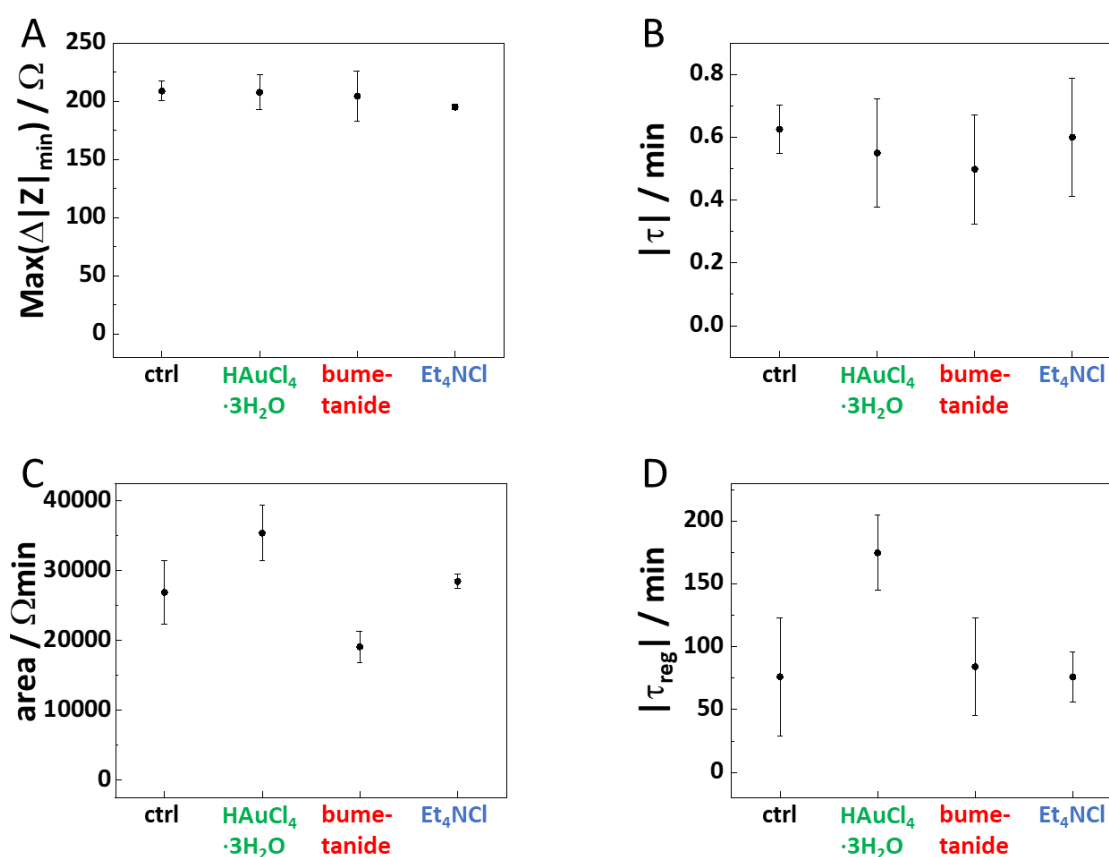


Figure 8.11 Quantitative evaluation of the QCM-based water flux experiments (fast-mode) on **hypertonic** stimulations of HTM-N cells demonstrated in Figure 8.10 A, in terms of the parameters maximum of the change of the minimal impedance $\text{Max}(\Delta|Z|_{\min})$ (A), magnitude of the time constant $|\tau|$ (B), area under the curve (C), and magnitude of the time constant of regeneration $|\tau_{\text{reg}}|$ (D) depending on the inhibitor applied. Inhibitors were $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (50 μM), bumetanide (10 μM), and Et_4NCl (7.5 mM), which were compared to control conditions without inhibitor. $\text{Max}(\Delta|Z|_{\min})$ was extracted from the local maximum after initial water flux at about 10 min. For determination of the area, the integration was performed between 0 min and 150 min and down to a value of -15Ω . Data are displayed as mean $\pm$ SD with the same number of replicates as for the respective QCM measurements ($n = 4 - 6(1 - 3)$).

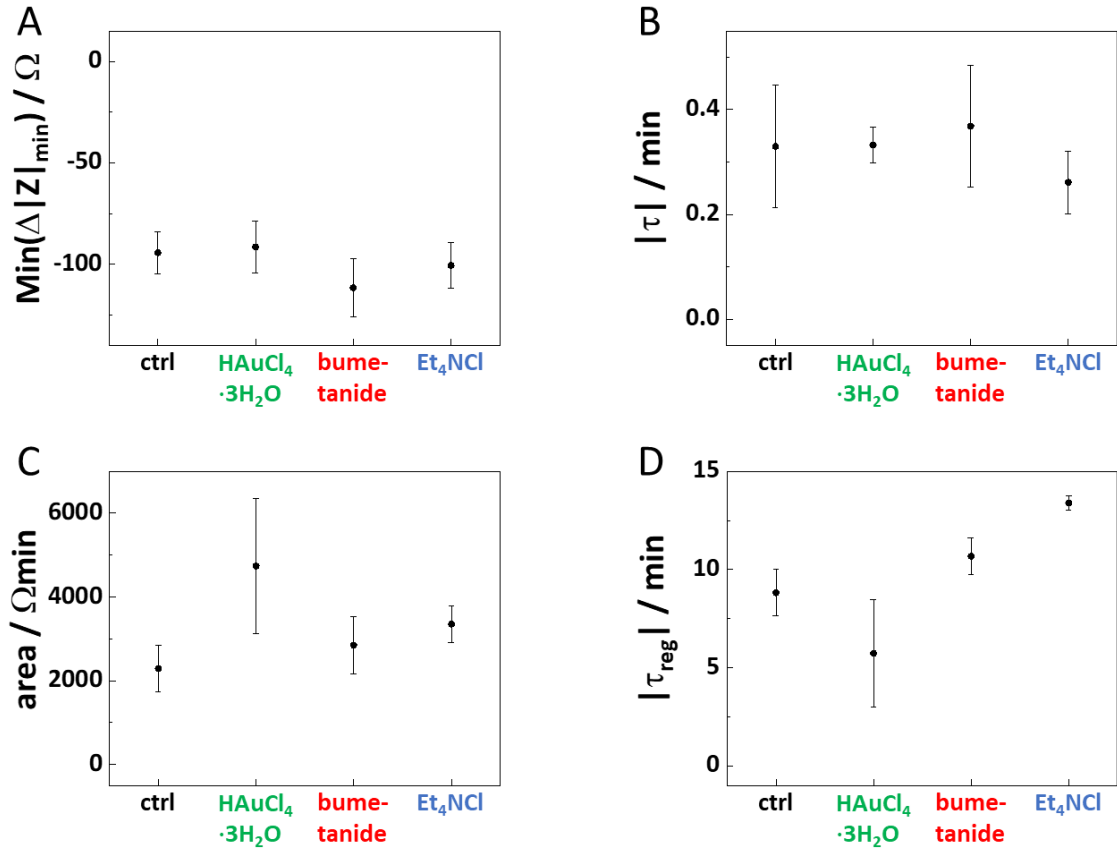


Figure 8.12 Quantitative evaluation of the QCM-based water flux experiments (fast-mode) on **hypotonic** stimulations of HTM-N cells demonstrated in Figure 8.10 B, in terms of the parameters minimum of the change of the minimal impedance $Min(\Delta|Z|_{min})$ (A), magnitude of the time constant $|\tau|$ (B), area under the curve (C), and magnitude of the time constant of regeneration $|\tau_{reg}|$ (D) depending on the inhibitor applied. Inhibitors were $HgAuCl_4 \cdot 3H_2O$ (50 μ M), bumetanide (10 μ M), and Et_4NCl (7.5 mM), which were compared to control conditions without inhibitor. For determination of the area, the integration was performed between 0 min and 60 min and up to a value of 45 Ω . Data are displayed as mean $\pm$ SD with the same number of replicates as for the respective QCM measurements ($n = 4 - 5(1 - 4)$).

Many of the error bars in Figure 8.11 and Figure 8.12 were relatively big, demonstrating a high standard deviation, especially for $|\tau|$ and $|\tau_{reg}|$, which demonstrated that the reproducibility within these experiments was not perfect. But the reproducibility between these experiments without inhibitor and those on HTM-N cells demonstrated in the previous section (section 8.2.2) was excellent since the $Max(\Delta|Z|_{min})$ of about 200 Ω (Figure 8.11 A) and $|\tau|$ of about 0.6 min (Figure 8.11 B) for the 200 mM sucrose solution without inhibitor and the $Min(\Delta|Z|_{min})$ of about -100Ω (Figure 8.12 A) and $|\tau|$ of about 0.35 min (Figure 8.12 B) for the hypo 1 solution without inhibitor were almost identical to the values presented in Figure 8.9. In comparison to the control without inhibitor, both $Max(\Delta|Z|_{min})$ and $Min(\Delta|Z|_{min})$ were found to be quite unaffected by the inhibitors (Figure 8.11 A and Figure 8.12 A). For $HgAuCl_4 \cdot 3H_2O$, this confirmed the result that the total amount of water flux was mainly unaffected by AQP1, which was already demonstrated for MDCK-I cells (see section 8.2.1). For bumetanide and Et_4NCl , these experiments indicated that the total

amount of water flux was also not influenced by $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporters and K^+ channels, respectively. Analyzing the inhibitors' influence on the time constant $|\tau|$ revealed, with respect to the relatively big error bars, no significant influence for both the hypertonic stimulation (Figure 8.11 B) and the hypotonic stimulation (Figure 8.12 B). Thus, the speed of water flux in HTM-N cells was independent of the presence of any of these inhibitors, which demonstrated for bumetanide and Et_4NCl that $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporters and K^+ channels did not contribute to the speed of water flux. But for the case of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, this was contrary to the results of MDCK-I-AQP1 cells, where the presence of this AQP inhibitor led to an increase in $|\tau|$ and, accordingly, to a reduction of the speed of water flux (see Figure 8.6 B and Figure 8.7 B). This indicated that the initial water flux in HTM-N cells after osmotic stimulations is not only carried out by AQP1. Although $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ is probably not selective for AQP1 (Niemietz & Tyerman, 2002), there might be other AQPs expressed in HTM-N cells, which can not be effectively inhibited by $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$. For example, AQP7 was also found in the trabecular meshwork (Tran *et al.*, 2012), which might ensure a constantly high rate of water flux despite of an AQP1 inhibition. This explanation would also fit to the finding from the previous section (section 8.2.2) that the speed of water flux in HTM-N cells was constantly very high and insensitive to osmolality (see Figure 8.9 B), but of course, has to be verified by further experiments.

Examination of the regeneration processes was performed by comparing the area under the curve and the time constant of regeneration $|\tau_{reg}|$ for the different inhibitors. These two evaluation parameters were, in contrast to $\text{Max/Min}(\Delta|Z|_{min})$ and $|\tau|$, observed to be influenced by the inhibitors (Figure 8.11 and Figure 8.12). The differences in contrast to the control without inhibitor were tested for statistical significance by the Student's *t*-test. First, a threshold of significance had to be selected. This was determined from the data of $\text{Max/Min}(\Delta|Z|_{min})$ and $|\tau|$, which were defined to be not significantly different for any of the inhibitors. From all these data of $\text{Max/Min}(\Delta|Z|_{min})$ and $|\tau|$, the value for $|\tau|$ for hypotonic stimulations in presence of Et_4NCl showed the biggest influence in comparison to the control (Figure 8.12 B). Thus, the Student's *t*-test was applied to this value (in comparison to the control) and the threshold of significance was selected so that this value was still regarded as not significantly different from the control. This resulted in a threshold of significance of 15%, which was used for testing all the values of the area and $|\tau_{reg}|$ for their statistical significance. For the hypertonic stimulation, the area was observed to be not significantly different in presence of Et_4NCl , but significantly higher for $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ and significantly lower for bumetanide (Figure 8.11 C). This confirmed the unmodified regeneration for Et_4NCl , the delayed regeneration for $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ and the earlier regeneration for bumetanide, as it was already indicated by the time courses of $\Delta|Z|_{min}$ in Figure 8.10 A. For the hypotonic stimulation, the area was observed to be slightly increased for bumetanide, a bit more increased for Et_4NCl , and even more increased for $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (Figure 8.12 C), which was determined to be significant in all three cases. These results confirmed the slightly delayed regenerations for bumetanide and Et_4NCl and the disturbed regeneration for $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, as observed in the time courses of $\Delta|Z|_{min}$ in Figure 8.10 B.

The values of $|\tau_{reg}|$ after hypertonic stimulation were very high (75 min – 175 min) (Figure 8.11 D) in comparison to those after hypotonic stimulation (5 min – 13 min) (Figure 8.12 D), confirming the very slow regeneration process of HTM-N cells after hypertonic stimulations. Whereas bumetanide and Et_4NCl did not influence $|\tau_{reg}|$ after hypertonic stimulation, it was significantly increased in presence of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (Figure 8.11 D), which demonstrated, additional to the delayed regeneration, also a slower regeneration. $|\tau_{reg}|$ after hypotonic stimulation was observed to be slightly increased for bumetanide and even more increased for Et_4NCl , whereas it was decreased in presence of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (Figure 8.12 D). All these differences of $|\tau_{reg}|$ after hypotonic stimulation were determined to be significant. But this decrease of $|\tau_{reg}|$ for $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was not meaningful due to the disturbed regeneration observed in the time course of $\Delta|Z|_{min}$ (Figure 8.10 B, green curve). In total, whereas none of the inhibitors had an effect on the total amount and speed of initial water flux, the regeneration processes were influenced by the inhibitors, which is discussed in the following for each individual inhibitor. The AQP inhibitor $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ led to a delayed (area, Figure 8.11 C) and slower ($|\tau_{reg}|$, Figure 8.11 D) regeneration after hypertonic stimulation and a disturbed (area, Figure 8.12 C) regeneration after hypotonic stimulation. This demonstrated the participation of aquaporins in both the regeneration processes after hypertonic and hypotonic stimulations of HTM-N cells, which might be inhibited by $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$. As already mentioned above, this was different to MDCK-I-AQP1 cells, where $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ reduced the speed of both the initial water flux and the regeneration. This was also different to literature, where the speed of water flux during hypertonically induced shrinking of plant cell vesicles was obtained to be decreased by $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (Niemietz & Tyerman, 2002). These differences were assumed to result from the different cell types used. The $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport inhibitor bumetanide showed changes of the area and $|\tau_{reg}|$ for the hypotonic stimulation, that were significant but still very small (Figure 8.12 C and D). Thus, a contribution of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporters to the regeneration after hypotonic stimulation remains unclear. When the potential contribution of RVI processes to the regeneration after hypertonic stimulation is analyzed, bumetanide was found to not influence the speed of regeneration ($|\tau_{reg}|$, Figure 8.11 D), but was observed to even lead to an earlier regeneration (area, Figure 8.11 C). The finding that bumetanide did not lead to a retardation of regeneration indicated that RVI processes do not appear in HTM-N cells, but there is no reasonable explanation for the even earlier regeneration. As already discussed above, the regeneration after hypertonic stimulations is only weakly pronounced and plays a minor role in HTM-N cells. This indicates that the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter, which is together with the Na^+/H^+ exchanger mainly responsible for RVI processes (McCarty & O’Neil, 1992; Lang *et al.*, 1998), is only barely expressed in HTM-N cells and can, therefore, not significantly be inhibited by bumetanide. Nevertheless, this result was different to literature, where RVI processes were obtained in primary HTM cells (O’Donnell *et al.*, 1995). These differences can maybe be resolved by future studies on performing QCM-based water flux experiments with primary HTM cells. The K^+ channel inhibitor Et_4NCl did not show an effect on the regeneration after hypertonic stimulation (Figure 8.11 C and D), which disproved the

contribution of K^+ channels to this process. Analyzing the potential contribution of RVD processes to the regeneration after hypotonic stimulation resulted in a delayed (area, Figure 8.12 C) and slower ($|\tau_{reg}|$, Figure 8.12 D) regeneration by the presence of Et_4NCl . This indicated the occurrence of RVD processes after hypotonic stimulation of HTM-N cells since these RVD processes are mainly accomplished by K^+ channels and Cl^- channels (McCarty & O'Neil, 1992; Lang *et al.*, 1998), which were successfully inhibited by Et_4NCl . Thus, this was in agreement with literature, where RVD processes were discovered in primary HTM cells (Mitchell *et al.*, 2002). But, Et_4NCl did not show an inhibition of AQP1 because $HAuCl_4 \cdot 3H_2O$, as already mentioned above, led to a delayed and slower regeneration after hypertonic stimulation by AQP inhibition, which was not obtained for Et_4NCl . Thus, this fitted to literature studies, which also disproved the AQP1 inhibition by Et_4NCl (Yang *et al.*, 2006; Søggaard & Zeuthen, 2008), but was contrary to others reporting an AQP1 inhibition by Et_4NCl (Detmers *et al.*, 2006; Müller *et al.*, 2008).

8.2.4 Microscopic Imaging of the Cytoskeletal Influence of Water Flux

In the previous sections, the QCM was applied to detect viscoelastic changes of HTM-N cells during hypertonically and hypotonically stimulated water flux to conclude on their concomitant volume changes. In the following, this was complemented by actin staining and subsequent CLSM imaging to gain additional structural information about the behavior of the actin cytoskeleton during these osmotic stimulations. CLSM images of the cells were taken for one hypertonic stimulation (200 mM) and one hypotonic stimulation (hypo 1) at different time points to represent the most important features obtained during recording of the minimal impedance in Figure 8.10. The osmolalities of these solutions are presented in Table 8.1. The time point of the (first) maximum (for hypertonic) and minimum (for hypotonic) was used to identify cytoskeletal changes concomitant with the total amount of initial water flux and a time point close to the endpoint of the QCM measurement was used to mirror the cells' state after regeneration. Accordingly, this was 10 min and 150 min for the 200 mM sucrose solution and 2 min and 60 min for the hypo 1 solution, while a comparison to control conditions was included for both stimulations by additionally taking images at 0 min. Confluent HTM-N cell layers were treated according to section 4.10.3 and images were taken according to section 4.10.2. For each condition, images were taken at three different spots with one image shown as representative for the respective condition in Figure 8.13 for the hypertonic stimulation and in Figure 8.14 for the hypotonic stimulation.

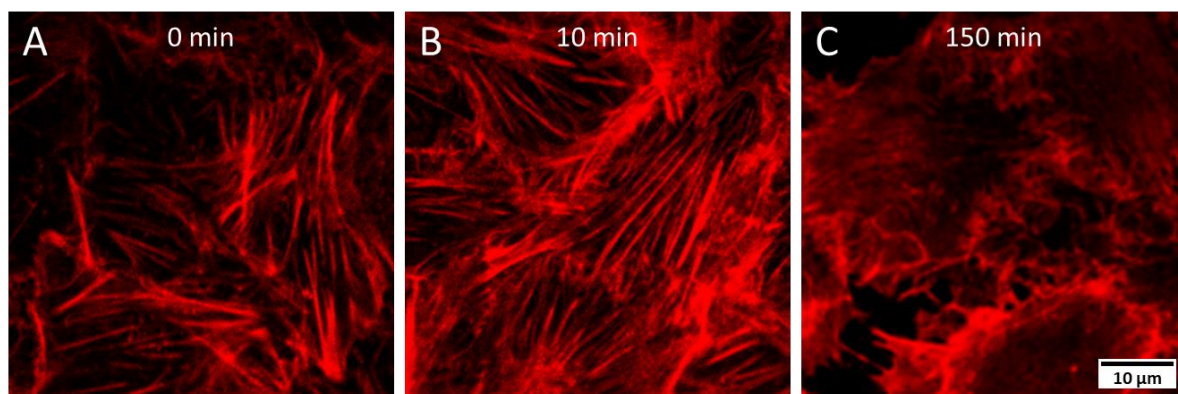


Figure 8.13 Confocal laser scanning microscopic images of confluent HTM-N cell layers with TRITC-phalloidin labeling of actin filaments (focal plane close to the substrate) taken during a water flux experiment on a **hypertonic** stimulation with the 200 mM sucrose solution. Cells were imaged for control conditions (**A**) and 10 min (**B**) and 150 min (**C**) after stimulation. Three images were taken for each condition, but only one image representative for the respective condition is presented here. $T = 37\text{ }^{\circ}\text{C}$.

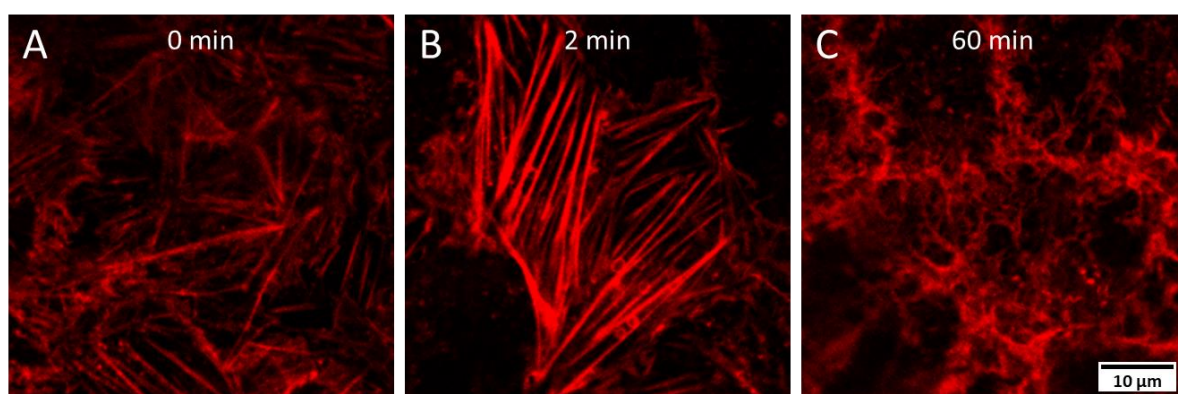


Figure 8.14 Confocal laser scanning microscopic images of confluent HTM-N cell layers with TRITC-phalloidin labeling of actin filaments (focal plane close to the substrate) taken during a water flux experiment on a **hypotonic** stimulation with the hypo 1 solution. Cells were imaged for control conditions (**A**) and 2 min (**B**) and 60 min (**C**) after stimulation. Three images were taken for each condition, but only one image representative for the respective condition is presented here. $T = 37\text{ }^{\circ}\text{C}$.

In most of the images in Figure 8.13 and Figure 8.14, the cell cortex was hardly visible, so that an alteration of cell size during water flux was not assessable here. But, as observed several times before in this thesis, HTM-N cells exhibited strongly pronounced stress fibers under control conditions (Figure 8.13 A and Figure 8.14 A). They underwent certain changes upon osmotic stimulations. For both 10 min after hypertonic stimulation (Figure 8.13 B) and 2 min after hypotonic stimulation (Figure 8.14 B), these thin and long fibers were observed to be even more pronounced. Since the images for these two conditions showed very similar cell responses, although different directions of minimal impedance change were observed in the QCM measurements (see Figure 8.10) according to different directions of the osmotic gradient, no correlation between stress fibers and cell stiffness was possible here.

Nevertheless, this finding of more pronounced stress fibers indicated a very tense and stressed cell status at these time points, where the cells were exposed to the maximal amount of water flux. Images taken 150 min after hypertonic stimulation (Figure 8.13 C) and 60 min after hypotonic stimulation (Figure 8.14 C) showed no stress fibers, indicating a relaxed cell status, which was in accordance with the regenerations observed in the QCM measurements (see Figure 8.10). But, these images reflecting the cells' state after regeneration (Figure 8.13 C and Figure 8.14 C) were clearly different to those for control conditions (Figure 8.13 A and Figure 8.14 A), which was contrary to expectations since a more or less complete regeneration of the minimal impedance was obtained in Figure 8.10. This confirmed observations from previous sections that the QCM signal does not correlate with stress fibers alone, but also with the cell cortex or the cytoplasmic viscosity. Further CLSM imaging experiments focusing on the cell cortex can possibly help verify stiffness changes and alterations of cell size during water flux.

Taken all together, the QCM was demonstrated as a powerful signal transducer for the investigation of osmotically triggered water flux. The advantage of this QCM-based approach over conventional (mostly optical) cell volume assessment techniques is gained by its quantitative, time-resolved, non-invasive, and very sensitive character. The rather low throughput of QCM measurements was partially compensated by the successful performance of serial additions. Using double-quartz-disks facilitated averaging of two parallelly generated readouts of the same cell layer with the possibility to perform an additional ECIS measurement. QCM results demonstrated a constantly very fast water flux in HTM-N cells, giving it a great potential to search and find glaucoma treatment strategies based on water flux into, out of, and through TM cells. But, AQP inhibition did not cause a reduced or decelerated initial water flux, which indicated the approach of reducing aqueous humor production and secretion in the ciliary body by AQP inhibition to be impractical, although an appropriate cell line was not investigated. Furthermore, HTM-N cells showed RVD processes, which could be inhibited by Et_4NCl , but no significant RVI processes according to the inability of bumetanide to interfere. But these barely occurring RVI processes contain the huge potential for a long-lasting cell shrinking (for example, by hypertonicity) associated with a sustained increase in TM permeability and a sustained decrease in IOP in glaucoma patients.

9 Summary / Zusammenfassung

9.1 Summary

This thesis deals with different approaches of non-invasive sensing techniques to contribute to current glaucoma treatment and to also find new glaucoma treatment strategies. The main focus was turned to its most prevalent form, primary open-angle glaucoma (POAG), where mechanical alterations of trabecular meshwork (TM) cells reduce the outflow of aqueous humor and, accordingly, increase the intraocular pressure (IOP) leading to a visual nerve damage. Studies were performed on 2D cell cultures, while cellular changes were recorded by the quartz crystal microbalance (QCM), Electric Cell-Substrate Impedance Sensing (ECIS), and confocal laser scanning microscopy (CLSM). Since the QCM is highly sensitive to stiffness changes / viscoelastic changes, this technique was mainly used to report on the relevant mechanical alterations, with the possibility to monitor mass changes simultaneously. ECIS was used to record cell shape changes, while most of the measurements were complemented by CLSM investigations to observe the concomitant cytoskeletal changes. Four distinct projects were investigated in this thesis, with the common aim of improving glaucoma treatment.

The first project of this thesis (see section 5) aimed at the mechanical characterization of cells by using polyacrylamide (PAAm) and polyethylene glycol (PEG) hydrogels as a model system. This correlation was possible due to similar behavior in terms of stiffness and viscoelasticity, although hydrogels are isotropic materials and cells are not. On the one hand, PAAm hydrogels were used to establish correlations between the Young's modulus measured by compression testing and the acoustic impedance recorded by QCM. These correlations were then used to calculate the Young's modulus of a variety of cells from their QCM response providing results similar to those reported for atomic force microscopy (AFM). Several advantages make this QCM-based approach even preferred over AFM, among them its cost-effective, time-resolved, non-invasive, and holistic nature, while avoiding the dependency on the tip size typically observed in AFM. On the other hand, equations correlating the complex shear modulus with QCM parameters published elsewhere were tested for PAAm and PEG hydrogels by calculation of the complex shear modulus from QCM data and comparison to values obtained from rheological measurements. Since the complex shear modulus is frequency-dependent and has not been studied in the MHz-region before, the results were plausible and in a reasonable order of magnitude. These equations were then applied to cells, which enabled a way to determine complex shear moduli in the MHz-region, which is hardly accessible by other techniques. This also enabled a method to determine the decay length of the shear wave for the cell line under study. Thus, as a complement to other established techniques, the QCM was demonstrated as a promising bioanalytical tool able to determine mechanical moduli of

cells, when correlations between these mechanical moduli and QCM parameters were constructed or tested for hydrogels in advance.

The second project (see section 6) addressed HTM-N cells as a model for the TM cells of the human eye and their stiffness changes induced by a stimulation with the connective tissue growth factor (CTGF) and the Rho-associated protein kinase (ROCK) inhibitor fasudil. CTGF is described to play a critical role in the pathogenesis of POAG. Fasudil was not yet approved for the treatment of POAG, although it was demonstrated in several previous studies to exhibit positive effects for the treatment of this disease. Cytoskeletal changes by CTGF, which would account for its role in the pathogenesis of POAG, were indicated by CLSM imaging, but could not be confirmed by QCM measurements. The observed differences to literature were mainly attributed to a different behavior of HTM-N cells and primary HTM cells. Fasudil was demonstrated to induce a relaxation and degradation of actin filaments in HTM-N cells, as indicated by the stiffness decrease observed in QCM measurements and the morphological changes observed in ECIS measurements and CLSM imaging. This suggested a reduction of outflow resistance in the TM by fasudil, leading to an increase in aqueous humor outflow and consequently to a reduction of IOP, the main risk factor of POAG. Thus, these results prove the suitability of fasudil for the treatment of POAG and may contribute to find new ROCK inhibitors and other drugs for glaucoma treatment.

The third project (see section 7) focused on the diffusion of the ROCK inhibitor fasudil through the vitreous body. To model the in-vivo situation of intravitreal fasudil injection followed by diffusion through the vitreous body and subsequent influence on TM cells, a transwell assay was developed in this thesis with photolithographic fabrication of the electrode arrays. This assay utilized continuous ECIS measurements to monitor the response of HTM-N cells to fasudil as well as the retardation by diffusion through the vitreous body in transwell filter inserts. Several experimental modifications were implemented to optimize the assay, among them the electrode layout and the way of fasudil application to the vitreous body. The cellular response to fasudil, as released from the vitreous body, was very similar to that obtained from a direct exposure, proving its relaxation effect even after previous diffusion. The resulting retardation by diffusion fitted quite well to calculations and diffusion tests with a colored substitute for fasudil. Additionally, results for fasudil previously encapsulated in poly(lactic-co-glycolic acid) microspheres and subsequently released over time proved the continued biological activity, suggesting an improvement of glaucoma therapy by such depot formulations for a prolonged drug release. Moreover, tests on agarose gels recommended their usage as a substitute for the vitreous body in in-vitro experiments. The successful performance of this transwell experiment demonstrated that it is complementary to existing organ-on-a-chip devices for the investigation of POAG and also suggests using this assay to screen for other ROCK inhibitors or different kinds of drugs, or to investigate even other diseases.

The last project of this thesis (see section 8) concerned the assessment of osmotically triggered water flux. Water flux induced by osmotic gradients led to changes of cell volume

associated with changes of intracellular viscoelasticity, which made it possible to detect water flux by QCM measurements. The QCM technique was demonstrated to be well-suited for investigation of water flux due to its quantitative, time-resolved, non-invasive, and very sensitive nature. After assay development using the easy-to-handle cell line MDCK, water flux was successfully monitored in HTM-N cells by the QCM. Differences between cell lines were attributed to the participation of an additional water flux across the basal membrane in case of epithelial cells with their tight intercellular junctions, which is associated with an alteration of the cell-substrate distance. Experimental modifications were included like a serial addition enhancing throughput, and using double-quartz-disks enabling the parallel generation of two signals of the same cell layer. Results for HTM-N cells demonstrated a constantly (independent of osmolality and aquaporin inhibition) very fast water flux, providing the great potential to find glaucoma treatment strategies based on water flux. For example, as already suggested in literature, hypertonically induced TM cell shrinking can increase TM permeability and decrease IOP in glaucoma patients. This effect may be long-lasting since it was revealed by studying different water flux inhibitors that HTM-N cells barely exhibit regulatory volume increase processes.

9.2 Zusammenfassung

Die vorliegende Doktorarbeit behandelt verschiedene Ansätze von nicht-invasiven Sensortechnologien, um zur gegenwärtigen Glaukombehandlung beizutragen und neue Strategien der Behandlung zu finden. Das Hauptaugenmerk lag dabei auf der meist verbreiteten Glaukom-Form, dem primären Offenwinkelglaukom (POAG), wobei mechanische Änderungen von Zellen im Trabekelwerk (TM) den Abfluss des Kammerwassers reduzieren, was zu einer Erhöhung des Augeninnendrucks (IOP) und zu einer Beschädigung des Sehnervs führt. Für die Untersuchungen in dieser Arbeit wurden zweidimensional wachsende Zellschichten verwendet. Zelluläre Veränderungen während der experimentellen Stimuli wurden mithilfe der Quarzkristallmikrowaage (QCM), der Methode des *Electric Cell-Substrate Impedance Sensing* (ECIS) und der Konfokalmikroskopie erfasst. Da die QCM hochsensitiv auf Änderungen der Steifigkeit oder der Viskoelastizität reagiert, wurden die für das Glaukom relevanten mechanischen Änderungen hauptsächlich mit dieser Messtechnik aufgezeichnet. Dabei bestand die Möglichkeit, gleichzeitig noch Massenänderungen aufzunehmen. ECIS wurde verwendet um Änderungen der Zellform zu detektieren, während begleitende Änderungen des Zytoskeletts konfokalmikroskopisch untersucht wurden. Diese Arbeit ist aufgeteilt in vier eigenständige Teilprojekte mit dem gemeinsamen Ziel die Glaukombehandlung zu verbessern.

Das erste Teilprojekt dieser Arbeit (Kapitel 5) zielte darauf ab, Zellen mechanisch zu beschreiben, dadurch dass Hydrogele aus Polyacrylamid (PAAm) und Polyethylenglycol (PEG) als Modell für die Zellen verwendet wurden. Dieses Modell ist wegen ähnlichen Eigenschaften im Sinne von Steifigkeit und Viskoelastizität gerechtfertigt, obwohl diese

Hydrogele im Gegensatz zu den Zellen isotrop sind. Einerseits wurden PAAm-Hydrogele verwendet, um Korrelationen zwischen dem durch Kompression gemessenen Youngschen Modul und der mit der QCM messbaren akustischen Impedanz aufzustellen. Diese Korrelationen wurden dann dazu verwendet, den Youngschen Modul für verschiedene Zelltypen auf Basis ihrer QCM-Parameter zu berechnen. Die entsprechenden Ergebnisse lagen sehr nahe an Literaturwerten, die mithilfe von Rasterkraftmikroskopie (AFM) gemessen wurden. Dieser auf der QCM basierende Ansatz hat etliche Vorteile gegenüber der AFM, darunter das kosteneffiziente, zeitaufgelöste, nicht-invasive und ganzheitliche Detektionsprinzip der QCM, während zusätzlich die Abhängigkeit von der Dicke der AFM-Spitze vermieden wird. Andererseits wurden aus der Literatur bekannte Gleichungen, die den komplexen Schubmodul mit QCM-Parametern in Beziehung setzen, an PAAm- und PEG-Hydrogelen getestet, indem deren komplexer Schubmodul aus QCM-Daten berechnet und mit rheologisch gemessenen Werten verglichen wurde. Da der komplexe Schubmodul frequenzabhängig ist und zuvor niemals im MHz-Bereich bestimmt wurde, waren die erhaltenen Ergebnisse sinnvoll und in einer angemessenen Größenordnung. Diese Gleichungen wurden dann auf Zellen angewandt, was es ermöglichte, den komplexen Schubmodul im MHz-Bereich zu bestimmen, was durch andere Methoden kaum erreichbar ist. Dies ermöglichte außerdem die Bestimmung der Eindringtiefe der Scherwelle für die untersuchten Zelllinien. Daher hat sich die QCM neben etablierten Methoden als ein vielversprechendes bioanalytisches Instrument erwiesen, mit dem mechanische Module von Zellen bestimmt werden können. Dazu müssen nur zuvor Korrelationen zwischen diesen mechanischen Modulen und QCM-Parametern mithilfe von Hydrogelen aufgestellt oder an Hydrogelen getestet werden.

Im zweiten Teilprojekt (Kapitel 6) ging es darum, HTM-N-Zellen als Modell für die TM-Zellen des menschlichen Auges zu verwenden und entsprechende Steifigkeitsänderungen im Zusammenhang mit dem Bindegewebswachstumsfaktor (CTGF) und dem Rho-Kinase-Inhibitor Fasudil zu untersuchen. Der Substanz CTGF wird eine entscheidende Rolle für die Entstehung des POAG zugeschrieben, wohingegen Fasudil trotz zahlreicher Hinweise auf einen positiven Einfluss auf den Krankheitsverlauf noch nicht für eine Behandlung des POAG zugelassen wurde. Konfokalmikroskopische Untersuchungen wiesen auf Änderungen des Zytoskeletts durch CTGF hin, die für dessen Rolle bei der Entstehung des POAG sprechen würden, was allerdings durch QCM-Messungen nicht belegt werden konnte. Dabei wurden die von der Literatur abweichenden Ergebnisse hauptsächlich dem unterschiedlichen Verhalten von HTM-N-Zellen und primären TM-Zellen zugeschrieben. Durch eine in QCM-Messungen beobachtete Abnahme der Steifigkeit und in ECIS-Messungen beobachtete morphologische Änderungen wurde gezeigt, dass Fasudil eine Entspannung und einen Abbau von Aktinfilamenten in den HTM-N-Zellen bewirkt, was konfokalmikroskopisch bestätigt wurde. Dieses Ergebnis legt eine Verringerung des Widerstandes im Abflusssystem des Trabekelwerkes durch Fasudil nahe, wodurch der Abfluss des Kammerwassers verstärkt und der IOP, der Haupt-Risikofaktor des POAG, reduziert werden würde. Folglich sprechen die gefundenen Ergebnisse für eine Eignung von Fasudil zur Behandlung des POAG und könnten

dazu beitragen, noch weitere Rho-Kinase-Inhibitoren oder andere Medikamente für die Glaukombehandlung zu entdecken.

Der Fokus des dritten Teilprojekts (Kapitel 7) lag auf der Diffusion des Rho-Kinase-Inhibitors Fasudil durch den Glaskörper. Die physiologische Situation einer intravitrealen Injektion von Fasudil, gefolgt von einer Diffusion durch den Glaskörper und einer anschließenden Beeinflussung der TM-Zellen, sollte nachgeahmt werden. Dafür wurde in dieser Arbeit ein neuer Transwell-Assay entwickelt, wobei die Elektroden-Arrays photolithographisch hergestellt wurden. Dabei wurde sowohl die Reaktion von HTM-N-Zellen auf Fasudil als auch die Verzögerung, die durch Diffusion durch den in Transwell-Filtereinsätzen gehaltenen Glaskörper verursacht wurde, durch ECIS-Messungen zeitaufgelöst verfolgt. Um den Assay zu optimieren, wurden etliche experimentelle Änderungen durchgeführt, was unter anderem das Elektrodenlayout und die Art der Zugabe von Fasudil zum Glaskörper betraf. Die Zellantwort auf Fasudil, das durch den Glaskörper diffundiert ist, war der einer direkten Stimulierung sehr ähnlich, was dessen relaxierenden Effekt auch nach vorheriger Diffusion nachweist. Die durch die Diffusion durch den Glaskörper auftretende Verzögerung stimmte ganz gut mit Berechnungen und Diffusionstests mit einem farbigen Ersatz für Fasudil überein. Fasudil, das zuvor in Poly(lactid-co-glycolid)-Partikeln eingekapselt war und anschließend mit der Zeit freigesetzt wurde, war nach der Freisetzung immer noch genauso biologisch aktiv. Dies spricht dafür, dass solche Depot-Formulierungen für eine verlängerte Freisetzung des Medikaments die Glaukombehandlung verbessern können. Außerdem wurde durch Tests mit Agarose Gelen gezeigt, dass diese in In-vitro-Studien als Ersatz für den Glaskörper geeignet sind. Die erfolgreiche Durchführung dieser Transwell-Experimente zeigt, dass dieser Assay komplementär zu bestehenden Organ-on-a-Chip-Ansätzen zur Untersuchung des POAG ist, und empfiehlt auch den Einsatz dieses Assays für die Suche nach weiteren Rho-Kinase-Inhibitoren und anderen Arten von Medikamenten, und sogar für die Untersuchung von anderen Krankheiten.

Das letzte Teilprojekt dieser Arbeit (Kapitel 8) handelte vom osmotisch ausgelösten Wasserfluss. Der durch osmotische Gradienten induzierte Wasserfluss hängt mit Änderungen des Zellvolumens und der intrazellulären Viskoelastizität zusammen, was eine Detektion mit der QCM ermöglichte. Die QCM-Technik hat sich zur Untersuchung des Wasserflusses als sehr geeignet erwiesen wegen ihrer quantitativen, zeitaufgelösten, nicht-invasiven und sehr sensitiven Messwert-Erfassung. Nachdem der Wasserfluss zuerst zur QCM-Assay-Entwicklung in den einfach handhabbaren MDCK-Zellen untersucht wurde, wurde er später auch in HTM-N-Zellen erfolgreich verfolgt. Unterschiede zwischen den Zelllinien wurden der Beteiligung eines zusätzlichen Wasserflusses über die Basalmembran bei Epithelzellen mit ihren starken Zell-Zell-Kontakten zugeschrieben, was mit einer Änderung des Zell-Substrat-Abstandes zusammenhängt. Die Experimente beinhalteten auch Optimierungen, wie zum Beispiel eine serielle Zugabe von Lösungen, die den Durchsatz der Methode erhöhte, oder die Verwendung von Doppel-Quarzen, die zwei Signale derselben Zellschicht parallel erzeugten. Die erzielten Ergebnisse zeigten einen konstant sehr schnellen Wasserfluss in HTM-N-Zellen auf, der unabhängig von Osmolalität und einer Aquaporin-

Inhibition war. Dies bietet die großartige Möglichkeit, Strategien der Glaukombehandlung zu finden, die auf Wasserfluss basieren. Zum Beispiel, wie es bereits in der Literatur vorgeschlagen wurde, könnte ein hypertone ausgelöstes Schrumpfen der TM-Zellen die Durchlässigkeit des Trabekelwerks erhöhen und den IOP in Glaukom-Patienten senken. Dieser Effekt könnte langanhaltend sein, da in Untersuchungen von verschiedenen Wasserfluss-Inhibitoren herausgefunden wurde, dass HTM-N-Zellen kaum regulatorische Prozesse zur Volumenerhöhung aufweisen.

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11 Appendix

11.1 Supplementary Information

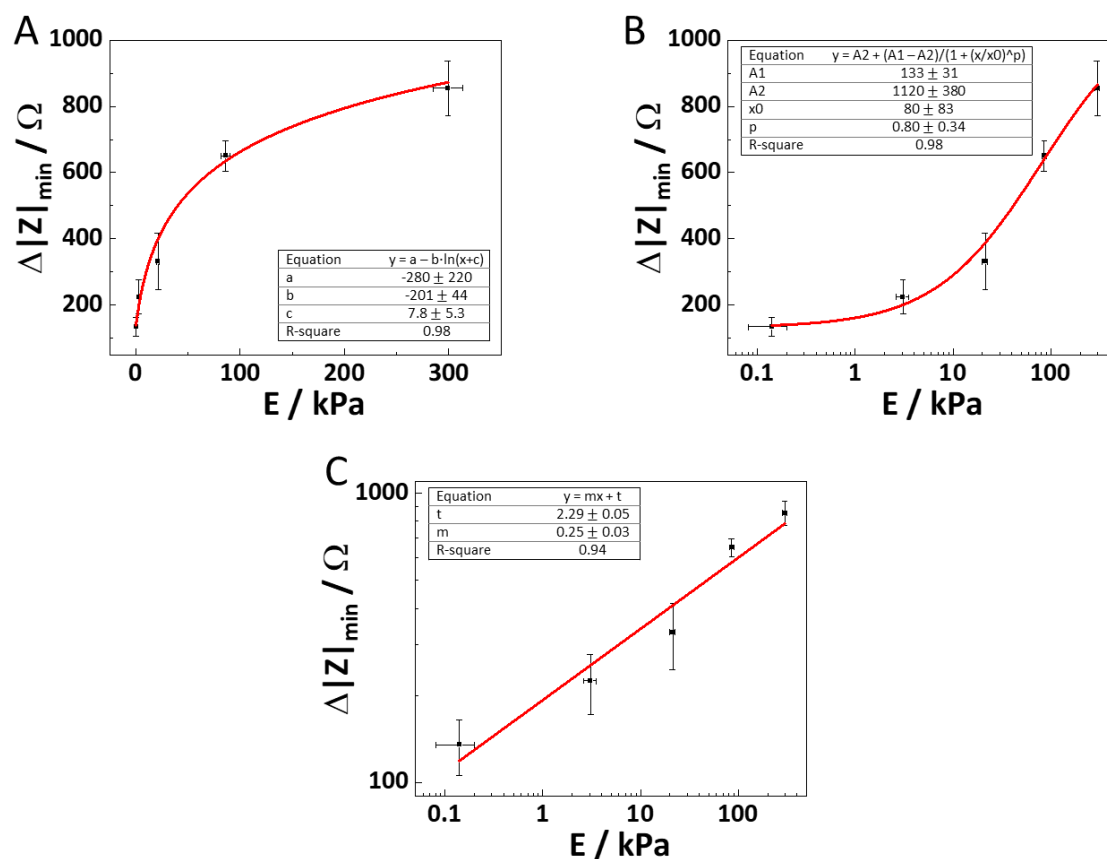


Figure S1 Summary of different plots and data fits tested for the correlation between QCM parameters (shown exemplarily for the change of the minimal impedance $\Delta|Z|_{min}$) and the Young's modulus E for PAAm hydrogels with both parameters on a linear scale and logarithmic data fit **(A)**, a semi-logarithmic scale and sigmoidal data fit **(B)**, and a double-logarithmic scale and linear data fit **(C)**. Data (black) were taken from Table 5.1, representing PAAm gels after 90 min of polymerization, and presented as mean $\pm$ SD with the number of replicates $n = 5$ for the Young's modulus and $n = 4 - 15$ for the change of the minimal impedance depending on PAAm gel type. Equations and parameters of the data fits (red) are given in the respective windows added to the graphs.

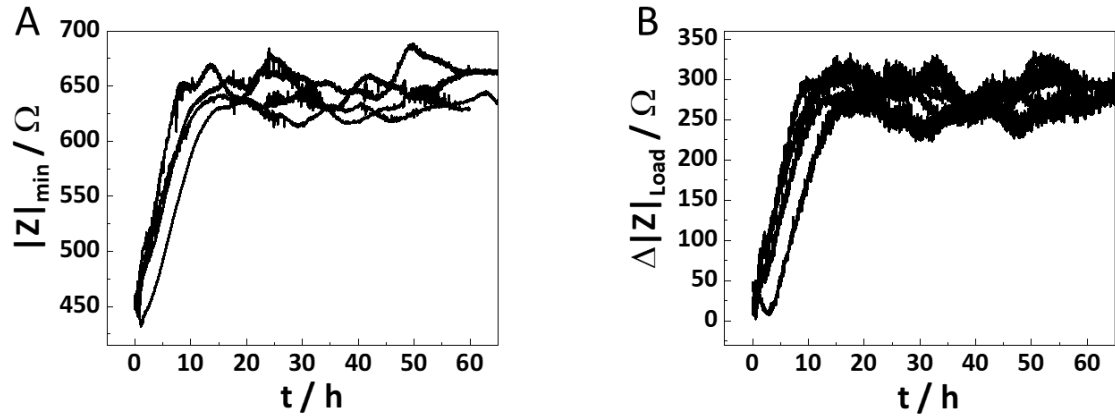


Figure S2 The minimal impedance $|Z|_{min}$ (A) and the load impedance $\Delta|Z|_{Load}$ (B) extracted from the same QCM measurements during HTM-N cell adhesion (seeded confluent) as shown in Figure 6.1. Measurements were started directly after addition of the cell suspension (including the 10 min storage in the laminar flow hood for settling down of cells), while each individual measurement is presented without normalization and averaging over the four total replicates with two biological replicates ($n = 4(2)$). $T = 37^\circ\text{C}$.

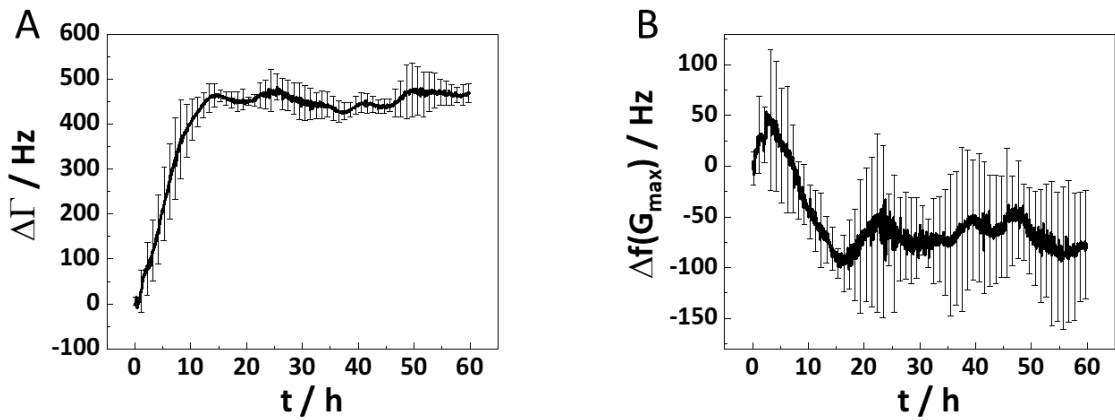


Figure S3 The change of the half-width-half-maximum of maximal conductance $\Delta\Gamma$ (A) and the change of the frequency of maximal conductance $\Delta f(G_{max})$ (B) extracted from the same QCM measurements during HTM-N cell adhesion (seeded confluent) as shown in Figure 6.1. Measurements were started directly after addition of the cell suspension (including the 10 min storage in the laminar flow hood for settling down of cells), while both QCM parameters were normalized to the measurement start. Data are displayed as mean $\pm$ SD out of four total replicates with two biological replicates ($n = 4(2)$). $T = 37^\circ\text{C}$.

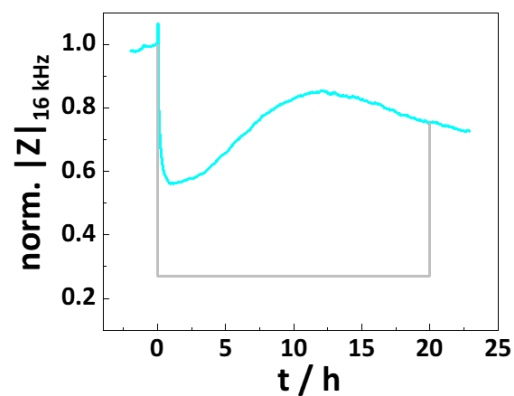


Figure S4 Determination of the area under the curve of a typical time course of the normalized impedance at 16 kHz $norm. |Z|_{16\text{ kHz}}$ showing the ECIS response of HTM-N cells seeded confluent in 8W10E arrays to 100 μM fasudil, which was added at time point 0 h (cyan). The area under the curve was determined down to a value of 0.27 representing cell-free conditions by integration from 0 h to 20 h (grey). The complete corresponding ECIS measurement is shown in Figure 6.6 and the results of this area determination are depicted in Figure 6.7. $T = 37\text{ }^{\circ}\text{C}$.

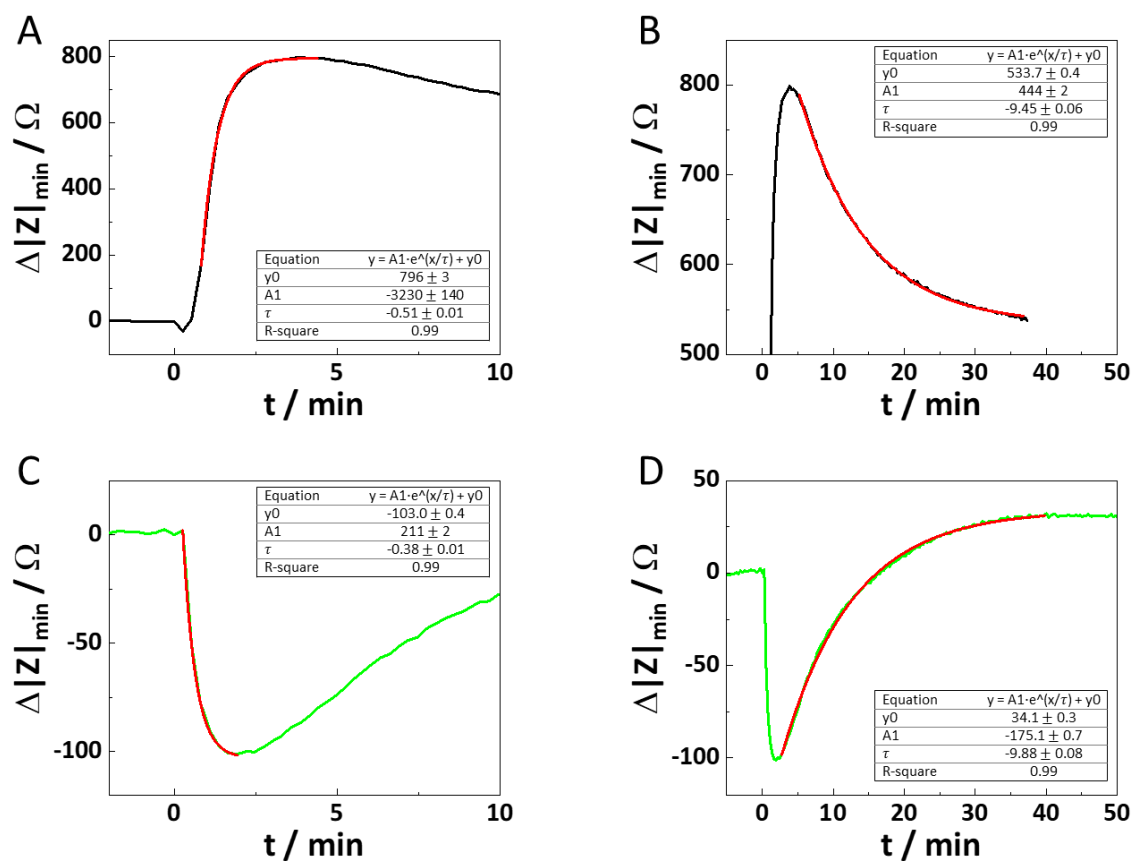


Figure S5 Determination of the time constant τ and the time constant of regeneration τ_{reg} for typical QCM measurements (fast-mode) during water flux experiments with clearly obtainable regeneration (RVC process) by exponential fits with Eq. (76). A typical hypertonic stimulation at 0 min (black) with its initial increase of $\Delta|Z|_{\min}$ (**A**) and subsequent regeneration (RVI process) (**B**) and a typical hypotonic stimulation at 0 min (green) with its initial decrease of $\Delta|Z|_{\min}$ (**C**) and subsequent regeneration (RVD process) (**D**) are displayed, while all four cases were fitted exponentially (red) with the same fit function, with the resulting fit parameters given in the respective windows. $T = 37^\circ\text{C}$.

11.2 Abbreviations

AAm	acrylamide	EDTA	ethylenediamine-tetraacetate
AC	alternating current	EMA	European Medicines Agency
AFM	atomic force microscopy	FDA	Food and Drug Administration
APS	ammonium persulfate	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
AQP	aquaporin	HPLC	high performance liquid chromatography
AT	ambient-temperature	HTM	human trabecular meshwork
BAAm	<i>N,N'</i> -methylenebisacrylamide	HTM-N	human trabecular meshwork (immortalized)
BSA	bovine serum albumin	HUVEC	human umbilical vein endothelial cells
BVD	Butterworth-Van Dyke	IC ₅₀	half maximal inhibitory concentration
CE	counter electrode	idf	inter-digitated finger
CLSM	confocal laser scanning microscopy/microscope	IOP	intraocular pressure
CPE	constant phase element	ITO	indium tin oxide
CTGF	connective tissue growth factor	MDCK-I	Madin-Darby canine kidney strain I
ctrl	control	MDCK-I-AQP1	Madin-Darby canine kidney strain I with overexpression of aquaporin-1
Cyt D	cytochalasin D	MDCK-II	Madin-Darby canine kidney strain II
DAPI	4',6-diamidino-2-phenylindole	MLC	myosin-light-chain
dd-H ₂ O	double-distilled water		
DMSO	dimethyl sulfoxide		
DSMZ	<i>Deutsche Sammlung von Mikroorganismen und Zellkulturen</i>		
ECIS	Electric Cell-Substrate Impedance Sensing		
ECM	extracellular matrix		

Appendix

MLCP	myosin-light-chain phosphatase	RVI	regulatory volume increase
		SD	standard deviation
MM	macromonomer	SPR	surface plasmon resonance
NRK	normal rat kidney	SV40	simian virus 40
PAAm	polyacrylamide	TEMED	tetramethylethylene-diamine
p.a.	per analysis		
PBCEC	porcine brain capillary endothelial cells	TGF- β	transforming growth factor- β
PBS ⁺⁺	phosphate buffered saline with Ca ²⁺ and Mg ²⁺	TM	trabecular meshwork
PBS ⁻⁻	phosphate buffered saline without Ca ²⁺ and Mg ²⁺	TRITC	tetramethylrhodamine isothiocyanate
		UV	ultraviolet
PEG	polyethylene glycol	v/v	volume to volume ratio
PFA	paraformaldehyde	WE	working electrode
PLGA	poly(lactic-co-glycolic acid)	w/v	mass to volume ratio
PMDA	Pharmaceuticals and Medical Devices Agency		
POAG	primary open-angle glaucoma		
QCM	quartz crystal microbalance		
Rho	Ras homolog		
rms	root mean square		
ROCK	Rho-associated protein kinase		
rpm	rounds per minute		
RT	room temperature		
RVC	regulatory volume change		
RVD	regulatory volume decrease		

11.3 Symbols

Quantities			
∂	partial derivative of any quantity	μ	reduced mass, in kg / arithmetic mean of any quantity
α	frequency-dependent resistance beneath the cells, in $\sqrt{\Omega} \cdot \text{cm}$	μ_q	shear modulus of AT-cut quartz, $2.947 \cdot 10^{10} \frac{\text{kg}}{\text{m} \cdot \text{s}^2}$
β	mass concentration, in $\frac{\text{g}}{\text{L}}$	ν	kinematic viscosity, in $\frac{\text{m}^2}{\text{s}}$
Γ	half-width-half-maximum of maximal conductance, in Hz	π	osmolality, in $\frac{\text{mosmol}}{\text{kg}}$
γ	specific conductivity, in $\frac{\text{mS}}{\text{cm}}$	ρ	density, in $\frac{\text{kg}}{\text{m}^3}$
Δ	change / shift of any quantity	ρ_L	liquid / load density, in $\frac{\text{kg}}{\text{m}^3}$
δ	decay length (or penetration depth), in m	ρ_q	quartz density, $2.648 \cdot 10^3 \frac{\text{kg}}{\text{m}^3}$
ε	strain, dimensionless	ρ_{sub}	specific resistivity beneath the cell layer, in $\Omega \cdot \text{cm}$
ε_0	vacuum permittivity, $8.854 \cdot 10^{-12} \frac{\text{S}}{\Omega \cdot \text{m}}$	σ	stress, in Pa / standard deviation of any quantity
ε_r	relative permittivity, dimensionless	τ	shear stress, in Pa / time constant, in min
η'	dynamic viscosity, in Pa · s	τ_{reg}	time constant of regeneration, in min
η'_L	dynamic viscosity of the liquid / load, in Pa · s	φ	angle / phase shift, in °
η''	storage viscosity, in Pa · s	φ_{max}	maximal phase shift, in °
η^*	complex viscosity, in Pa · s	ω	angular frequency, in Hz
λ	wavelength, in m	ω_s	angular series resonant frequency, in Hz
λ_{em}	emission wavelength, in m	A	area, in m^2
λ_{ex}	excitation wavelength, in m		

a_0	amplitude of the shear wave at the quartz-liquid interface, in m	d_q	quartz thickness, in m
		E	Young's modulus, in Pa
A_{CPE}	area-related resistance / capacitance of a CPE, in $\frac{F \cdot s^{n-1}}{cm^2}$ ($n = [0,1]$)	F	force, in N
		f	frequency, in Hz
$a(z)$	amplitude of the shear wave, in m	f_0	(fundamental) resonance frequency, in Hz
B	susceptance, in S	f_A	antiresonant frequency, in Hz
b	decay constant (or propagation constant), in $\frac{1}{m}$	$f(G_{max})$	frequency of maximal conductance, in Hz
C	capacitance, in F	f_P	parallel resonant frequency, in Hz
c	(molar) concentration, in $\frac{mol}{L}$ (= M)	f_R	resonant frequency, in Hz
C_0	parallel capacitance, in F	f_S	series resonant frequency, in Hz
C_c	cell capacitance on transcellular pathway, in F	f_{sens}	most sensitive frequency, in Hz
C_m	average cell membrane capacitance of one membrane, in $\frac{\mu F}{cm^2}$	$f_{ Z _{max}}$	frequency of maximal impedance, in Hz
		$f_{ Z _{min}}$	frequency of minimal impedance, in Hz
C_q	capacitance of a quartz resonator, in F	G	conductance, in S
CI	cytotoxicity index, dimensionless	g	gravitational acceleration, $9.81 \frac{m}{s^2}$
D	dissipation factor, dimensionless / diffusion coefficient, in $\frac{m^2}{s}$	G'	storage modulus, in Pa
		G''	loss modulus, in Pa
d	capacitor plate distance, in m	G^*	complex shear modulus, in Pa
d_L	load thickness (rigid mass layer), in m	$ G^* $	magnitude of the complex shear modulus, in Pa

G_{max}	maximal conductance, in S	n	overtone number / number of replicates, dimensionless
h	cell distance from the electrode, in m	q	charge, in C
I	current, in A / fluorescence intensity, dimensionless	R	(ohmic) resistance, in Ω
$\hat{i}$	current amplitude, in A	r	radius, in m / damping coefficient, in $\frac{\text{kg}}{\text{s}}$
$i(t)$	AC current, in A	R_b	paracellular barrier resistance, in $\Omega \cdot \text{cm}^2$
$\underline{i}(t)$	complex current, in A	R_{bulk}	electrolyte resistance, in Ω
$Im(\underline{Z})$	imaginary part of complex impedance, in Ω	R_c	cell resistance on paracellular pathway, in Ω
$Im(\underline{Z}_{Load})$	imaginary part of the load impedance, in Ω	r_c	cell radius, in m
j	imaginary unit, $\sqrt{-1}$	r_{el}	radius of circular gold film electrode, in m
K	electromechanical coupling constant for AT-cut quartz, $K^2 = 7.899 \cdot 10^{-3}$	R_L	load resistance, in Ω
k	Hooke's constant, in $\frac{\text{kg}}{\text{s}^2}$	R_q	resistance of a quartz resonator, in Ω
k_B	Boltzmann's constant, $1.381 \cdot 10^{-23} \frac{\text{J}}{\text{K}}$	$Re(\underline{Z})$	real part of complex impedance, in Ω
L	inductance, in H	$Re(\underline{Z}_{Load})$	real part of the load impedance, in Ω
l	length, in m	s	mean displacement, in m
l_0	original length, in m	S_f	integral mass sensitivity (or Sauerbrey constant), in $\frac{\text{m}^2}{\text{kg} \cdot \text{s}}$
L_L	load inductance, in H	T	temperature, in K / period, in s
L_q	inductance of a quartz resonator, in H	t	time / duration, in s
m	mass, in kg	U	voltage, in V
M_q	constant factor for quartz (5 MHz AT-cut) material properties, $0.104 \frac{\Omega \cdot \text{m}^2 \cdot \text{s}}{\text{kg}}$	$\hat{u}$	voltage amplitude, in V
		$u(t)$	AC voltage, in V

$\underline{u}(t)$	complex voltage, in V	$ Z _{max}$	maximal impedance, in Ω
V	volume, in m^3	$ Z _{min}$	minimal impedance, in Ω
v	velocity, in $\frac{m}{s}$	$\underline{Z}_R$	impedance contribution of a resistor, in Ω
v_q	propagation velocity of shear waves in AT-cut quartz, $3336 \frac{m}{s}$	$\underline{Z}_s$	static impedance, in Ω
X	reactance, in Ω	$\underline{Z}_{sm}$	surface mechanical impedance, in $\frac{kg}{m^2 \cdot s}$
x	displacement, in m / distance in x-direction, in m / total number of replicates, dimensionless		
y	distance in y-direction, in m / number of biological replicates, dimensionless		
$\underline{Y}$	complex admittance, in S		
Z	impedance, in Ω		
z	distance in z-direction, in m		
$\underline{Z}$	complex impedance, in Ω		
$ Z $	impedance magnitude, in Ω		
$\underline{Z}_C$	impedance contribution of a capacitor, in Ω		
$\underline{Z}_{CPE}$	area-related impedance contribution of a CPE, in $\Omega \cdot cm^2$		
$\underline{Z}_L$	impedance contribution of an inductor, in Ω		
$\underline{Z}_{Load}$	load impedance, in Ω		
$\underline{Z}_m$	motional impedance, in Ω		
$\underline{Z}_m^0$	motional impedance of an unloaded resonator, in Ω		

Units

°	degree	V	volt
°C	degree Celsius		
%	percent		
Ω	ohm		
A	ampere		
Å	angstrom		
C	coulomb		
d	day		
Da	dalton		
F	farad		
g	gram		
H	henry		
h	hour		
Hz	hertz		
J	joule		
K	kelvin		
L	liter		
m	meter		
min	minute		
mmHg	millimeters of mercury		
mol	mol ($= 6.022 \cdot 10^{23}$ particles)		
N	newton		
Pa	pascal		
S	siemens		
s	second		

11.4 List of Solutions and Reagents

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich Chemie GmbH, Munich, Germany
4',6-diamidino-2-phenylindole dihydrochloride (DAPI 2HCl)	Sigma-Aldrich Chemie GmbH, Munich, Germany
acetone	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
acrylamide (AAM) solution	Sigma-Aldrich Chemie GmbH, Munich, Germany
adhesion promoter TI Prime	Microchemicals GmbH, Ulm, Germany
agarose	Sigma-Aldrich Chemie GmbH, Munich, Germany
ammonium persulfate (APS)	Merck KGaA, Darmstadt, Germany
argon (Ar)	Linde plc, Pullach, Germany
bovine serum albumin (BSA)	Sigma-Aldrich Chemie GmbH, Munich, Germany
bumetanide	Sigma-Aldrich Chemie GmbH, Munich, Germany
calcium chloride (CaCl_2)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)	Sigma-Aldrich Chemie GmbH, Munich, Germany
carbon dioxide (CO_2)	Linde plc, Pullach, Germany
cytochalasin D	Sigma-Aldrich Chemie GmbH, Munich, Germany
D-glucose	Sigma-Aldrich Chemie GmbH, Munich, Germany
dimethyl sulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich Chemie GmbH, Munich, Germany

ethylenediaminetetraacetate (EDTA)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Elma Clean 10 solution	Elma Schmidbauer GmbH, Singen, Germany
ethanol (EtOH)	CSC JÄKLECHEMIE GmbH & Co. KG, Nuremberg, Germany
fetal bovine serum (FBS)	VWR International, Radnor, PA, USA
hydrogen peroxide (H ₂ O ₂)	Merck KGaA, Darmstadt, Germany
hydrogen tetrachloroaurate trihydrate (HAuCl ₄ ·3H ₂ O)	Sigma-Aldrich Chemie GmbH, Munich, Germany
hygromycin B	InvivoGen, San Diego, CA, USA
iodine (I ₂)	Merck KGaA, Darmstadt, Germany
isopropyl alcohol	Merck KGaA, Darmstadt, Germany
L-glutamine (L-Gln) solution	Sigma-Aldrich Chemie GmbH, Munich, Germany
magnesium sulfate (MgSO ₄)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Minimum Essential Medium Eagle (MEM)	Sigma-Aldrich Chemie GmbH, Munich, Germany
nitrogen (N ₂)	Linde plc, Pullach, Germany
<i>N,N'</i> -methylenebisacrylamide (BAAm) solution	Sigma-Aldrich Chemie GmbH, Munich, Germany
paraformaldehyde (PFA)	Sigma-Aldrich Chemie GmbH, Munich, Germany
penicillin solution	Sigma-Aldrich Chemie GmbH, Munich, Germany
phosphate buffered saline with Ca ²⁺ and Mg ²⁺ (PBS ⁺⁺)	Sigma-Aldrich Chemie GmbH, Munich, Germany
phosphate buffered saline without Ca ²⁺ and Mg ²⁺ (PBS ⁻⁻)	Sigma-Aldrich Chemie GmbH, Munich, Germany
photopolymer AZ [®] ECI 3027	Microchemicals GmbH, Ulm, Germany

polyvinylpyrrolidone-iodine solution	B. Braun Melsungen AG, Melsungen, Germany
potassium chloride (KCl)	Merck KGaA, Darmstadt, Germany
potassium dihydrogen phosphate (KH_2PO_4)	Merck KGaA, Darmstadt, Germany
potassium iodide (KI)	Merck KGaA, Darmstadt, Germany
resazurin	Sigma-Aldrich Chemie GmbH, Munich, Germany
sodium bisulfate (NaHSO_4)	Merck KGaA, Darmstadt, Germany
sodium chloride (NaCl)	VWR International, Radnor, PA, USA
sodium hydroxide (NaOH)	Fisher Scientific GmbH, Schwerte, Germany
streptomycin solution	Sigma-Aldrich Chemie GmbH, Munich, Germany
sucrose	Sigma-Aldrich Chemie GmbH, Munich, Germany
sulfuric acid (H_2SO_4)	Sigma-Aldrich Chemie GmbH, Munich, Germany
tetraethylammonium chloride (Et_4NCl)	Sigma-Aldrich Chemie GmbH, Munich, Germany
tetramethylethylenediamine (TEMED)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
TRITC-phalloidin	Sigma-Aldrich Chemie GmbH, Munich, Germany
Triton X-100 solution	Sigma-Aldrich Chemie GmbH, Munich, Germany
trypan blue solution	Sigma-Aldrich Chemie GmbH, Munich, Germany
trypsin solution	Sigma-Aldrich Chemie GmbH, Munich, Germany
Tween 20	SERVA Electrophoresis GmbH, Heidelberg, Germany

11.5 List of Materials, Instrumentation, and Software

8W10E arrays	Applied Biophysics, Troy, NY, USA
8-well-corpus	workshop of precision engineering, University of Regensburg, Germany
96-well plates	Greiner Bio-One International GmbH, Kremsmünster, Austria
argon plasma cleaner PDC 32G-2	Harrick Plasma Inc., Ithaca, NY, USA
autoclave DX-45	Systec GmbH, Nuremberg, Germany
black inkjet dye	HARKE Services GmbH, Mühlheim, Germany
Bürker chamber	LO - Laboroptik Ltd., Lancing, UK
camera	Nikon Corporation, Tokyo, Japan
centrifugation tubes	Greiner Bio-One International GmbH, Kremsmünster, Austria
centrifuge 5415 R	Eppendorf AG, Hamburg, Germany
confocal laser scanning microscope Eclipse 90i	Nikon Corporation, Tokyo, Japan
cryogenic tank GC – BR 2150M	Jutta Ohst german-cryo GmbH, Jüchen, Germany
cryoscopic osmometer (Osmomat 030)	Gonotec GmbH, Berlin, Germany
cryovials	TPP Techno Plastic Products AG, Trasadingen, Switzerland
culture flasks	Greiner Bio-One International GmbH, Kremsmünster, Austria
double-quartz-disks	KVG Quartz Crystal Technology GmbH, Neckarbischofsheim, Germany
drying cabinet Heraeus T6	Thermo Fisher Scientific, Hanau, Germany
drying cabinet Memmert BE400	Gemini B.V., Apeldoorn, Netherlands
ECIS software	Applied Biophysics, Troy, NY, USA

ECIS® Zθ	Applied Biophysics, Troy, NY, USA
electrical conductivity meter inoLab Multi 720	WTW, Weilheim, Germany
Filtropur S filters	Sarstedt AG & Co. KG, Nümbrecht, Germany
fluorescence reader GENios	Tecan Group AG, Männedorf, Switzerland
glass rings	workshop of precision engineering, University of Regensburg, Germany
glass slides	Paul Marienfeld GmbH & Co. KG, Lauda- Königshofen, Germany
gold sputtering target	General Electric Company, Munich, Germany
Heraeus Multifuge 1S-R	Thermo Fisher Scientific, Hanau, Germany
HPLC device 1260 Infinity II LC	Agilent Technologies, Inc., Santa Clara, CA, USA
ImageJ	developed by Wayne Rasband (National Institutes of Health, Bethesda, MD, USA)
impedance analyzer 1260A	Solartron Analytical, Farnborough, UK
incubator Heraeus BB 15	Thermo Fisher Scientific, Hanau, Germany
incubator MCO-170AICUVH	Panasonic Healthcare Co., Ltd., Sakata, Japan
incubator New Brunswick Galaxy 48 S	Eppendorf AG, Hamburg, Germany
inkjet film	Folex Coating GmbH, Cologne, Germany
inkjet printer SureColor SC-P400	Epson, Suwa, Japan
Instron 5542	Artisan Technology Group, Champaign, IL, USA
LabVIEW-based ECIS measurement software	programmed by Prof. Wegener (University of Regensburg, Germany)
LabVIEW-based QCM fitting software	programmed by Prof. Wegener (University of Regensburg, Germany)

LabVIEW-based QCM measurement software	programmed by Prof. Wegener (University of Regensburg, Germany)
laminar flow hood HERAsafe KS/KSP class II biological safety cabinet	Thermo Fisher Scientific, Hanau, Germany
Lexan® plates	GE Healthcare, Munich, Germany
micro test tubes	Eppendorf AG, Hamburg, Germany / Sarstedt AG & Co. KG, Nümbrecht, Germany / VWR International, Radnor, PA, USA
microwave oven OMW 210-S	Venalisia Import GmbH, Kösching, Germany
Origin	OriginLab, Northampton, MA, USA
parafilm	Pechiney Plastic Packaging, Inc., Chicago, IL, USA
Pasteur pipettes	VWR International, Radnor, PA, USA
Petri dishes	Greiner Bio-One International GmbH, Kremsmünster, Austria
phase contrast microscope Diaphot	Nikon Corporation, Tokyo, Japan
pipettes	BRAND GmbH & Co. KG, Wertheim, Germany / Eppendorf AG, Hamburg, Germany
pipettes (disposable)	Greiner Bio-One International GmbH, Kremsmünster, Austria
pipette tips	Sarstedt AG & Co. KG, Nümbrecht, Germany
PowerPoint	Microsoft, Redmond, WA, USA
quartz disks	KVG Quartz Crystal Technology GmbH, Neckarbischofsheim, Germany
relay	workshop of electronics, University of Regensburg, Germany
rheometer Kinexus lab+	Malvern Panalytical, Kassel, Germany
silicone glue	BAUHAUS, Regensburg, Germany
silicone grease	Kurt Obermeier GmbH & Co. KG, Bad Berleburg, Germany

silver conductive paint	Busch GmbH & Co. KG, Viernheim, Germany
software EZ-C1	Nikon Corporation, Tokyo, Japan
spin coater WS-650-23B	Laurell Technologies Corporation, North Wales, PA, USA
sputter coater Balzers SCD 050	BalTec AG, Pfäffikon, Switzerland
Teflon® QCM chambers	workshop of precision engineering/electronics, University of Regensburg, Germany
Teflon® vessels (for gel preparation and microscopy)	workshop of precision engineering, University of Regensburg, Germany
transwell filter inserts	Corning Inc., Corning, NY, USA
ultrasonic bath Sonorex Digitec DT 31 H	BANDELIN electronic GmbH & Co. KG, Berlin, Germany
UV exposure device BEL14002	isel Germany AG, Eichenzell, Germany
vortex mixer REAX 1R	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
water bath TW12	Julabo GmbH, Seelbach, Germany

Danksagung

Der allergrößte Dank geht natürlich an meine Eltern für die bedingungslose Unterstützung während meines gesamten Studiums. Obwohl von allen Seiten Druck kommt, nach über 10 Jahren an der Uni endlich richtig zu arbeiten, habt ihr euch daran nie wirklich beteiligt und habt mir damit keinen zusätzlichen Stress gemacht. Also danke für eure uneingeschränkte Liebe, Unterstützung und Geduld!

Nicht weniger danken möchte ich natürlich meinem Doktorvater Prof. Dr. Joachim Wegener. Joe, zuerst danke ich dir für die Vergabe dieses spannenden Promotionsthemas und deine genialen Ideen und Vorschläge, vor allem wann immer irgendetwas nicht funktioniert hat. Noch wichtiger ist meiner Meinung nach die menschliche Seite, bei der du mit deiner coolen Art eine überragende Arbeitsatmosphäre geschaffen hast, wie man sie sonst nirgends antreffen wird.

Ebenfalls bedanken möchte ich mich bei Prof. Dr. Miriam Breunig. Danke dir für das unkomplizierte Übernehmen des Zweitgutachtens, deine Impulse im Nanoglaukom-Projekt und deine überaus sympathische Art.

Das führt mich auch direkt zu den nächsten Leuten, bei denen ich mich bedanken möchte, und zwar all denen, die am Nanoglaukom-Projekt beteiligt waren. Dazu gehören neben den bereits erwähnten Prof. Dr. Joachim Wegener und Prof. Dr. Miriam Breunig vor allem noch Prof. Dr. Rudolf Fuchshofer, Dr. Raphael Mietzner und Dr. Franziska Frömel. Ich danke euch für die regelmäßigen spannenden Diskussionen und Anregungen im Nanoglaukom-Meeting, die mir bei der Bearbeitung meines Projekts sehr geholfen haben.

Großer Dank gebührt auch meiner Betreuerin Dr. Judith Stolwijk. Danke dir dafür, dass du mir sowohl während meiner Masterarbeit, als auch in den Anfängen meiner Doktorarbeit, enorm geholfen hast durch deine ausgesprochene fachliche Kompetenz.

Die obengenannte überragende Arbeitsatmosphäre wurde natürlich auch wesentlich durch meine Kollegen aus der Arbeitsgruppe Wegener geprägt. Ich danke euch für die freundschaftliche Atmosphäre, die durch die stetige gegenseitige Unterstützung, die regelmäßigen Mensagänge und auch die außeruniversitären Unternehmungen geschaffen wurde.

Außerdem möchte ich mich bei meinen Praktikantinnen Synthia Yemata Ndeti und Anna Gebhard und Bachelorandinnen Sophia Baumann und Christine Drießlein bedanken, die bei einigen in dieser Arbeit präsentierten Ergebnissen mitgewirkt haben und entscheidende Beiträge geleistet haben. Eure Betreuung und die Zusammenarbeit mit euch hat mir sehr viel Spaß gemacht.

Meinen Dank verdienen auch Dr. Heike Lehr und Dr. Christian Ziegler vom Lehrstuhl Göpferich. Danke, dass ihr mir ermöglicht habt, Kompressionsmessungen bzw. rheologische Messungen meiner Gele bei euch durchzuführen.

Zu guter Letzt möchte ich mich bei Simone, Leo und Tommi bedanken, die diese Doktorarbeit zur Korrektur gelesen haben. Danke euch, dass ihr einen Teil eurer kostbaren Zeit investiert habt, euch da „durchzufuchsen“, und mir dadurch sehr geholfen habt.

Curriculum Vitae



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Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

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Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

