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Novel liquid application systems for poorly soluble drugs

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meiner Familie

Ausdauer wird früher oder später belohnt – meistens aber später.

Wilhelm Busch

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Chapter 1

Introduction and goals of the thesis

1.1. Ophthalmic drug delivery

The human eye is a very unique and isolated organ containing the optical apparatus which is essential for vision (Fig. 1 [1]). The lens divides it into the anterior and posterior segment. The anterior segment consists of cornea, aqueous humor and iris ciliary body and the posterior part contains the retina and the vitreous body. Due to the missing blood vessels within the cornea, lens and vitreous body, the delivery of oxygen and nutrients is via diffusion in the aqueous humor. The eye can further be divided into three main layers; an outer fibrous layer (sclera and cornea), a middle vascular layer (choroid, ciliary body and iris) and an inner nervous layer (retina) [2].

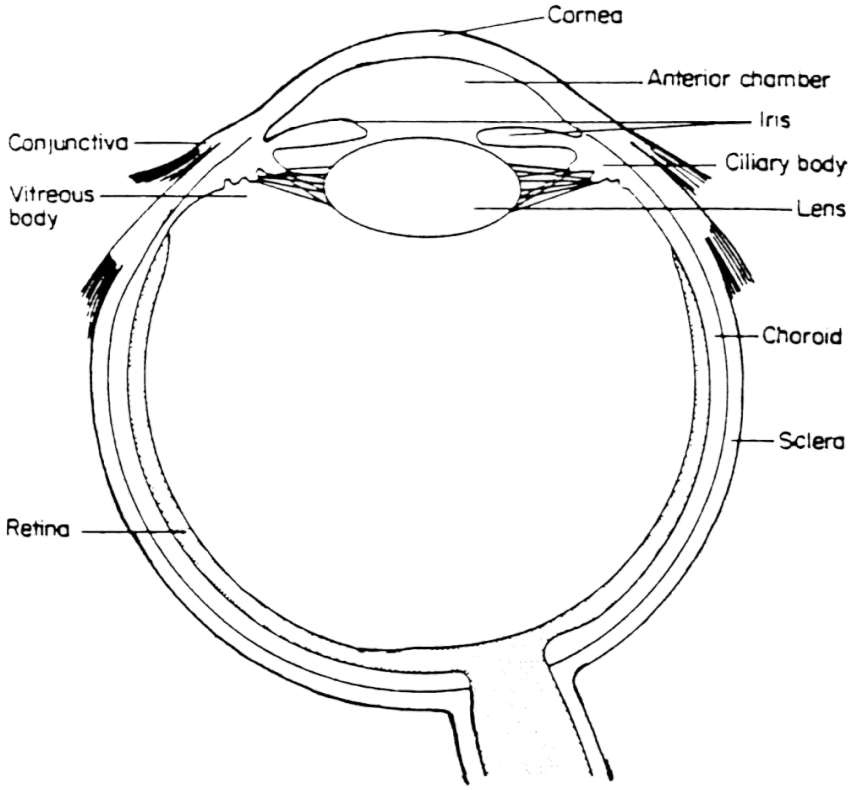

Fig. 1: Horizontal section through the eye [1]

There are various diseases affecting the eye. Examples are blepharitis, allergic, bacterial or viral conjunctivitis, keratitis, or dry eye syndrome at the anterior eye. Furthermore, there are proliferative vitreoretinopathy, retinitis pigmentosa or macular

degeneration at the posterior segment leading to severe patient discomfort [1]. For an efficient treatment drugs can be applied via different routes; topical, intravitreal, scleral or systemic. The topical administration is the most common route for diseases affecting the anterior eye, however, due to a high drug loss in the precorneal area, it is poorly suitable for treatment of complications in the posterior part [3]. To reach this section, intravitreal or sclera injections are preferred, even if they are associated with a high risk of complications [4]. After systemic application therapeutic drug levels can be reached in ophthalmic tissue, however, only 1 – 2 % of the plasma concentration is achieved and hence, toxic systemic concentrations can easily be reached and side effects may occur [3].

In the last two decades the main focus of ocular therapy was set in two arms. On the one hand, there was research for new effective drugs for the treatment of poorly controlled diseases, on the other hand, the improvement of ocular dosage forms for already existing drugs became a major issue [1].

The most common approach for the administration of ophthalmic drugs is the delivery through simple aqueous eye drops. However, due to the physiological ocular barriers, those come with important disadvantages in terms of biocompatibility, bioavailability and toxicity [4, 5]. There are several barriers that hamper the corneal or scleral uptake of drug upon instillation. With the basal tear flow and the nasolacrimal drainage, which are usually responsible for the clearance of foreign particles from the eye surface, more than 95 % of the applied drug is directly washed out [3]. Furthermore, the amount of drug which is then unavailable at the eye surface may easily become systemically available and cause toxic effects, as for example known for the potent drug timolol after topical administration [6,7]. The cornea itself consists of different hydrophilic and lipophilic layers and thus causes difficult

conditions for its permeation by drugs. The final drug levels in corneal tissue and the bioavailability are discussed to be smaller than 1 – 5 % of the initially instilled drug dosing [8–10]. Another example of an ocular barrier in terms of drug resorption is the ocular – blood barrier [11]. It prevents the targeting of the posterior section of the eye through topical administration by stopping the travelling of drugs from scleral tissue over the retinal capillaries [11].

Thus, in recent years much effort was put in research to develop novel ophthalmic application systems to reach a higher bioavailability in combination with minimal to no further systemic side effects of potent drugs, the need of which increases steadily for prevalent diseases like dry eye disease. One approach was the preparation of in-situ gelling systems, which showed an increase in viscosity upon instillation and therefore prolonged the residence time of drug at the ocular surface [2]. Besides these, microemulsions, microsuspensions or colloidal systems like liposomes, nanoemulsions or nanoparticles were developed [2]. A benefit of these formulations was the possibility to incorporate higher levels of hydrophobic drugs compared to aqueous systems [2]. By choice of suitable surface characteristics of these systems it was possible to gather muscoadhesive properties, for example by charge, and thus to slow down the time of evaporation and to increase the bioavailability of the drug. Fialho et al showed a microemulsion system for 0.1 % of dexamethasone with double of the maximum drug absorbance, a faster penetration through the corneal layers and with twice the area under the curve as known for comparable conventional systems [12]. However, up to now it is not clear, if for example positively charged surfaces may not lead to toxic effects when applied over longer period [13, 14]. A different strategy is followed by the preparation of ocular inserts or minitablets. These systems are inserted in the conjunctival cul de sac and show tremendous potential to

increase the bioavailability by prolonged contact time and sustained drug release and furthermore to reduce side effects and the frequency of application. However, they show a lot of disadvantages like foreign body sensation, discomfort, a difficult handling or blurred vision which finally leads to discontinuation in therapy [15–17]. Nanosuspensions combine benefits of aqueous and solid systems by dispersion of drug nanoparticles in an aqueous environment. Due to the submicron size of their particles (200 – 600 nm), they improve the saturation solubility of the drug and hence, its bioavailability. Furthermore, they are also as functional as sustained release systems, which is a big step towards the safety and efficiency of drugs. Unfortunately, to prepare the particles, nanosuspensions suffer from a cumbersome manufacturing using preparation methods like for example the solvent displacement method or the ionic gelation technique or milling [18].

Besides all benefits which were gathered by the various novel formulation approaches, their compositions and manufacturing are far from simplicity and biocompatibility. Therefore, there is still a huge need for formulations which combine simplistic production and biocompatibility with enhanced drug solubility and an increase in bioavailability to have efficient tools for the treatment of the various ophthalmic diseases.

1.2. Dry eye disease

Dry eye disease (DED) is a severe medical problem with billions of people affected, and thus, a highly important issue for research. Over the last twelve years the number of publications has nearly increased by a factor of 6 (Fig. 2) [19].

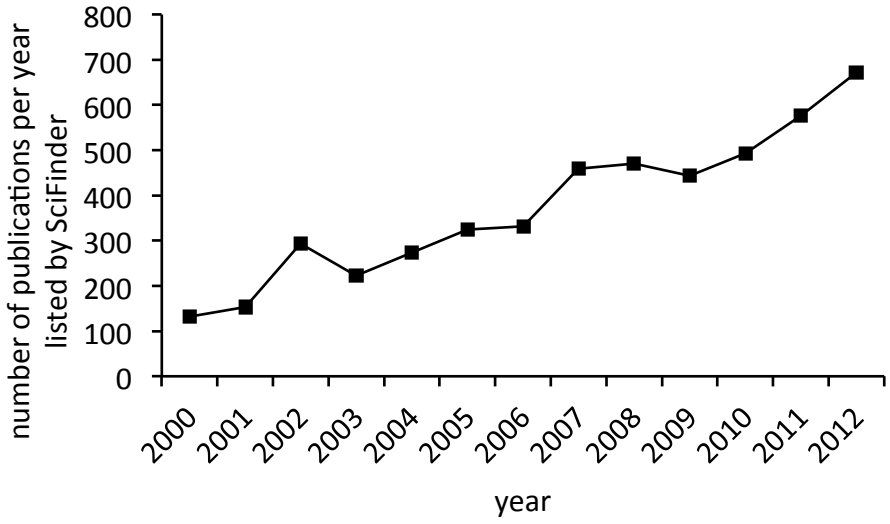

Fig. 2: Trend in number of publications for the key words “dry eye” listed by SciFinder® between 2000 and 2012 [20];

Due to current knowledge from research on hyperosmolarity and ocular surface inflammation in dry eye and the effect of dry eye on visual function, the definition of DED was re-defined by the International Dry Eye Workshop in 2007 as *“… a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface* [21]*.”*

DED affects the lacrimal functional unit (LFU), which is the lacrimal glands, the ocular surface (cornea, conjunctiva, meibomian glands), the lids and the sensory and motory nerves connected to them. The overall function of the LFU is to preserve the tear film, the transparency of the cornea and the quality of the image projected onto the retina [22]. Diseases or damage of any of its components can destabilize the tear film and lead to DED.

The vicious circle of DED sketched by the International Dry Eye Workshop is shown in Fig. 3 [21]. Its core mechanisms, which initiate, amplify and potentially change its character, are tear hyperosmolarity and tear film instability.

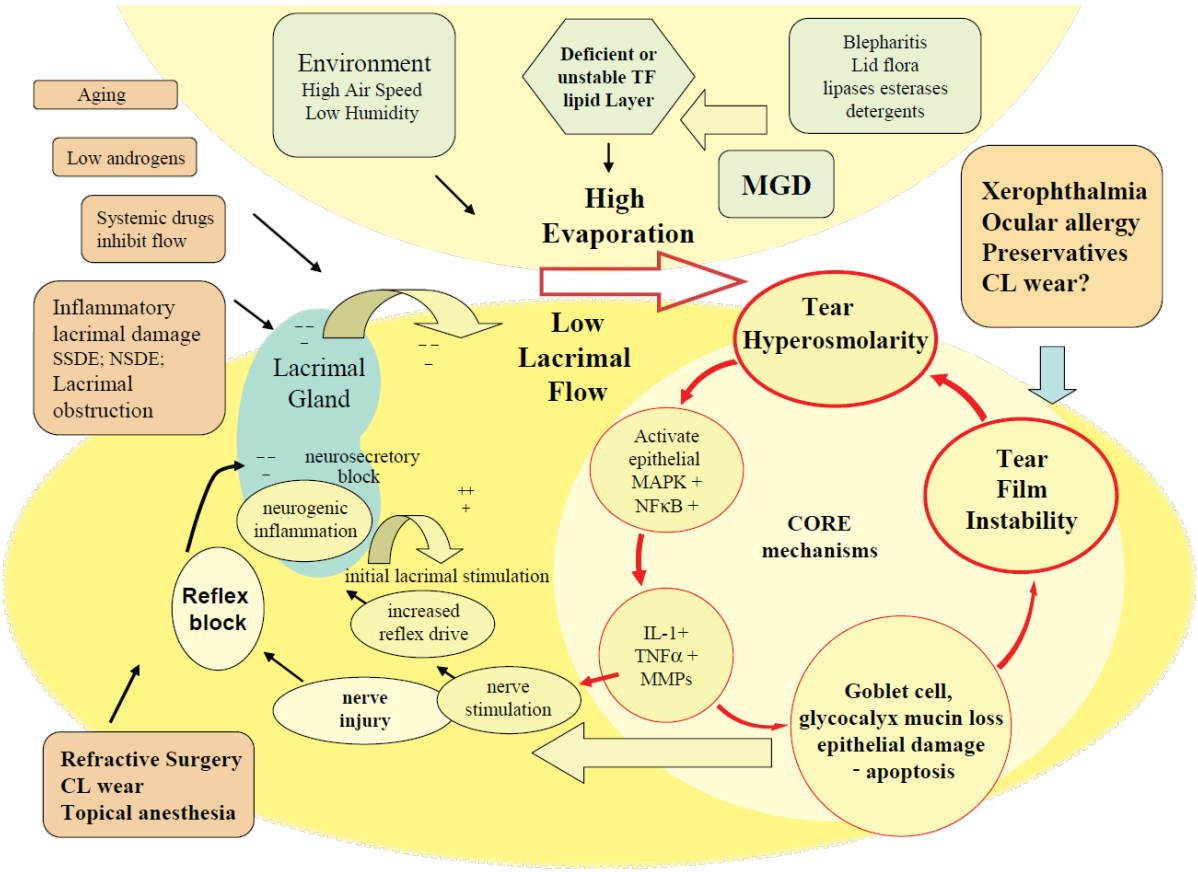

Fig. 3: Vicious circle of the multifactorial disease dry eye syndrome according to the International Dry eye Workshop 2007; core mechanisms (tear hyperosmolarity and tearfilm instability), inflammatory process and exogenous amplifying factors; [21]

Hyperosmolarity of the tearliquid is the central mechanism causing ocular surface inflammation and damage. It typically arises via evaporation of water of epithelial surface cells and stimulates a cascade of inflammatory events (MAP kinases and NFκB signaling pathways) and the generation of inflammatory cytokines (IL-1α; IL-1β), tumor necrosis factor (TNF) α and matrix metalloproteinases (MMP-9)[23,24], which directly leads to apoptotic cell death of epithelial cells like mucin secreting goblet cells [25].

Unrelated to prior tear hyperosmolarity, in some forms tear film instability is the indicating event of DED, such as after LASIK surgery or disturbances of the ocular surface mucins like xerophthalmia or allergic eye disease [21].

As a result of different epidemiologic studies on dry eye the consistent risk factors are for example age, gender with higher prevalence in women, postmenopausal estrogen therapy, low omega 3 and omega 6 fatty acid uptake or an antihistamine medication. The prevalence of DED in these studies varies dependent on the study criteria and the investigated group between 5.2 % and 63 % [26]. About 4.9 million Americans with an age over 50 suffer from DED and tens of millions have less severe but related syndromes [27]. The incidence of DED increased between 1990 and 1998 at 57.4 %. In comparison cataract increased at 16.4 %. Regarding to the US-Census Bureau until 2050 the population between 65 and 84 years will increase at 100 % and the 85 years and elder ones at even 333 % [28]. Thus, this will further increase the incidence of DED.

Dry eye patients often report symptoms like distinct pain, irritation, foreign body sensation, grittiness, excess tearing or in more severe case a decrease of their visual ability. Untreated, clinical course may proceed up to infection, corneal ulcer or even blindness [14]. Hence, DED is a tremendous problem concerning the patients' quality of life. About 60 % of the patients feel hindered in their everyday life and even 37.5 % report to be less efficient at work in consequence of their affection [26, 29].

Upon these facts, regarding the prevalence, the incidence and also the adverse affects the patients have to deal with, there is a huge need for an efficient therapy. In general, the therapeutic options are twofold; palliative therapy and causal treatment. The main part of DED therapy is still palliative, what means that the disease is treated symptomatically. Several possibilities are reported, such as tear

supplementation, tear retention or tear stimulation by use of for example pilocarpine [30]. Tear retention using punctum plugs to retard the tear clearance and gather a more efficient treatment with eye drops is an option first reported in 1975 by Freeman et al. [31]. However, the most common treatment is tear supplementation to lubricate the eye, replace missing tear fluid, reduce the elevated tear osmolarity, wash out inflammatory agents and especially improve the patient comfort and the quality of life [32].

Despite the symptomatic improvements using palliative therapeutics, the inflammation, which is part of nearly each form of DED stays untreated. Therefore, an anti-inflammatory therapy is more and more proclaimed [26]. Various options have been investigated like treatment with 15(S)-hydroxyeicosatetraenoic-acid, local androgens, corticosteroids or tetracyclines. However, cyclosporin A was finally found to be the most promising option in terms of a causal treatment. Unfortunately, until today there is just one formulation approved by the US Food and Drug Administration (FDA) for the treatment of DED in the US only [26,32].

Thus, as a result of the course of the disease and the current therapeutic options there is a tremendous lack of drug products using potent drugs like, for example, cyclosporin A which would be efficient in causal treatment of dry eye disease and would lead to a significant increase of the patients' quality of life.

1.3. Cyclosporin A: a potent but poorly soluble drug

The cyclic undecapeptide cyclosporin A (CsA) is produced by Tolypocladium inflattum Gams and other fungi imperfecti [33]. It is successfully used as an oral immunosuppressant drug in transplantation medicine since the 1970s when it was discovered in the labs of Sandoz in Switzerland. Cyclosporin A binds to the

immunophilin cyclophilin and this complex inhibits the dephosphorylation of transcription factors especially the nuclear factor of activated T-lymphocytes (NFAT) and its subsequent translocation from the cytoplasm to the nucleus in an IL-2 mediated process. Thereby the activation of promoters of the T-cell activation and the immune response are inhibited [34].

Besides its use after graft surgery, it was shown for cyclosporin A, that it can reach ocular tissue via intravenous or oral administrations. It is effective in treatment of inflammations affecting the posterior segment of the eye as well as, for example, in peripheral ulcerative keratitis, severe Grave's ophthalmopathy, autoimmune uveitis, atopic keratoconjunctivitis or dry eye disease [33]. In treatment of dry eye disease the mechanism of increasing the tear production is not completely unraveled, but it seems to be related to the immunomodulatory activity of cyclosporin A, which decreases the local inflammation [35]. However, to gather sufficiently high local tissue levels, high systemic concentrations have to be applied [33,36]. Unfortunately, if exceeding therapeutic levels, CsA may cause severe side effects like especially acute or chronic nephrotoxicity. Further toxic effects are hypertension, hyperlipidemia, gingival hyperplasia, neurotoxicity or thrombotic microangiopathy [37].

To overcome these severe problems, a local therapy of ophthalmic diseases would be preferential because much less CsA would transit into the blood stream. Furthermore, in DED this would mean a direct targeting of the site of disease. As patients, who received systemic CsA as standard therapy after transplantation, showed an increased tear production, a first study on topical CsA as a therapy for dry eye was conducted in 1994. Gunduz et al showed a significantly prolonged tear film break up time (TFBUT) and a decrease in rose Bengal staining. However, after 2

months of treatment with a 2 % CsA olive oil eye drop solution no improvement in Schirmer-I-test was found compared to the vehicle [38]. In following years numerous studies proved the inflammatory background of dry eye disease and the potential efficiency of topical cyclosporin A administration [22,39–41]. In 2000, Sall et al conducted two multicenter, randomized studies with twice a day local instilled 0,05 % and 0,1 % CsA emulsions over 6 months on 877 patients who showed mild to severe DED. He could show significant improvements with the corneal staining test and the Schirmer-I-test. Therefore, he proved the efficiency and safety of the formulations and found further that the higher concentrated emulsion was not beneficial compared to the lower concentrated one [42]. Other studies were able to observe a decrease of proinflammatory cytokine levels (interleukin (IL) -6) [43]. Small et al. finally reported that under a long term twice a day local treatment with those emulsions no relevant CsA levels were detected in blood [44]. Besides all these objective improvements gathered by first local treatments with CsA, it was not less important that patients reported to be more satisfied by this kind of therapy and had to use less tear substitutes [45].

However, in contrast to these first and promising results, further research on novel formulations has been hampered by severe hurdles. Unfortunately, cyclosporin A suffers from high lipophilicity (log P = 3.0 [46]) and with 6,6 µg/ml an extremely poor solubility in water [47] and thereby, cannot be administered via common ophthalmic formulations like aqueous eye drops. Thus, in the past in most studies CsA was administered dissolved in vegetable oils or emulsions, also containing an oil phase [42,48,49]. However, due to a high affinity of the lipophilic drug to the oil vehicle, the bioavailability of drug in corneal tissue was quite low [46]. Furthermore, oily vehicles

are poorly tolerated at the eye. Patients report burning, stinging and foreign body sensation after treatment with such systems [36,50].

Therefore, a lot of research was conducted in recent years to fit the requirements of a ideal topical ophthalmic formulation. Such a perfect system should be well tolerated, easy in manufacturing and administration; it should prolong the drug residence time at the eye, have a long shelf life and finally avoid the systemic absorption [33]. Cyclodextrins, micellar systems, emulsions, liposomes and also nanoparticular systems were investigated. Promising results such as high drug loading in conjugation with an increased solubility, prolonged residence time and especially high corneal tissue levels were reported [20,51–55]. Unfortunately, many of these recently developed systems suffer from cumbersome manufacturing protocols or unclear biocompatibility [13,14,54,55].

Thus, there is still an immense need for formulations which are able to combine a high drug load with good biocompatibility and good bioavailability.

1.4. Goals of the thesis

This thesis is focused on the development of innovative liquid application systems for the poorly water soluble drugs cyclosporin A, budesonide and beclometasone for ophthalmic use. One of these formulations is an in-situ nanosuspension (INS). An INS is a clear and colorless solution consisting of drug, a non aqueous water miscible solvent, a surfactant and an amount of water that does not induce precipitation of the drug. Upon application and contact with the aqueous tear fluid the drug precipitates in the form of nanoparticles and is taken up by tissue (Fig. 4). A second approach for drug administration is the design of a self- assembling micellar formulation with special focus on a simplistic manufacturing and high biocompatibility. For the

development of such systems it was necessary to gather detailed information on the characteristics of solvents, additives and the drugs and especially on their interactions in terms of a controlled precipitation on the one hand and drug solubilization on the other hand. The challenge was to optimize such systems concerning simplicity, stability, biocompatibility and bioavailability in-vitro as well as in-vivo.

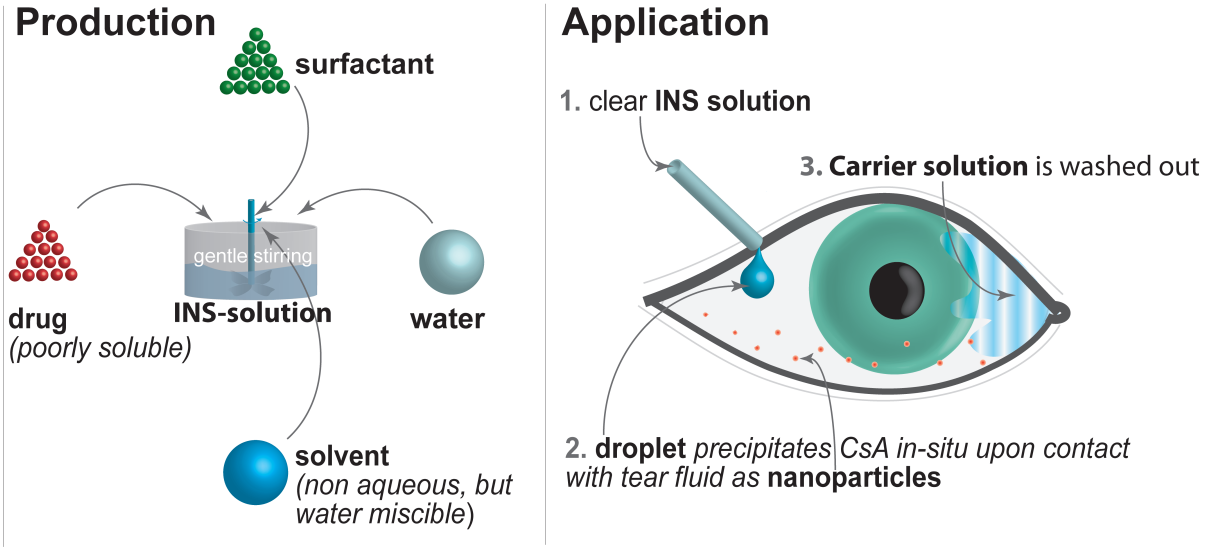

Fig. 4: A poorly soluble drug, a surface active additive and the maximum possible amount of water that doesn't induce a precipitation are solved in a non aqueous, but water miscible solvent to generate a clear solution by gentle stirring. In combination with the tear fluid, the administered droplet exceeds the critical water- content and with that the point of precipitation. The drug spontaneously precipitates into nanoparticles and is taken up by the tissue.

First, the field of possible solvents and additives was investigated to limit the options for the formulation process to few promising compounds **(Chapter 2)** to find the optimal parameters for the development of an INS. To this end, poly(ethylene glycol) – based solvents were researched in terms of their qualities to solubilize lipophilic drugs and additives. They were furthermore physicochemically characterized and their compatibility with polymers occurring usually for manufacturing, primary packaging or filter membranes was tested. Finally, a turbidimetric high throughput

(tHTP) analytical method was developed and installed to obtain detailed information on the precipitation behavior of the drugs upon contact with water.

In detailed tHTP studies, a defined composition of a cyclosporin A containing INS was designed **(Chapter 3)**. As size and charge are highly important parameters for an ophthalmic formulation of nanoparticles [14,18,20], the nanoparticles resulting from the INS were characterized in these regards. Moreover, a first stability study was conducted. As information on biocompatibility of a formulation and further on the bioavailability of the applied drug are essential, to this end, analytical models, methods and equipment for the in-vitro studies on tolerability towards primary human epithelial cornea cells (HCEpiC) and drug resorption at enucleated porcine eyes were developed and described.

In **Chapter 4** a second approach for the administration of poorly soluble drugs at the anterior eye was described for a self- assembling micellar system with simple manufacturing. In a first study, suitable non-ionic surfactants were characterized and selected towards the development of preclinical formulations. According to the INS, the biocompatibility of these micellar solutions was proven at HCEpiC cells. Finally, their efficiency in terms of drug delivery into corneal tissue was investigated with the established porcine cornea model.

As it is known from literature that surfactant micellar systems may be less stable than polymeric micelles [56,57], a long- term stability study was conducted to show to the stability and efficiency of the simplistic formulation **(Chapter 5)**.

Upon the information on biocompatibility and bioavailability, gathered by former in-vitro studies, in the final study **(Chapter 6)**, both formulations, the INS and the micellar solution were tested in-vivo on male rabbits to complete the set of pre-

clinical data. Their biocompatibility was investigated for placebo and drug- loaded preparations and the drug cornea levels reached for the drug- loaded samples were analyzed thereafter by the established analytical setup and methods.

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Chapter 2

Poly(ethylene glycol)-based solvents for a novel ophthalmic application system

Abstract

For the delivery of poorly soluble drugs and especially for novel ophthalmic application systems like an in-situ nanosuspension (INS), where the drug should precipitate upon contact with water, there is a high demand of suitable solvents. Therefore, in the present study we investigated the properties and suitability of different liquid poly(ethylene glycols) (PEG) and ether derivatives thereof. All solvents showed excellent drug solubilizing qualities, in which the dimethyl ether PEGs (DME) dissolved the highest concentrations of the observed drugs budesonide, beclometasone and cyclosporin A (CsA). Among the additives only Solutol HS15, a non ionic surfactant, produced perfectly clear and liquid solutions in all solvents. The lowest viscosity was observed for DME500. Due to their negligible amounts of water, all solvents, as obtained from the manufacturer, were generally suitable for an INS. Furthermore, they showed resistance towards different plastics used with primary packing materials or syringes. Especially for regenerated cellulose (RC), used as filter membrane, a good wetting as well as no drug adsorption during filtration was observed. By use of a turbidimetric high throughput screening and light microscopy, the INS concept and its dependency of the used drug and solvent could be proven. Unfortunately, a modulation or controlling of the precipitation with Solutol was not possible.

2.1. Introduction

The choice of suitable solvents or solvents for pharmaceutical applications has a tremendous impact on highly important parameters like biocompatibility, toxicity and, if a drug is present, also on resorption kinetics and, therefore, bioavailability [1,2]. Drugs with a poor solubility in water, like cyclosporin A (CsA), are often formulated with oily or organic excipients [3–5]. Unfortunately, irritations and low bioavailability are typical problems, especially after topical administration of such carriers [6]. Hence, on the one hand, new strategies and on the other hand, different kinds of solvents have to be investigated to enhance the efficiency of the applied drugs, and to keep patient compliance.

For topical and ophthalmic administration, various types of poly(ethylene glycols) (PEG) are known from literature. PEG and its derivatives, such as ethers or fatty acid esters, offer great properties like very good biocompatibility and water solubility. They show no immunogenicity, no reproductive-, developmental- or genotoxicity and also no carcinogenic properties. Beneficial features of this class of substances are moderate to no ocular or dermal irritations and an extremely low acute and chronic toxicity [7,8]. PEG400, for example, is found as plasticizer in ocular inserts. PEGs are also an ingredient in lubricant eye drops for the treatment of dry eye disease (DED) like in Systane® Ultra (Alcon Laboratories) [9,10]. Bain et al used an amount of 10 % PEG to decrease the gelation temperature of methyl cellulose to create an in-situ gelling system with prolonged retention time at the anterior eye [11]. The methyl ether derivative (MPEG) is found as a part of co-polymers like MPEG-hexyl-substituted polylactide which was used to solubilize CsA in polymeric micelles [8]. Due to their excellent transparency, a completely different approach was followed by Brandl et al, who used PEG based hydrogels as biodegradable intra-ocular drug

delivery systems [12]. Furthermore, Lennikov et al used liquid PEG to dissolve Astaxantin and investigated its UV protective properties towards the eye surface of mice in-vivo [13].

Based on this rich body of applications described for PEG, we decided to develop a novel kind of liquid, aqueous formulation using the advantages of PEG to overcome solubility barriers for hydrophobic drugs. Our intention was to overcome the use of oily vehicles and to enhance the presence of the instilled drug at the anterior eye. Our approach was to make use of the solubilizing qualities of PEG, to dissolve poorly water soluble drugs and then incorporate water up to a critical amount, where the solution is still “stable”. If such a system gets into contact with water, like for example after application at the eye with the tear fluid, a spontaneous precipitation of drug would be induced. In the present work, we investigated the suitability of different liquid poly(ethylene glycols) towards such an in-situ precipitating system for the lipophilic model drugs budesonide, beclometasone and cyclosporin A.

2.2. Materials and methods

2.2.1. Materials

Cyclosporin A (CsA), budesonide and beclometasone were supplied by the Pharma Stulln GmbH (Stulln, Germany). Solutol® HS 15, Soluplus®, Methocel® E15, Kollidon® 17PF and Kollidon® 12PF were a kind gift from the BASF (Ludwigshafen, Germany). Polyglycol 400 a poly(ethylene glycol) (PEG) (M_W = 400 g/mol), Polyglycol M250, M350 and M500, three mono-methoxy-PEGs (M_W = 250 g/mol, 350 g/mol, 500 g/mol) and Polyglycol DME250 and DME500, two dimethoxy-PEGs (M_W = 250 g/mol and 500 g/mol), were supplied by Clariant Produkte GmbH (Frankfurt a. M., Germany). Deionized water was obtained from a Milli-Q water purification system from Millipore (Schwalbach, Germany). MeOH and MeCN (both HPLC grade) were purchased from Merck (Darmstadt, Germany).

2.2.2. Solubility assessment of drugs

500 mg of drug, budesonide, beclometasone and cyclosporin A were each dissolved in 1 ml of the solvents PEG400, M250, M350 and DME250. The samples were gently stirred for 24 hours at room temperature to make sure that the solutions were truly saturated with a sediment of drug still present. After centrifugation at 16.000 g for 15 min, 100 µl of each sample were diluted with 900 µl of MeOH. 100 µl of the already diluted CsA samples were diluted once more with 900 µl of MeOH for analysis.

The quantitative analysis of the residual drug content was carried out using high pressure liquid chromatography (HPLC). For each drug, a stock solution (SL) with a concentration of 5 mg/ml was prepared in MeOH. The SLs were diluted to

concentrations of 5, 1, 0.5, 0.1, 0.05 and 0.01 mg/ml with MeOH. The external standards were stored at -80°C until further analysis.

HPLC analytics of budesonide: The Shimadzu chromatographic equipment (Shimadzu Deutschland GmbH, Duisburg, Germany) consisted of a SCL-10A_{VP} controller and a LC-10AT_{VP} pump that operated with a flow rate of 1 ml/min. Further, a SIL-10AD_{VP} auto injector with a sample volume of 10 µl, a CTO-10AS_{VP} oven with a temperature of 22 °C and the UV detector SPD-10A_{VP} at a wavelength of λ = 244 nm. As solid phase a reversed phase octadecyl column Luna 3µ C18(2) 100A with 100 x 4.6 mm was used (Phenomenex Ltd., Aschaffenburg, Germany). Isocratic elution was carried out with the methanol – water mixture (80:20, v/v) over 5 min.

HPLC analytics of beclometasone: Chromatographic conditions were in general as described for budesonide using slightly different parameters. The flow rate was set to 1,5 ml/min, the oven temperature was kept at 30 °C and UV detection was carried out at 238 nm. An isocratic elution was conducted with the MeCN – H_2O mixture (60:40, v/v) over 10 min.

HPLC analytics of cyclosporin A: Chromatographic conditions were again as described for budesonide with slightly different parameters. The flow was set to 1.0 ml/min, the oven temperature was kept at 75 °C and UV detection was carried out at 210 nm. The injection volume was 15 µl. The mobile phase consisted of 75 % MeCN and 25 % H_2O (v/v). A gradient over 5 min to 90 % MeCN and 10 % water was applied. After 12.5 min the column was again re-equilibrated to the initial conditions until the end of the run at 15 min.

2.2.3. Solubility of additives

20 mg, 50 mg and 100 mg of the additives Methocel E15, Solutol HS15, Soluplus, Kollidon 17PF and Kollidon 12PF were dispersed in 1 ml of each solvent and stirred using a Variomag® Multipoint stirrer (2mag-USA, Daytona Beach, FL, USA) for 24h at room temperature in transparent glass vials. The mixtures were visually inspected in terms of transparency, homogeneity and eventual presence of particles or not dissolved material.

2.2.4. Viscosity

The temperature- dependent viscosity of the solvents was recorded using an AR 2000 rheometer (TA Instruments, Eschborn, Germany) equipped with a plate – cone steel geometry with an angle of 2° and a diameter of 60 mm. The co-solvent was casted on the bottom plate of the rheometer and the cone was lowered to a gap size of 1000 µm. The system was shielded by a solvent trap, to avoid evaporation or other influences of the environment. At a constant shear rate of 100 s^{-1} the temperature ramp from 4 to 40 °C over a period of 20 min was started and viscosity was recorded. The measurements were performed in triplicate.

2.2.5. Water- content of PEG

The water- content of the solvents as contracted from the manufacturer was determined using Karl Fischer titration. Measurements were performed using a Moisturemeter MCI Model CA-02 (Mitsubishi Chemical Industries Limited, Tokyo, Japan). A 1 ml syringe was filled with sample and weighed. About one third of the syringe's volume was injected into the reaction vessel and the mass of water was measured. The syringe with the remaining sample was weighed to determine the

injected mass. Measurements were performed in triplicate for each liquid. Afterwards the water- content was calculated as

$$x\ \%\ (w/w)\ = \frac{m_{H2O}}{m_o} \cdot 100,$$

where $x\ \%\ (w/w)$ is the water- content, m_{H2O} is the measured mass of water and m_o is the mass of the injected sample.

2.2.6. Plastics resistance towards the solvents

Discs of about 1x1 cm were cut out of polypropylene (PP), polyamide (PA) and celluloseacetate (CA) bars (Probesammlung zur Kunststoffkunde, Verband Kunststofferzeugende Industrie e.V., Frankfurt a.M.). A filter membrane made of regenerated cellulose (RC) was excised of a Spartan™ 13 mm syringe filter (pore size = 0.2 µm) (Whatman GmbH, Dassel, Germany). The samples were transferred in glass vials and covered with 5 ml of PEG400, M250, M350 and DME250, respectively. Further, 4 BD Discadit™ II syringes (Becton Dickinson GmbH, Heidelberg) of PP (tube) / poly ethylene (PE) (plunger) / oleamid (anti-blocking agent) were filled with the solvents. All samples were stored at room temperature under light exclusion for 15 days. They were examined visually every day in terms of transparency of the solution and transformation of the slices. At day 15, UV – spectra were taken of the pure solvents and the samples over a range from 190 to 900 nm using a UVIKON 941 double beam UV/VIS spectrophotometer (KONTRON Instruments, Neufarn, Germany) to detect the potential dissolution of the different polymers. The samples were prepared and measured in triplicate.

2.2.7. Contact angle measurements

The contact angle of water and solvents (PEG400, M250, M350 and DME250) was measured on a RC filter membrane, excised of a Spartan™ 32mm syringe filter, using a Dataphysics contact angle system OCA 15 Plus (Dataphysics Instruments GmbH, Filderstadt, Germany). One droplet of sample was deposited on the membrane and the contact angle was recorded in triplicate.

2.2.8. Drug adsorption on RC-filter membranes

Solutions with a concentration of 1 mg/ml of drug in PEG400 were prepared with budesonide and CsA by gentle stirring. The transparent solutions were filtered through a Spartan™ 13mm syringe filter with a pore size of 0.2 µm. 100 µl of the non-filtered solutions and the filtered solutions, respectively, were diluted with 900 µl MeOH in HPLC vials. The quantitative analysis was carried out by HPLC as described in 2.2.2.

2.2.9. Turbidimetric high throughput screening

The turbidimetric high throughput (tHTP) screening was performed to determine the critical water- content and hence, the solubility of budesonide and beclometasone in ternary as well as in quaternary systems (Tab. 2.1). The turbidity was measured at a single wavelength of λ = 550 nm using a Titertek Plus plate reader (ICN Biomedicals Ltd, High Wycombe, UK). All measurements were performed triplicate in a 96 well quartz microplate (Hellma, Müllheim, Germany). The initial sample volume was 50 µl per well. The first well in each row was a blank solution. The plate was alternately measured at λ = 550 nm, titrated with 2 µl water per well, gently vortexed at level 3 using a Vortex-Genie®2 equipped with a 3-inch platform with rubber cover (Scientific

Industries, Inc., Bohemia, USA) and again measured at λ = 550 nm. This cycle was repeated until each well showed a precipitation. The absorbance of the increasing concentration of H2O (% (w/v)) was plotted against the drug concentration (% (w/v)). The point of precipitation (PoP) was defined as the concentration of water at which the absorbance starts to increase by at least 30 % with respect to the mean of the five previous absorbance values.

2.2.9.1. Sample preparation for measurements in ternary systems (drug, solvent, water)

The solvents PEG400, M250, M350 and DME250 were investigated with CsA and budesonide. In combination with budesonide additionally M500 and DME500 were investigated. With every solvent a colorless, transparent stock solution of 10 mg/ml budesonide and 20 mg/ml beclometasone was produced by gentle stirring. The stock solutions were diluted with the appropriate solvent to concentrations of 1, 0.75, 0.5, 0.25, 0.1 % budesonide (w/v) and 2.0, 1.5, 1.0, 0.5, 0.25, 0.1 % beclometasone (w/v) (Tab. 2.1).

2.2.9.2. Sample preparation for measurements in quaternary systems (drug, solvent, surfactant, water)

Solubility in quaternary systems was investigated for budesonide only (Tab. 2.1). Two different initial concentrations of the non-ionic surfactant Solutol were tested. Two stock solutions were produced: stock solution 1 (SL_1) containing 2 % (w/v) budesonide, stock solution 2 (SL_2) containing 40 % (w/v) Solutol and 10 % (w/v) Solutol, respectively. Stock solution 3 (SL_3) with c(budesonide) = 1 % (w/v) and c(Solutol) = 20 % (w/v) and accordingly c(Solutol) = 5 % (w/v) was received by mixing 1 ml SL_1 with 1 ml SL_2. SL_4 was obtained by dissolving 200 mg/ml and 50 mg/ml

Solutol, respectively, in the different solvents. The SL_3 was diluted with the corresponding SL_4 to 5 concentrations of 1, 0.75, 0.5, 0.25, 0.1 % budesonide (w/v).

Tab. 2.1: tHTP-screening; overview of the sample preparation and measurement cycle for the determination of the PoPs with budesonide (BUD) and beclometasone (BEC); left side: composition of all ternary systems; right side: composition of the quaternary systems; All concentrations are displayed (w/v);

PEG400	M250	M350	M500	DME250	DME500
ternary systems					
				quaternary systems	
				+ Solutol	
				5%	20%
+ BUD	+ BEC			+ BUD	+ BEC
1.00 %	2.0 %			1.00 %	1.00 %
0.75 %	1.5 %			0.75 %	0.75 %
0.50 %	1.0 %			0.50 %	0.50 %
0.25 %	0.5 %			0.25 %	0.25 %
0.10 %	0.25 %			0.10 %	0.10 %
	0.10 %				
+ stepwise 2 µl H_2O; vortexing; turbidimetric measurement at λ = 550 nm					
calculation of the PoPs					

2.2.10. Light microscopy of a budesonide precipitate

A 0.5 % (w/v) budesonide solution in DME250 was prepared by gentle stirring. 400 µl of this solution were precipitated with 600 µl of H_2O, to exceed the PoP. 15 µl of the dispersion were put on a glass slide and spread by adding a coverslip. Light microscopy pictures were taken using a Leica DM IRB microscope equipped with a PL Fluotar 10x (NA 0.3; PH 1) objective (Leica Microsystems Inc, Deerfield, IL, USA) and the EclipseNet Software (Laboratory Imaging, s.r.o., Praha, Czech Republic).

2.2.11. pH and osmotic pressure measurements of carrier mixtures

Aqueous stock solutions of M500 and DME500 were prepared with concentrations of 10 % and 20 % (w/v) for each solvent. The samples were prepared by addition of 0, 5, 10 and 20 % (w/v) Solutol to each stock solution. pH was measured at room temperature under gentle stirring using a Sartorius PB-11 basic pH meter equipped with a pH combination electrode PY-P11 (Sartorius AG, Goettingen, Germany). The osmotic pressure was determined using a Knauer – semi micro osmometer K7400 (KNAUER Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin, Germany).

2.2.12. Short term stability of budesonide and cyclosporin A

Aqueous solutions of 85 % M500 and DME500, respectively, containing 0.1 % (w/v) budesonide and 1 % CsA, respectively, were prepared by gentle stirring. Due to a known lowered stability of CsA in the presence of ionic additives, like sodium chloride [14], or phosphate buffered systems (data not shown), the solutions were not buffered. The 1 ml samples were stored at 25°C and 65 % relative humidity. Budesonide samples were drawn at day 0, 2, 4, 8, 23, 36 and 43. CsA samples were taken at day 0, 1, 3, 5, 8, 15, 29 and 43. All samples were centrifuged at 16000 g for 15 min. 100 µl of the supernatant were diluted with 900 µl of MeOH and stored at -80°C until further analysis. The quantitative analysis was carried out by HPLC as described in 2.2.2. All samples were prepared in triplicate.

2.3. Results and discussion

2.3.1. Solubility of drugs

As a first essential feature, the solubilizing capacities of the solvents PEG 400, M250, M350 and DME250 were investigated for the three poorly soluble drugs budesonide, beclometasone and cyclosporin A. The results are displayed in Fig. 2.1. All solvents showed excellent solubilizing properties. Budesonide which has a solubility in water of about 14 µg/ml [15] was dissolved in levels of at least 12.5 ± 0.6 mg/ml in M350 and at a maximum of 21.8 ± 0.8 mg/ml in DME250, which is a 900 fold increase of the amounts in water. The dissolved beclometasone concentrations were between 24.4 ± 4.3 mg/ml and 55.4 ± 6.5 mg/ml. In comparison, its solubility in water is approximately 2 µg/ml [16]. Cyclosporin A, with a solubility in water of 6.6 µg/ml [17], showed the highest solubility increase. There were at least 100 mg/ml soluble in PEG400 and up to 293.5 ± 9.3 mg/ml in DME250. Due to problems with the purification for the HPLC samples, the exact values for CsA in PEG400 could not be determined. In all experiments the solutions of a minimum of 100 µg/ml were perfectly transparent.

The soluble concentrations of the three drugs exceed clearly the typically used amounts in various formulations like for example suspensions, ethanolic solutions or micreoemulsions [1,18–22]. Hence, due to their solubilizing qualities, all solvents seemed to be promising options for the INS.

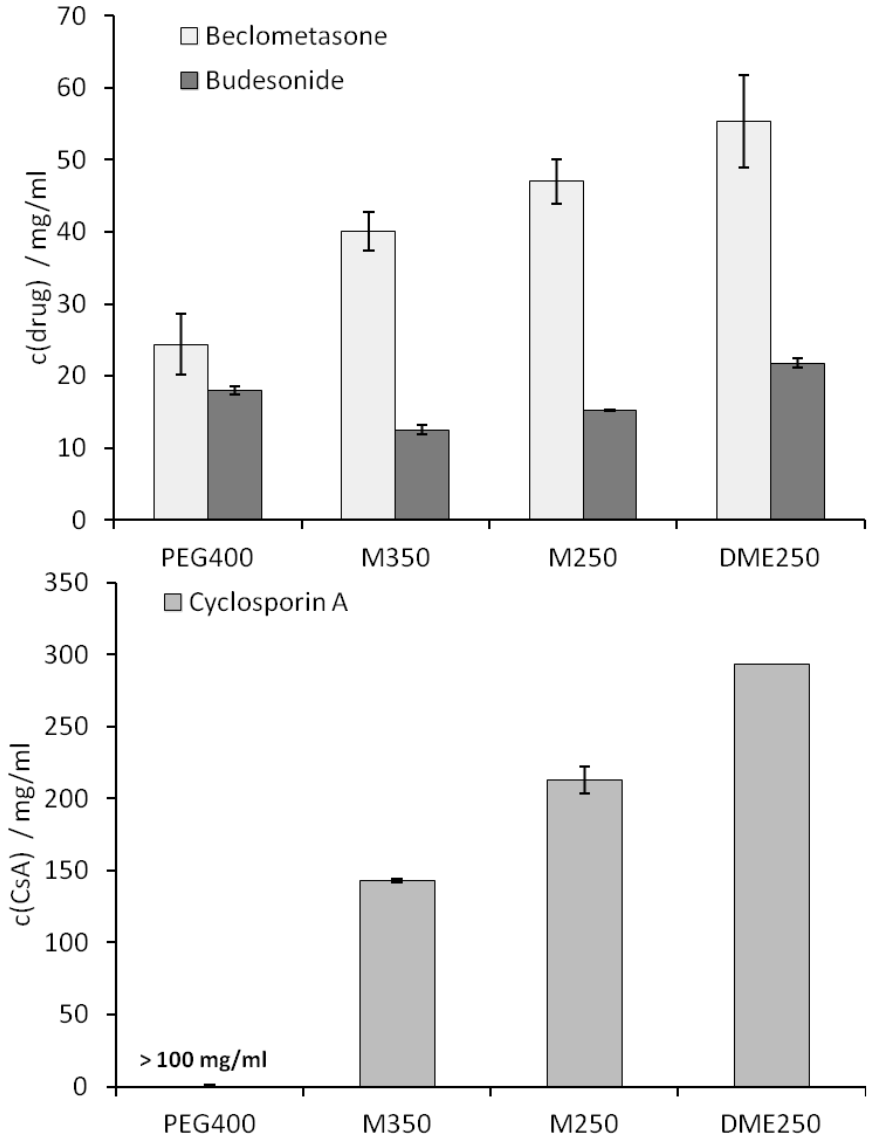


Fig. 2.1: Solubility of (upper panel) Budesonide (■), beclometasone (□) and (lower panel) cyclosporin A (□) in liquid poly(ethylene glycols) determined by HPLC; (n=3)

2.3.2. Solubility of additives

In order to modulate and control an INS in terms of particle growth and size following precipitation in water as well as their retention time at the site of application, such as the eye surface, additives like viscosity enhancers (VE), surfactants (AS) or other solubilizing agents (OS) were regarded as an option. Hence, the solubility of various additives in the solvents was investigated. The results of the screening are shown in

Tab. 2.2. With Methocel E15 a VE and Soluplus an AS only opaque dispersions were observed. Neither at low concentrations of 2 % nor at high concentrations of 10 % both substances could be dissolved. Both, Kollidon 12PF and 17PF, two OS, showed transparent and colorless solutions in all liquids despite the di-methylether derivatives DME250 and DME500. This may be due to their higher lipophilicity stemming from their methoxy groups. The only additive which showed perfectly clear solutions in all solvents was the non-ionic surfactant Solutol HS15. At all concentrations generated transparent mixtures were obtained. Due to its compatibility with all media, Solutol was used as a model additive for all further investigations.

Tab. 2.2: Solubility of viscosity- and solubility-enhancing additives in different concentrations in the co-solvents; (● = clear, transparent and colorless solution)

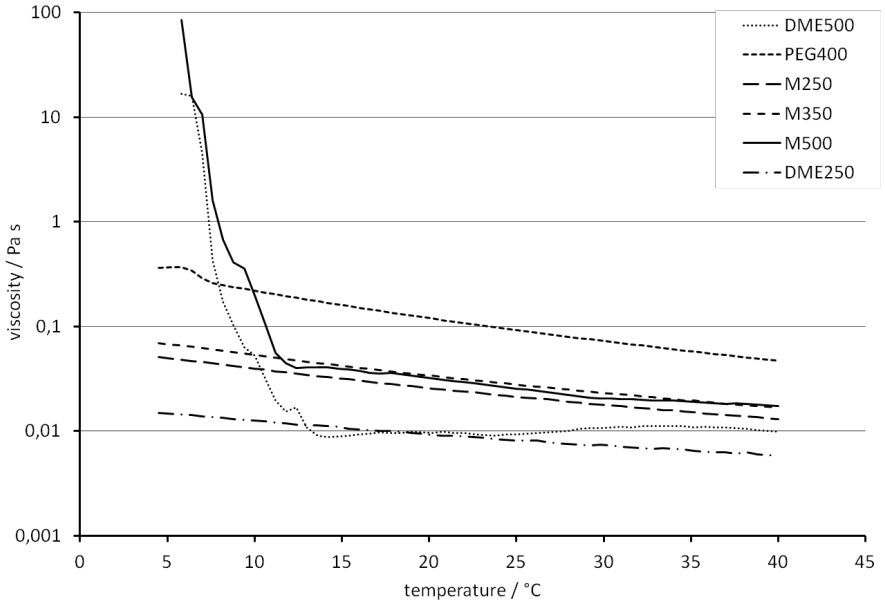
	PEG400			M250			M350			M500			DME250			DME500		
c(add)/%	2	5	10	2	5	10	2	5	10	2	5	10	2	5	10	2	5	10
Methocel E15																		
Solutol	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Soluplus																		
Kollidon 17PF	●	●	●	●	●	●	●	●	●	●	●	●						
Kollidon 12PF	●	●	●	●	●	●	●	●	●	●	●	●						

2.3.3. Viscosity of the solvents

The viscosity of the pure solvents was determined at temperatures from 4 to 40°C. The results and corresponding curves are displayed in Fig. 2.2. The solvents showed a temperature dependent decrease of the viscosity, which is typical for liquids, due to the lower molecular interactions at higher temperatures [23]. Only for DME500 a

nearly constant viscosity of around 10 mPa s was determined between 13 and 40 °C. Furthermore, the viscosity showed a decrease with declining molecular weight on the one hand, and with end-capping, on the other hand. Hence, for PEG400 the highest levels and for DME250 the lowest levels were observed. At a temperature below 13 °C, M500 and DME500 showed a tremendous viscosity increase to levels higher than 16 Pa s at 4 °C. This increase is due to melting temperature of 12°C and 13°C for M500 and DME500, respectively, reported by the supplier for the two solvents with the highest molecular weight. Due to their lower melting point, the other solvents were less prone to viscosity changes when lowering the temperature to 4°C. At a temperature of 34°C, typical of the eye surface [24], the viscosity of all solvents was between 7 and 20 mPa s except for PEG400 which had a viscosity of 60 mPa s. As reported by Han et al, the ideal viscosity of lubricant eye drops for the treatment of dry eye disease is around 6 – 12 mPa s [25]. Thus, the pure solvents nearly were in this range, but there would still be options for the formulation process, especially because Han et al reported levels at room temperature, which were slightly higher. Values reported for PEG400 in the literature fitted the observed viscosity levels and proved the results [25]. Further, the data reported in the material safety data sheet of the supplier matched the results at the comparable temperatures.

In conclusion, all the solvents showed good fluidity, but also enough viscosity to be able to be retained on the eye surface. Due to its obviously missing temperature dependency between 13 and 40°C but constantly increased viscosity, DME500 showed very interesting features for an ophthalmic formulation.



	4°C	Lit	25°C	Lit	34°C	Lit (35°C)	MSDS Clariant		
	mPa s	mPa s	mPa s	mPa s	mPa s	mPa s	mPa s	T /°C	Solidification / °C
PEG400	364	-	92	94.4	60	55.6	116	20	4
M250	51	-	21	-	16	-	25	20	-26
M350	69	-	28	-	20	-	11	50	-6
M500	84630	-	25	-	20	-	22	50	12
DME250	15	-	8	-	7	-	8	20	-23
DME500	16660	-	9	-	11	-	16	40	13

Fig. 2.2: Viscosity as a function of temperature from 4°C to 40°C of liquid poly(ethylene glycols) PEG400, M250, M350, M500, DME250 and DME500; n = 3;

2.3.4. Moisture of non-aqueous solvents

As the INS preparation should precipitate spontaneously upon contact with water such as in the case of the aqueous tear fluid, it is of utmost importance to control the water- content of the formulations exactly. The water- content of the contracted solvents was determined by Karl-Fischer titration. All solvents showed only traces of water which decreased with end-capping and increasing chain length (Fig. 2.3). This

is in accordance with the results determined by Cohen et al. [26] who found a decreasing hygroscopicity for polyethylene glycols with an increasing molar weight.

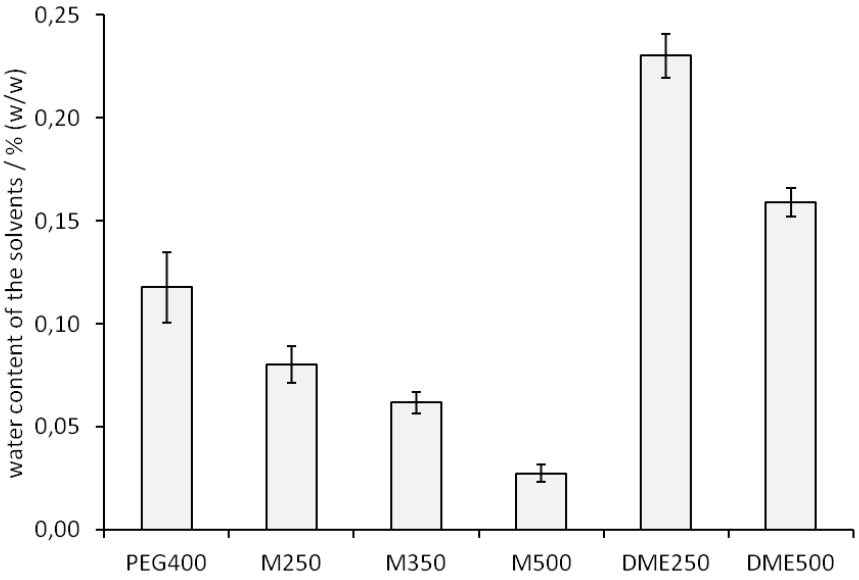


Fig. 2.3: Water- content of the different liquid polyethylene glycol co-solvents determined by Karl-Fischer titration;

The insignificantly low concentrations of water in the pure solvents were neglected for the further development of the INS.

2.3.5. Compatibility of the solvents towards plastics and filter membranes

In the process of production and packaging pharmaceuticals get into contact with many devices made of polymers. In order to prove the compatibility, we investigated the UV spectra of the solvents before and after incubation with polymers bottles, syringes or filter membranes are made of. The UV spectra are displayed in Fig. 2.4. The spectra after storage with packing materials like PP or PA as well as the spectra after storage in a PP / PE / oleamid syringe did not differ from the spectra of the pure solvents. Also the filter membrane built of RC did not cause a change of the solvent's spectrum, no matter which solvent was used. Only the filter membrane made of cellulose acetate (CA) was dissolved by all solvents. An obvious opacity was

determined visually and all the spectra showed clear changes in comparison to the pure solvents and the other investigated plastics. These results fit the data in the literature. CA is known to be instable with solvents and RC is offered as a solvent-resistant alternate material [23].

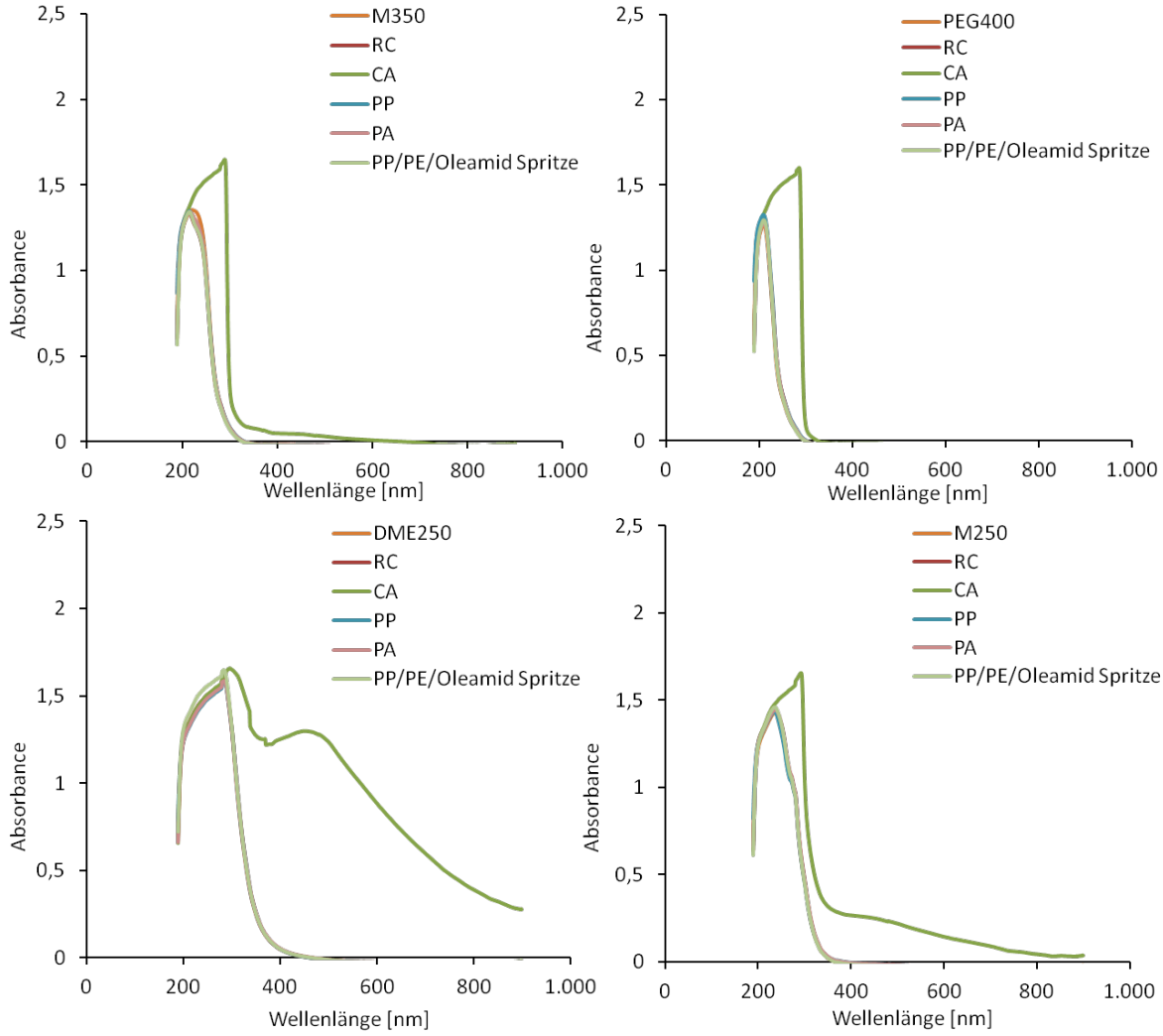


Fig. 2.4: Compatibility of the co-solvents with different plastics of possible filter membranes, primary packing materials or syringes proven by UV-spectra; pure co-solvent, RC, CA, PP, PA, PP/PE/oleamid syringe; n = 3;

In order to investigate the suitability of RC as a filter membrane for sterile filtration, the contact angle as a measure for the wettability with the solvents was determined. The results are shown in Fig. 2.5 (A). Nearly similar angles between 20° and 35° at the RC surface were observed for all solvents and for water which was used as a

reference. Due to the fact that the observed angles were very low, we expected a quite good filterability from these results.

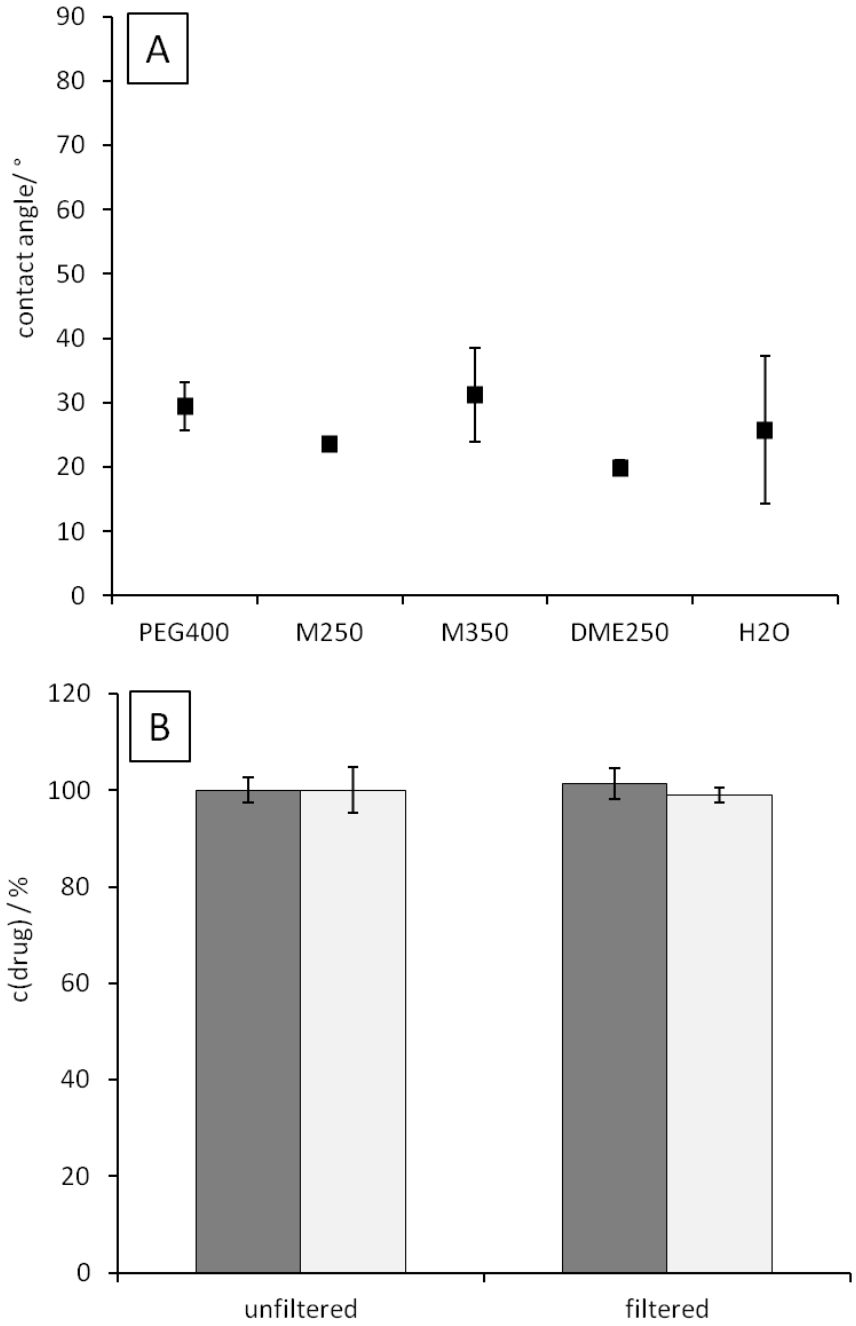


Fig. 2.5: (A) Contact angle on a RC filter membrane of liquid poly(ethylene glycols); (n=3); (B) drug adsorption on a RC filter membrane; ■ budesonide, □ cyclosporin A; (n=3);

The RC membrane was further investigated in terms of drug adsorption. Solutions of 1 mg/ml budesonide and cyclosporin A in PEG400 were analyzed by HPLC before

and after filtration. The data is displayed in Fig. 2.5 (B). As RC membranes are usually known to have low tendency towards drug adsorption and high recovery rates [27], the membrane accordingly showed a recovery of 100 % for CsA, as well for budesonide. From this data, it can be concluded that materials such as PP, PA or PE as well as filter membranes out of RC should be suitable for use in production or primary packing of the INS.

2.3.6. Proof of the 'in-situ Nanosuspension' concept

As the INS concept is based on a spontaneously induced, but defined drug precipitation upon contact with a physiological liquid like the tear fluid, the essential task was to investigate this precipitation in terms of its reproducibility, the dependency of different solvents, drugs and their concentrations. The PoPs, determined by a turbidimetric HTP screening, are shown in Fig. 2.6.

As expected, the critical amounts of water increased with decreasing drug levels. PEG400, M250 and M350 thereby showed the lowest PoPs and their behavior was nearly identical over the whole range. With DME250, however, the PoPs were obviously higher. For 0.03 % beclometasone and 0,04 % budesonide, the critical amounts of water were up to 55.4 ± 0.7 % and 70.2 ± 0.0 %, respectively. Due to its higher critical amounts of water, budesonide was additionally investigated with M500 and DME500 as solvents and with levels of 74.5 ± 0.1 % and 77.2 ± 0.2 % water the critical amounts of water were even higher than for DME250. In general, beclometasone clearly showed lower PoPs than budesonide, no matter which solvent was used. This may be due to the lower solubility of beclometasone in water, as mentioned in 2.3.1. Due to the fact as it was shown in preliminary experiments for CsA (data not shown), that surface active agents are suitable to change the

precipitation in terms of particle size and distribution, the influence of 5 % or 20 % Solutol on the critical water- content was investigated for budesonide.

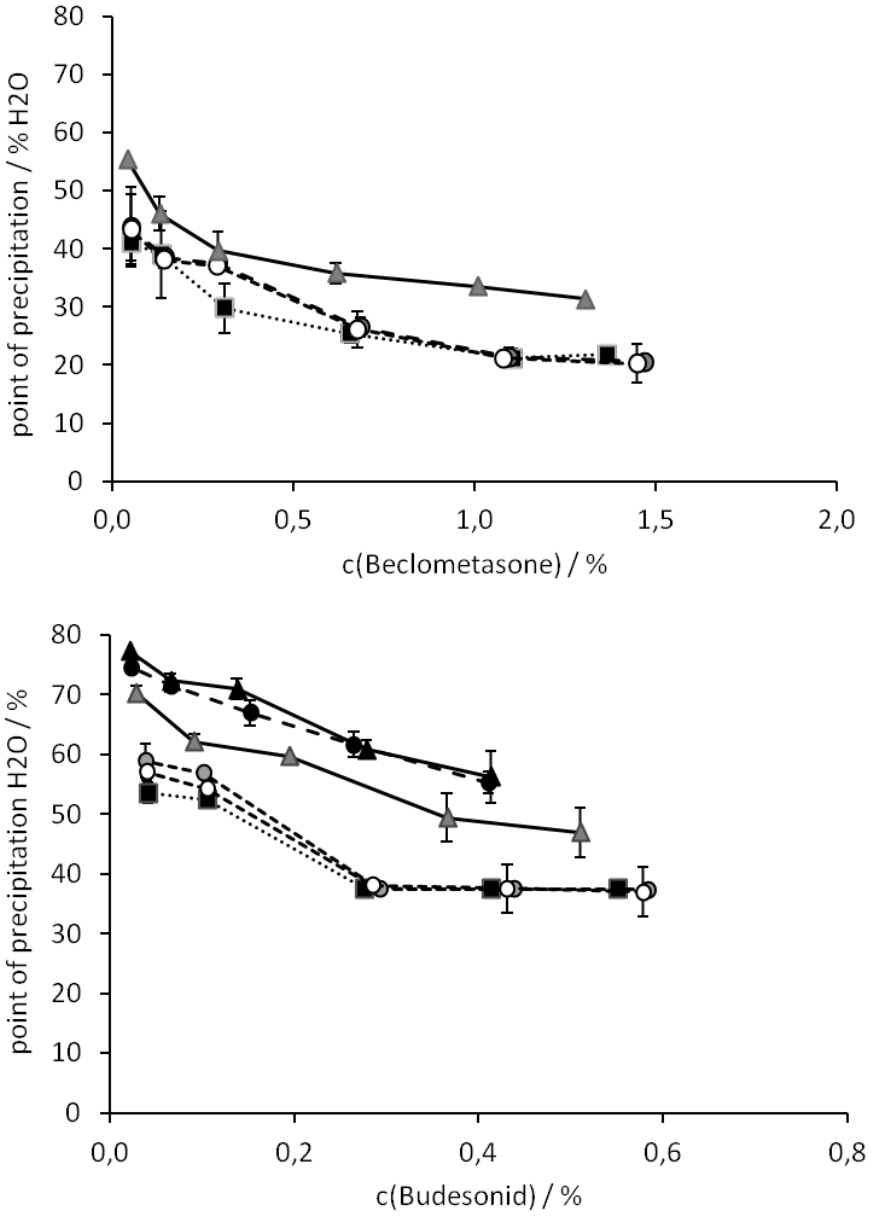


Fig. 2.6: Drug solubility and determination of the points of precipitation in ternary mixtures of drug, co-solvent and H_2O for beclometasone (upper panel) and budesonide (lower panel); PEG400 (■), M250 (○), M350 (◌), M500 (●), DME250 (△), DME500(▲);

Because of their excellent behavior in the ternary systems, M500 and DME500 were chosen as solvents for these experiments in quaternary systems. Unfortunately, the tHTP screening in the quaternary systems of drug, solvent, surfactant and water

showed no further precipitation. The solutions stayed stable and formed most likely micellar structures that encapsulated the drugs.

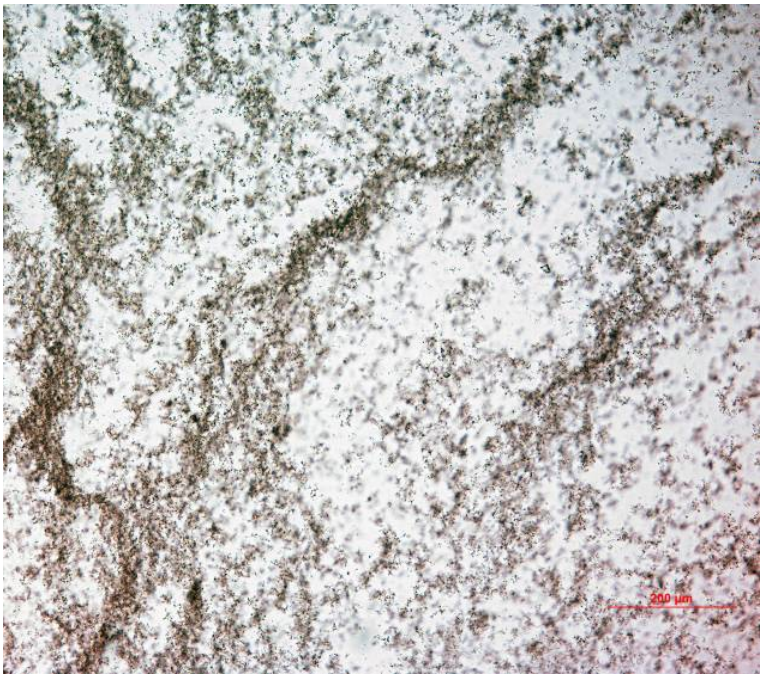


Fig. 2.7: Light microscopic picture of the precipitate of a 0.5 % budesonide in DME250 at 10fold magnification; scale bar 200 µm;

Finally, to visualize the precipitated structures of the ternary systems, light microscopy pictures were taken. Fig. 2.7 shows the microscopic picture of a 0.5 % budesonide in DME500 precipitate as an example. The structures were “cloudy” and agglomerated. No individual, defined structures were detectable, as expected due to the missing stabilizing additive. In conclusion, the functionality of the basic concept of the INS could be proven for poorly soluble drugs. The small standard deviations of the precipitation experiments show that it was possible to precipitate the drugs reproducibly at defined critical water amounts. Furthermore, by use of suitable solvents, high levels of water could be incorporated until precipitation occurred which would be very beneficial in terms of physiological compatibility. Regrettably, the addition of Solutol in combination with budesonide had no further influence on the critical water amounts and on the precipitated particles and their size. It even

inhibited the whole precipitation. Therefore, to control an INS containing budesonide, efficient additives still have to be identified.

2.3.7. Physicochemical characterization of INS carrier mixtures

Placebo carriers of the INS containing additive (Solutol), solvent (M500 or DME500) and water were investigated in terms of osmotic pressure and pH, to estimate their physiological tolerance. The pH of all mixtures was in a range from 3,9 to 6,7 (Fig. 2.8). It increased with increasing levels of Solutol. At higher solvent concentrations, for example 50 % or 80 % without Solutol, the pH was clearly higher.

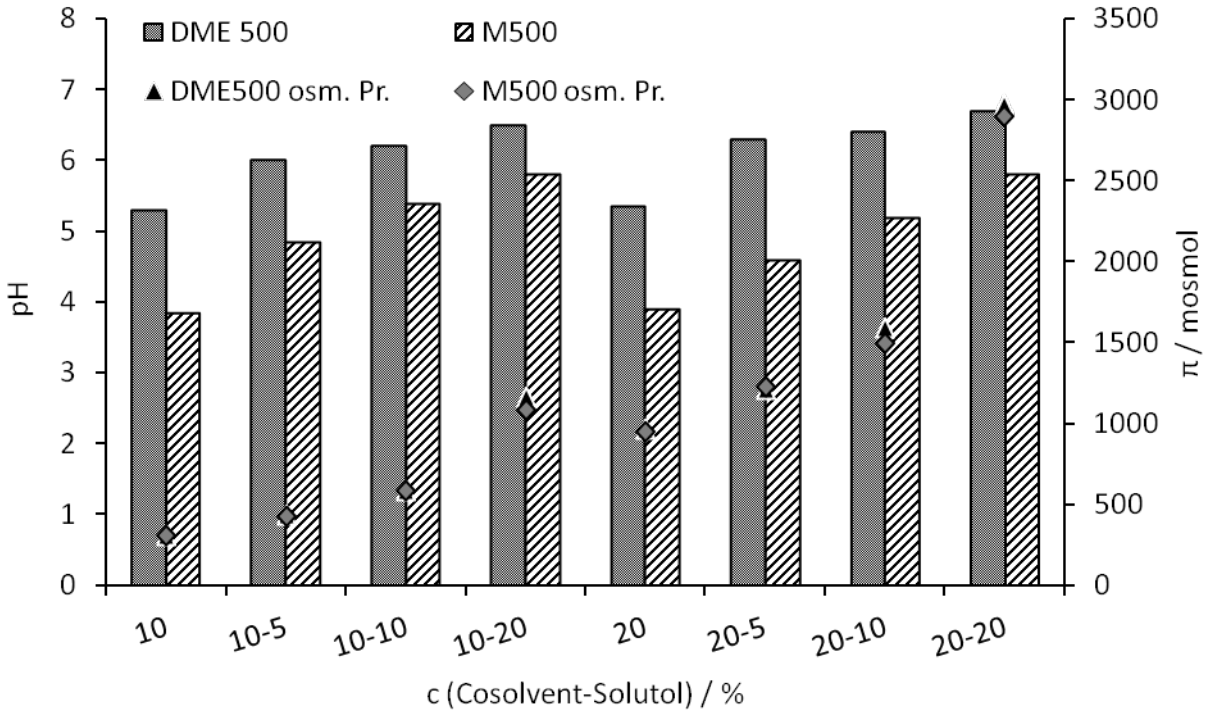


Fig. 2.8: Osmotic pressure (M500(◆), DME500(▲)) and pH (M500(□), DME500(■)) of different aqueous co-solvent - solubilizer combinations;

The values were 6,8 and 8,4 (data not shown). Mixtures with DME500 showed clearly higher values than with M500. The osmotic pressure ranged from nearly isotonic solutions of 10 % solvent with 304 and 307 mosmol to clearly hypertonic

solutions of 20 % solvent and 20 % Solutol with 2915 and 2895 mosmol for DME500 and M500, respectively. In general, all mixtures were hypertonic. Levels of 572 mosmol are reported to be usually tolerated at the anterior eye without side effects [28]. In addition, it was disclosed in the suppliers' material safety data sheet (MSDS) that even the pure solvents showed no irritation at rabbits' eyes (Method: OECD 405).

As a result of the tHTP screening (2.3.6) an INS would have a level of 20 % or even more solvent. However, in terms of a formulation, it has to be proven in-vivo if the high osmolarities will be tolerated.

2.3.8. Short term stability of budesonide and CsA

In order to assess the compatibility of the solvents with the drug, the stability of budesonide and CsA was investigated with M500 and DME500 at increased solvent concentrations over a period of 43 days at room temperature. As shown in Fig. 2.9, the concentration of budesonide, dissolved in M500, starts to decrease with day 23 down to less than 80 % of the initial concentration at day 36. Dissolved in DME500, the degradation starts right from the beginning down to less than 40 % of the initial concentration on day 36. It is reported in the literature for the stability of budesonide that the optimum pH range is 4 – 5. The more the pH reaches higher values, budesonide degrades rapidly [29]. The solutions had a pH of 6.7 and 8.5 for M500 and DME500, respectively. That seemed to be one reason for the more rapid loss of budesonide with DME500. But also the pH in M500 was higher than the pH in the stability optimum of budesonide, which may be an explanation for the decrease after 23 days. Due to this strong decrease, the measurements were stopped after 36 days for both solvents. The use of DME500 seemed not to be possible for INS in

combination with budesonide. With M500, a defined pH adjustment and further compatibility study need to be conducted to obtain stable formulations and exclude effects of the solvent itself on the degradation of the drug.

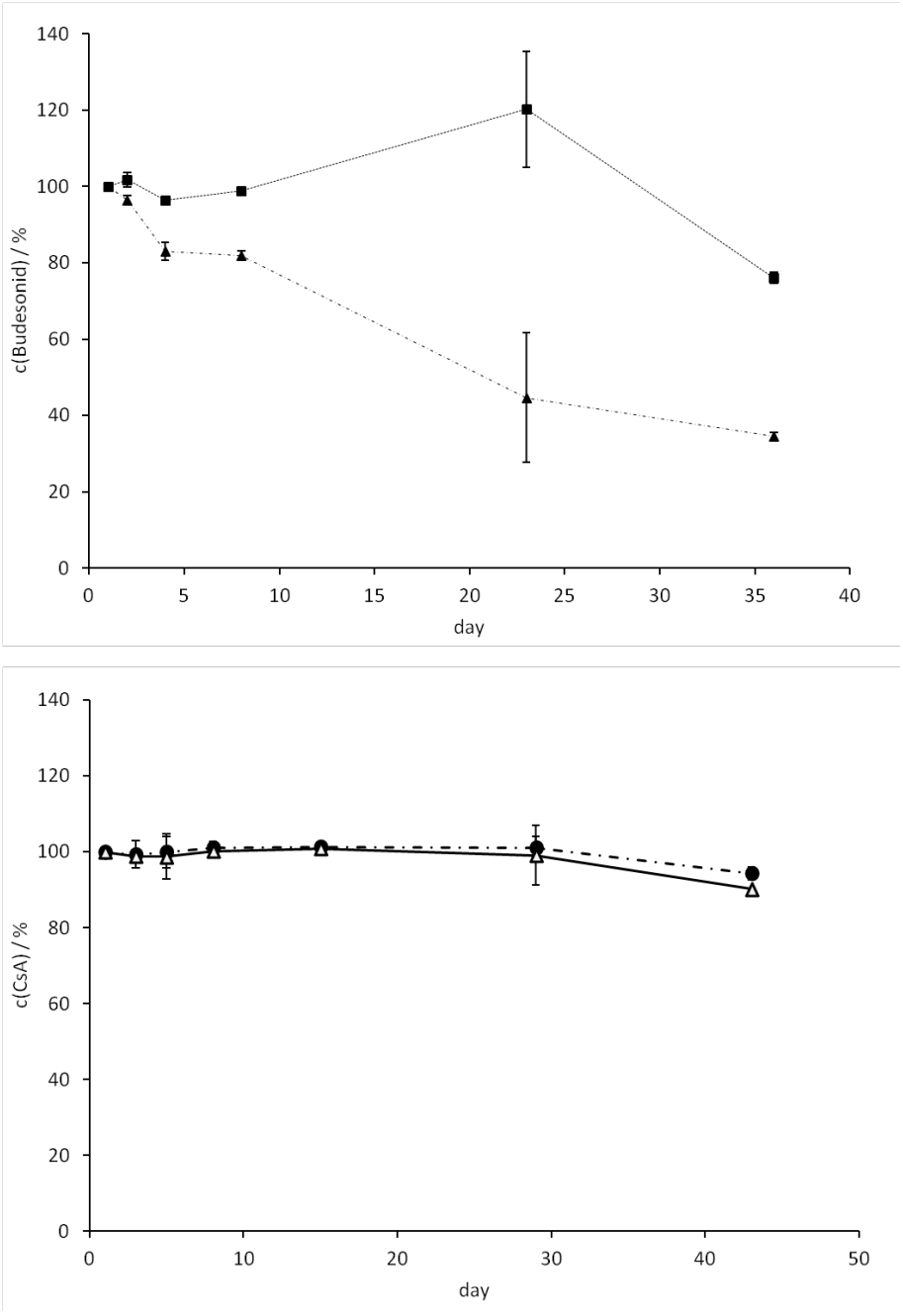


Fig. 2.9: Drug stability of Budesonide (upper panel) and CsA (lower panel) in 85 % M500 (▲) and 85 % DME500 (■) over a period of 36 and 43 days; measurements were performed in triplicate;

The CsA solutions had a pH of 8.5 and 7 for DME500 and M500, respectively. A pH related instability as seen for budesonide was not expected. CsA showed stability over the whole period for both formulations. The residual amounts were above 90 % of the initial CsA concentration. Slightly higher levels of drug were detected in DME500. Due to its cyclic structure and lipophilic character, CsA is basically known to be very stable over a wide range of experimental conditions [30]. With a later formulation for the anterior eye in mind the solvents seemed not to be a limiting factor in terms of drug stability and their influence may even be less problematic based on the presumably lower concentrations used in the final formulations.

2.4. Conclusion

The investigated solvents showed many suitable characteristics, as for example a high solubilizing potential, low water- content and good compatibility with polymers. This makes them good candidates for the solvent component of an in-situ precipitating formulation for water insoluble, lipophilic drugs. We could show that the concept of an INS with defined points of precipitation worked. Unfortunately, a modulation or controlling of the precipitation to with additives like Solutol was not possible. Due to the hyperosmolarity of the solvents in higher concentrations, biocompatibility studies have to be conducted in-vitro and in-vivo. Even if there was a clear trend among the solvents, the detailed characteristics were very special for each system consisting of drug, solvent and water. Based on this data the INS has to be further investigated for cyclosporin A (Chapter 4). As in presence of Solutol no further precipitation could be induced, the option of clear aqueous formulations using non-ionic surfactants like Solutol should be observed as a second promising approach (Chapter 4).

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Chapter 3

Developing an in-situ nanosuspension: a novel approach towards the effective administration of poorly soluble drugs at the anterior eye

submitted to *European Journal of Pharmaceutical Science*

Abstract

With about 50-60 billion only in the US, dry eye disease (DED) is a severe medical problem. Even though there are potent drugs like cyclosporin A (CsA), due to their poor solubility in water, there is still huge lack in causal treatment. We developed an in-situ nanosuspension (INS) as a novel approach towards the administration of CsA at the anterior eye. The INS solution precipitates upon contact with the tear fluid and creates the active system right at the site of application in form of drug nanoparticles. Therefore we selected two suitable solvents (Polyglycol M500; Polyglycol DME500) out of different liquid poly(ethylene glycols). Aqueous solutions of these and a surfactant were well tolerated by primary human epithelial cells in vitro. To determine the critical water- content for a precipitation, the solubility of CsA was investigated in quaternary systems of drug, solvent, surfactant and water. Further, particle size and distribution were screened as a function of drug- and surfactant concentration. Finally, DME500 showed the best qualities as solvent for an INS and four different INS solutions were developed and characterized. The best solution showed a particle size of 505 ± 5 nm, a polydispersity index (PdI) of 0.23 ± 0.0 and a neutral zeta potential of -0.07 ± 0.05 mV. After single instillation at porcine eyes in-vitro, 3345 ± 744 ng_{CsA}/g_{cornea} were detected in corneal tissue, while the levels of Restasis® with 545 ± 137 ng_{CsA}/g_{cornea} were significantly lower ($P < 0.01$). Upon these results, the INS proved our hypothesis and marked a promising, novel approach towards the causal treatment of inflammatory diseases at the anterior eye.

3.1. Introduction

Many highly potent drugs for the therapy of ophthalmic diseases suffer from a poor solubility in aqueous media. Cyclosporin A (CsA), for example, is highly efficient in the treatment of inflammation affecting the anterior eye. CsA was successfully applied in vernal conjunctivitis [1], dry eye syndrome [2], corneal graft rejection after transplantation [3], uveitis [4], necrotizing scleritis [5], Sjogrens syndrome [6], Behcet's disease [7] and thyroid ophthalmopathy [8].However, CsA has a high lipophilicity [9] and is with a solubility of 6.6 µg/ml practically insoluble in water [10]. Therefore, CsA has been administered in oily formulations [11–13] or ointments [14,15]. Unfortunately, such vehicles suffer from two major disadvantages. Since they come along with a burning sensation, an irritative effect on the cornea and blurred vision [16,17], patient compliance of such preparations is low. Furthermore, due to its higher affinity for an oily phase of a formulation compared to the aqueous milieu of the cornea [9], only small amounts of CsA become available in tissue [18]. To overcome this limitation high concentrations, commonly about 2.0 % of CsA must be applied to reach a therapeutic effect [19].

Substantial effort has been devoted to research on colloidal formulations to overcome these problems. Besides an increased apparent solubility of drug in an aqueous environment, colloidal systems offer a number of options to deliver drugs into the cornea by processes such as adhesion to cells or penetration into the inter cellular space [20–24]. A variety of colloidal systems, like drug- loaded cyclodextrins [25], micelles [18,26], liposomes [27] or nanoparticles [28–30] have been considered as suitable carriers for CsA, but only a 0.05 % CsA castor oil based microemulsion (Restasis®, Allergan, USA), developed by Ding et. al [31] made it to the US market for the therapy of Keratoconjunctivitis sicca (KCS) to date [17,32]. However,

Restasis® shows typical side effects like burning and stinging sensation [33,34] and suffers from low tissue concentration levels [35].

We hypothesize that CsA nanocrystals could overcome this limitation and concomitantly avoid the typical side effects of oily components. To create such a nanosuspension in-situ could be an innovative approach since it would avoid the frequently cumbersome manufacture and handling of drug nanocrystals [36,37] and excel colloidal formulations in terms of bioavailability at the same time. We developed our in-situ nanosuspension system (ISN) such that CsA is dissolved in a solvent / water mixture from which the drug precipitates spontaneously in nanoparticular structures upon contact with the tear fluid.

In this study we describe the development of an INS for CsA and the availability of drug in excised porcine cornea.

3.2. Materials and methods

3.2.1. Materials

Cyclosporin A (CsA) and BLUyal® UD an aqueous 0.15 % sodium hyaluronate solution were supplied by the Pharma Stulln GmbH (Stulln, Germany). Cyclosporin D (CsD) was a kind gift of Prof. Dr. F. Kees (University of Regensburg, Regensburg). Solutol® HS 15 was a kind gift from the BASF (Ludwigshafen, Germany). Polyglycol 400 a poly(ethylene glycol) (PEG) (M_W = 400 g/mol), Polyglycol M250, M350 and M500, three methoxy-PEGs (M_W = 250 g/mol, 350 g/mol, 500 g/mol) and Polyglycol DME250 and DME500, two dimethoxy-PEGs (M_W = 250 g/mol and 500 g/mol), were supplied by Clariant Produkte GmbH (Frankfurt a. M., Germany). Deionized water was obtained from a Milli-Q water purification system from Millipore (Schwalbach, Germany). Primary human cornea epithelial cells (HCEpiC) and corneal epithelial cell medium (CEpiCM) were purchased from Sciencell Research Laboratories (Carlsbad, USA). Methanol and acetonitrile (both HPLC grade) were purchased from Merck (Darmstadt, Germany). 0.1 N hydrochloric acid was obtained from Carl Roth (Karlsruhe, Germany).

3.2.2. Turbidimetric precipitation survey

3.2.2.1. Sample preparation for measurements in ternary systems (drug, solvent, water)

The six non-aqueous solvents were investigated. With every solvent a colorless, transparent stock solution of CsA (10 % (w/v)) was produced by gentle stirring. The stock solutions were diluted with the appropriate solvent to nine different concentrations of 10, 8, 6, 4, 2, 1, 0.5, 0.25 and 0.1 % CsA (w/v) (Tab. 3.1).

3.2.2.2. Sample preparation for measurements in quaternary systems (drug, solvent, surfactant, water)

Solubility in quaternary systems was investigated with M500 and DME500, respectively. Two different initial concentrations of the non-ionic surfactant Solutol were tested. Two stock solutions were produced: stock solution 1 (SL_1) containing 6 % (w/v) CsA, stock solution 2 (SL_2) containing 40 % (w/v) Solutol and 10 % (w/v) Solutol, respectively. Stock solution 3 (SL_3) with c(CsA) = 3 % (w/v) and c(Solutol) = 20 % (w/v) and accordingly c(Solutol) = 5 % (w/v) was received by mixing 1 ml SL_1 with 1 ml SL_2. SL_4 was obtained by dissolving 200 mg/ml Solutol in the different solvents. The SL_3 was diluted with the SL_4 to 5 different concentrations of 3, 2, 1, 0.5, and 0.1 % CsA (w/v) (Tab. 3.1).

3.2.2.3. Turbidimetric measurement cycle

The turbidimetric high throughput (tHTP) screening was performed to determine the solubility of CsA in ternary, as well as in quaternary systems. The turbidity was measured at a single wavelength of λ = 550 nm using a Titertek Plus plate reader (ICN Biomedicals Ltd, High Wycombe, UK). All measurements were performed in triplicate in a 96 well quartz microplate (Hellma, Müllheim, Germany). The initial sample volume was 50 µl per well. The first well in each row was a blank solution. The plate was alternately measured at λ = 550 nm, titrated with 2 µl H_2O per well, gently vortexed at level 3 using a Vortex-Genie®2 equipped with a 3-inch platform with rubber cover (Scientific Industries, Inc., Bohemia, USA) and again measured at λ = 550 nm. This cycle was repeated until each well showed a precipitation. The absorbance of the increasing concentration of H2O (% (m/m)) was plotted against the c (CsA) (% (m/m)). The point of precipitation (PoP) was defined as the water

concentration the absorbance started to increase with at least 30 % with respect to the mean of the five previous absorbance values.

Tab. 3.1: tHTP-screening; schematic overview of the sample preparation and the measurement cycle for the determination of the PoPs; left side: composition of the ternary systems; right side: composition of the two selected quaternary systems;

<table>
<tr><th colspan="6">ternary systems</th><th colspan="2">quaternary systems</th></tr>
<tr><td>PEG400</td><td>M250</td><td>M350</td><td>M500</td><td>DME250</td><td>DME500</td><td>M500</td><td>DME500</td></tr>
<tr><td rowspan="11"></td><td>10 %</td><td rowspan="11"></td><td rowspan="11"></td><td rowspan="11"></td><td rowspan="11"></td><td rowspan="3">+ Solutol</td><td>5%</td></tr>
<tr><td>8 %</td><td>20%</td></tr>
<tr><td>6 %</td><td>(w/v)</td></tr>
<tr><td>4 %</td><td rowspan="8">+ CsA</td><td>3 %</td></tr>
<tr><td>2 %</td><td>2 %</td></tr>
<tr><td>1 %</td><td>1 %</td></tr>
<tr><td>0.5 %</td><td>0.5 %</td></tr>
<tr><td>0.25 %</td><td>0.1 %</td></tr>
<tr><td>0.1 %</td><td>(w/v)</td></tr>
<tr><td colspan="8">+ stepwise 2 µl H_2O; vortexing; turbidimetric measurement at λ = 550 nm</td></tr>
<tr><td colspan="8">calculation of the PoPs</td></tr>
</table>

3.2.3. Tolerability towards HCEpiC cells in-vitro

3.2.3.1. Cell culture

Compatibility with HCEpiC cells in vitro was determined by observation of the cell volume. HCEpiC were cultured in the delivered corneal epithelial cell medium (CEpiCM) according to the supplier's instructions. The cells were seeded on 13 mm glass slides and cultured until they showed confluence.

3.2.3.2. Cell volume observation

Due to the predominantly hyperosmotic conditions, the shift in cell volume was used as a significant parameter to investigate the compatibility of the solvents with the

HCEpiC. Therefore, the following samples were tested: an aqueous solution containing 10 % (w/v) Solutol, aqueous solutions containing 10 % (w/v) or 20 % (w/v) M500 or DME500, respectively. A commercial 0.15 % sodium hyaluronate solution used as a lubricant for DED patients served as negative control. Volume detection was carried out using a superfusion system containing an Axiovert 40CFL microscope (Zeiss, Jena, Germany), a Coolsnap EZ camera (Fotometrix, Tucson, USA) and a perfusion chamber. The flow was set to 250 ml/h. The medium turnover took place within 30 s at a continuous flow. The period of observation was 25 min. It was split into 5 min flow of reference solution, a switch to sample solution for the next 10 min and after this a switch back to the reference solution for 10 min. The pictures were taken over the whole period (1 frame = 3 s). The data was processed at the following time points: 0, 30, 150, 300, 330, 450, 600, 750, 930, 1200, 1350, 1500 s. The cell area served as a measure for the volume. The data were processed using the ImageJ software (National Institutes of Health, United States).

The obtained data were statistically analyzed using a One Way RM ANOVA in combination with a Holm-Sidak method for pair wise multiple comparison at a significance level of $p < 0.05$ using SigmaPlot 11.0 (Systat Software Inc., San Jose, California, USA).

3.2.4. Particle size measurements

The particle size of the in-situ precipitate occurring upon contact with water was measured using dynamic light scattering (DLS). Samples were prepared in M500 and in DME500, respectively. The following combinations of drug and surfactant content were screened: 1 %, 5 %, 10 %, 15 % and 20 % (w/v) Solutol; 0 %, 0.1 %, 0.5 %, 1.0 %, 2.0 % and 3.0 % (w/v) CsA. 30 µl of each sample dilution were filled in 1.5 ml

eppendorf cups (n = 3). These samples were precipitated with an excess of 770 µl H_2O under gentle mixing. 400 µl of each sample were used to measure the particle size and the polydispersity index (PdI) with a Zetasizer Nano ZS (Malvern, Herrenberg, Germany).

3.2.5. Scanning electron microscopy

In order to visualize the resulting particles, a 3 % (w/v) solution of CsA in DME500 (A) without surfactant and a 3 % (w/v) solution of CsA in DME500 (B) stabilized with 10 % Solutol (w/v) were precipitated as described in 3.2.4. The samples were prepared on a Spartan™ syringe filter membrane with a pore size of 0.2 µm by filtration of the dispersions, to exclude the solvents without any thermal impact on the system. The filter membranes were fixed on aluminum stubs using conductive carbon tape. Subsequently, micrographs were obtained by SEM using a Quanta 200 from FEI (Hillsboro, Oregon, USA).

3.2.6. Solutions for in-vitro tissue uptake studies

3.2.6.1. Preparation

Tab. 3.2: INS solution design: INS solution at the PoP after instillation; compositions of the 4 INS solutions before application with varying amounts of water and DME500;

	composition at the eye / PoP	INS solutions			
		# 1	# 2	# 3	# 4
CsA	1.00 %	1.30 %	1.30 %	1.30 %	1.30 %
DME500	54.0 %	71.7 %	65.0 %	60.0 %	55.0 %
Solutol	10.0 %	13.7 %	13.7 %	13.7 %	13.7 %
Water	35.0 %	13.3 %	20.0 %	25.0 %	30.0 %

Four different solutions were prepared by dissolving 13 mg of CsA and 137 mg of Solutol in DME500 under gentle stirring. After a clear solution was obtained, the appropriate amount of water was added under constant stirring until the final formulation was transparent, colorless and visually free of air bubbles. The amounts of DME500 (A) and water (B) are shown in Tab. 3.2. The pH of the formulations was adjusted to 7.4 using 0.1 N HCl.

3.2.6.2. Size and Charge

The samples for the particle size measurement were prepared by precipitating 300 µl of each INS solution with 100 µl H_2O and gentle mixing in a 1.5 ml eppendorf cup. Particle size and size distribution were measured by DLS. The samples for the zeta potential measurement were prepared by precipitating 750 µl of each sample with 250 µl H_2O and gentle mixing in a 1.5 ml eppendorf cup. The zeta potential was measured by laser doppler electrophoresis using a Zetasizer Nano ZS.

3.2.6.3. Fluorescence microscopy

In order to visualize the precipitated structures, the lipophilic dye Nile Red (NR) was added to the following samples: (A) H_2O and (B) solution #1; Sample (C) was obtained by precipitation of 300 µl (B) with 100 µl H_2O. From the precipitate of sample (C), sample (D) was kept after purification by centrifugation at 10.000 g over 10 min and re-dispersion with 50 µl H_2O. Microscopic pictures were taken with an Axiovert 200M inverse microscope equipped with a LSM510 laser scanning device, a Plan-Apochromat 63x (NA 1.4) objective and the AIM 4.2 software (Zeiss, Jena, Germany). An argon laser at a excitation wavelength of 488 nm was used. Emission was detected using a 580-615 nm band-pass filter.

3.2.7. In-vitro model for drug resorption

Restasis® and a 2 % CsA olive oil solution served as reference solutions for the in-vitro resorption studies. Solution # 1 (INS 1.0) and an INS containing 0.4 % CsA (INS 0.4) were used as samples (Tab. 3.2). The INS 0.4 was prepared in analogy to the INS 1.0. The 2 % CsA olive oil solution was prepared by dissolution of CsA under gentle stirring for 2h at room temperature. The pH was adjusted to 7.4 with 0.1 N HCl.

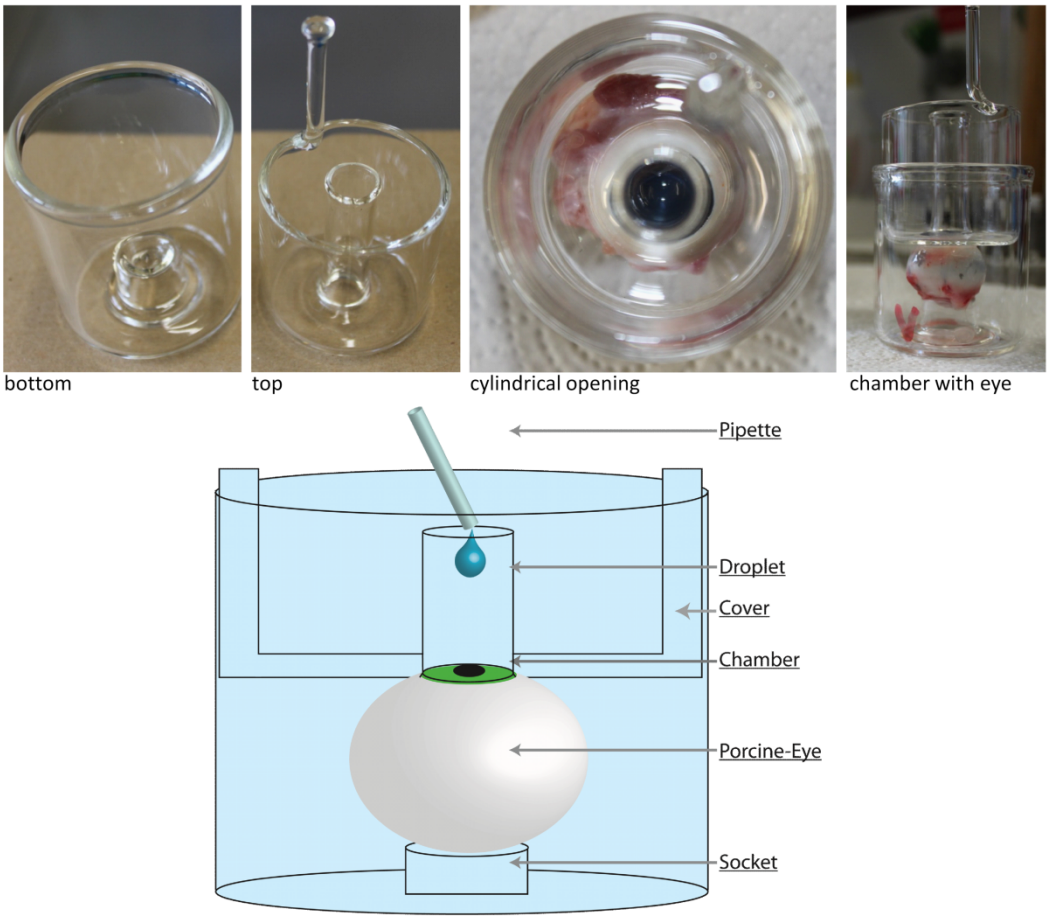

Fig. 3.1: Custom glass resorption chamber: the bottom glass is equipped with a little socket to place the eye; the top glass fixes and seals the eye; the cylindrical opening offers the option to instill preparations locally to the cornea;

The enucleated pig eyes were supplied from a local abattoir and stored in isotonic, sterile sodium chloride solution until use. Custom made glass resorption chambers

(CRC) were manufactured by the University glassblower (Fig. 3.1). An eye was placed in the bottom well of the CRC and fixed with the top glass insert, so that the cornea was centered in the cylindrical opening (CO) of the upper part of the incubation chamber. The glass and the wet eye surface sealed the contact area so that no liquid from the CO was lost. 200 µl of the reference solutions were placed on the plane cornea. 150 µl of the INS solution were placed on the cornea together with 50 µl of H_2O to induce precipitation (the ratio 150 : 50 is equivalent to a 30 µl eye drop instilled into 10 µl of tear fluid at the anterior eye). The eyes were incubated with the solutions for 30 min at RT. Afterwards the top glass was removed and each eye was washed four times with 1 ml of a 5.2 % aqueous mannitol solution. The cornea was then excised with a surgical knife and homogenized by use of a tissue pulverizer (made by the university machine shop in analogy to the Bessman Tissue Pulverizer) and liquid nitrogen. The homogenized cornea were transferred into glass vials and weighed. To extract CsA, the tissue pieces were incubated under gentle stirring with 1 ml MeOH, spiked with 100 ng/ml CsD as internal standard. After the extraction the suspension was centrifuged at 16.000 g for 15 min. 500 µl of the supernatant were filled in HPLC vials and stored at -80°C until further use. All samples were prepared and measured in triplicate.

3.2.8. CsA analytics

The UHPLC-MS analytics were performed in accordance to Rodriguez-Aller et al (2011) [38]. An Agilent Technologies UHPLC system equipped with a 6540 quadrupole time of flight (Q-TOF) LC/MS system fitted with an Agilent jet stream electron spray ionization (AJS ESI) interface (Agilent Technologies Deutschland GmbH, Böblingen, Germany) was used. The chromatographic equipment consisted further of a high performance (HiP) autosampler G4226A with a sample volume of

2 µl and a storage temperature of 5 °C, a binary pump G4220A at a flow rate of 0.6 ml/min and a column compartment G1316C with a column temperature of 70 °C. The AJS ESI was used in positive ion mode. Separation was performed on a Waters Aquity BEH c18, 1.7 u, 2.1 x 50 mm column (Waters Corporation, Milford, MA, USA). The mobile phase consisted of 40 % (A) H_2O with 0,1 % formic acid (FA) and 60 % (B) methanol (MeOH) with 0.1 % FA. A gradient was applied for 3 min to concentrations of 0 % (A) and 100 % (B). Afterwards a re-equilibration was performed for 3 min. MassHunter Workstation software was used for data acquisition (Agilent Technologies Deutschland GmbH, Böblingen, Germany). Data handling and calculation were done using Microsoft Excel 2007 and Dintest 2004 software (ARVECON GmbH, Walldorf, Germany).

The obtained data were analyzed using the t – test at a significance level of $p < 0.05$ using SigmaPlot 11.0.

3.3. Results and discussion

3.3.1. CsA solubility in ternary systems (drug, solvent, water)

First we investigated the solubility of the drug in ternary mixtures of CsA, PEG and water. Of special interest was the determination of the point of precipitation (PoP) i.e. the maximum water- content at which CsA would still be dissolved (Fig. 3.2).

All solvents were able to solubilize at least 10 % (w/v) CsA, which is way far enough for an effective ocular dose which is discussed to be between 0.05 % (w/v) and 2.0 % (w/v) [14,19,39]. DME250 allowed for the highest water- content before a precipitation occurred. The lowest PoP was detected for PEG400. All other solvents ranged between these extremes. As expected, the critical water-content, on the one

hand increased with decreasing levels of CsA. On the other hand, it depended on the hydrophobicity and molar mass of the PEGs. The higher the degree of etherification and the lower the chain length, the more water was needed for a precipitation of CsA.

In terms of tolerability, high levels of incorporated water are beneficial for a final formulation since this will lead to a lower osmotic pressure. Since tolerated osmotic pressure levels are limited (572mOsm [40] were reported) and lower for PEGs with higher molecular weight [41], we selected DME500 and M500 for all further studies.

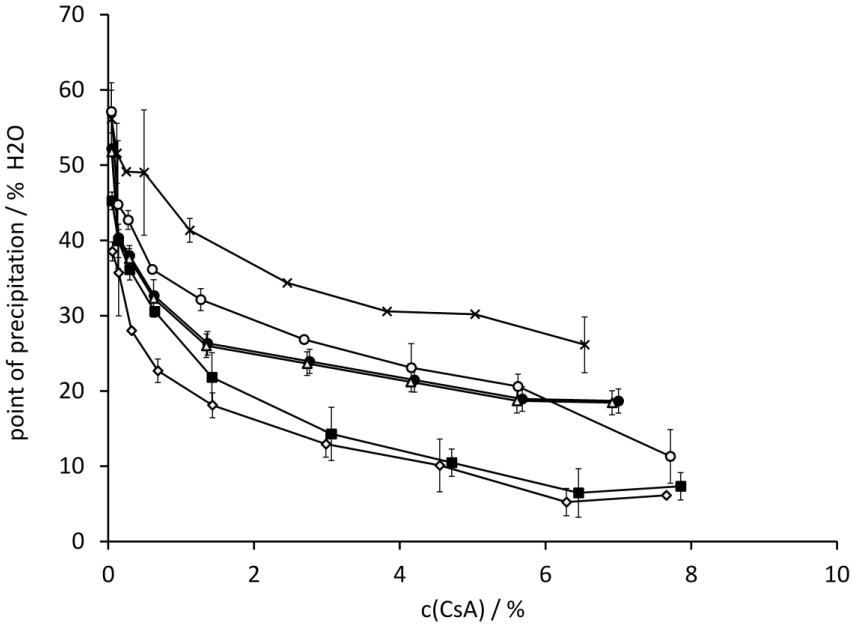

Fig. 3.2: Drug solubility: ternary systems of CsA, solvent and water at their PoP for different compositions; (◇) PEG400, (●) M350, (△) M250, (x) DME250, (■) M500, (○) DME500;

3.3.2. CsA solubility in quaternary systems (drug, solvent, surfactant, water)

To take advantage of the fact that a non-ionic surfactant may be beneficial for reducing the drug particle size after precipitation we investigated the solubility of CsA in quaternary systems of drug, solvent, surfactant (Solutol) and water (Fig. 3.3).

Mixtures with M500 as solvent showed no difference in the critical water-content with or without the surfactant for CsA concentrations above 1 % (w/v) (Fig. 3.3 A).

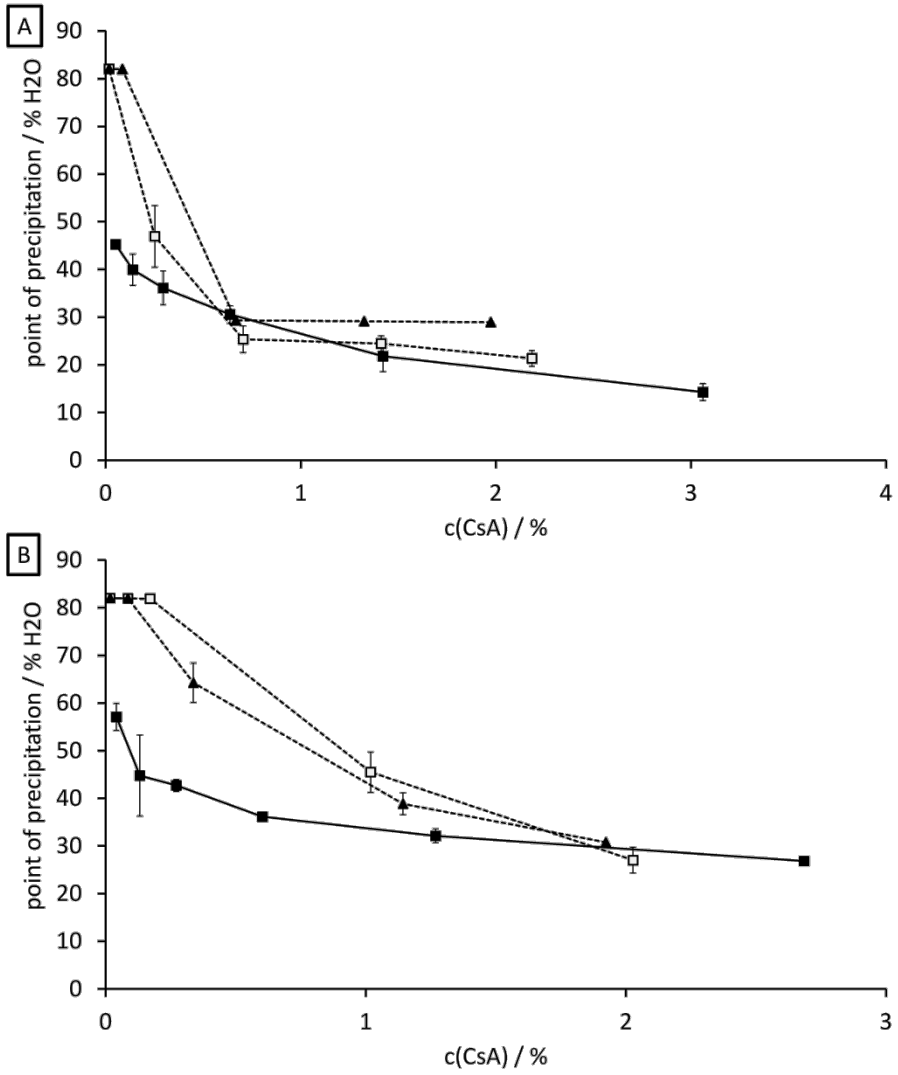

Fig. 3.3: Drug solubility: quaternary systems, consisting of drug, surfactant, solvent and water; [A] system with M500 as solvent; [B] system with DME500 as solvent; Solutol as a surfactant was used in concentrations of 0 % (■), 5 % (□) and 20 % (▲);

At lower concentrations, the critical water-content could even be increased up to 80 % (w/v). The maximum water-content was also independent of the surfactant concentration in a range between 5 % (w/v) and 20 % (w/v) Solutol. Mixtures with DME500 as solvent, in contrast, showed a slightly different behavior (Fig. 3.3 B). Below CsA concentrations of 2 % (w/v) the surfactant increased the critical water-content continuously compared to the ternary system. The highest levels were again about 80 % (w/w) water. The PoP was again independent of the surfactant concentration in a range between 5 % (w/v) and 20 % (w/v) Solutol.

3.3.3. Compatibility towards HCEpiC cells in-vitro

The cells showed a decrease in cell area upon exposition to any solution even classical Ringer's solution (data not shown). Only the cell culture medium delivered with the cells was accepted without any loss in cell area. Fig. 3.4 A shows the decrease in the cell area of a commercially available 0.15 % sodium hyaluronate solution (SHS) and an aqueous solution of 10 % (w/v) Solutol. For both solutions after 1500 seconds a shrinking down to 76.1 ± 2.1 % and 71.6 ± 3.8 %, respectively, of the initial cell area could be detected. In a next step aqueous mixtures of Solutol and solvent were compared with SHS. Fig. 3.4 B shows the change of cell area with time upon exposition to the four different solutions. The first differences to SHS were observed with the medium change to the sample solution at 330 s, what seemed to be due to their hypertonicity. This also explained the more abrupt decrease with [b] and [d] containing 20 % (w/v) of solvent [42]. [b] showed here the most significant decrease. Until 930 s, the cell area declined continuously, but much less with [c] (75.9 ± 3.2 %) and [d] (78.1 ± 1.9 %) compared to [a] (69.6 ± 2.9 %) and [b] (69.2 ± 4.4 %). After medium change back the cell area stayed constant. At 1500 s, the endpoint of observation, sample [d] showed the significantly highest levels (Tab. 3.3). An initial loss of water and the corresponding decrease in cell area within the first few seconds is typical for hypertonic stress on cells [43]. After this response to the "osmotic stress", the cell area decrease equilibrated as described by Zhou et al [42].

Due to less shrinking upon contact with the solutions containing DME500, it seemed to be the most promising solvent in terms of a later application at the anterior eye.

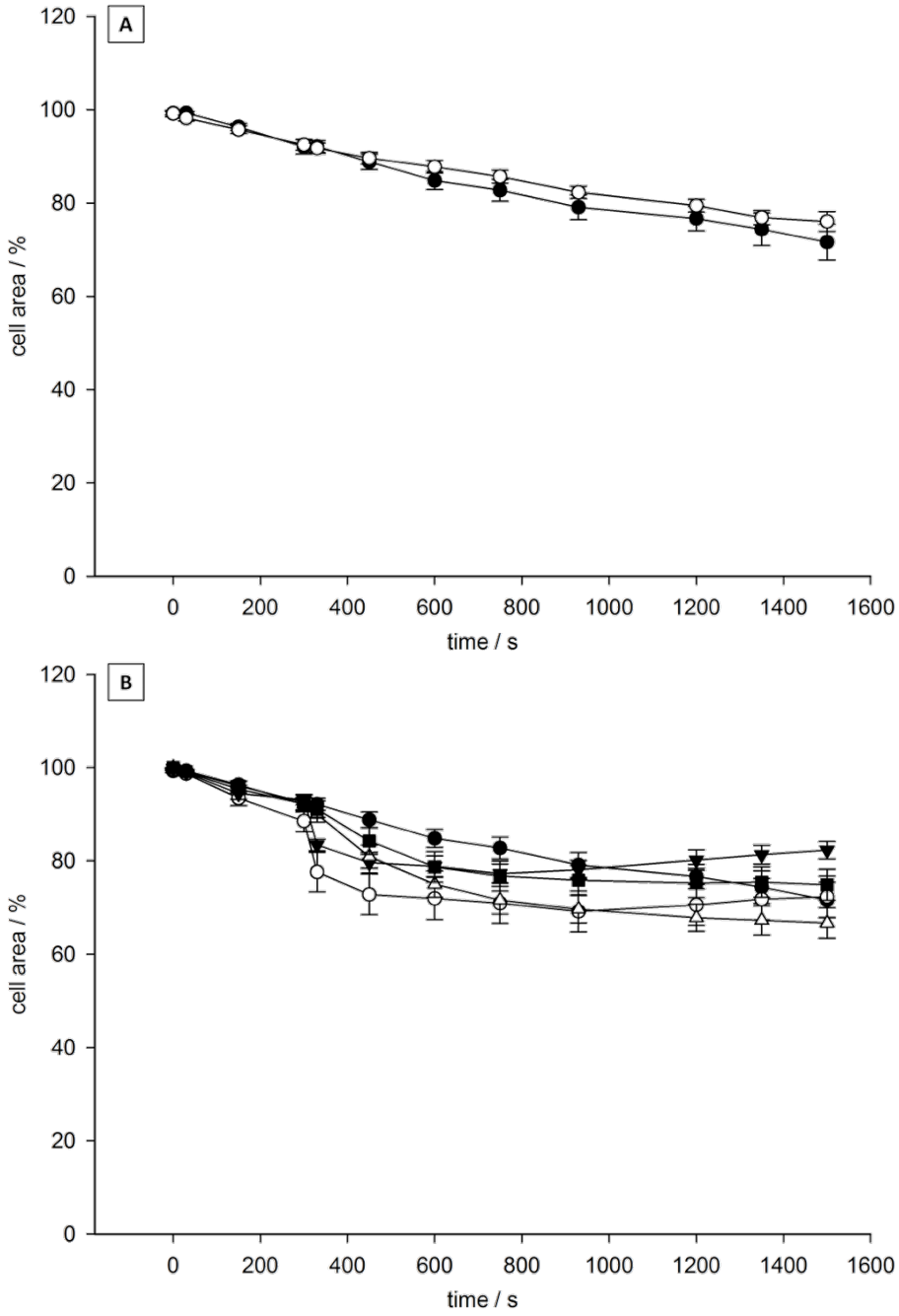

Fig. 3.4: Tolerability: [A] effect on the cell volume caused by the exposure with the aqueous mixtures of 10 % Solutol (○) compared to a aqueous 0.15 % sodium hyaluronate solution (●); [B] comparison of the change of cell volume caused by the exposure with aqueous mixtures of M500 and Solutol, and DME500 and Solutol with the aqueous 0.15 % sodium hyaluronate solution: [a] 10 % (w/v) Solutol / 10 % (w/v) M500 (△); [b] 10 % (w/v) Solutol / 20 % (w/v) M500 (○); [c] 10 % (w/v) Solutol / 10 % (w/v) DME500 (▲); [d] 10 % (w/v) Solutol / 20 % (w/v) DME500 (●);

Tab. 3.3: Tolerability: significant differences determined by one way RM ANOVA at the medium switch (300s), after the medium switch (330s), at the medium switch back (930s) and at the end of observation (1500s) for [A] 10% M500 / 10% Solutol [B] 20% M500 / 10% Solutol, [C] 10% DME500 / 10% Solutol, [D] 20% DME500 / 10% Solutol and [R] the reference eye drop solution BLUyal®; *** = p < 0,05 (One Way RM ANOVA);

time / sec	**[A]** *vs.* **[R]**	**[A]** *vs.* **[B]**	**[A]** *vs.* **[C]**	**[A]** *vs.* **[D]**	**[B]** *vs.* **[R]**	**[B]** *vs.* **[C]**	**[B]** *vs.* **[D]**	**[C]** *vs.* **[R]**	**[C]** *vs.* **[D]**	**[D]** *vs.* **[R]**
300										
330		***			***	***				
930							***			
1500				***			***		***	***

3.3.4. Particle size screening

In order to prove the concept of the INS we investigated the particle size of CsA crystals upon precipitation. The CsA concentration ranged from 0 %, to detect the micelle size in the drug- free system, to 3.0 % (w/v). The Solutol concentrations ranged between 1 % and 20 % (w/v). M500 and DME500 were used as solvents. A high excess of water was added to guarantee a complete precipitation. With few exceptions all samples showed average particles with sizes below 250 nm (Fig. 3.5 A, B). In general it could be shown, that increasing concentrations of drug led to increasing particle sizes. The most consistent results for both solvents in this setup were achieved using a surfactant concentration of 5 % Solutol. With M500 as solvent (Fig. 3.5 A, C) the particle size distributions as reflected by the PdI were broader. The samples made with DME500 (Fig. 3.5 B, D) showed similar particle sizes but substantially narrower particle size distributions with mean PdIs below 0.3. At 0.1 % (w/v) CsA the particles showed nearly the same size as the plain micelles. In the presence of 20 % (w/v) Solutol even the 0.5 % (w/v) CsA sample only showed this

size. Due to the inhomogeneous PdIs (Fig. 3.5 A, C), this clear separation between precipitation and micellar structures could not be shown with M500. Instead, tremendous differences in terms of a reproducible, homogeneous precipitation were observed for the two solvents. The variation of the surfactant concentration showed no significant impact on the particle size [44]. As shown for the solubility in quaternary systems, it was more the presence than the concentration of surfactant that had a remarkable effect on the wetting of the precipitate.

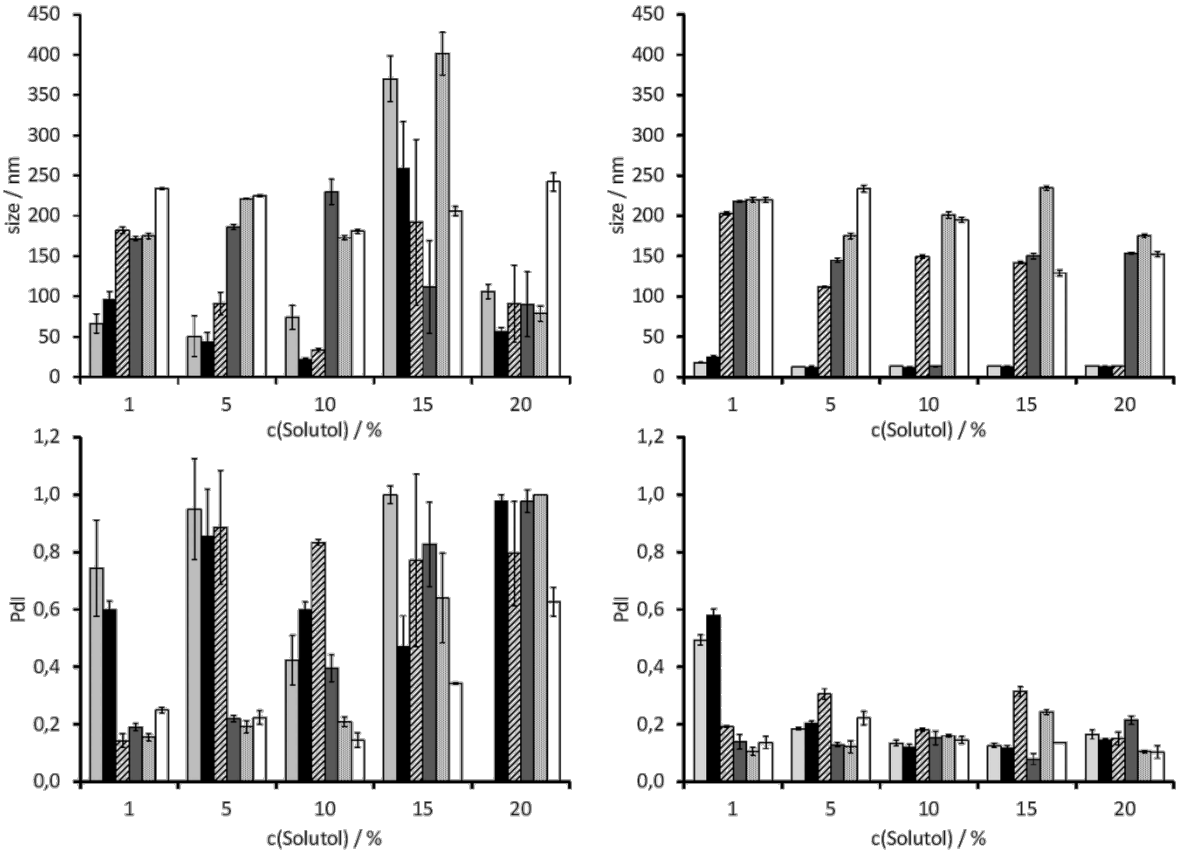

Fig. 3.5: Particle size screening: particle size (upper row) and corresponding polydispersity indices (lower row) of the precipitated structures as a function of drug- and surfactant concentration; left panel: formulations with M500 as a solvent; right panel: formulations with DME500 as a solvent; (□) 0 % CsA, (■) 0.1 % CsA, (□) 0.5 % CsA, (■) 1.0 % CsA, (■) 2.0 % CsA, (□) 3.0 % CsA;

Only at the lowest (1 %) or highest concentration (20 %) differences were visible, but not necessarily significant. The most deciding factor with impact on the particle size was the concentration of CsA. In general, as known from literature, with higher

amounts of CsA, larger particles occurred [44]. With M500 as solvent (Fig. 3.5 A, C) the particle size distributions as reflected by the PdI were broader. The samples made with DME500 (Fig. 3.5 B, D) showed similar particle sizes but substantially narrower particle size distributions with mean PdIs below 0.3. At 0.1 % (w/v) CsA the particles showed nearly the same size as the plain micelles. In the presence of 20 % (w/v) Solutol even the 0.5 % (w/v) CsA sample only showed this size. Due to the inhomogeneous PdIs (Fig. 3.5 A, C), this clear separation between precipitation and micellar structures could not be shown with M500. Instead, tremendous differences in terms of a reproducible, homogeneous precipitation were observed for the two solvents. The variation of the surfactant concentration showed no significant impact on the particle size [44]. As shown for the solubility in quaternary systems, it was more the presence than the concentration of surfactant that had a remarkable effect on the wetting of the precipitate. Only at the lowest (1 %) or highest concentration (20 %) differences were visible, but not necessarily significant. The most deciding factor with impact on the particle size was the concentration of CsA. In general, as known from literature, with higher amounts of CsA, larger particles occurred [44].

The effect of the surfactant Solutol was proven, using environmental scanning electron microscopy (eSEM). Fig. 3.6 illustrates a non stabilized sample (A) and sample (B) stabilized with 10% Solutol. Precipitate (A) showed a compact layer of precipitate. The filter membrane was not visible. No single, defined structures were detected. Precipitate (B) visualized aggregates of single spherical particles with a size above 200 nm (Fig. 3.6, white circles). The aggregates seemed to be due to the filtration pressure during the preparation. Because of the fact, that the bulk of the precipitate could be filtrated and thus, was not visible on the membrane, the single particles were presumably sized below 200 nm.

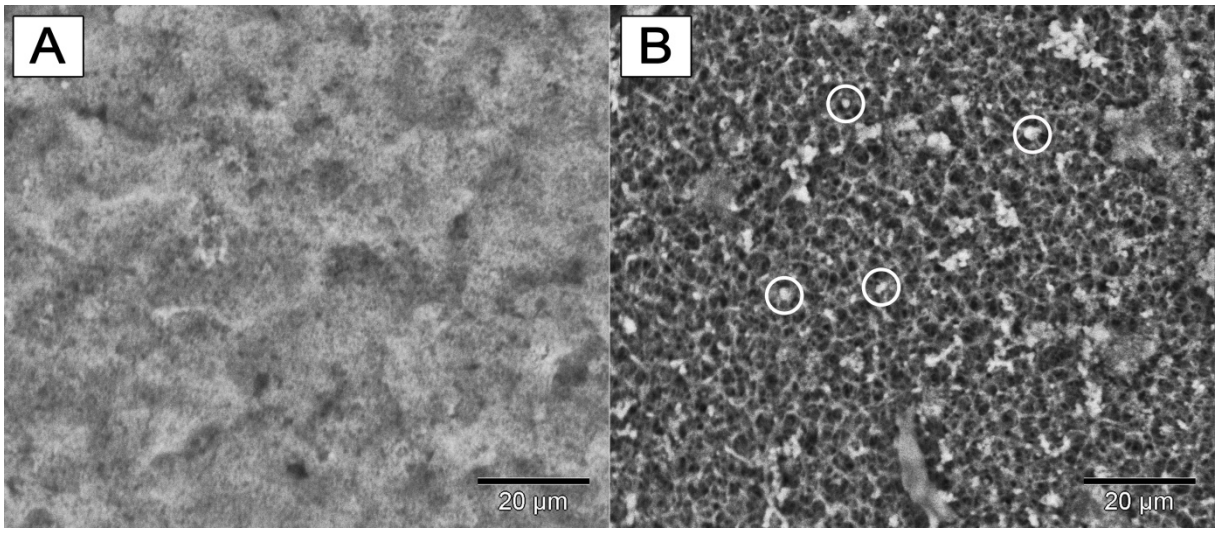

Fig. 3.6: Particle size screening: eSEM pictures of the precipitate of (A) 3% CsA/DME500 without a stabilizing surfactant and (B) 3% CsA/DME500 with 10% Solutol as stabilizing surfactant on a 0.2 µm filter membrane; the white spots mark some single particles and agglomerates exemplarily;

This fitted the results of the PCS with a size of 194.97 ± 3.27 nm (Fig. 3.5). The presence of surfactant had a tremendous impact on the precipitation. With Solutol homogeneous dispersed systems, without, non defined, agglomerated cloudy structures occurred. Due to a re-dissolving of the particles while the sample preparation after the evaporation of the aqueous phase and thus an excess of solvent, the observation using a non environmental SEM was not possible.

3.3.5. Development and characterization of INS solutions for in-vitro resorption studies

In order to obtain a promising composition for subsequent in-vitro investigations in a porcine model, four INS solutions were designed and characterized with respect to their particle size, size distribution and zeta- potential.

3.3.5.1. Development of the INS solutions

The design of the solutions was based on the solubility studies in quaternary systems using DME500 as solvent. Due to the missing knowledge on precipitation and

resorption in-vivo, a PoP concentration of 1 % (m/m) CsA was chosen for the development. Its composition at the eye / PoP is shown in Tab. 3.2. Upon this composition, the four different solutions were calculated by lowering the water-content to reach a varying water excess at the PoP. The calculation is based on the hypothesis that a tear volume of about 10 µl is present at the ocular surface and a droplet of 30 µl is instilled [45,46]. Sample #1 was configured by subtraction of this amount of tear volume from the aqueous fraction at the PoP. The samples #2, #3 and #4 were configured by lowering the content of DME500 and an increase of the amount of water.

3.3.5.2. Characterization of the INS solutions

The four different solutions were characterized in terms of particle size, size distribution and zeta potential using dynamic light scattering and laser doppler electrophoresis, respectively. The results are shown in Tab. 3.4.

Tab. 3.4: Solution characterization: particle size, size distribution and zeta potential of the four solutions with varying amounts of DME500 and H_2O upon precipitation;

INS solution	Z_{AV} / nm	PdI	η-potential / mV
# 1	697 ± 19	0.110 ± 0.051	-0.01 ± 0.11
# 2	885 ± 15	0.111 ± 0.048	-0.115 ± 0.1
# 3	505 ± 5	0.230 ± 0.029	-0.07 ± 0.05
# 4	487 ± 7	0.440 ± 0.075	-0.05 ± 0.13

All precipitated samples showed particle sizes below 900 nm. The initial water-content of the solutions had a huge impact on the particle size. With a higher excess of water at the PoP, smaller particles were detected. Solutions #1 and #2 with 13 % and 20 % H_2O, respectively, showed particles with a size of 697 ± 19 nm and 885 ±

15 nm. However, the solutions #3 and #4 with higher amounts of water obviously led to smaller particles of 505 ± 5 nm and 487 ± 7 nm, respectively. Due to their submicron size, as previously reported these particles might be suitable for endocytotic uptake into cornea cells [47]. They could then act like a reservoir, and release the drug to the surrounding tissues [17]. In contrast to the size, the PdI increased with higher water- content, similarly as reported by Ford et al. (1999) for CsA nanoparticles out of an acetonic solution [44]. Hence, solution #4 with the smallest Z_{AV} showed the highest PdI. Solution #1 (PdI = 0.110 ± 0.051) and #2 (PdI = 0.111 ± 0.048) had a nearly monomodal size distribution. In general, solution #3 showed the best performance with a size of 505 ± 5 nm and a PdI of 0.230 ± 0.029. As expected and due to the missing charges in the molecules, a nearly neutral zeta potential was determined for all solutions.

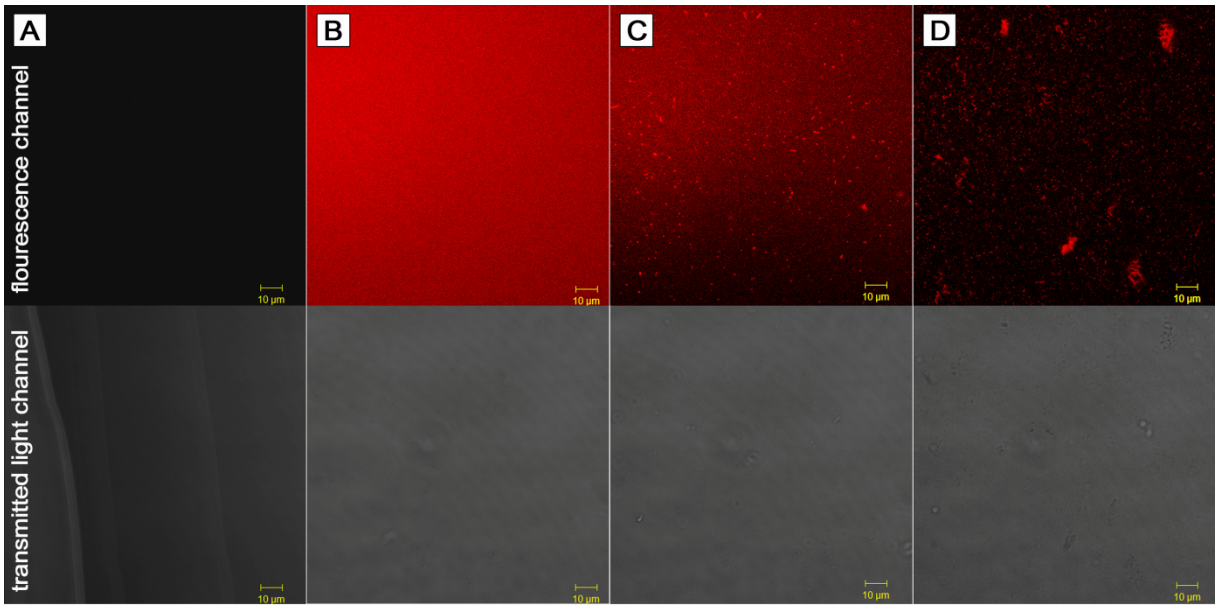

Fig. 3.7: Solution characterization: fluorescence microscopic pictures of Nile Red (NR) stained samples; (A) H_2O + 0.01% NR; (B) INS #1 + 0.01 % NR before precipitation; (C) INS #1 + 0.01 % NR after precipitation; (D) sample (C) after centrifugation and re-dispersion in H_2O;

In order to visualize the precipitate as well as the homogeneity of the unprecipitated solution, fluorescence microscopic pictures were taken (Fig. 3.7). Nile Red, which

shows fluorescence only in lipophilic environment, was used as dye, as similarly done previously as hydrophobic model drug for micellar systems [26]. Due to the missing hydrophobic environment in water, the blank (A) showed neither fluorescence, nor structures in the transmitted light channel. Solution #1 (B) showed a homogeneous fluorescence all over the sample. This was belike to the micelle formation of Solutol. Solutol micelles were typically around 13 nm (data not shown) as also reported in literature [48]. After (B) was precipitated, besides homogeneous background fluorescence, red dots ranging from some nanometers to aggregates of a few micrometers were observed (C). It could be possible, that NR precipitated on its own and was stabilized by Solutol. However, because of the very low amounts of NR used, it might be more suitable, that it was attached to the lipophilic CsA-precipitate. The aggregates may be due to the mechanical influence while the preparation on the glass slides. (C) was centrifuged and the precipitate was re-dispersed in water (D). Due to the centrifugation, some agglomerates in a range of about 10 µm were observed. Besides these, no further background fluorescence was disturbing. In contrast to (C) with many aggregates of a few micrometers, less of those but much more single particles in the upper nanometer scale appeared. As expected, the transmitted light channel just showed the big fuzzy agglomerates. With respect to the mechanical influence while sample preparation for microscopy, in comparison to the sample preparation for DLS, where nearly no mechanical influence occurred, the results from (D) and the PCS of solution #1 (Z_{AV} = 697 ± 19 nm) fit and support each other.

3.3.5.3. Drug resorption using a porcine in vitro model

To prove the suitability of the INS as a therapeutic application system for the anterior eye, solution #1 was investigated in terms of corneal CsA uptake in-vitro using a

enucleated porcine eye model. To gather further information on the concentration dependent uptake and to see if a further dose reduction would be feasible, an INS containing only 0.4 % CsA was additionally investigated.

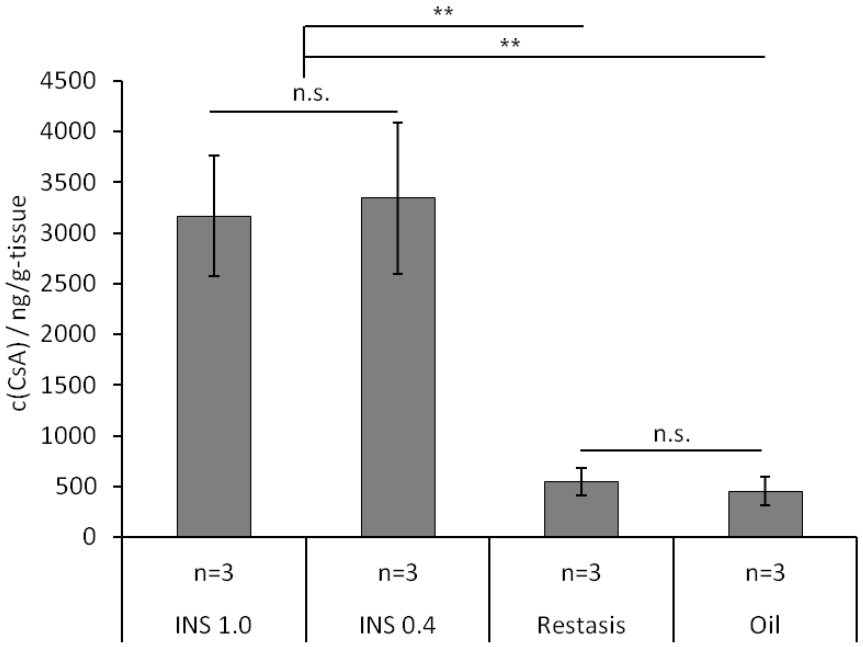

Fig. 3.8: In-vitro resorption: amount of CsA taken up by porcine-cornea from 200µl sample solution over a period of 30 min; samples: INS 1.0 (1 % CsA), INS 0.4 (0.4 % CsA) Restasis® (0.05 % CsA microemulsion), Oil (2 % CsA olive oil solution); ** = P < 0.01(t-test); screening was performed in triplicate;

The INS solutions were compared to Restasis® and a 2 % CsA olive oil solution (Fig. 3.8). The final sample volume was 200 µl to get a complete wetting of the cornea. After 30 min, a typical half life time after single instillation [35], the mean corneal drug concentrations were 3165 ± 597 ng_{CsA}/g_{cornea} and 3345 ± 744 ng_{CsA}/g_{cornea} for the 1 % INS and the 0.4 % INS, respectively. Both were significantly above the amounts detected for Restasis® (545 ± 138 ng_{CsA}/g_{cornea}) and the oily solution (452 ± 143 ng_{CsA}/g_{cornea}). Despite its very high CsA concentration, which is a fifth of the 0.4 % INS content and 40 fold of Restasis®, the oily solution did not show significantly different tissue levels compared to Restasis®. The 0.4 % INS already seemed to reach maximum possible cornea levels, which did not significantly differ

from those reached by the 1 % INS. This may be due to the smaller size of the nanoparticles resulting from lower CsA concentrations of the INS (compare results of the particle size screening, 3.3.4)

Compared to in-vivo studies with rats, where Restasis® did not show quantifiable CsA levels in corneal tissue after twice a day administration over 5 days, [35], we could detect CsA after administration of Restasis®, but far below the levels after an administration of the INS solutions. Daull et al detected 20 min after an instillation of 50 µl in-vivo only slightly higher levels for Restasis® (748 ± 129 ng_{CsA}/g_{cornea}) then our group. Further, after one week with instillations of 50 µl Restasis® twice a day, the tissue levels reached not more than 1072 ± 369 ng_{CsA}/g_{cornea} [49]. Therefore, we assume for our INS solutions after instillation of a typical droplet (25 µl) in vivo corneal levels significantly above those of Restasis® and the therapeutic tissue levels for CsA (50 – 300 ng_{CsA}/g_{cornea}) as reported by Kaswan et al [50]. It seems, Restasis® and the oily solution, suffered from the higher affinity of CsA to the lipophilic vehicle compared to the hydrophilic milieu of the cornea [9]. Even thus the instilled volume (200 µl) of our in-vitro investigations had to be higher than a usual eye drop to guarantee a complete wetting of the corneal surface, the similar tissue levels of Restasis® detected by our group and Daull et al proved the comparability of our in-vitro system. Hence, our INS is a promising approach to increase the bioavailability of CsA and eventually lower the number of instillations per day significantly. This may help to increase the patients' compliance [34,35,51].

3.4. Conclusion

In the present work we developed an “in-situ nanosuspension” to overcome the problems associated with the administration of poorly soluble drugs, such as CsA, at the anterior eye. The INS is a clear, transparent preparation, without a cumbersome production and a simple application regimen. It is able to solubilize high amounts of CsA and precipitates CsA into nanosized, stabilized particles upon contact with aqueous fluids. The compatibility with a corneal cell line was observed as good as ophthalmic solutions like a commercial aqueous 0.1 % sodium hyaluronate solution. After single instillation of the INS in a porcine cornea model in-vitro promisingly high CsA levels were obtained in the tissue.

3.5. References

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Chapter 4

A self- assembling, colloidal system for the ocular administration of poorly soluble, lipophilic drugs

to be submitted to a peer reviewed journal

Abstract

The efficient treatment of diseases at the anterior segment of the eye with potent, but poorly soluble drug is a major issue in research. In this study we developed a self-assembling micellar system for budesonide and cyclosporin A (CsA), which was investigated in terms of its solubilizing qualities, particle size and morphology, compatibility and drug resorption into porcine cornea in-vitro. The clear and colorless solutions dissolved both drugs in a concentration dependent manner just by use of gentle stirring and without further organic solvent or mechanical influences in significant higher concentrations compared to their solubility in water. The poly(ethylene glycol) fatty alcohol ethers Sympatens AS and Sympatens ACS showed the best performance. The micelles had a size from 9.4 ± 0.03 nm to 12.7 ± 0.04 nm, in which Solutol led to the biggest micelles and the CsA loaded ones, no matter which surfactant was used, were slightly smaller, than the budesonide loaded and drug- free micelles. The size and spherical shape was proven by transmission electron microscopy. The solutions were tolerated by primary human epithelial cornea cells in-vitro as well as the control. Finally, in an in-vitro porcine cornea model, the 0.05 % CsA micellar solution showed with 1557 ± 407 ng_{CsA}/g_{cornea} significantly higher tissue levels then Restasis® (545 ± 137 ng_{CsA}/g_{cornea}) and a typically used 2 % CsA oily solution (452 ± 142 ng_{CsA}/g_{cornea}). In conclusion, the data demonstrate a promising and powerful approach for the application of poorly soluble drugs towards the treatment of diseases like for example dry eye disease or age-related macular degeneration (ArMD).

4.1. Introduction

Local therapy at the anterior eye for the treatment of inflammatory diseases, such as dry eye disease (DED), is still a highly unmet need. Contemporary research focuses on increasing the bioavailability and the therapeutic efficiency of drugs and on avoiding their systemic side effects [1–4]. Two examples for highly potent anti-inflammatory drugs are budesonide and cyclosporin A (CsA). Due to its potential to inhibit the expression of vascular endothelial growth factor (VEGF), budesonide, with a 200 fold higher affinity for the corticoid receptor than dexamethasone [5], might be an option in the local therapy of age related macular degeneration (ARMD) [6]. CsA on the other hand, is successfully used in the treatment of uveitis [7], necrotizing scleritis [8] or thyroid ophthalmopathy [9] and is one of the most promising substances for the treatment of dry eye disease [10]. Unfortunately, both potent drugs suffer from their insufficient solubility in aqueous media (budesonide, 14 µg/ml in water [11]; CsA, 6.6 µg/ml in water [12]) and their high octanol-water-coefficient of logP = 3.2 [6] and logP = 3 [13] for budesonide and CsA, respectively. Therefore, there have been numerous formulation efforts to administer these drugs for a number of treatments. Budesonide, which is typically used in treatment of pulmonary diseases is usually administered via a dry powder inhaler (Pulmicort® Turbohaler®) or a suspension (Pulmicort® Suspension; Pulmicort® Topinasal®). Sahib. et al developed a sterically stabilized micellar system, which is able to dissolve approximately 600 µg/ml budesonide, but suffers from a cumbersome way of production [14]. Many different options, with a main focus on colloidal systems like micelles [15,16], liposomes [17] or various nanoparticular formulations [18–20] were investigated to solubilize CsA. A cationic nanoemulsion is currently in clinical trial phase III and Restasis® (Allergan) a 0.05 % CsA microemulsion is the only approved

product, however, on the US market only [21]. Hence, dry eye syndrome is typically treated with 2 % CsA oily eye drops, using vegetable oils as solvent [22,23]. But, all systems containing an oily phase usually come with side effects like burning, stinging sensation and blurry vision. Due to this fact, the patient compliance is very low [24].

Even though there was a lot of research done in the past, there is still a tremendous need for a simple, but yet efficient aqueous colloidal system, which does not need any oil phase. Made of inexpensive surfactants it should self assemble in the presence of water and drug of which it should solubilize large amounts. Such a formulation would come with a tremendous increase of the patient compliance and would be a major option in the causal treatment of various diseases at the anterior segment of the eye.

The aim of our study was to develop such a self-assembling micellar system. Of high interest was, thereby, its in-vitro tolerability and drug resorption in a porcine in-vitro model.

4.2. Materials and methods

4.2.1. Materials

Cyclosporin A (CsA), budesonide and BLUyal® UD a 0,15 % aqueous sodium hyaluronate solution were supplied by the Pharma Stulln GmbH (Stulln, Germany). Cyclosporin D (CsD) was a kind gift of Prof. Dr. F. Kees (University of Regensburg, Regensburg). Solutol® HS 15 was a kind gift from the BASF (Ludwigshafen, Germany). Sympatens AS/200G (AS) and Sympatens ACS/200G (ACS) were provided by KOLB (Hedingen, Switzerland). Nile Red (NR) and pyrene were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Mannitol was purchased from Caesar & Loretz GmbH (Hilden, Germany). Deionized water was obtained from a Milli-Q water purification system from Millipore (Schwalbach, Germany). Primary human cornea epithelial cells (HCEpiC) and corneal epithelial cell medium (CEpiCM) were purchased from Sciencell Research Laboratories (Carlsbad, USA). Methanol (MeOH), acetonitrile (MeCN), isopropanol (all HPLC grade) and chloroform p.a. were purchased from Merck (Darmstadt, Germany). 0.1 N hydrochloric acid and 0.1 N sodium hydroxide solution were obtained from Carl Roth (Karlsruhe, Germany).

4.2.2. Determination of the critical micelle concentration (CMC)

The critical micelle concentration of the three surfactants was determined using a fluorescence spectroscopy method [25,26]. To determine the critical micelle concentration pyrene aliquots of 1 mg per vial were prepared. Hence, 120 mg of pyrene were dissolved in 3000 µl of chloroform. 120 glass vials (2 ml Screw top vial; BGB Analytik AG, Rheinfelden, Germany) were filled with 25 µl of this solution using a positive displacement pipette (Multipette® plus; Eppendorf AG, Hamburg,

Germany) and the solvent was evaporated using a stream of nitrogen gas. The vials were caped and stored under exclusion of light at room temperature until further use.

Aqueous stock solutions (1 ml) of the three surfactants with concentrations of 0.1 % (w/v) for Solutol and 1.0 % (w/v) for AS and ACS were prepared. They were diluted with water to 6 different concentrations: 0.1, 0.05, 0.01, 0.005, 0.001, 0.0001, 0 % (w/v) for Solutol; 1.0, 0.1, 0.01, 0.001, 0.0001, 0.00001 % (w/v) for AS and ACS.

200 µl of each dilution was filled in a vial containing a pyrene aliquot (n=3). The vials were incubated under shaking for 24 h at room temperature. Afterwards, 150 µl of each vial were transferred to eppendorf cups and centrifuged at 12.000 g for 10 min with a Centrifuge 5415 R (Eppendorf AG, Hamburg, Germany). 100 µl of the supernatant were transferred in a 96 multiwell plate. Pyrene was exited at a wavelength of 339 nm and its emission was recorded at 390 nm using a PerkinElmer LS55 Fluorescence Spectrometer (PerkinElmer LAS GmbH, Rodgau-Jügesheim, Germany). The mean fluorescence intensity was plotted against the log of the surfactant concentration of AS, ACS and Solutol.

4.2.3. Solubility enhancement

Aqueous stock solutions of the surfactants were prepared at concentrations of 5, 10 and 20 % (w/v) under gentle stirring. 30 mg of CsA and 5 mg budesonide, respectively, were added to 1 ml of the surfactant solutions (n=3) and stirred for 24 h at room temperature in glass vials. Afterwards, the dispersions were transferred into Eppendorf cups and centrifuged at 16.000 g for 10 min. 100 ml of the supernatant were diluted with 900 µl of MeOH in HPLC vials (2 ml Screw top vial; BGB Analytik AG, Rheinfelden, Germany) and stored at -80 °C (HERAfreeze; Thermo Fischer Scientific GmbH, Ulm, Germany) until further use.

As an external standard, concentrations of CsA in MeOH of 10 %, 7.5 %, 2.5 %, 1.0 %, 0.75 %, 0.5 %, 0.25 % and 0.1 % (w/v) were used. The external standard concentrations of budesonide were 1 %, 0.75 %, 0.25 %, 0.10 %, 0.075 %, 0.05 %, 0.025 % and 0.01 % (w/v). The quantitative analysis of the residual drug content was carried out using high pressure liquid chromatography (HPLC).

For the cyclosporin A analytics, a Shimadzu chromatographic setup consisting of a SCL-10A_{VP} controller, a LC-10AT_{VP} pump at a flow rate of 1 ml/min, a SIL-10AD_{VP} autoinjector with a sample volume of 15 µl, a CTO-10AS_{VP} oven with a temperature of 75°C and a SPD-10A_{VP} UV detector at a wavelength of 210 nm were used (all from Shimadzu Deutschland GmbH, Duisburg, Germany). A reversed octadecyl column Luna 3µ C18(2) 100A with 100 x 4.6 mm served as solid phase (Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of 75 % MeCN and 25 % H_2O and was linearly decreasing to 90 % MeCN and 10 % H2O over 5 min. These concentrations were kept until 12.5 min and afterwards the column was re-equilibrated to the initial conditions until the end of the run at 15 min.

The chromatographic conditions for the budesonide analytics were in general as described for CsA with slightly different parameters. The injection volume of the autoinjector was set to 10 ml, the oven temperature was set to 22 °C and UV detection was carried out at 244 nm. An isocratic elution was carried out with a methanol – water mixture (80:20, v/v) over 5 min.

4.2.4. Drug- loaded micelle preparation

100 ml of a isotonic 5.2 % (w/v) mannitol aqueous solution (MAS) were prepared by gentle stirring. 30 ml of surfactant stock solutions (SSS) with concentrations of 5 % (w/v) AS, 5 % (w/v) ACS and 10 % (w/v) Solutol, respectively, were prepared by

dissolving the surfactants in MAS under gentle stirring. 100 mg CsA and 10 mg budesonide, respectively, were dissolved in 10 ml of each SSS by gentle stirring. The pH was adjusted to 7.4 and the SSS were stored at 4 °C until use.

4.2.5. Micelle size and zeta- potential measurements

The particle size and the polydispersity index, as a measure of size distribution of the micelles was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern, Herrenberg, Germany). The sample volume in the poly styrene semi-micro cuvettes was 400 µl. The zeta potential was measured in folded capillary cells (Malvern, Herrenberg, Germany) with a sample volume of about 1 ml by laser doppler electrophoresis using a Zetasizer Nano ZS.

The obtained data were analyzed using a One Way RM ANOVA in combination with a Holm-Sidak method for pair wise multiple comparison at a significance level of $p < 0.05$ using SigmaPlot 11.0 (Systat Software Inc., San Jose, California, USA).

4.2.6. Micelle characterization by transmission electron microscopy

Micelle size and shape of a drug- free of 5 % ACS aqueous solutions was visualized by transmission electron microscopy (TEM) using a negative staining technique. Therefore, 2 µl of the sample solution were dried on the surface of carbon coated copper grids (400 mesh; Plano GmbH, Wetzlar, Germany). The sample was stained using a 2 % (w/v) uranylacetate aqueous solution. Images were recorded using a Philips CM12 transmission electron microscope (FEI Electron Optics, Eindhoven, Netherlands) equipped with a slow scan CCD camera TEM1000 (TVIPS, Tietz GmbH, Gauting, Germany). Particle size was determined by measuring 100 micelles using the ImageJ software (National Institutes of Health, United States).

4.2.7. Entrapment efficiency

Four different surfactant concentrations were prepared to determine their entrapment efficiency of 0.1 % drug. To solubilize CsA aqueous solutions of 1 %, 0.7 %, 0.3 % and 0.1 % (w/v) AS and ACS, respectively, for budesonide concentrations of 3 %, 2 %, 1 % and 0.7 % (w/v) of the surfactants were prepared by gentle stirring.

1 ml of each concentration was spiked with 1 mg of CsA and budesonide, respectively, and stirred for 24 h at room temperature (n=3). The dispersions were centrifuged at 16.000 g for 10 min using eppendorf cups. 100 µl of the supernatant were diluted with 900 µl MeOH in HPLC vials (2 ml Screw top vial; BGB Analytik AG, Rheinfelden, Germany). The samples were stored at -80 °C (HERAfreeze; Thermo Fischer Scientific GmbH, Ulm, Germany) until further analysis.

The CsA and budesonide concentrations were determined by HPLC measurements as described under 4.2.3.

The entrapment efficiency E was calculated as

$$E[\%] = \frac{\frac{c_{n1}}{c_{o1}} + \frac{c_{n2}}{c_{o2}} + \frac{c_{n3}}{c_{o3}}}{3} \cdot 100$$

were c_n was the determined concentration in the sample, which was divided through $c_{o,}$ the initial concentration of 0.1 % drug for each of the three measurements. The average was calculated by division through n = 3 and finally, the relative entrapment efficacy E was held by multiplication with 100.

4.2.8. Compatibility of micelles with cornea cells

Compatibility with HCEpiC cells, a primary human cornea epithelial cell line, was determined in vitro by observation of the volume control of the cells. HCEpiC were

cultured in the delivered corneal epithelial cell medium (CEpiCM) according to the supplier's instructions. The cells were seeded on 13 mm glass slides and cultured until they showed confluence.

An aqueous solution containing 10 % (w/v) Solutol, an aqueous solution of 5 % (w/v) AS and an aqueous solution of 5 % (w/v) ACS were investigated as colloidal vehicles. BLUyal®, a 0.15 % sodium hyaluronate solution was used as negative (i.e. biocompatible) control. Volume detection was carried out using a superfusion system containing an Axiovert 40CFL microscope (Zeiss, Jena, Germany) equipped with a Coolsnap EZ camera (Fotometrix, Tucson, USA) and a perfusion chamber. The flow rate was set to 250 ml/h. The medium turnover took place within 30 s at a continuous flow. The period of observation was 25 min. It was split into 5 min flow of reference solution, a switch to sample solution for the next 10 min and after this a switch back to the reference solution for 10 min. The pictures were taken over the whole period (1 frame = 3 s). The cell area served as a measure for the volume. The data were processed using the ImageJ software.

The obtained data were analyzed using a One Way RM ANOVA in combination with a Holm-Sidak method for pair wise multiple comparison at a significance level of $p < 0.05$ using SigmaPlot 11.0.

4.2.9. In-vitro model for drug resorption

The experiments were conducted as described by Luschmann et al 2013 [27]. In brief: Enucleated pig eyes were supplied from a local abattoir and stored in isotonic, sterile sodium chloride solution until use. An eye was placed in the CRC and fixed. 200 µl of the solutions were placed on the plane cornea. The eyes were incubated for 30 min at room temperature. Each eye was washed four times with 1 ml of aqueous

5.2 % (w/v) mannitol solution. The cornea were excised with a surgical knife and homogenized by use of a tissue pulverizer (made by the university machine shop in analogy to the Bessman Tissue Pulverizer) and liquid nitrogen. To extract CsA, the tissue pieces were incubated for 2 hours under gentle stirring with 1 ml MeOH, and spiked with 100 ng/ml CsD as internal standard. After the extraction the suspension was centrifuged at 16.000 g for 15 min. 500 µl of the supernatant were filled in HPLC vials and stored at -80°C until further use. All samples were prepared and measured in triplicate.

4.2.10. Chromatographic method and equipment

The UHPLC-MS analyses were performed as described by Luschmann et al 2013[27]. In brief: An Agilent Technologies UHPLC system equipped with a 6540 quadrupole time of flight (Q-TOF) LC/MS system (Agilent Technologies Deutschland GmbH, Böblingen, Germany) was used. Separation was performed on a Waters Aquity BEH c18, 1.7 u, 2.1 x 50 mm column. The mobile phase consisted of 40 % (A) H_2O with 0.1 % formic acid (FA) and 60 % (B) methanol (MeOH) with 0.1 % FA. A gradient was applied for 3 min to concentrations of 0 % (A) and 100 % (B). Afterwards a column re-equilibration was performed for 3 min.

The obtained data were analyzed using the t – test at a significance level of $p < 0.05$ using SigmaPlot 11.0.

4.3. Results and discussion

4.3.1. Manufacture of drug- loaded micelles

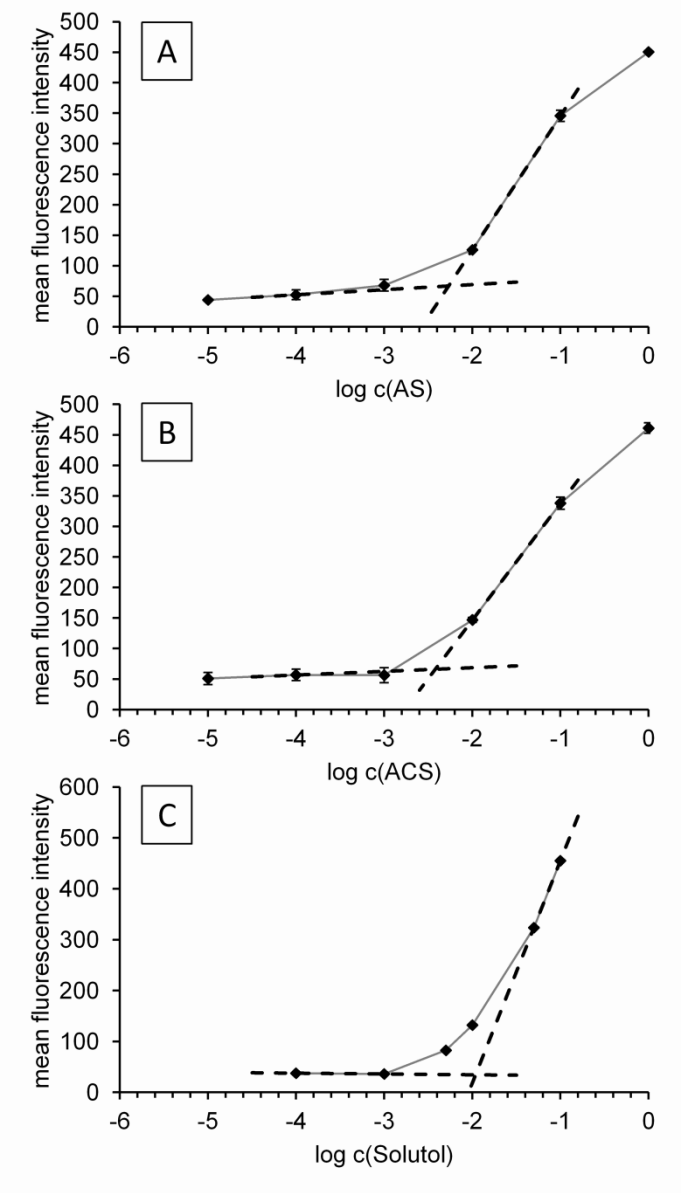

Fig. 4.1: Micelle characterization; determination of the CMC of [A] Sympatens AS and [B] Sympatens ACS and [C] Solutol by use of a pyrene fluorescence assay;

First of all we determined the surfactant concentration that was to be exceeded to obtain micelles. The observed CMCs were 0.0054 %, 0.0038 % and 0.0109 % (w/v) for AS, ACS and Solutol, respectively (Fig. 4.1).This is for Solutol in accordance with

the literature values [28], but for AS and ACS about 10 times higher than reported by the manufacturer.

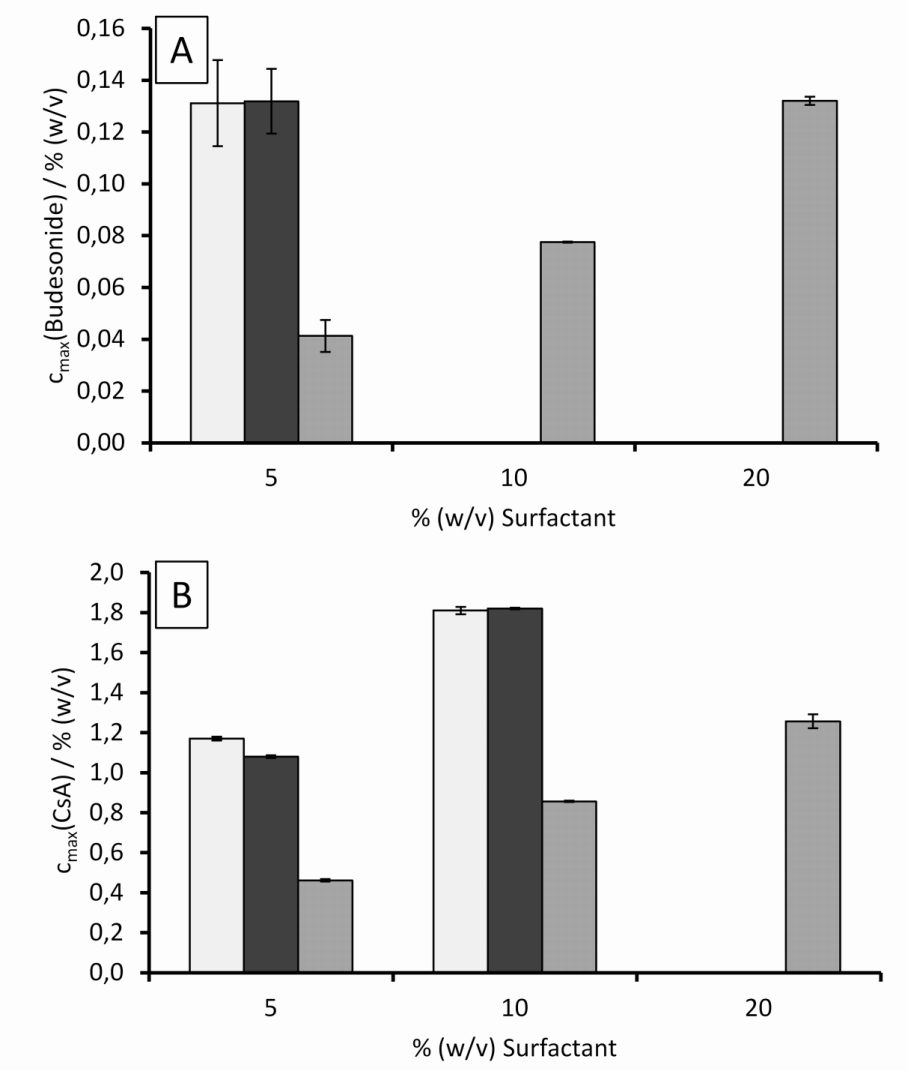

Fig. 4.2: Solubility: maximum solubility of [A] budesonide and [B] cyclosporin A by use of different concentrations of the non-ionic surfactants Sympatens AS (□), Sympatens ACS (■) and Solutol (▣);

Depending on the formulation, CsA and budesonide are typically applied in concentrations of 0.05 – 2,0 % (w/v) [29–31] and 0.025 – 0.05 % (w/v) [32], respectively. Since the solubility of both drugs in water is considerably lower, we explored the potential of the surfactants to increase drug solubility in water.

In general, all surfactants solubilized CsA and budesonide in a concentration dependent manner (Fig. 4.2). The highest levels for budesonide of 0.132 % (w/v), which are far above the typical therapeutic dosing, were reached with a surfactant concentration of 20 % Solutol, however, this was already possible with 5 % (w/v) of AS and ACS, respectively. At these surfactant concentrations approximately 1.2 % (w/v) of CsA could be solubilized, which was in the upper therapeutic range. With 10 % (w/v) of AS and ACS, respectively, it was nearly possible to reach the maximum applied dosing of 2 % (w/v) CsA dissolved in oil. In comparison to the soluble concentrations in water, this meant a 2700 fold increase of CsA and a 90 fold increase for budesonide [11,12]. Finally, AS and ACS solubilized four times higher amounts of each drug compared to Solutol and thus, showed a significantly higher potential as a surfactant for ophthalmic use.

4.3.2. Micelle characterization

4.3.2.1. Micelle size and charge

Due to their paramount significance for the tissue intrusion micelle size and zeta potential were determined.

All micelles showed very small and similar particles sizes (Fig. 4.3). AS and ACS formed micelles of around 10 nm, Solutol of about 12 nm. Micelle size was nearly unaltered after loading with CsA or budesonide, as reported in literature for drug-loaded polymeric micelles [33,34]. Irrespective of the surfactant CsA loaded micelles were smaller than budesonide loaded ones or the drug-free control. All preparations had a narrow size distribution, with a PdI < 0.16 and, a nearly neutral zeta potential with no significant differences between the drug-free and drug- loaded micelles.

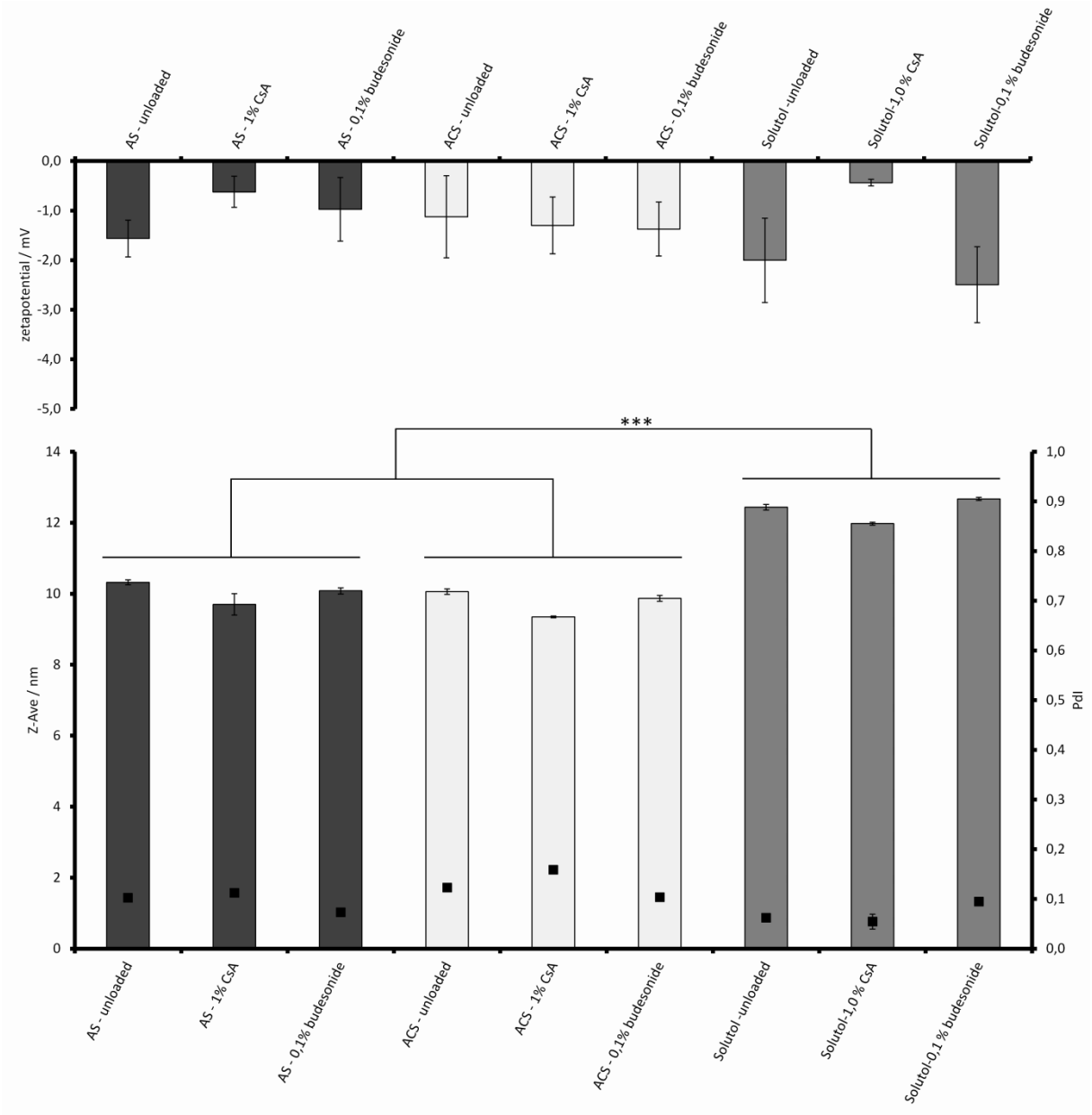

Fig. 4.3: Micelle characterization: Size, size distribution (PdI (■)) and Zeta potential of drug-loaded and drug-free micellar systems of Sympatens AS (■), Sympatens ACS (□) and Solutol (■); the drug loading was 1% (w/v) CsA or 0.1 % (w/v) budesonide; *** = P < 0.001

While on the one hand that meant that the particles would not be able to stick to the negatively charged tissue surface via electrostatic interactions [35]. There may be on the other hand a benefit on positively charged colloidal particles in terms of biocompatibility [17,36]

4.3.2.2. Shape and distribution

To visualize the micellar structures, and to confirm the PCS results, TEM pictures were exemplarily taken of a 5 % Sympatens AS aqueous solution. The images are shown in Fig. 4.4, [A] + [B]. A homogeneous distribution of the colloidal structures with a size of 9.2 ± 1.2 nm could be observed. The micelles were of nearly spherical shape. These results agree with the data determined by dynamic light scattering and data by Di Tommaso et al [37].

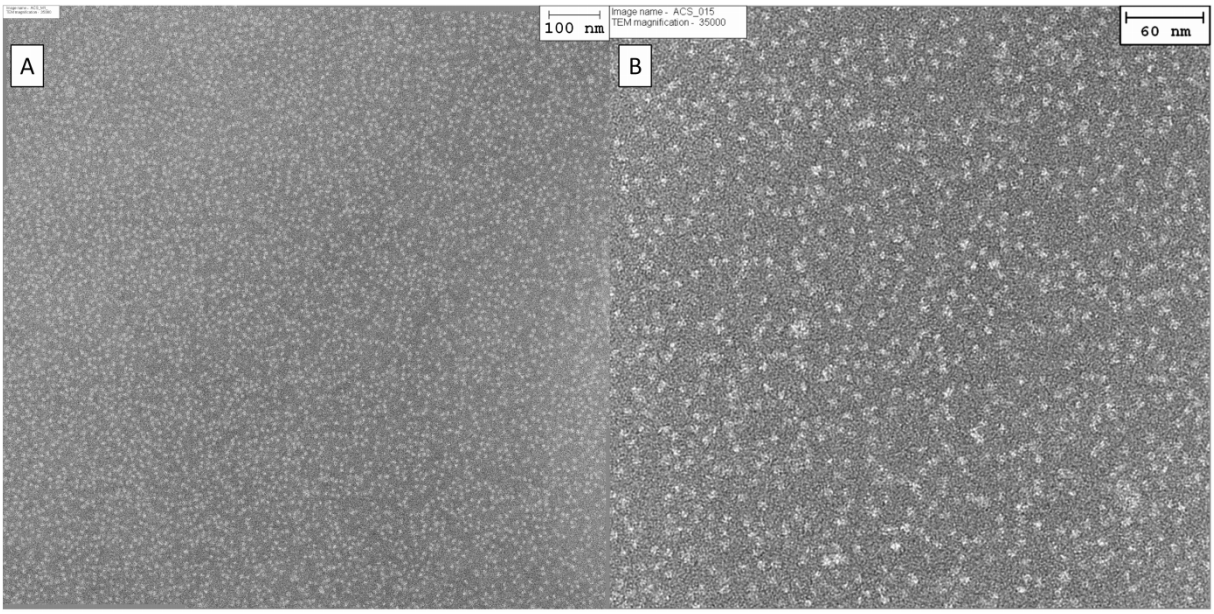

Fig. 4.4: Micelle characterization: [A]+[B] TEM images of 5 % Sympatens ACS micellar system;[A] overview over the homogeneous preparation; [B] single micellar structures;

4.3.2.3. Entrapment efficiency at therapeutically dosing

Upon the drug solubility study, the goal was now to identify the minimum amount of surfactant needed for therapeutically relevant aqueous formulations. Due to their superior solubilization potential, only Sympatens AS and ACS were investigated.

Fig. 4.5 [A] shows the encapsulation efficiency for 0.1 % (w/v) budesonide. Surfactant concentrations were ranging from 0.75 % to 3.0 %. None of the

concentrations could encapsulate 100 % of the initial drug amount. The highest rate was 64 % (w/v) with 3 % AS and ACS, respectively.

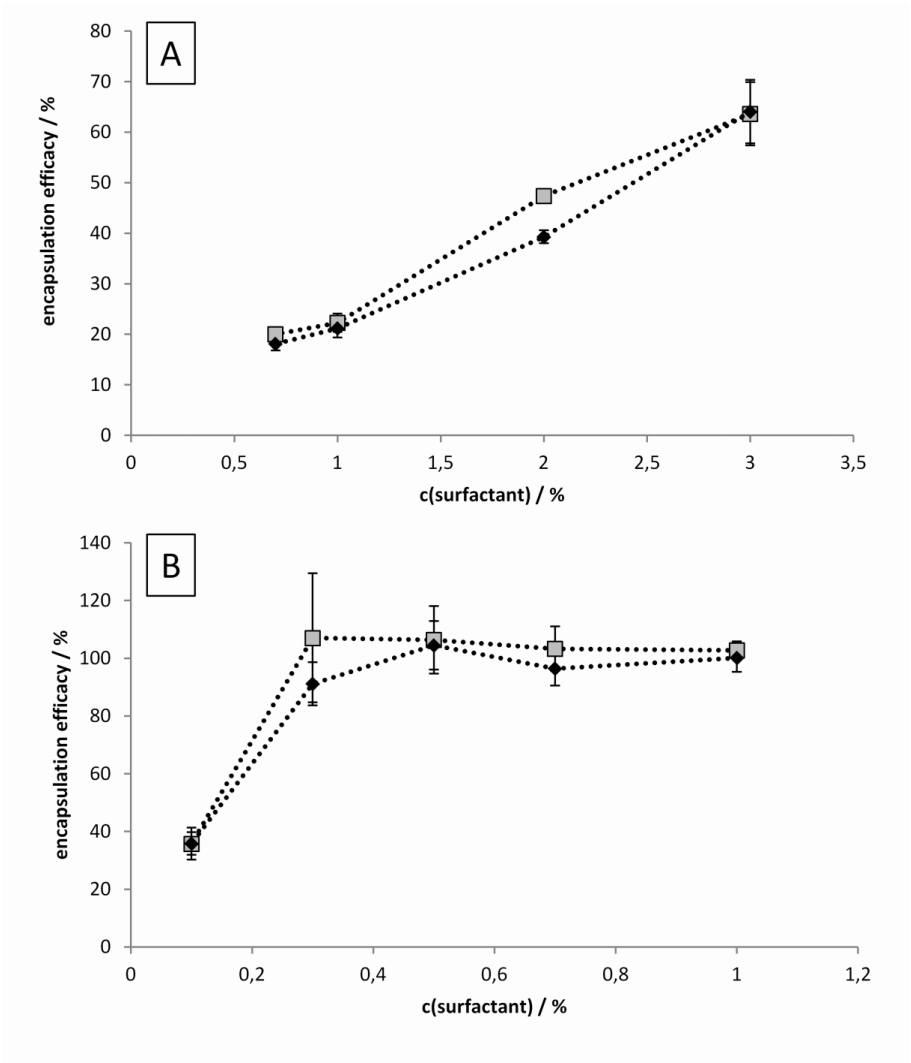

Fig. 4.5: Encapsulation efficacy: encapsulation of an initial amount of [A] 0.1 % (w/v) budesonide and [B] 0.1 % (w/v) cyclosporin A by micellar solutions of Sympatens AS (□) and ACS (◆) at different concentrations;

Due to the therapeutic doses from 0.025 % (w/v) to 0.05 % (w/v) of budesonide [32], surfactant concentrations between of at least 1.0 % (w/v) of AS and ACS were required. Similar solubilization qualities were reported by Sahib et al for a 3 % (w/v) PEG_{5000}-DSPE micellar solution [14].

The surfactant concentrations for cyclosporin A ranged from 0.1 % to 1.0 % (w/v) Fig. 4.5 [B] and the initial drug amount was set to 0.1 % (w/v), according to Restasis® (0.05 % CsA) [38]. With at least 0.3 % (w/v) and 0.5 % (w/v) of AS and ACS, respectively, the encapsulation efficiency was 100 % of the initial amount of drug, but at 0.3 % (w/v) of ACS it was still 91 ± 7.5 %. Hence, to prepare a formulation with 0.05 % (w/v) CsA, the amount of surfactant, could be reduced down to 0.3 % (w/v). In contrast, to prepare a 0.066 % CsA solution, Di Tommaso et al needed 3.0 % MPEG-hexPLA copolymer [16].

4.3.3. Cornea cell compatibility

To test the cell compatibility of formulations we used primary human epithelial cornea cells. Due to their sensitivity, the volume control of the cells was used as a measure for tolerance towards the surfactant solutions [39].

In all cases we did not observe any adverse reaction or loss of cells in the monolayer. Fig. 4.6 shows the changes in cell volume for the treatment with a 5 % aqueous solution of Sympatens AS, a 5 % aqueous solution of ACS and a 10 % aqueous solution of Solutol, all compared to a 0.15 % sodium-hyaluronate solution as control. The cells showed a nearly constant volume decrease down to about 70 % of the initial cell volume for all liquids towards the endpoint of observation. Only the cell culture medium was tolerated without any cell volume change. Even with classical ringer solution a similar decrease could be detected (data not shown). Only the last 2 values of the sample in panel [B] showed an unexpected decrease, which seemed be due to a layer focus problem of the camera after medium change and hence, the highly blurry cell membrane.

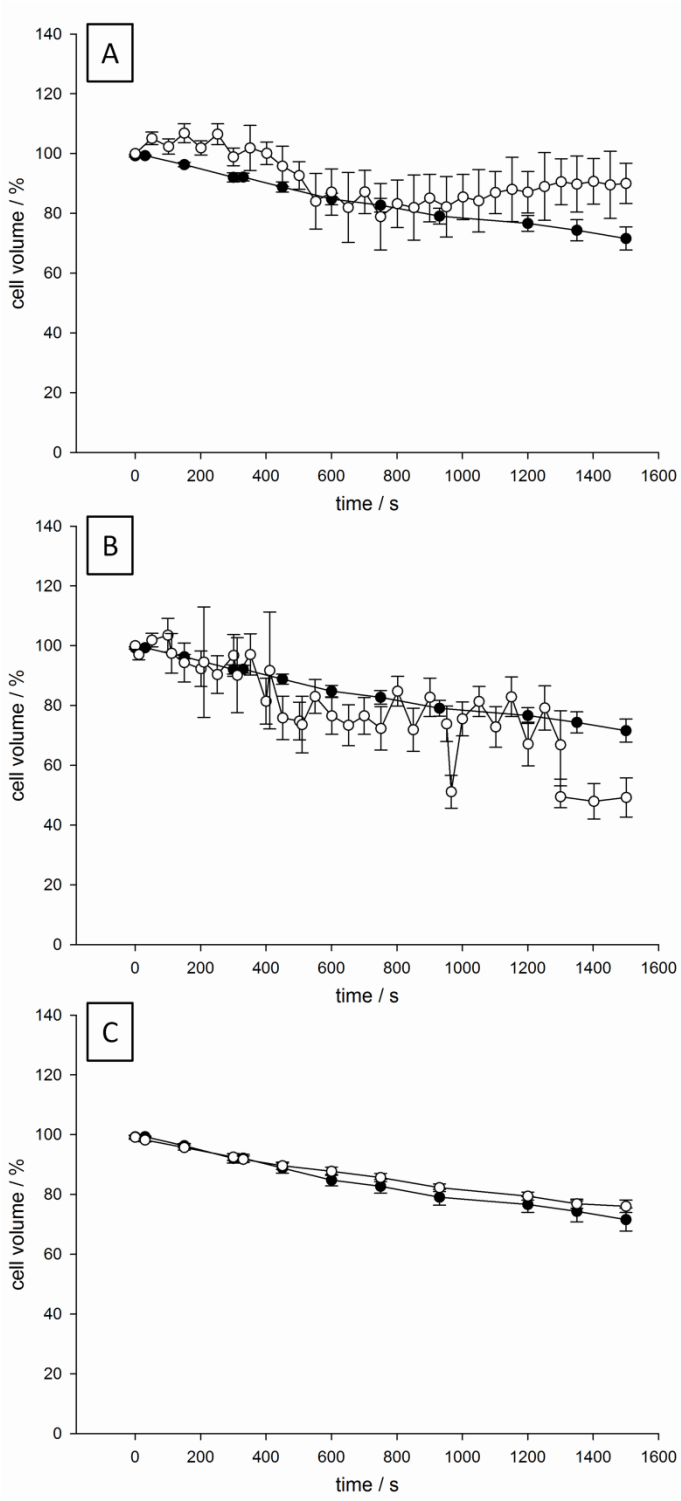

Fig. 4.6: Tolerability: effect towards the volume control of HCEpiC cells in-vitro as a measure of tolerability of the micellar solutions; [A] 5 % (w/v) Sympatens AS (○), [B] 5 % (w/v) Sympatens ACS (○) and [C] 10 % (w/v) Solutol (○); SHS was used as control in all panels (●); timescale: 0-300 s reference solution; 300-930 s switch to sample solution, 930-1500 s switch back to the control;

The differences between the curves of sample AS and ACS in comparison to the curves of the reference and Solutol were supposably detected by reason of the two different batches of HCEpiC cells used. Due to the fact, that the HCEpiC were primary cells, a higher variation between the single batches and experiments was expected.

In general, it could be shown, that none of the highly concentrated surfactant solutions had a significantly effect on the cell volume beyond that of a commercially available, biocompatible, ocular lubricant.

4.3.3.1. Drug resorption in-vitro

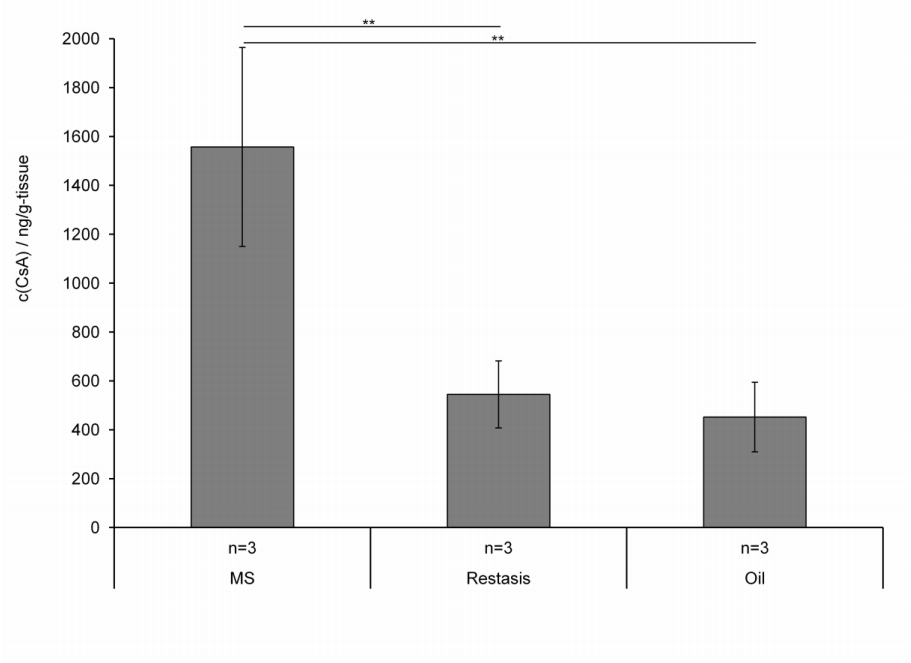

Fig. 4.7: In-vitro resorption: amount of CsA taken up by porcine-cornea from 200µl sample solution over a period of 30 min; sample solutions: MS (0.05 % CsA micellar solution), Restasis® (cyclosporine ophthalmic emulsion 0.05 %), Oil (2 % (w/v) CsA olive oil solution); ** = P < 0.01 (t-test); measurements were performed in triplicate; results are shown as average ± standard errors;

To explore the potential of our self- assembling colloidal systems to serve as a therapeutic formulation for the anterior eye, we investigated the CsA uptake in porcine corneal tissue of the 0.05 % CsA micellar solution.

We compared a 0.05 % micellar CsA solution to Restasis® and a 2 % CsA olive oil solution, which are the currently used parameters for ocular CsA administration (Fig. 4.7). The mean drug concentration in the cornea for the MS was 1557 ± 407 ng_{CsA}/g_{cornea}. It was significantly higher than those, reached with Restasis® (545 ± 137 ng_{CsA}/g_{cornea}) or the 2 % CsA oily solution (452 ± 142 ng_{CsA}/g_{cornea}). These values agree well with literature data. Daull et al. observed comparable levels 20 min after single administration of a cationic 0.05 % CsA nanoemulsion and Restasis® in rabbits [35]. Hence, even though the volume of 200 µl, that was needed in our study to ensure a total wetting of the porcine cornea, was above the volume of a typically administered eye drop (25-50 µl), the in-vitro model seemed suitable to predict the behavior of different types of formulations quite well. The 2 % CsA oily solution, did not show any significant differences compared to Restasis®. The average concentration was even less. Despite of its high drug loading, which was a 40 fold of our MS and also of Restasis®, the oily solution showed the worst tissue levels. This, and also the significant lower levels of Restasis® in comparison to the 0.05 % CsA MS, seemed to be due to the expected partition of CsA, which is known to have a clearly higher affinity to lipophilic vehicles than to the hydrophilic milieu of the cornea [13]. Referring to the therapeutic tissue levels of CsA reported by Kaswan et al (50 – 300 ng/g_{tissue}) [20] and the results of this in vitro investigation, the 0.05 % CsA MS developed by our group, should easily be able to cause therapeutic levels in the cornea which are significantly higher than those of Restasis®. In comparison to the levels of Daull and Di Tommaso et al, it would be possible to lower the number of applications per day towards Restasis®, which is at least administered twice a day [24,35,38]. Thus in combination with an increased therapeutic option, the 0,05 % CsA MS would enhance further the patients' compliance.

The distribution of budesonide into the posterior segment of the eye either by topical or intraocular administration has to be proven in further investigations.

4.4. Conclusion

We summarize from the present work, our self- assembling micellar solutions are a highly interesting and promising approach to overcome problems, which usually occur with the formulation and administration of poorly water drugs. With suitable non-ionic surfactants the drug solubility was enhanced significantly in comparison to pure water. At therapeutic drug loadings only small amounts of surfactant were necessary to create transparent, liquid systems. The tremendous advantage of the AS and ACS micelles was their self assembly and drug loading directly in the aqueous medium by use of only gentle stirring, which led to small sized and neutrally charged colloidal structures, The solutions were as perfectly tolerated by HCEpiC cells in-vitro. Finally, for CsA, the porcine in-vitro resorption model showed big advantages compared to typically used and also to approved formulations. The mucoadhesive qualities of the PEG surface of our micelles might help to increase this effect further in-vivo [20].Upon these results our self- assembling micellar solutions are a novel option in the treatment of diseases at the anterior segment of the eye with poorly soluble drugs.

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Chapter 5

A self-assembling, colloidal system for poorly soluble drugs: a stability study

Abstract

Colloidal, micellar systems, especially those assembled by classical surfactants are repeatedly reported to be challenging systems concerning their stability. With the present study the long term stability at 5 °C over a 12 month- period of self-assembling micellar solutions was investigated. The drug- free samples were perfectly stable in terms of micelle size and size distribution. Also no tremendous changes in pH and osmotic pressure, whether buffered or non-buffered, were observed. CsA was stable in a non-buffered solution. Constant particle size (Z_{AV} = 7.7 – 8.6 nm) and distribution (PdI <0.07) as well as a quite stable pH with a variation within one unit were detected. No loss of CsA was observed for 12 months. Budesonide, with a stability optimum at a slightly acid pH, showed in two of the non-buffered systems a loss of 13 % of the initial drug content. Furthermore, a slight increase of particle size was detected. However, when stored in isotonic buffered solution at pH of 6.1, only one sample showed a drug loss of 5 %. The solutions are a highly promising approach towards the treatment of diseases at the anterior or posterior segment of the eye.

5.1. Introduction

Many different approaches have been developed in recent years to deliver hydrophobic, poorly soluble drugs to their site of action. Nano-emulsions, liposomes, nano-associates or micelles are just a few examples for colloidal delivery systems [1–5] that have been identified as a promising approach. Micelles, formed by amphiphilic structures above a substance specific concentration, the so called critical micelle concentration (CMC), are nanoparticles with a hydrophobic core and a hydrophilic shell [6]. Two types of micelles are prevalent in literature: so called polymeric micelles, formed by copolymers and surfactant micelles, formed by classical surfactant molecules [7,8]. Both types have the ability to form small colloidal structures below 200 nm and to incorporate hydrophobic drugs into their hydrophobic core [6]. Chauhan et al, for example, prepared surfactant micelles loaded with the hydrophobic ketoconazole [9], Mondon et al., reported on cyclosporin A (CsA) loaded polymeric micelles [5]. However, it was claimed that polymeric micelles have a superior stability compared to surfactant micelles [10,11].

In this thesis a colloidal system with a high drug loading for the poorly soluble drugs budesonide (14 µg/ml [12]) and cyclosporin A (6.6 µg/ml [13]) was developed. Non-ionic surfactants of the poly(ethylene glycol) fatty alcohol ether type were used to prepare micellar solutions with a particle size of 8 to 10 nm. A very important topic with respect to a drug product is the stability of such formulations [14]. According to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), the purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product changes with time under the influence of a variety of environmental factors such as temperature and humidity [15].

Hence, upon the very promising data already shown in this thesis, the aim of the present research was to investigate the stability of drug- loaded surfactant micelles in terms of shelf life, particle size and residual drug content in a long term stability study according to the ICH guidelines for “Drug substances intended for storage in a refrigerator” at 5°C ± 3°C over 12 months [15].

5.2. Materials and methods

5.2.1. Materials

Cyclosporin A (98.5 %) (CsA) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Budesonide (99.7 %) was purchased at the European Directorate for the Quality of Medicines and Healthcare (Strasbourg, France). Sympatens AS/200G (AS) and Sympatens ACS/200G (ACS) were kindly provided by KOLB (Hedingen, Switzerland). Water for injection in bulk was obtained according to the European Pharmacopeia by an in-house purification system (Stulln, Germany). Disodiumhydrogenphosphate (99.5 %), sodiumchloride (99.8 %) and phosphoric acid (89 %) were purchased at Merck (Darmstadt, Germany). Methanol (MeOH), acetonitrile (MeCN), isopropanol (all HPLC grade) and trifluoric acid (99.9 %) were obtained from Merck (Darmstadt, Germany).

5.2.2. Sample preparation

The clear and colorless micellar solutions were prepared by dissolving the drug and / or the surfactant in water under gentle stirring for 24 h. The different compositions are shown in Tab. 5.1. Besides placebo solutions, three different drug concentrations were made: a placebo, a low concentration and a high concentration. Furthermore, the two surfactants AS and ACS were investigated. Budesonide samples were prepared non-buffered as well as buffered. CsA samples were only produced non-buffered.

Budesonide: An amount of 100 g stock solution was prepared by dissolving 5 g of surfactant together with 0 mg, 50 mg and 100 mg budesonide, respectively, in water. 50 g of each solution were buffered with 52 mg disodiumhydrogenphosphate,

isotonized with 360 mg of sodium chloride and the pH was adjusted to 6.0 using phosphoric acid. Each solution, the non-buffered and the buffered one, was filled into 15 poly(ethylene) eye drop bottles (2 ml).

Cyclosporin A: An amount of 50 g stock solution was prepared by dissolving 2.5 g of surfactant together with 0 mg, 100 mg and 500 mg CsA, respectively, in water. The solutions were filled into 15 poly(ethylene) eye drop bottles (2 ml).

Tab. 5.1: Sample composition: micellar solutions of budesonide and cyclosporin A for stability storage over 12 months using two different surfactants (s) and two different drug (d) concentrations; budesonide samples were prepared non-buffered (n.b.) and buffered (b.) with disodiumhydrogenphosphate; buffered samples were further isotonized (i.) with sodiumchloride;

		Budesonide			**Cyclosporin A**		
#	**Surfactant**	c(d) / %	c(s) / %		c(d) / %	c(s) / %	
1	AS	0	5	n.b.			
2	AS	0.05	5	n.b.			
3	AS	0.1	5	n.b.			
4	ACS	0	5	n.b.			
5	ACS	0.05	5	n.b.			
6	ACS	0.1	5	n.b.			
7	AS				0	5	n.b.
8	AS				0.2	5	n.b.
9	AS				1.0	5	n.b.
10	ACS				0	5	n.b.
11	ACS				0.2	5	n.b.
12	ACS				1.0	5	n.b.
13	AS	0	5	b./i.			
14	AS	0.05	5	b./i.			
15	AS	0.1	5	b./i.			
16	ACS	0	5	b./i.			
17	ACS	0.05	5	b./i.			
18	ACS	0.1	5	b./i.			

5.2.3. Storage conditions and sample treatment

The samples were stored under exclusion of light over a period of 12 months at 4 °C. The sampling points were set to 0, 1, 3, 6, 9 and 12 months. At each point 3 bottles were withdrawn for analysis and equilibrated to room temperature for 24 h. One bottle was used for particle size measurements. The two other bottles were combined and pH and tonicity were assessed. Afterwards the samples were prepared for high performance liquid chromatography (HPLC) analysis.

5.2.4. Physicochemical characterization

The pH of the samples was measured at room temperature using a SevenMulti^{TM} pH meter with a sampling volume of 2 ml from Mettler-Toledo GmbH (Gießen, Germany). The osmotic pressure was determined using a Knauer – semi-micro osmometer K7400 (KNAUER Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin). The sample volume was 150 µl.

5.2.5. Chromatographic analysis

The residual amount of drug was determined using the Hitachi LaChrome Elite chromatographic equipment, which consisted of the HTA L-2130A pump, the HTA L-2200 autoinjector, the HTA L-2300 column oven and the HTA L-2400 UV detector. If the samples were opaque they were filtered before analysis using a Durapore syringe filter with a pore size of 0.2 µm (Millipore Corporation, Billerica, MA, USA).

For the budesonide analytics as solid phase a reversed phase octadecyl column YMC-Pack ODS-AQ, 3µm, 150 x 4.6 mm was used. The mobile phase consisted of (A) 65 % “buffer solution pH 3.2” and (B) 35 % acetonitrile. To obtain (A) 7.2 g disodiumhydrogenphosphate were dissolved in 1800 ml water. 200 ml of an aqueous

solution of 2.5 g/l phosphoric acid were added to these mixtures. After pH adjustment the solution was filtered through a 0.2 µm membrane. After 22 min a gradient over 5 min to 50 % (A) and 50 % (B) was applied and these levels were kept till the end of observations at 40 min. Afterwards the column was equilibrated over 10 min to obtain the starting conditions. Budesonide was detected at 240 nm using UV detection. The flow was set to 1.6 ml/min, the injected volume was 20 µl and the column temperature was set to 60°C. As reference solution 25 mg budesonide was dissolved in 15 ml (B) and diluted to 100 ml with (A) to obtain a drug concentration of 0.25 mg/ml. 1 ml sample solution was diluted to 2 ml with (A). Reference and samples were prepared in duplicate.

For the cyclosporin A analytics as solid phase a reversed phase octadecyl column Waters SunFire C18, 3.5µm, 100 x 3 mm was used. The mobile phase consisted of (A) 38 % H_2O, (B) 60 % acetonitrile and (C) 2 % isopropanol, each containing 0.01 % trifluoroacetic acid. A gradient was applied. Until 6.5 min the concentrations changed to 24 / 67 / 9 % (A / B / C). Within the next 0.5 min the amounts changed further to 25 / 70 / 5 % (A / B / C). These levels were kept till 9 min. Afterwards, the column was re-equilibrated over 6 min. CsA was detected at 220 nm using UV detection. The flow was set to 1.0 ml/min, the injected volume was 20 µl and the column temperature was set to 60°C. As reference solution (S1) 50 mg CsA were dissolved in 50 ml MeOH to obtain a drug concentration of 1.0 mg/ml. To obtain (S2) and (S3) 1 ml of (S1) was diluted to 10 ml and 100 ml with MeOH, respectively. 1 ml sample solution was diluted to 10 ml with MeOH. Reference and samples were prepared in duplicate.

5.2.6. Particle size and distribution

The particle size of the self- assembling micelles, was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern, Herrenberg, Germany). The hydrodynamic diameter (Z_{AV}), the polydispersity index (PdI), the intensity weighted diameter (d_I) and the amount of micelles at the given d_I (% d_I) were assessed. The sample volume in the poly styrene semi-micro cuvettes was 400 µl. The measurements were performed in triplicate.

5.3. Results and discussion

5.3.1. Physicochemical characteristics

As basic stability parameters the pH and the osmotic pressure were investigated at each sampling time. The non-buffered budesonide samples (Fig. 5., left panel) showed a quite stable pH with a small variation within one pH unit. Initially, the samples prepared with AS (#1-3) had a slightly lower pH than the samples prepared with ACS (#4-6) (Tab. 5.2). After 12 months the pH ranged between 7.5 and 5.6. However, there was neither a clear dependency observed in terms of the used drug or surfactant concentration nor in terms of the surfactant type. The samples #13-18 (Fig. 5., right panel), buffered with disodiumhydrogenphosphate, showed a constant pH throughout the whole period without any significant variation. As reported in literature budesonide had its stability optimum in the acidic range (pH 4 – 5) and higher levels would lead to degradation [16]. Therefore, the buffered samples were adjusted to pH = 6.1 to have a milieu which would be tolerated by budesonide as well as by the anterior eye (pH of the tearfilm: 7.1-7.6 [17]). Due to the presence of bicarbonate ions in the tear film and hence, its buffer capacity, the European

Pharmacopeia (Pharm. Eur. 6.0) allows the application of euhydric liquids [18]. Thus, even with the non-buffered samples, the pH was always in an acceptable range.

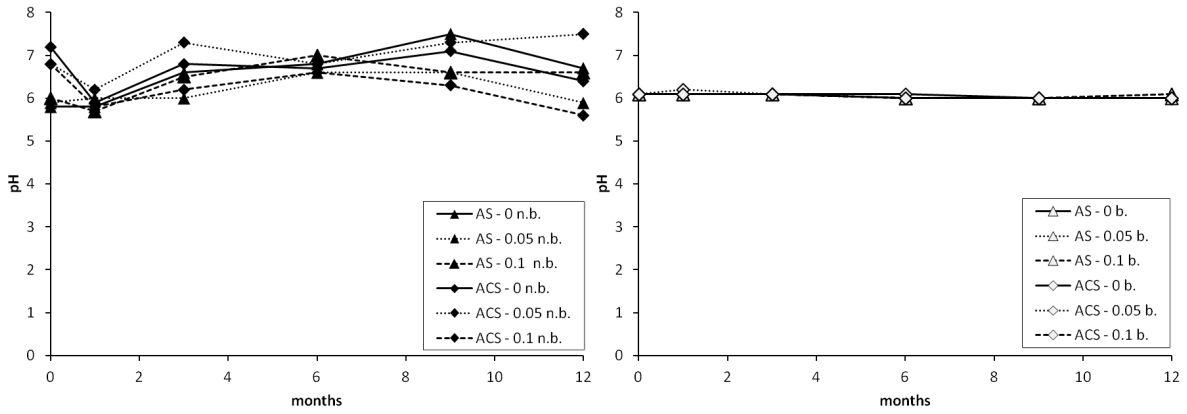

Fig. 5.1: Physicochemical stability: pH over 12 months of budesonide micellar solutions; *left panel:* samples # 1-6, non-buffered; *right panel:* samples #13-18, buffered;

The samples #1-6 were neither buffered nor isotonized. They showed a very low, but constant osmotic pressure over the 12 months (Fig. 5.2, left panel). The osmotic pressure of the samples #13-18 was adjusted with sodium chloride and was constant throughout the experiment in a range around 280 mosmol. Despite the same treatment, only sample # 16 showed constantly higher values (Fig. 5.2, right panel). As a mean osmolarity in human tears, William et. al observed 318 mosmol in his study on 324 tear samples [19]. Even if it was shown that slightly hypotonic eye drops would have no disadvantage compared to isotonic solutions with dry eye patients [20], for stronger hypotonic conditions a swelling of cells and even an occurring opacity can be expected [21,22]. Therefore, it will be necessary to adjust at least the osmotic pressure to physiological levels to guaranty an application without any irritations.

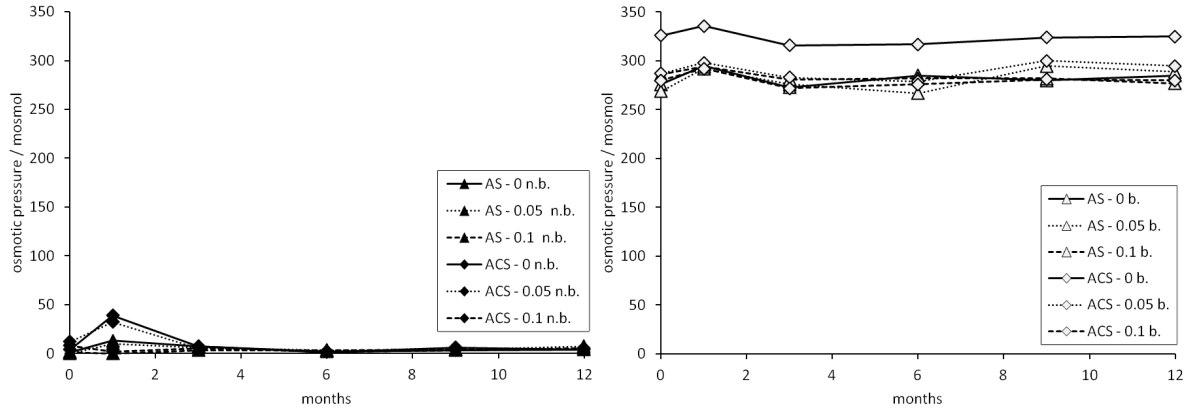

Fig. 5.2: Physicochemical stability: osmotic pressure (π) over 12 months of budesonide micellar solutions; *left panel:* samples # 1-6, non-buffered; *right panel:* samples #13-18, buffered;

Due to a known instability with ionic additives, the CsA micellar solutions (#7-12) were only investigated non-buffered and non-isotonized [23]. Similar to budesonide, their pH (Fig. 5.3, left panel) was quite constant over the time of observation. Initially the values were around pH 7 and decreased over 12 months to levels between pH 5 and 7. The decrease after one month seemed to be due to a measurement problem because it affected all samples. A pH < 5.8 or > 11.4 is known to cause irritations with a possibility of 99 % [24]. However, many drugs are used in solutions with a pH around 5. Hence, the final pH of a solution should be a compromise between the stability or functional optimum of the drug and the physiological needs of an application. Due to the fact that CsA is known to be stable over a wide range of experimental conditions [25], it would be beneficial to adjust the pH of the subsequent formulations more in the range of the physiological pH.

The osmotic pressure of the samples #7-12, as seen for budesonide, was very low but constant (Fig. 5.3, right panel). As discussed above, an adjustment to isotonic levels using, for example, mannitol as non-ionic additive would be preferable.

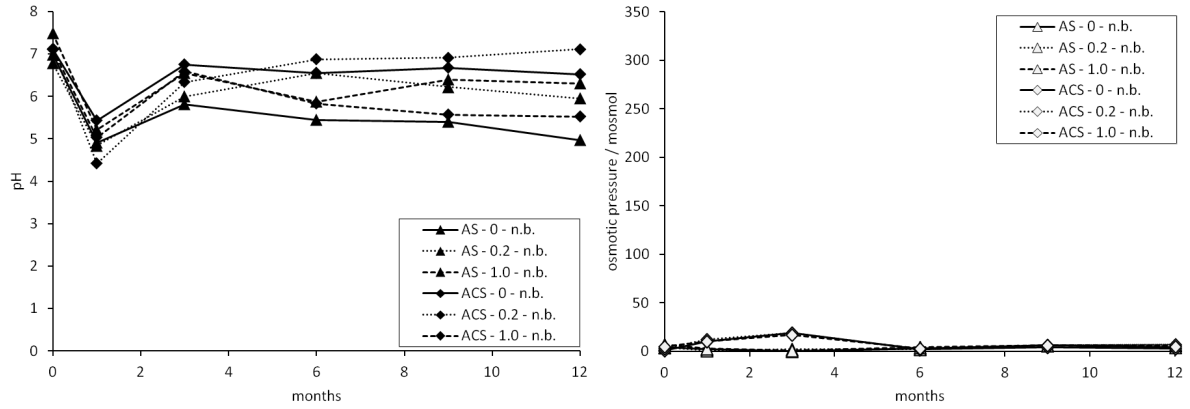

Fig. 5.3: Physicochemical stability: CsA micellar solutions, samples #7-12, non- buffered; *left panel:* pH; *right: panel:* osmotic pressure (π);

5.3.2. Particle size and distribution

The particle size of the micelles was determined to observe agglomeration or micelle growth over time. Fig. 5.4 shows the Z_{AV} and PdI (upper panel) as well as the d_I $\%d_I$ (lower panel) for the budesonide micellar solutions (#1-6). In general, a very constant size of the colloidal structures was observed. The Z_{AV} for all samples was between 8.4 nm and 10.1 nm over the whole period. Even if there were differences detectable, at the end of observation all samples showed a very similar and very small Z_{AV}. Even though the PdI which is a measure for the homogeneity of the size distribution increased slightly over time, it still was below 0.25. At the end of observation a slight increase of the Z_{AV} (< 9.5 nm) and PdI (< 0,25) was observed for the non-buffered drug- loaded samples (#2, #3, #5). Overall, the samples prepared with ACS (#4-6), contained smaller micelles than samples prepared with AS (#1-3).

For the intensity weighted diameter and its distribution similar results were observed. In contrast to the Z_{AV}, the d_I could show that the general size of the micelles was stable throughout the period of 12 months. The increased Z_{AV} and PdI correlated with a decrease of the $\%d_I$ at 9 and 12 months. While the d_I was stable, its amount at the

end of observation was slightly decreased; however, at least 95 % of the micelles in every sample were stable.

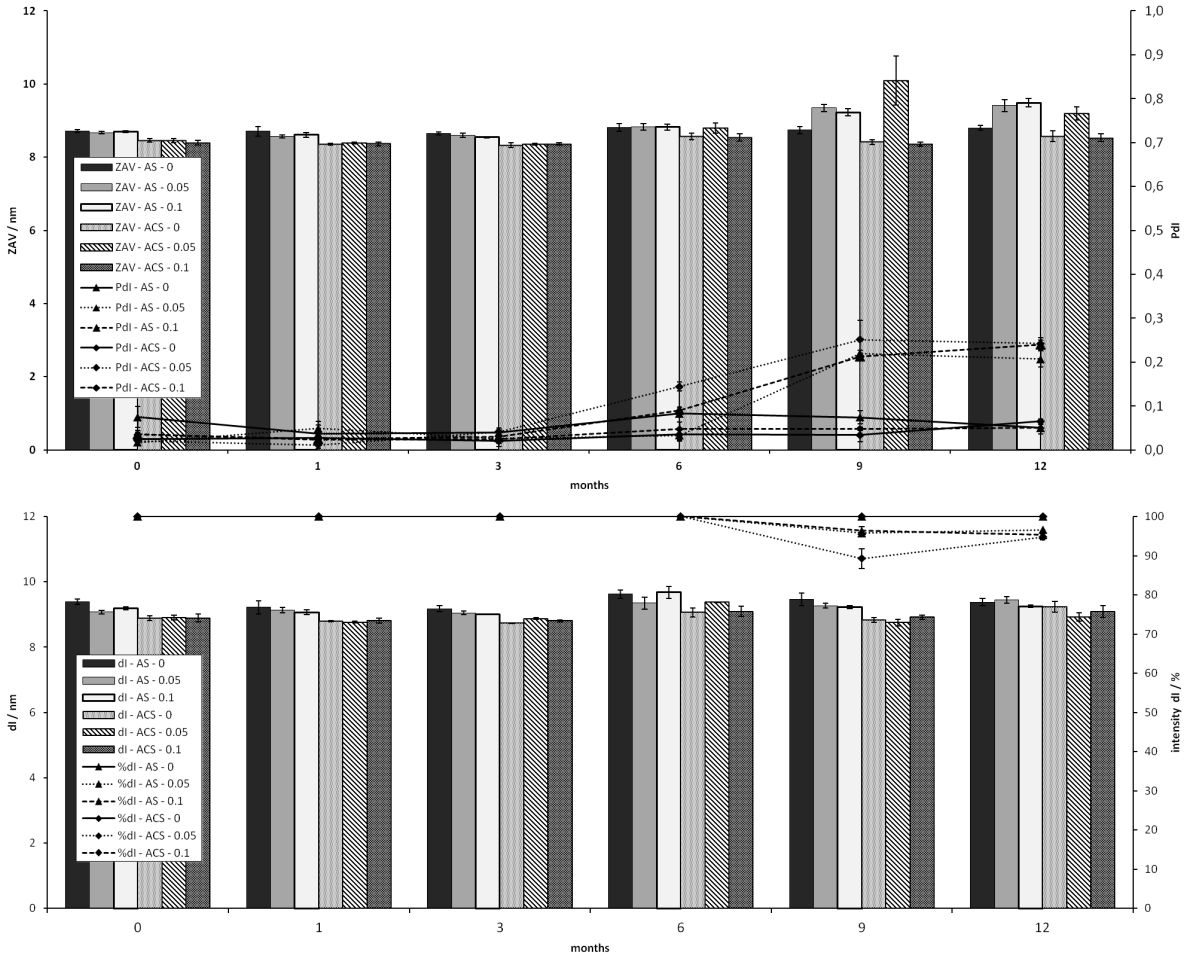

Fig. 5.4: Micelle stability: size and size distribution of the non- buffered budesonide samples #1-6; *upper panel:* hydrodynamic diameter (Z_{AV}) and polydispersity index (PdI); *lower panel:* intensity weighted diameter (d_I) and amount of micelles with the given d_I (d_I / %);

Fig. 5.5 shows the results of the particle size measurement of the CsA micellar solutions (#7-12). Each sample had a perfectly stable Z_{AV} and PdI over 12 months. The Z_{AV} was between 7.6 nm and 8.9 nm and the PdI was < 0.1, reflecting a very homogeneous size distribution. At higher drug loading slightly smaller micelles were observed. As already seen for budesonide, the micelles prepared with AS were slightly larger than the ones prepared with ACS. The d_I, as already seen for the Z_{AV},

was constant over the period of observation. The small PdI values from the upper panel were confirmed by the $\%d_I$.

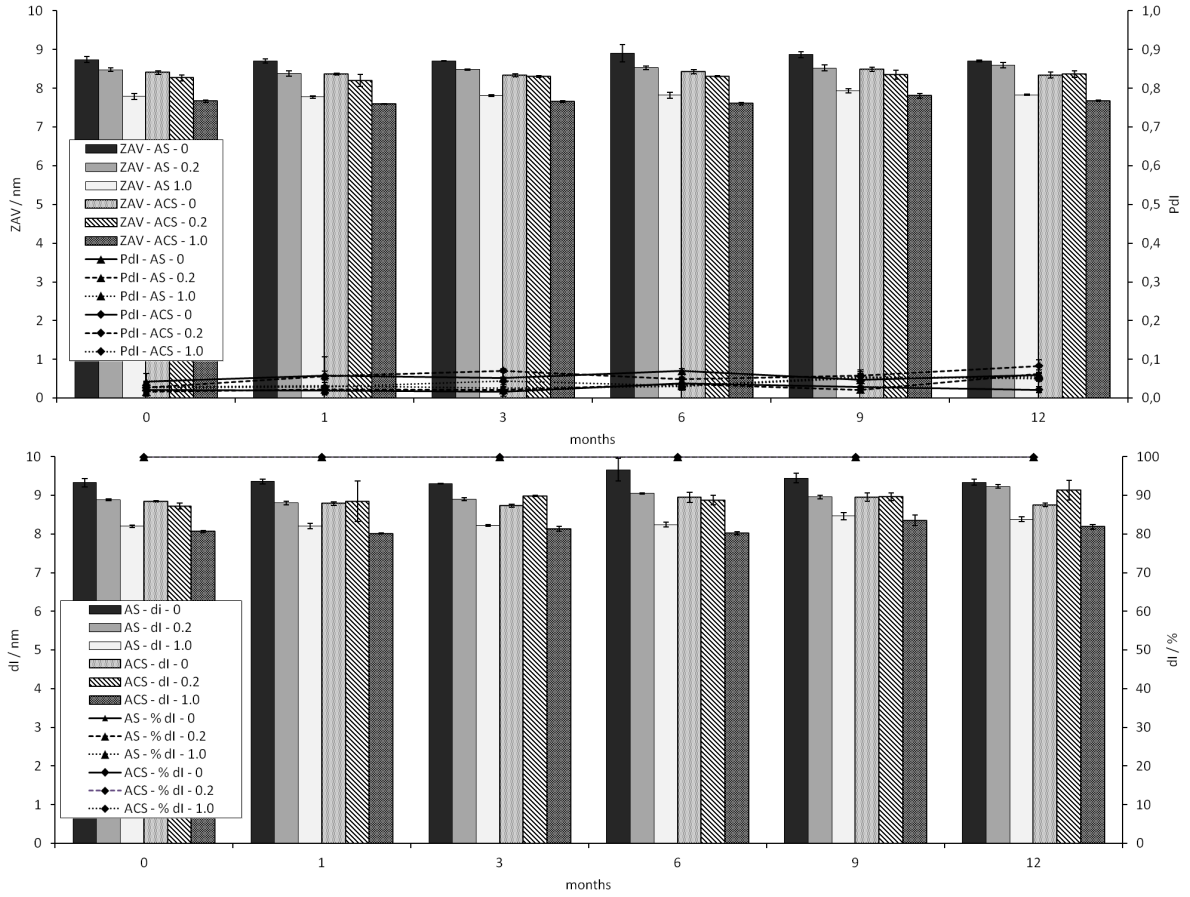

Fig. 5.5: Micelle stability: size and size distribution of the non- buffered CsA samples #7-12; *upper panel:* hydrodynamic diameter (Z_{AV}) and polydispersity index (PdI); *lower panel:* intensity weighted diameter (d_I) and amount of micelles with the given d_I (d_I / %);

An important parameter closely related to the stability of micelles is their critical micelle concentration [10, 26]. As shown in this thesis, both surfactants have very low CMCs that seemed to be small enough, to solubilize the systems for at least one year [27]. Despite the fact, that the stability of micelles typically increases, when loaded with hydrophobic drugs [10, 26], this was not observed for CsA or budesonide. This may be due to the very stable drug- free micelles. In contrast, for budesonide few slightly larger micelles or aggregates thereof occurred. In comparison to already reported stability data where at 4°C with CsA loaded micelles

bigger particles in a size of 150 - 220 nm were detected, our micellar system seemed very stable [5]. We observed that the micelles loaded with budesonide did not differ in size from the drug- free ones. Incorporation of CsA led even to smaller micelles with increasing drug load. This proved as already shown in this thesis [27] that the micelle size seemed to be dependent on the encapsulated drug. On the one hand Mondon et al. as well as Elsahaby et al reported no variations for the size of their micelles, whether loaded or not [5,28]. On the other hand Dane et al showed an increase in size of their micellar system when loaded with mometasone, but also a decrease with tacrolimus and rapamycin [29].

Hence, in terms of size and size distribution at 5°C we could observe a very stable micellar system. This would have a tremendous impact towards consistent characteristics regarding the behavior and resorption at the site of application.

5.3.3. Drug content

As a final and perhaps most important parameter of the stability study, the solubilized amount of drug at 5°C over time was monitored. The results were obtained using HPLC-MS. Fig. 5.6 shows the residual content of budesonide in the non-buffered (left panel) and buffered (right panel) samples. The non-buffered preparations made with AS (#2, 3) were quite stable over 6 months, but decreased to 87 % and 88 %, thereafter. However, the samples prepared with ACS (#5, 6) ended up after 12 months with drug amounts of 100 % and 97 %, respectively. For the samples #2 and #3 it seemed that the decrease of the budesonide concentration correlated with the simultaneously decreasing $\%d_I$ (Tab. 5.2), however, sample #5 also showed this decrease in $\%d_I$ without any loss of drug.

The buffered samples (#14, #15, #17, #18) were all perfectly stable over 12 months. In comparison to the non-buffered samples the buffered ones showed clearly less variation of drug content over time. It seemed that the more stable pH conditions led to more consistent concentrations.

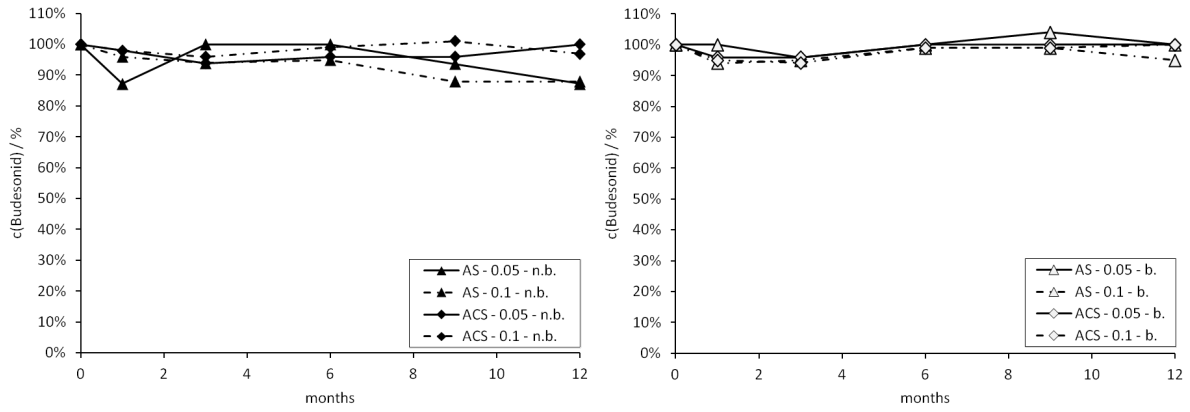

Fig. 5.6: Drug content: residual drug amount of budesonide at 5°C over a period of 12 months; *left panel:* samples #1-6, non- buffered; *right panel:* samples #13-18, buffered;

The residual drug concentration of CsA was monitored only in non-buffered and non-isotonized formulations, due to known incompatibilities with ionic additives (Fig. 5.7) [23]. As CsA was found to be stable under various experimental conditions [25], none of the slightly acid to neutral environments showed a significant impact on drug stability. Further, there was no dependency observed on the surfactants used for preparation. All formulations showed perfectly stable drug concentrations over 12 months at 5°C. A summarizing overview on the results is given in Tab. 5.2.

As reported also by other groups, colloidal, micellar systems, but preferably polymeric micellar systems were observed to be stable formulations for poorly soluble drugs when stored under suitable conditions. Polymeric micelles formed by a copolymer of methoxy poly(ethylene glycol) and hexyl – substituted - polylactide, loaded with CsA, stored at 4°C over 12 months showed a drug loss of less than 7 % [5]. Also stored at 4°C the mixed micelles of Wang et al solubilized paclitaxel over 6

months with a final loss of 2.9 % drug [30]. Further, propofol could be prepared as a stable formulation at 25°C over 6 months with mPEG-PLA/Solutol mixed micelles by use of polymeric and surfactant components [31]. Without a perfectly fitting surfactant or combinations thereof micellar systems were shown to lose their dissolved hydrophobic drug much earlier [32].

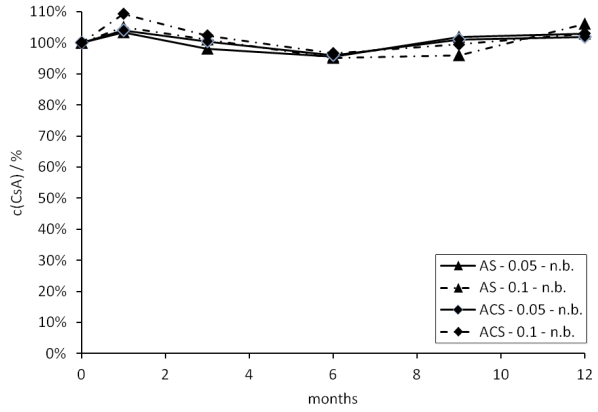

Fig. 5.7: Drug content: residual drug amount of CsA at 5°C over a period of 12 months; samples #7-12, non- buffered;

In comparison and as expected, budesonide showed better stability characteristics in buffered solution while CsA was found to be stable in non-buffered formulations. Only two samples at all fell slightly below the critical 90% mark of drug content after 12 months. Based on these results, further investigations using sterile sample preparations, the adjustment of the tonicity with Mannitol of the CsA solutions, and temperature levels of 5°C, 25°C and 40°C need to be conducted in the future.

Tab. 5.2: Result overview: summary and comparison of the values at the beginning ($_o$) and the end ($_{12}$) of the stability study; samples #1-6: budesonide micellar solutions, non- buffered; samples #7-12: CsA micellar solutions, non- buffered; samples #13-18: budesonide micellar solutions, buffered; the grey shaded lines mark the samples with a final drug amount below 90 %; *n.d.* = not determined;

#	pH_o	pH_{12}	π_o	π_{12}	Z_{AVo}	Z_{AV12}	PdI_o	PdI_{12}	dI_o	dI_{12}	$\%dI_o$	$\%dI_{12}$	$c(d)_o$	$c(d)_{12}$
			mosmol		nm				nm		%		%	
1	5.8	6.7	1	4	8.7	8.8	0.08	0.05	9.4	9.4	100	100	-	-
2	5.9	5.9	0	7	8.7	9.4	0.02	0.21	9.1	9.4	100	96.8	100	87
3	6.0	6.6	1	5	8.7	9.5	0.04	0.14	9.2	9.2	100	95.3	100	88
4	7.2	6.4	4	4	8.5	8.6	0.03	0.07	8.9	9.2	100	100	-	-
5	6.8	7.5	12	4	8.5	9.2	0.02	0.24	8.9	8.9	100	94.8	100	100
6	6.8	5.6	8	5	8.4	8.5	0.04	0.05	8.9	9.1	100	100	100	97
7	7.0	5.0	4	4	8.7	8.7	0.04	0.06	9.3	9.3	100	100		
8	7.5	6.3	3	7	8.5	8.6	0.02	0.06	8.2	8.4	100	100	100	103
9	6.8	6.0	7	6	7.8	7.8	0.03	0.06	8.9	9.2	100	100	100	106
10	7.1	6.5	5	3	8.4	8.3	0.02	0.02	8.8	8.8	100	100		
11	7.1	7.1	4	7	8.3	8.4	0.03	0.08	8.1	8.2	100	100	100	102
12	7.1	5.5	1	5	7.7	7.7	0.03	0.05	8.7	9.1	100	100	100	103
13	6.1	6.0	276	285	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	-	-
14	6.1	6.1	269	289	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	100	100
15	6.1	6.1	286	277	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	100	95
16	6.1	6.0	326	325	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	-	-
17	6.1	6.0	287	295	*n.d*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	100	100
18	6.1	6.0	280	280	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	100	100

5.4. Conclusion

With the present work a self- assembling colloidal micellar system was investigated in terms of its stability at 5 °C over a 12 months period. For budesonide, and cyclosporin A suitable conditions for stable surfactant micelles were found. Depending on the incorporated drug, either a buffered or non-buffered environment was necessary. The solutions were stable in terms of size and size distribution as well as in their residual drug content. None of the stable formulations decreased to a final drug concentration below 95 % after 12 months. Based on these results further optimization and stability studies according to the ICH Guidelines at 25°C / 65 % RH and 40°C / 70 % RH have to be done. With the present long term stability study the hypothesis could be supported that the self- assembling surfactant micelles for the application of poorly soluble drugs are a very promising approach towards the treatment of for example inflammatory diseases at the anterior eye.

5.5. References

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Chapter 6

Ocular delivery systems for poorly soluble drugs: an in-vivo evaluation

to be submitted to a peer reviewed journal

Abstract

For highly potent but poorly soluble drugs like cyclosporine A, the development of formulations providing an increase of corneal drug tissue levels and thus of bioavailability, in combination with a rise of patient compliance is still a challenge. Therefore, we designed two liquid application systems, an in-situ nanosuspension (INS) and a micellar solution (MS) and tested both formulations in vivo on rabbits regarding their tolerability and the tissue uptake of CsA. The INS evoked minimal to no irritations. The MS on the other hand, was perfectly tolerated. After an observation period of 180 min, the rabbits were sacrificed and the corneal tissue levels of CsA were analyzed. The INS and the MS both showed high levels of 1683 ± 430 ng_{CsA} / g_{cornea} and 826 ± 163 ng_{CsA} / g_{cornea}, respectively, and exceeded drug tissue levels reported for Restasis® (350 ng_{CsA} / g_{cornea}) and cationic emulsions (750 ng_{CsA} / g_{cornea}). Furthermore, the data correlated well with in-vitro studies using porcine eyes. Upon these results our INS and MS marked outstanding novel approaches for the treatment of inflammatory ophthalmic diseases.

6.1. Introduction

The cyclic undecapeptide cyclosporin A (CsA) shows pharmacological activities, that makes it most attractive for a number of ophthalmic applications [1]. As a potent immunosuppressant, it is widely used to prevent corneal graft rejection and to fight diseases involving pre-inflammatory cytokines, like dry eye disease (DED) or autoimmune uveitis [2,3]. For DED the mechanism of CsA action has not been completely unraveled, but it seems to be related to its immune-modulatory activity, which decreases local inflammation [4].

Due to the fact, that CsA is a highly lipophilic and poorly water soluble compound (6.6 µg/ml [5]; logP = 3 [6]), it is frequently administered as an oily solution. Unfortunately, such preparations are poorly tolerated and lead to low bioavailability because of higher attraction of the drug by the lipophilic vehicle, rather than the more hydrophilic tissue [6–10]. However, as known from the literature, therapeutic tissue levels have to be at least 50 – 300 ng_{CsA}/g_{cornea} [11]. After one week with five instillations per day in-vivo tissue levels of approximately 500 ng_{CsA}/g_{cornea} have been reached [12]. This suggests that therapeutic tissue levels are just reach at the expense of a large number of instillations and, therefore, questionable patient compliance. The first and only US Food and Drug Administration (FDA) approved product Restasis®, a 0.05 % CsA emulsion, did not increase the CsA concentrations in corneal tissue. In contrary, after twice a day administration over one week Di Tommaso et al showed levels below the limit of quantification (LOQ = 2 ng/ml) [13], while Daull et al detected approximately 350 ng_{CsA}/g_{cornea} [14] 3 hours after a single instillation of 50 µl Restasis®.

It is therefore, little surprising that more recently polymeric micelles [13,15] and cationic emulsions [14] emerged as promising options for the ocular CsA delivery with significantly increased tissue levels after single and also multiple dosing in-vivo. Unfortunately, some of these formulations still come with problems like a cumbersome manufacturing or unclear tolerability and toxicity over a midterm or long-term period [15–18]. To overcome these problems, our group developed two novel ophthalmic CsA delivery systems based on simple manufacturing and well known ingredients only. An in-situ forming nanosuspension (INS) made of liquid poly(ethylene glycols) (PEG) that avoid the disadvantages of lipophilic solvents [19], and on the other hand a simple micellar solution (MS) containing non ionic surfactants that increase drug solubility [20]. Both formulations were already shown to have a tremendous potential to increase CsA resorption in-vitro [19,20]. The aim of the current study was to investigate the INS and our MS in terms of biocompatibility and bioavailability in corneal tissue in-vivo.

6.2. Materials and methods

6.2.1. Materials

Cyclosporin A (98.5 %) (CsA) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Cyclosporin D (CsD) was a kind gift of Prof. Dr. F. Kees (University of Regensburg, Regensburg). Sympatens AS/200G (AS) was kindly provided by KOLB (Hedingen, Switzerland). Solutol® HS 15 was a kind gift from the BASF (Ludwigshafen, Germany). Polyglycol DME500, a dimethoxy-poly(ethylene glycol) (M_W = 500 g/mol), was supplied by Clariant Produkte (Deutschland) GmbH (Frankfurt a. M., Germany). Mannitol was purchased from Caesar & Loretz GmbH (Hilden, Germany). Deionized water was obtained from a Milli-Q water purification system from Millipore (Schwalbach, Germany). Methanol (MeOH) (HPLC grade) was purchased from Merck (Darmstadt, Germany). Fumaric acid (FA), 0.1 N hydrochloric acid and 0,1 N sodium hydroxide solution were obtained from Carl Roth (Karlsruhe, Germany).

6.2.2. Preparation and characterization of the in-situ Nanosuspension

In-situ nanosuspensions were prepared as liquid, transparent and colorless solutions, from which drug nanostructures precipitate upon contact with the aqueous tear fluid. Sample #1, a high concentrated INS placebo solution, was prepared of 55 % DME500, 13 % Solutol and 32 % water by gentle stirring over night. Sample #2, a low concentrated INS placebo solution, was prepared from 39 % DME500, 5 % Solutol and 56 % water gentle stirring over night. Sample #3, a low concentrated INS CsA solution was prepared in analogy from 39 % DME500, 5 % Solutol and 0.4 % After 24 h, 31.6 % water were added to each solution under stirring for 1 hour, which did not trigger precipitation but helped to reduce the content of non-aqueous solvent.

The pH of the samples was adjusted to 7.4 with 0.1 N hydrochloric acid using a Sartorius PB-11 basic pH meter equipped with a pH combination electrode PY-P11 (Sartorius AG, Goettingen). The final solutions were sterilized using a FP 30 syringe filter unit containing a regenerated cellulose membrane with a pore size of 0.2 µm (Whatmann GmbH, Dassel, Germany). The filtered solutions were stored in sterile vials at 5°C until further use.

6.2.3. Preparation and characterization of the micellar solution

The clear and colorless self- assembling micellar solutions consisted of a non-ionic surfactant, drug and water. For the placebo solution (sample #4), 0.3 % Sympatens AS and for the drug-loaded preparation (sample #5) 0.3 % Sympatens AS and 0.05 % cyclosporine A were dissolved with an isotonic aqueous 5.2 % mannitol solution under gentle stirring over night. The pH was again adjusted to 7.4 as described above. The tonicity was determined using a Knauer – semi micro osmometer K7400 (KNAUER Wissenschaftliche Gerätebau GmbH, Berlin, Germany). Sterile filtration and storage thereafter was under the same condition as for the INS solutions.

6.2.4. Size and zeta-potential measurements

300 µl of sample #3 were precipitated with 100 µl water under gentle mixing in an 1.5 ml eppendorf cup. The micellar solutions were used as prepared above. The particle size was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern, Herrenberg, Germany). The sample volume used in the polystyrene semi-micro cuvettes was 400 µl. The zeta potential was measured in folded capillary cells (Malvern, Herrenberg, Germany) with a sample volume of about 1 ml by laser doppler electrophoresis using again a Zetasizer Nano ZS.

6.2.5. Animals

For the investigations male chinchilla-bastard rabbits, weighing 3 kg, were used. Before starting the experiments, the ocular surface was evaluated visually for any damage using a Kowa SL-15 hand slit lamp (b o n Optic Vertriebsgesellschaft mbH, Lübeck, Germany). The experiments were conducted exactly as outlined in the experimental protocol approved by the University ethics committee and the District Government of Upper Palatinate (reference number: 54-2532.1-21/10).

6.2.6. Tolerability studies

5 rabbits each were used for the investigation of the INS solutions, and for the micellar solutions, respectively.

The tolerability investigation was a two step process. In a first experiment 25 µl of the sample #1 were instilled into the right eye and 25 µl of a sodium hyaluronate lubricant solution serving as negative control were instilled into the left eye of an animal. The eyes were evaluated visually using a hand slit lamp immediately after the application and 15, 30, 45, 60, 120 and 180 min thereafter. Four weeks later, the eyes were evaluated again prior to a second set of experiments to make sure the formulations had been tolerated. Then 25 µl placebo sample #2 was instilled in the left eye and 25 µl of the drug- loaded sample #3 were instilled in the right eye of an animal. The eyes were again examined as outlined above. The evaluation and scoring was performed as described by Ballantyne B. [21].

Micellar solutions were characterized as shown above for the INS. Initially sample #4 and the lubricant were instilled. Four weeks later sample #4 and #5 were used.

6.2.7. Drug resorption studies

3 hours after the treatment with the drug- loaded samples, the rabbits were sacrificed by intravenous injection of 200 mg/kg_{animal} pentobarbital sodium. Both eyes were enucleated and the cornea were excised. The harvested tissue were homogenized using liquid nitrogen in combination with a tissue pulverizer (made by the University machine shop in analogy to the Bessman Tissue Pulverizer). Each homogenized cornea was transferred to a glass vial and weighed. The cornea were incubated for 2h under gentle stirring with 0.5 ml MeOH, containing 0.1 µg/ml CsD as internal standard. The extract was centrifuged and the supernatant stored in HPLC vials at -80°C until analysis.

6.2.8. Chromatographic equipment and conditions

CsA analysis was carried out by ultra high performance liquid chromatography in combination with mass spectrometry (UHPLC-MS) according to Rodriguez-Aller et al (2011) [22]. An Agilent Technologies UHPLC system equipped with a 6540 quadrupole time of flight (Q-TOF) LC/MS system fitted with an Agilent jet stream electron spray ionization (AJS ESI) interface (Agilent Technologies Deutschland GmbH, Böblingen, Germany) was used. The further equipment consisted of a high performance (HiP) autosampler G4226A with a sample volume of 2 µl, a binary pump G4220A and a column compartment G1316C kept at 70 °C. The AJS ESI was used in positive ion mode. Separation was performed on a Waters Aquity BEH c18, 1.7 u, 2.1 x 50 mm column at a flow rate of 0,6 ml/min (Waters Corporation, Milford, MA, USA). The mobile phase consisted of (A) water and (B) methanol each containing 0.1 % formic acid. A linear gradient was applied from 40 % A to 0 % A over 3 min. After each analysis the column was allowed to equilibrate for 3 minutes to the starting

conditions. The MassHunter Workstation software was used for data acquisition (Agilent Technologies Deutschland GmbH, Böblingen, Germany). Data handling and processing were done using Microsoft Excel 2007 and the Dintest 2004 software (ARVECON GmbH, Walldorf, Germany).

6.2.9. Statistics

The obtained data were analyzed using a t – test at a significance level of $p < 0.05$ using SigmaPlot 11.0.

6.3. Results and discussion

6.3.1. Characterization of the samples

Tab. 6.1: Sample characterization: chart of the different samples used for tolerability and resorption studies with male rabbits; pH, tonicity, particle size (Z_{AV}) and zeta- potential (ZP) were determined;

#	Sample	Composition	pH	Osmotic pressure	Z_{AV}	PdI	ZP
				mosmol	nm		mV
1	High concentrated INS - Placebo	55 % DME500 13,7 % Solutol 32.3 % H_2O	7.4	>> 3000	-	-	-
2	Low concentrated INS - Placebo	39 % DME500 5 % Solutol 56 % H_2O	7.4	> 3000	-	-	-
3	Low concentrated INS - Verum	39 % DME500 5 % Solutol 55.6 % H_2O 0.4 % CsA	7.4	> 3000	385 ± 5	0.2 ± 0.03	-0.06 ± 0.04
4	Micellar Solution-Placebo	0.3 % AS 5.2 % Mannitol H_2O ad 100	7.4	270	10.1 ± 0.03	0.07 ± 0.008	-0.4 ± 0.1
5	Micellar Solution-Verum	0.3 % AS 5.2 % Mannitol 0.05 % CsA H_2O ad 100	7.4	271	9.7 ± 0.05	0.1± 0.003	-0.6 ± 0.3

Prior to the in-vivo experiments the solutions were characterized physicochemically. Tab. 6.1 shows their pH, tonicity, particle size and zeta potential. Since they are drug-free, no precipitation could be induced with the placebo solutions and thus neither particle size nor a zeta potential could be measured. After precipitation of sample #3 colloidal particles of 385 nm, a PdI of 0.2 ± 0.03 and a zeta- potential of – 0.06 ± 0.04 mV were obtained. The drug-free and drug-loaded MS showed a tonicity of 270 mosmol and 271 mosmol, a size of 10.1 ± 0.03 nm and 9.7 ± 0.05 nm, and a zeta potential of – 0.4 ± 0.1 mV and – 0.6 ± 0.3 mV, respectively. The PdI was below 0.1 for all MS.

6.3.2. Biocompatibility of the placebo preparations

Fig. 6.1 shows the average scoring and the standard error for five male rabbits treated with the high concentrated INS placebo solution (sample #1). Such a preparation would able to dissolve as much as 1.0 % CsA [19]. Initially, all rabbits showed a reddening of the conjunctiva, a moderate swelling of the lids and only few slightly opaque spots on the cornea. The initial total score up to 15 min was 10.6 ± 1.6 scoring points (SP) which is a fifth of the maximum score (54 SP). The animals rubbed their eyes and behaved nervous, which was supposably due to a considerable discomfort. After 45 min only two of the rabbits still showed an effect, after 60 min there was only one left (1.4 ± 1.4 SP). Thus, the initial reaction was reversible within one hour, which is in very good agreement with the material safety data sheet (MSDS) of the supplier, based on OECD guideline 405 [23]. According to the document pure DME500 showed no irritations at the rabbits' eye 1, 24, 48 and 72 hours after application.

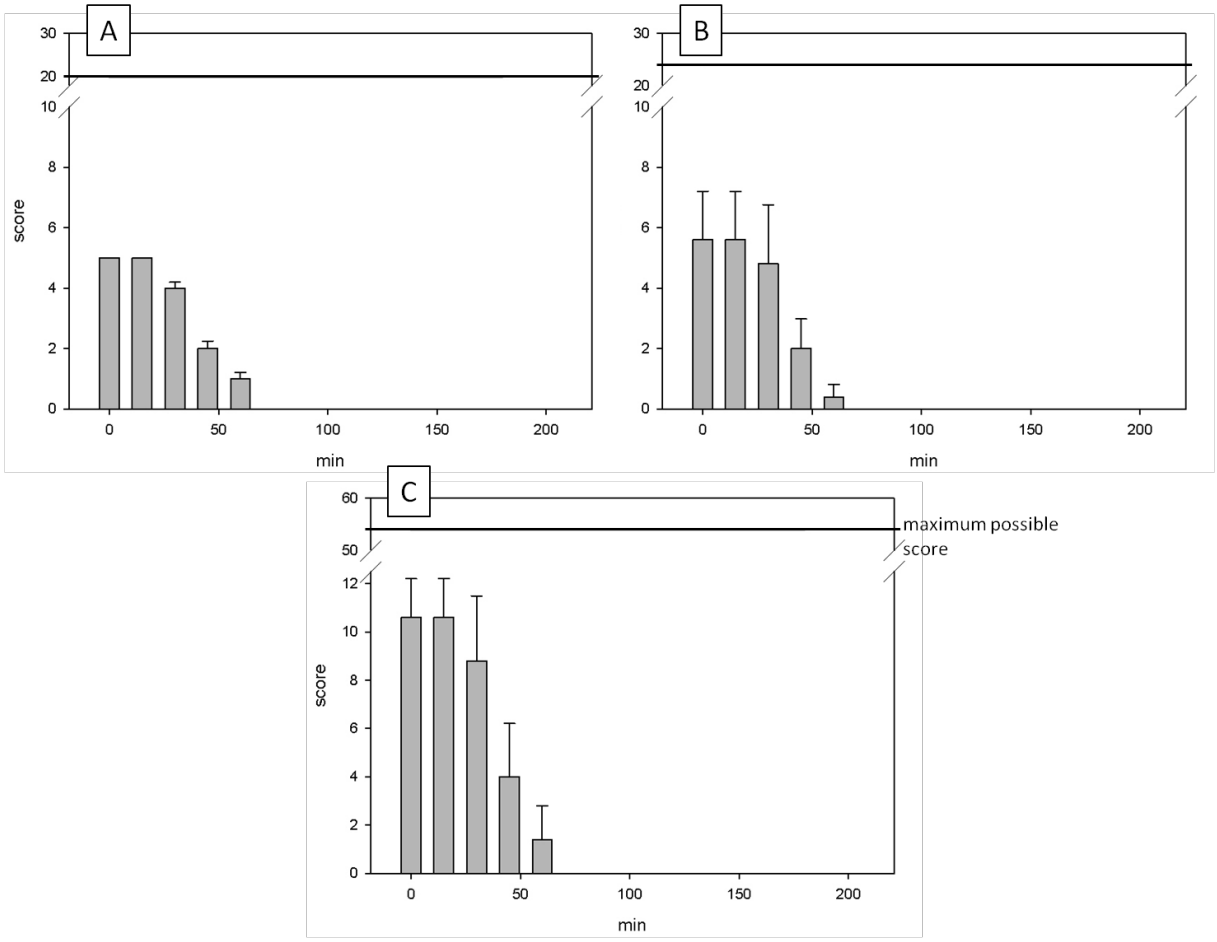

Fig. 6.1: Tolerability score of 5 male rabbits over a period of 180 min after instillation of a 25 µl droplet of the high concentrated INS placebo solution (55% DME500 / 13.7% Solutol/ 32.3% H_2O); (A) cornea, (B) conjunctiva/lids; (C) total score; data is plotted against the maximum possible score for each panel; Data is shown as average with standard error (n=5);

Due to the unfavorable tolerability of sample solution #1, four weeks later, the tolerability of the low concentrated INS placebo solution (sample #2), which would still be able to carry 0.4 % CsA was investigated to assess the benefit of the lower DME500 concentration (Fig. 6.2). Initially, a minimal reddening of the conjunctiva was observed, but in a way, that it could only be seen by use of the slit lamp. The cornea showed no reaction. The initial score was 1.8 ± 0,2 out of 54 SP. No eye rubbing or any abnormal behavior could be observed. After 15 min, only one rabbit showed a minimal reaction leading to a total scoring of 0.4 ± 0.4. After 30 min no further effect was detected. Compared to highly concentrated INS placebo solution, the low concentrated one showed a tremendous increase in biocompatibility.

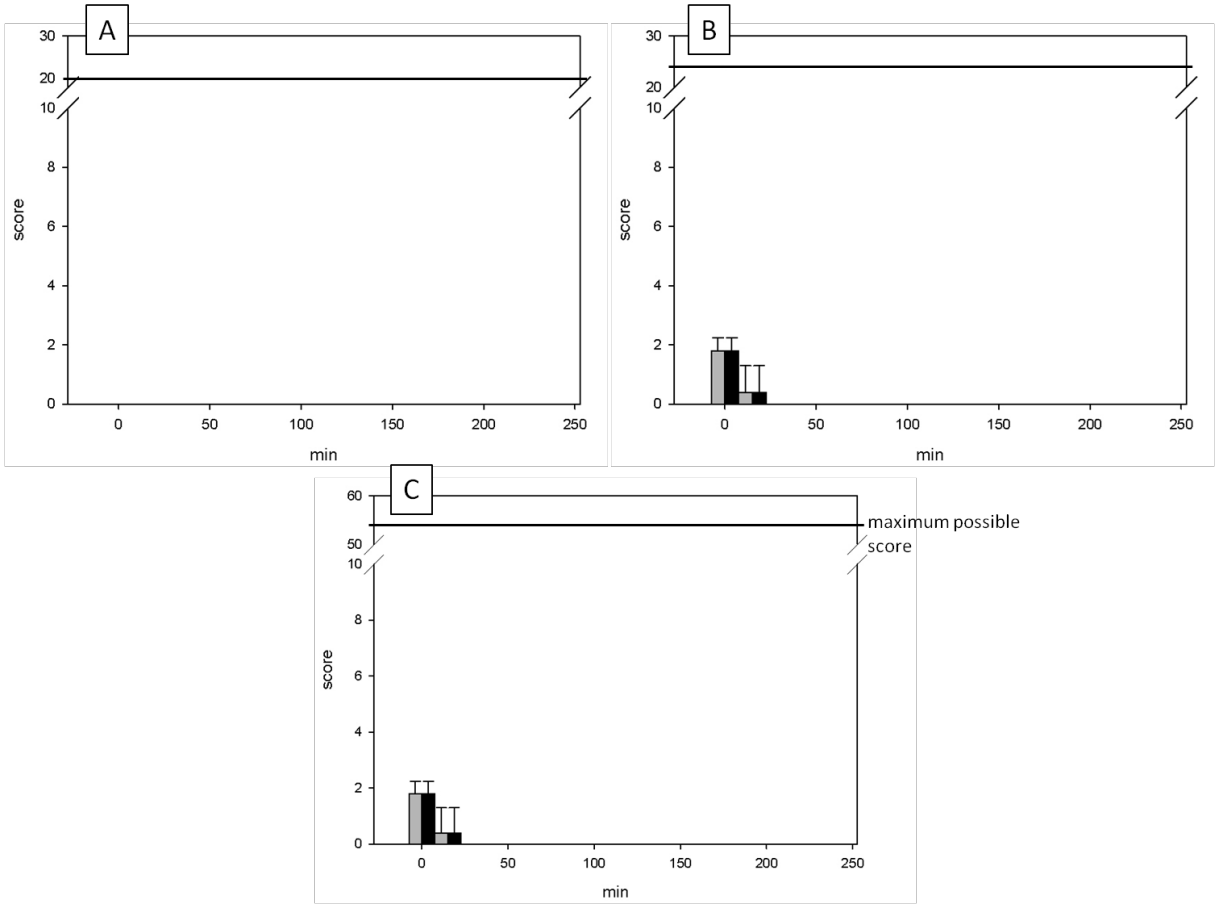

Fig. 6.2: Tolerability score of 5 male rabbits over a period of 180 min after instillation of a 25 µl droplet of the low concentrated INS placebo (□) and 0.4 % CsA solution (■) (39% DME500 / 5% Solutol/ 55.6% H_2O); (A) cornea, (B) conjunctiva/lids; (C) total score; data is plotted against the maximum possible score for each panel; data is shown as average with standard error (n=5);

To visualize the nearly perfect tolerability of sample #2, the eyes of the five treated rabbits, 15 min after instillation are shown in Fig. 6.3. The lids showed a normal, healthy rosy color, without any swelling. Even though a clearly hypertonic solution was instilled, the conjunctiva and cornea showed no signs of pathological alterations.

Finally, the placebo MS (sample #4) was investigated (Fig. 6.4). None of the animals showed any kind of response. Neither the cornea, nor the conjunctiva or the lids showed any alteration. No behavioral reaction nor any kind of eye rubbing was observed. The solution was perfectly tolerated.

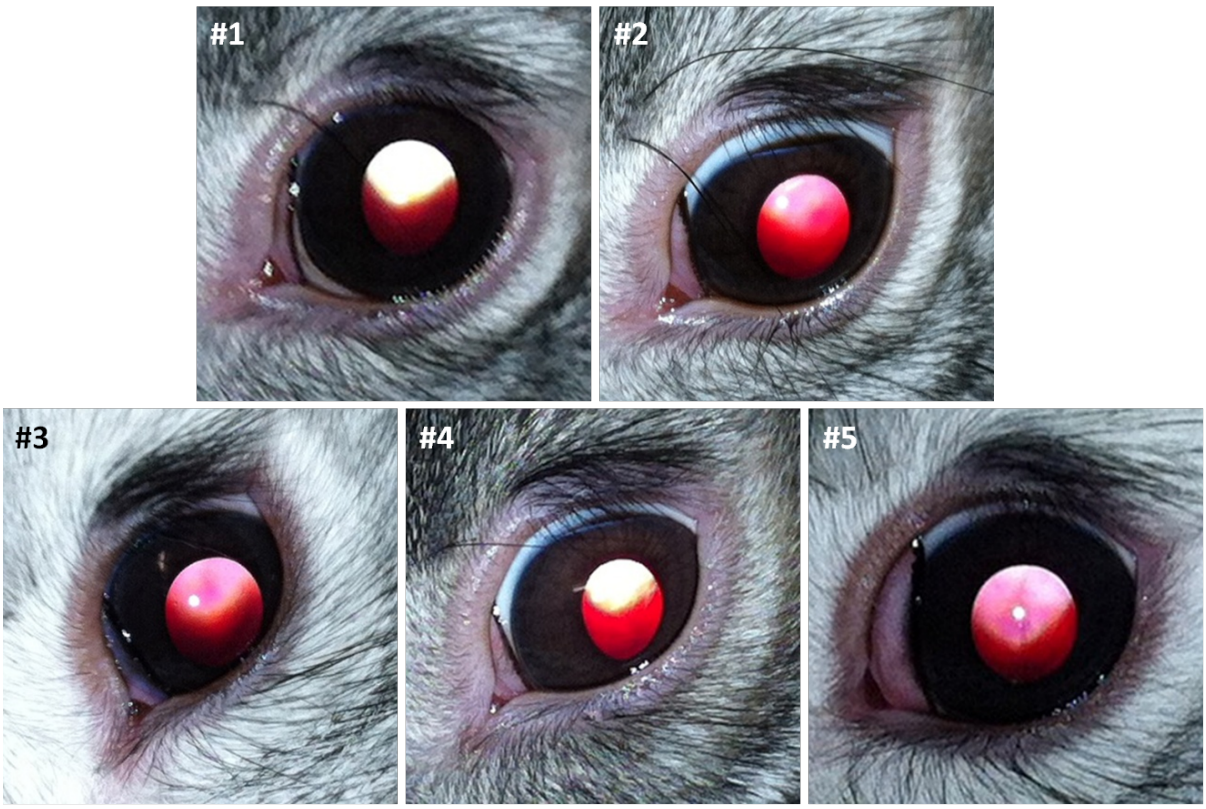

Fig. 6.3: Pictures of 5 male rabbit eyes (left eye) 15 min after treatment with 25 µl of the low concentrated INS 0.4 % CsA solution (39% DME500 / 5% Solutol/ 55.6% H_2O);

6.3.3. Biocompatibility of the drug- loaded preparations.

After the placebo solutions the drug-loaded solutions (sample #3 and #5) were investigated (Fig. 6.2 and Fig. 6.4). No abnormalities could be detected and both solutions behaved exactly as the respective placebo preparation. The drug- loaded samples confirmed the good biocompatibility of the INS and the MS placebo solutions. This is in accordance with data published by di Tommaso et al, who could not show a significant difference between their 0.05 % CsA loaded micellar system and an isotonic saline solution [24].

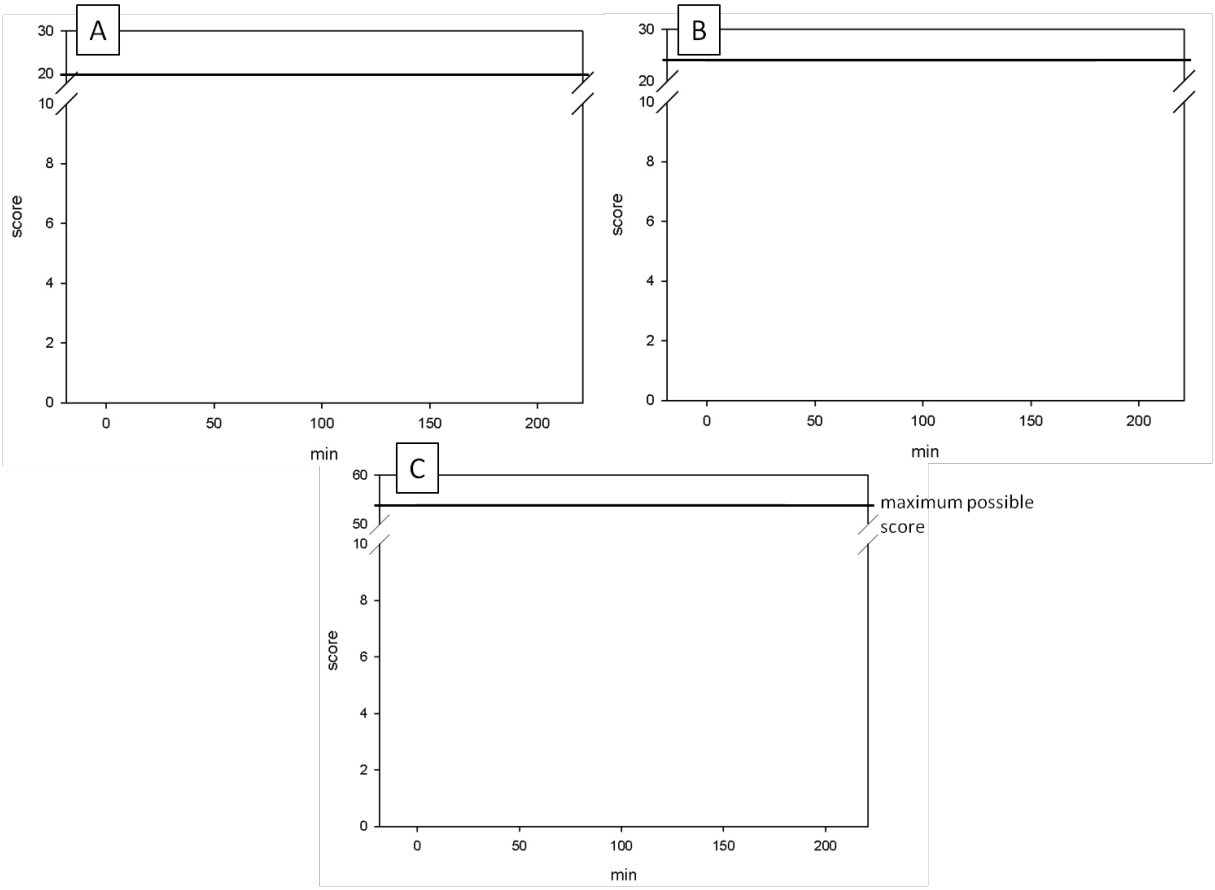

Fig. 6.4: Tolerability score of 5 male rabbits over a period of 180 min after instillation of a 25 µl droplet; initial instillation of the MS placebo (□); MS placebo (▒) and MS 0.05% CsA solution (■) after 4 weeks; (A) cornea, (B) conjunctiva/lids; (C) total score; data is plotted against the maximum possible score for each panel; Data is shown as average with standard error (n=5);

6.3.4. Bioavailability

The most important parameter to assess was the absorbed amount of CsA in the cornea upon instillation. Based on the compatibility study we tested INS sample #3 and MS sample #5. The corneal tissue levels of CsA were determined three hours after instillation (Fig. 6.5). For the INS (0.4 % CsA) a value of 1683 ± 430 ng_{CsA} / g_{cornea} was determined. The MS (0.05 % CsA) produced tissue levels of 826 ± 163 ng_{CsA} / g_{cornea}. In the contra lateral eyes significantly lower amounts of 207 ± 77 ng_{CsA} / g_{cornea} and 151 ± 102 ng_{CsA} / g_{cornea} for the INS and the MS, respectively were detected. These concentrations correspond to approximately 18 % and 12 % of the concentrations found in the treated eyes. Such significant CsA

levels in the contra lateral, untreated eye were first reported by Wiederholt et al (13 %) [25]. Vernillet et al later confirmed the passage of CsA from the treated to the non-treated eye [26].

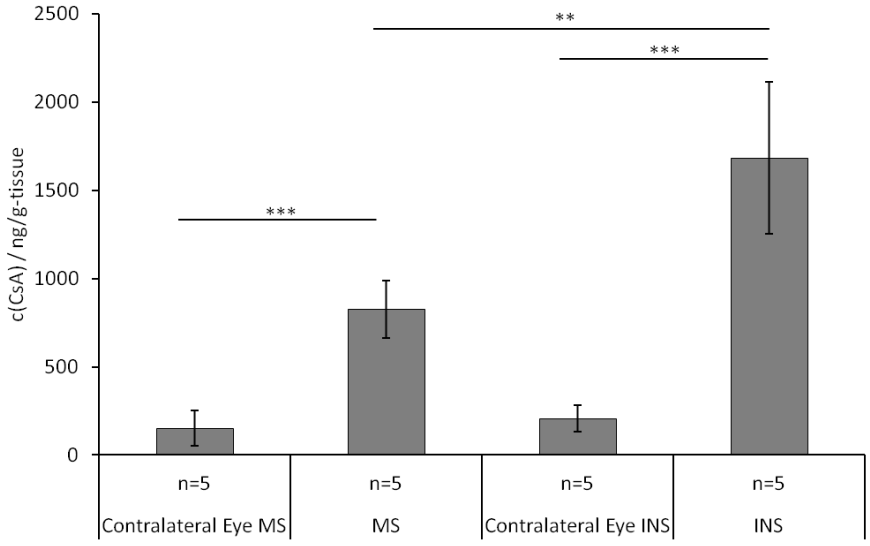

Fig. 6.5: Amount of CsA taken up by the rabbits' corneal tissues; INS 0.4% CsA, INS placebo, MS 0.05% CsA solution and MS placebo were instilled (25 µl); the left eyes of the rabbits were treated with the drug- loaded solutions, the right eyes with placebo; n = 5; ** = P < 0.01 / *** = P <0.001;

Both samples, the INS as well as the MS simply reached outstandingly high CsA tissue levels. The 1683 ng_{CsA} / g_{cornea} of the INS were significantly higher than levels reached by a 0.1 % CsA cationic emulsion (1200 ng_{CsA} / g_{cornea}) [14]and a 0.05 % CsA polymeric micelle formulation after twice a day administration over one week (1540 ng_{CsA} / g_{cornea}) [13]. The 826 ng_{CsA} / g_{cornea} observed for the MS were comparable to concentrations reported for cationic emulsions containing 0.05 % CsA. They are known to show a longer retention at the cornea, because of their positive charge. Despite its neutral surface charge our micellar solution generated even slightly higher tissue levels than the positively charged emulsion after 3 hours (750 ng_{CsA} / g_{cornea}), which was administered with a drop volume of 50 µl [14], compared to 25 µl in this study. That neither the INS, not the MS carry positive charges on their surfaces, is definitely an advantage with respect to long-term compatibility [17,18].

The in-vivo data for the MS and the INS solution are well in line with precious in-vitro studies. Using porcine eyes we found after 30 min in-vitro 3345 ± 745 ng_{CsA} / g_{cornea} for the 0.4 % CsA INS [19] and 1557 ± 407 ng_{CsA} / g_{cornea} for the MS [20]. This is exactly twice the concentrations measured in-vivo after 3 h. According to these results, our porcine in-vitro model seems to be highly suitable to predict corneal in-vivo levels and thus it may help to decrease the number of in-vivo investigations on animals.

The plethora of data found in the literature in conjugation with our data, allows for a direct comparison with Restasis® and oily formulations without any need for further experiments. In-vitro we could already show significant higher levels for the INS and the MS compared to Restasis® (0.05 % CsA emulsion) and a 2 % CsA oily solution, while there was no significantly difference between them [19,20]. Since the in-vitro model seems to be highly predictive for in-vivo tissue levels, we expect significantly lower concentrations for Restasis® and the oily eye drops on the rabbit eye. This is confirmed by the study of Daull et al who reported for a single instillation of 50 µl Restasis® at rabbit eyes in-vivo after 180 min tissue levels of about 350 ng_{CsA} / g_{cornea} [14]. This is only just a fifth of the levels reached by our INS with a droplet size of 25 µl. For a 0.5 % CsA oil formulation administered five times a day over one week levels of only 580 ng_{CsA} / g_{cornea} were reached at rats in-vivo [12]. This lower bioavailability of CsA delivered by formulations containing an oil-phase, which is a perfect solvent for the highly lipophilic drug, seemed to be due to the higher affinity of the CsA to the oily vehicle, than to the hydrophilic environment of the corneal tissue [27,28]. We could overcome these problems with our preparations, which are rather simple in manufacturing. They offer an option to decrease the number of instillations per day and thus, improve patient compliance.

6.4. Conclusion

The INS and the MS are both preparations with a high therapeutic potential. With no irritations at all, the MS marked the perfect system for an application at the anterior eye. Also for the INS only a minimal initial reaction was observed. Finally, both formulations were capable to reach very high drug levels in corneal tissue. Besides the outstanding tissue levels, the formulations offer big advantages regarding simple manufacturing from only well known compounds. Hence, both novel delivery systems mark highly promising approaches for the efficient treatment of inflammatory ophthalmic diseases and may help to increase the patient compliance by lowering the number of instillations per day.

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Chapter 7

Summary and conclusion

This thesis was focused on the development of efficient novel liquid formulations for poorly water soluble drugs for the treatment of inflammatory ophthalmic diseases. With Restasis® there is currently only one drug product approved by the FDA, in the US only, for the treatment of dry eye syndrome. It still suffers from low bioavailability, bad biocompatibility and thus a low patient compliance, as well as cumbersome manufacturing. Hence, there is a tremendous lack in the options for a causal therapy **(Chapter 1)**. For this reason, two novel approaches, which could be finally prepared at room temperature only by gentle stirring from well known compounds, were investigated; an in-situ nanosuspension (INS), a clear solution which precipitates into nanoparticles upon contact with the tear fluid, and self- assembling micellar solution (MS) consisting of suitable no-ionic surfactants, water and drug only.

Initially, a selection of poly(ethylene glycol)-based solvents was investigated in terms of their ability to solubilize the lipophilic drugs cyclosporin A, budesonide and beclometasone as well as different viscosity enhancing or surface active additives **(Chapter 2)**. The solvents showed outstanding solubilizing qualities for the drugs and the non-ionic surfactant Solutol® HS15 and further good compatibility with polymers. A turbidimetric high throughput method to determine the exact concentrations of water, at which a precipitation occurred, could be established. Thereby, with all solvents the concept of an in-situ precipitating formulation could be proven, with unique characteristics for each formulation. Unfortunately, in combination with budesonide and beclometasone it was not possible to vary or even control the precipitation. In presence of Solutol no further precipitation occurred. However, this made Solutol a promising candidate for development of micellar solutions (**Chapter 4**). As budesonide and beclometasone were not suitable for further INS investigations, they were exclusively conducted with cyclosporin A.

Upon this basic characterization study and the proof of the INS concept, a defined INS system containing CsA could be prepared and investigated with the solvent DME500, a poly(ethylene glycol) ether with an average molecular mass of 500 Da **(Chapter 3)**. It was possible to control the amount of water necessary for a precipitation as well as the size of the resulting nanoparticles. The formulation used for biocompatibility and bioavailability studies showed a particle size of 697 ± 19 nm, a polydispersity index (PdI) of 0.1 ± 0.05. The particles carried no surface charge. By use of a flow through cell culture system in combination with a microscope equipped with a camera, the change in cell volume of primary human epithelia cornea cells (HCEpiC) by contact with the formulations was used as a measure for biocompatibility, and was found to be as good as a commercial aqueous solution of sodium hyaluronate. The study on drug uptake into porcine cornea was performed using custom made glass resorption chambers (CRC). Outstanding high tissue levels of cyclosporin A (3345 ng_{CsA}/g_{cornea}) which significantly exceeded those of Restasis® (545 ng_{CsA}/g_{cornea}) and an oily CsA solution (452 ng_{CsA}/g_{cornea}) were detected. To this end, the INS concept was shown to be functionally, biocompatible and able to reach high drug tissue levels in-vitro.

The second approach investigated and developed in this thesis was a self-assembling micellar solution. To reach this goal, by sorting of different non-ionic surfactants, Sympatens AS and Sympatens ACS, two poly(ethylene glycol) fat-alcohol ethers, were observed to be most suitable, due to their high efficiency in terms of drug loading **(Chapter 4)** and stability **(Chapter 5)**. Only very low concentrations were necessary to reach therapeutic drug loadings for budesonide and cyclosporin A. The non-charged micelles had depending on their drug load a size from 8 to 10 nm and a monomodal size distribution (PdI < 0.1). They were

perfectly tolerated by the established HCEpiC model in-vitro and a first preclinical formulation with 0.05 CsA and 0.3 % Sympatens AS reached significantly higher in-vitro drug tissue levels (1557 ng_{CsA}/g_{cornea}) in porcine cornea than Restasis® (545 ng_{CsA}/g_{cornea}) and an oily solution of CsA (452 ng_{CsA}/g_{cornea}) **(Chapter 4)**. Furthermore, conditions could be achieved for non-loaded as well as drug-loaded systems at which the micellar system could be proven to be stable over a period of at least 12 months at 5 °C **(Chapter 5).**

With the last study, shown in **Chapter 6**, the two novel application systems were investigated for their compatibility and efficiency in-vivo. This allowed concomitantly to investigate the significance of the porcine in-vitro model. Both formulations, the INS and the MS, were well tolerated by the rabbits. With no abnormal reaction at all, the MS marked the perfect system and even exceeded the INS which caused only a minimal initial irritation. The tissue levels reached by the INS and the MS after 3 h were outstandingly high and exactly half of the levels detected for both formulations with the in-vitro model after 30 min. Furthermore, the in-vivo concentrations were significantly higher than those of Restasis® and a cationic emulsion with charge prolonged cornea retention.

In summary, it can be said that the INS and the MS could be shown to be formulations with simple manufacturing, high stability and biocompatibility. Both reached outstanding drug tissue levels. The established porcine in-vitro model turned out to reliably predict in-vivo drug cornea levels and thus, avoid unnecessary animal experiments. Both liquid application systems for poorly soluble drugs could be developed up to a pre-clinical form which are ready to enter clinical tests as new drug products for the causal treatment of inflammatory ophthalmic diseases such as the dry eye syndrome.

Appendix

Abbreviations

ACS.....Sympatens ACS / 200 G

ARMD .....age related macular degeneration

AS .....Sympatens AS / 200 G

b.i. .....buffered and isotonized

BEC.....beclometasone

BUD .....budesonide

CA .....cellulose acetate

CEpiCM.....corneal epithelial cell medium

CL .....contact lens

CMC.....critical micelle concentration

CO.....cylindrical opening

CRC .....custom made glass resorption chamber

CsA .....cyclosporin A

CsD .....cyclosporin D

DED .....dry eye disease

d_I .....intensity weighted diameter

DLS .....dynamic light scattering

DME .....di-methoxy-poly(ethylene glycol)

eSEM .....environmental scanning electron microscopy

ESI .....electron spray ionization

FDA.....U.S. Food and Drug Administration

HCEpiC .....primary human corneal epithelial cells

HCl .....hydrochloric acid

HPLC .....high pressure liquid chromatography

ICH.....International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use

IL .....interleukin

INS .....in-situ nanosuspension

KCS.....keratoconjunctivitis sicca

LFU .....lacrimal functional unit

LOD.....limit of detection

Log P.....octanol water coefficient

LOQ .....limit of quantification

M .....mono-methoxy-poly(ethylene glycol)

MAPK.....mitogen-activated protein kinase

MAS .....mannitol aqueous solution

MeCN.....acetonitrile

MeOH.....methanol

MGD.....meibomian gland dysfunction

MMP.....matrix metalloproteinase

MPEG .....mono-methoxy-poly(ethylene glycol)

MS.....mass spectroscopy

MS.....micellar solution

MSDS.....material safety data sheet

M_W.....molar weight

n.b. .....non-buffered

NaOH .....sodium hydroxide

NFAT.....nuclear factor of activated T-lymphocytes

NFκB .....nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

NR.....nile red

NSSDE.....non-Sjogrens syndrome dry eye

OS.....other solubilizing agent

PA .....polyamide

PCS.....photon correlation spectroscopy

PdI.....polydispersity index

PE .....polyethylene

PEG .....poly(ethylene glycol)

PEI .....polyethylenimine

PLA .....polylactide

PoP .....point of precipitation

PP .....polypropylene

Q-TOF.....quadrupole time of flight

RC.....regenerated cellulose

RM ANOVA.....repeated measurements analysis of variance

SEM .....scanning electron microscopy

SHS.....sodium hyaluronate solution

SL.....stock solution

SP .....scoring point

SSDE .....Sjogrens syndrome dry eye

SSS.....surfactant stock solution

TEM .....transmission electron microscopy

TF.....tear film

TFBUT .....tear film break up time

tHTP.....turbidimetric high throughput

TNFα.....tumor necrosis factor α

UHPLC.....ultra high pressure liquid chromatography

VE .....viscosity enhancer

VEGF .....vascular endothelial growth factor

Z_{AV}.....hydrodynamic diameter

π.....osmotic pressure

Curriculum vitae

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List of publications

Publications in scientific journals

Luschmann C., Tessmar J., Strauß O., Framme C., Luschmann K., Goepferich A.; Developing an in-situ nanosuspension: a novel approach towards the effective administration of poorly soluble drugs at the anterior eye; submitted to European *Journal of Pharmaceutical Science* **(Chapter 3)**

Luschmann C., Tessmar J., Strauß O., Luschmann K., Goepferich A.; A self-assembling, colloidal system for the ocular administration of poorly soluble, lipophilic drugs; *to be submitted to a peer reviewed journal* **(Chapter 4)**

Luschmann C., Herrmann W., Strauß O., Luschmann K., Goepferich A.; Ocular delivery systems for poorly soluble drugs: an in-vivo evaluation; *to be submitted to a peer reviewed journal* **(Chapter 6)**

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C. Luschmann., C. Framme, K. Luschmann, J. Tessmar, A. Göpferich: A high throughput strategy to investigate the controlled precipitation of poorly watersoluble drugs out of nonaqueous media in situ. 7th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Malta (2010).

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Patents

PCT/EP 2012/06 4895; A. Göpferich, **C. Luschmann**; Wässrige Lösungen von lipophilen Substanzen, insbesondere Arzneistofflösungen;

WO 2010/060989 A1; A. Göpferich, **C. Luschmann**; Lösungen von lipophilen Substanzen, insbesondere Arzneistofflösungen;

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbst verfasst habe und keine anderen als die angegebenen Hilfsmittel verwendet habe

Regensburg, den 20. Februar 2013

Christoph Luschmann