Adhesive polymer films for the prevention of postsurgical adhesions

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Abbreviations and Symbols

%	percent
°	degree
°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer
ANOVA	analysis of variance
ASTM	American Society for Testing and Materials
$C_{12}H_{10}Ca_3O_{14}$ 4 H_2O	calcium citrate tetrahydrate
CA	contact angle
$CaCl_2$	calcium chloride
$CaCO_3$	calcium carbonate
$CaHPO_4$	calcium phosphate
$CaSO_4$	calcium sulfate
cm	centimeter
CO_2	carbon dioxide
D_2O	deuterium oxide
Da	Dalton
DCM	dichloromethane
DIAD	diisopropyl azodicarboxylate
DMEM	Dulbecco's modified eagle's medium
DMTMM	4-(4.6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
DTP	Dithiopropionic acid dihydrazide
DTT	1,4-Dithiothreitol
EDC	N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EMEM	Eagle`s minimal essential medium
e-modulus	elastic modulus
eq	equivalent

FCS	fetal calf serum
g	gram
G`	Elastic modulus
G``	Viscous modulus
G-block	Guluronic acid block
GDL	gluconic acid δ-lactone
GPC	gel permeation chromatography
h	hour
H_2O_2/ HOOH	hydrogen peroxide
H_2SO_4	sulfuric acid
HA	hyaluronic acid
HA-SH	thiolated hyaluronic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
High G alginate	alginate with high amount of guluronic acid
I_2	iodine
ITC	isothermal titration calorimeter
kDa	kilo Dalton
kg	kilogram
KI	potassium iodide
kPa	kilopascal
l	liter
Low G alginate	alginate with low amount of guluronic acid
M	molar
m	mass
MDa	mega Dalton
mg	milligram
$MgSO_4$	magnesium sulfate
min	minute
ml	milliliter
mm	millimeter
mM (mmol)	millimolar
M_n	number average

mol	molar
MPa	megapascal
MTT-Assay	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay
MW	molecular weight
M_w	weight average molecular weight
M_w/ M_n	polydispersity index
N	Newton
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometer
NMR	nuclear magnetic resonance
Pa	pascal
PBS	phosphate buffered saline
PEG	poly (ethylene glycol)
pH	negative logarithmic value of the hydrogen ion concentration
PLA	Poly (L-lactide-co-D,L-lactide; 70:30)
PLA PEG PLA (PLA35kDa-PEG10kDa-PLA35kDa)	lipophilic triblock copolymer of poly(lactic acid) and poly(ethylene glycol)
PPh3	triphenlyphosphine
rpm	revolutions per minute
SD	Substitution degree
SDS	sodium dodecyl sulfate
sec	second
SEM	scanning electron microscopy
THF	tetrahydrofuran
Tris	Tris(hydroxymethyl)aminomethane
UV-Vis	ultraviolet-visible
W0	dry weight
WST	Water-soluble Tetrazolium salt
wt	wet weight

1. Barrier films for adhesion prevention: What can we learn from wound dressings

This chapter gives an overview of polymers that can be used as wound dressings and are therefore good candidates for the preparation of films for the prevention of peritoneal adhesions.

1.1 Introduction

In the modern wound care natural and synthetic polymers are often used for wound dressings due to specific beneficial characteristics, like the ability to regulate a moisture imbalance by removing wound exudate and maintaining a moist wound environment or the ability to donate moisture [1,2]. Advanced non-adherent hydrogel dressings can be furthermore removed pain free without causing damage to the injured tissue [2], which promotes the desired wound healing and enhances the restoration of the skin defect. To prevent peritoneal adhesions after surgery similarly designed barrier films made of different degradable and non-degradable polymers already showed excellent clinical results due to an appropriate separation of the damaged tissues during the wound-healing period. Accordingly, a closer look onto the characteristics of polymers, which are used for effective wound dressings, should allow the identification of new candidates for the preparation of alternative films for adhesion prevention and moreover improve currently used systems for this application.

In order to appropriately compare the two applications and learn from each other, the differences in the healing of skin defects and defects of the peritoneum and the resulting polymer requirements, which are used for the preparation of the medical devices, will be highlighted in more detail.

Modern wound dressings for the treatment of acute and chronic external wounds are made of polymers, which fulfill many different clinical functions, like for example the uptake of wound exudate or the maintenance of a humid wound environment to enhance the healing process. The established polymers for this application, like for example alginate and hyaluronic acid, already showed many benefits in the treatment of skin defects. But also for the prevention of peritoneal adhesions hyaluronic acid is already used in the application form of a gel or a film, because the separation of the traumatized tissues is currently considered to be the most effective way to prevent the formation of adhesions.

For both types of wounds biomaterial characteristics like biocompatibility, biodegradability, non-immunogenicity, hemostatic activity, as well as the absence of toxic, carcinogenic or thrombogenic activities are essential to fulfill the criteria of suitable devices to treat patients [3,4]. Accordingly many polymers have already been used for both applications, for wound

dressings, as well as for films for adhesion prevention, with different success and a different focus of their application.

The research of George D. Winter in 1962 firstly demonstrated the beneficial effect of a moist environment on the wound healing process, because wounds that are exposed to air form a scab that retards epithelization. Wounds, covered with a polyethylene film to keep them moist do not form a scab and the epithelization is more rapid [5]. Since this time, many wound dressings made of different hydrogel forming polymers, which create a moist environment for healing, were invented and successfully brought to the clinic. Besides a moist environment these hydrogels have many more beneficial advantages. They are non-adherent on the wound and therefore can be removed from the wound without causing additional pain and further damage to the newly grown skin epithelium [6]. On the other hand, exuding wounds need a wound dressing that is able to incorporate large amounts of wound exudate, which can be accomplished by wound dressings made of alginate [7], a natural polymer that is known to absorb large amounts of liquid resulting in the formation of stable hydrogels. Another quite similar polysaccharide, which is used for wound dressings due to its ability to reduce scar formation and alternatively already applied for adhesion prevention as a biodegradable and biocompatible hydrogel, is hyaluronic acid, which only differs slightly in structure and functional groups (Figure 1).

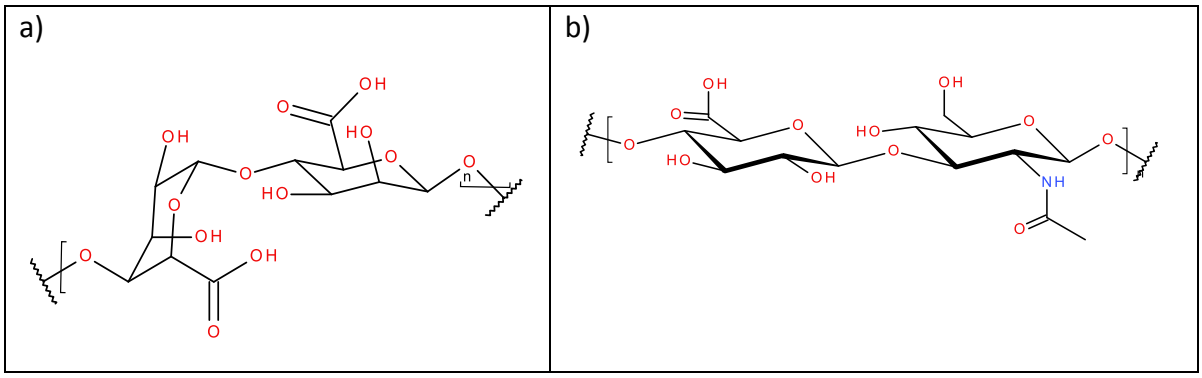


Figure 1: a) alginic acid, b) hyaluronic acid

To prevent the formation of peritoneal adhesions, a reduction respectively inhibition of scar formation is also highly desirable. Therefore hyaluronic acid seems to be an advantageous polymer for the preparation of films for adhesion prophylaxis. In the peritoneal cavity the effective uptake of wound exudates or bacteria by a polymer like alginate should not be expected to be that helpful. However, alginate also showed promising results in the

prevention of peritoneal adhesions due to some other beneficial characteristics like its mucoadhesive properties [8].

1.2 Differences of the two application sites

To identify the essential prerequisites for the two application sites, differences in structure and function should be highlighted and compared in more detail. Furthermore, it is also necessary to understand the differences in two wound healing processes as well as the resulting requirements for wound dressings and adhesion barriers.

1.3 Structure and function of skin and peritoneum

The largest and most important organ of the human body is the skin. It protects the human body from microbes and chemical agents. Furthermore it helps to regulate the body temperature. The skin consists of three different layers the epidermis, the dermis and the hypodermis. The epidermis is the outer layer of the skin. It provides physical protection and works as a waterproof barrier. The dermis, as well as the hypodermis consists of connective tissue consisting of collagen and glycosaminoglycans. Furthermore the dermis contains sweat glands and hair follicles and the hypodermis fat [9].

The peritoneum is a protective membrane, too. Unlike the skin, its assignment is not to protect the human body from environmental influences, but to cover the organs and allow them to move upon each other without friction [10]. It consists of a sheet of connective tissue and a single layer of mesothelial cells that are anchored to the basement membrane [11,12].

Both the skin as well as the peritoneum can like any other tissue or organ lose their functionality by the formation of scar tissue after trauma. Furthermore injured peritoneum can form peritoneal adhesions. Therefore it is of utmost importance to enhance the wound healing and reduce the scar formation to retain their functionality.

1.4 Differences in wound healing

The wound healing of a skin defect differs from that of a peritoneal defect. The healing of a skin defect can be divided into the following stages. In the first stage, called the immediate, the hemostasis occurs. The initiation of the coagulation cascade leads to the formation of a fibrin clot and platelet aggregation. In the second stage, the inflammation takes place with

the activation of the complement cascade, which comes along with the invasion of neutrophils and macrophages. In the third stage, the proliferation, the formation of granulation tissue occurs. Fibronectin, hyaluronan and collagen are produced by migrated fibroblasts and the wound is closed by epithelization. In case of skin defects, the epidermalization takes place from the borders of the wound until the wound is closed. The final stage is the remodeling stage that leads to the scar maturation. In this stage fibronectin and hyaluronan are broken down and replaced by collagen. In open wounds the wound healing is finished with a contraction step [1,13–15].

In contrast to the skin, the entire surface of a peritoneal wound becomes epithelialized simultaneously and not gradually from the borders like in skin defects. When new mesothelial cells are attracted to the site of injury by chemotactic messengers that are released by blood clots, platelets or leukocytes, they form multiple individual islands on the peritoneal defect. From these islands they divide until the surface of the injury is completely covered by a new layer of mesothelial cells [16]. Therefore, the time needed for the healing process is more or less the same for smaller and larger peritoneal defects, since no cell migration is necessary and cell division is initiated from multiple healing sites. In contrast skin defects typically need longer healing times depending on the size and depths of the wound.

During the peritoneal healing injured parts of the peritoneum can be connected due to the formation of stable fibrin bands, which is enhanced due to the immediate contact of the insured tissues. In normal peritoneal healing these fibrin bands are subsequently resolved by fibrinolysis and the natural tissue organization is restored. If the bands are not effectively resolved due to impaired fibrinolytic activity, these weak fibrous bridges can be later infiltrated with fibroblasts, followed by vascularization and collagen deposition, which ultimately results in the formation of persisting adhesions. As a consequence different side effects can occur like pain, small-bowel obstruction, secondary infertility and furthermore [16–18].

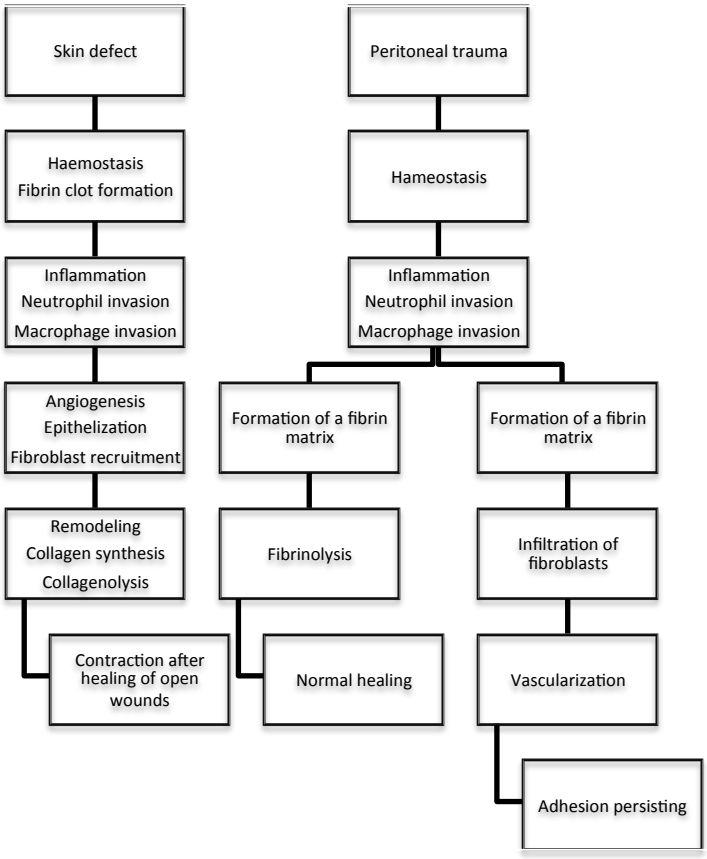


Figure 2: Wound healing of skin and peritoneum

Both healing processes of skin and peritoneum have much in common. They can result in the formation of scared tissue that ultimately lost its functionality and causes discomfort or even pain for the patient. For the application of a wound dressing as well as for a film for adhesion prevention, the aim should be to reduce the scar formation and moreover the formation of adhesive bonds in case of the peritoneum.

1.5 Different Requirements on wound dressings and barriers for adhesion prevention

Wound dressings as well as barrier films should obviously be non-toxic, non-immunogenic and biocompatible. The also essential preparation of sterile wound dressings and barrier films comes along with the difficulties that most natural and synthetic polymers partially degrade or chemically suffer during autoclaving or irradiation procedures, resulting in altered device properties as well as undesirable breakdown products. Therefore, it is also essential to find a way to prepare sterile products or at least to be able to sterilize them as gentle as possible after preparation of the device.

The most significant requirements for the wound dressings depend on the actual type of wound that has to be covered. For an appropriate wound healing an optimal environment is necessary, which may be different for every type of wound. Most wounds need a moist environment. This is naturally given in the peritoneal cavity and moreover should be effectively protected from bacteria, which could cause inflammation and result in further complications of the wound healing process. In order to keep a moist environment, a dry or desiccated skin defect should be wetted and kept in this state by the wound dressing. Whereas a skin defect, that produces large amounts of wound exudate should be ideally covered with a wound dressing that is able to take up the exudate and provide a surrounding that stabilizes the healing skin and prevents the elimination of the newly grown epithelium [1,19].

For the peritoneal application the main task of the barrier film is consequently not the protection of the wound from environmental influences and the maintenance of the moist environment, but to effectively separate the damaged tissue long enough to prevent the undesired formation of peritoneal adhesions. However, for both applications it is beneficial, if the surgeon is able to cut the device into the desired size and shape in order to effectively cover different defects. For the application with the peritoneum it is also desirable, if the device can be implanted via laparoscopic intervention and is still flexible enough to cover uneven surfaces of the small intestine or other organs within the patient.

An external wound dressing is most commonly fixed via the adhesive parts of the wound dressing composite itself or via additionally applied external fixing materials like bandages or adhesive tape. An intestinal adhesion barrier on the other hand can only be fixed with the help of sutures or staples, which in consequence can disadvantageously lead to the formation of adhesions and therefore should be most effectively avoided by the preparation of devices or materials, which stick to the treated tissue on their own without causing undesired connections or bands, which later result in the formation of new connections of the adjacent tissue sites. Even more important than the fixation is the removal of both types of wound dressings. This should generally be possible without causing further damage to the wounded skin or peritoneum. For a skin defect, the wound dressing can easily be removed from the wound, if it does not stick to it. Therefore devices are beneficial, which maintain a thin layer of liquid between the tissue and the device, ultimately resulting in an easy removal

from the skin. In the peritoneal cavity the removal of the anti-adhesive barrier film has obviously to be done by a second surgical intervention, this again may cause new adhesion sites. To avoid this, peritoneal barrier films should be biodegradable and consequently be removed from the peritoneal cavity by either enzymatic or hydrolytic degradation or just by simple dissolution.

1.5.1 Purity

Polymers, which are used for wound dressings, as well as adhesion barriers, should generally be non-toxic and non-immunogenic, since they both get in close contact with the patient's tissue and consequently with individual cells, as well as the patient's immune system [20]. As adhesion barriers the polymers are applied parenterally and therefore have to be of even higher purity than the polymers that are used for wound dressings, where the polymer is usually not resorbed and only remains on the outer skin of the patient until it is removed by the doctor or just falls off. Before the preparation of any barrier film or wound cover, it should be additionally considered, that a subsequent sterilization can have dramatic effects on natural macromolecules, as well as synthetic polymers [4]. Therefore it is always important to know, if the used polymer can be sterilized before film preparation or if the final product has to be sterilized, obviously without altering its properties or molecular structure. To remove toxic chemicals that have been applied for the synthesis of the polymer like solvents, cross-linkers or unreacted monomers, the obtained polymer should always be sufficiently purified with the help of solvent washing or dialysis [21] before it can be used for the preparation of wound dressings or barrier films. The efficient purification is even more important for natural polymers from plants or bacteria, like alginate [22] and hyaluronic acid, which have to be intensively purified before parenteral application to remove for example residues that remain from the leaves of the algae.

1.5.2 Degradability

Regarding the degradation of polymer devices at the application site, several different pathways are discussed, which all ultimately have to lead to water-soluble breakdown products that can be metabolized or eliminated from the body. Based on the chemical structure of the device two main steps of the degradation have to be distinguished. The first applies to the erosion of a device due to the separation or dissolution of the whole polymer

chains that are only loosely fixed by entanglements or weak ionic interactions. A second process involves the breakdown of few chemical bonds, if cross-linked networks of water-soluble polymers are degraded, or even the breakage of the whole polymer chain into monomers, if water insoluble polymers are applied.

Accordingly, devices made of water insoluble polymers, like polyesters or polyanhydrides, can undergo surface erosion or bulk erosion, depending on the diffusion speed of water into the polymer device, this ultimately determines the kinetics of polymer breakdown [23]. The degradation of devices made of biodegradable polymers can furthermore involve the hydrolytic or an enzymatic cleavage of the polymer chain, which may also lead to different kinetics of polymer erosion [24]. Hydrolytically degradable polymers, which carry functional groups, like esters or anhydrides, only degrade based on the amount of water and steric effects of neighboring groups [24]. On the opposite, enzymatically degradable polymers, like collagen, albumin, fibrin or others are cleaved by specific enzymes, which further enhance the polymer's elimination based on their local amount and activity [24].

The overall degradation of polymers can be largely influenced by secondary modifications of the polymer chains or additional side functionalities. Alginate itself is a hydrophilic polymer, but in a humid environment the alginate chains do not degrade efficiently and devices made of alginate only undergo erosion based on the speed of dissolution of the loose chains. With the help of partial oxidation using sodium periodate, aldehyde groups can be introduced, which render the polymer more susceptible to hydrolysis and oxidation [25]. When a wound dressing made of a hydrogel is removed from the wound area, polymer parts may also still stick to the wound, but they can be subsequently washed away during a necessary cleaning of the wound area. Nevertheless it might still be possible, that during the healing process small amounts of the hydrogel have been incorporated in the granulation tissue and will be later removed by biodegradation or can be incorporated in the newly formed tissues, as for example in case of hyaluronic acid hydrogels.

If a peritoneal barrier device will not be removed from the body by a second surgery, the used polymers have to be fully biodegradable or at least the prepared device should completely erode into fragments that can be eliminated from the body. Biodegradable polymers that are used for all kinds of parenteral applications can be eliminated unaltered or metabolized into endogenous compounds, which can be metabolized or also eliminated [20].

For example barrier films that are prepared of polylactic acid like Surgiwrap® degrade via hydrolytic degradation ultimately into bioresorbable monomers like lactic acid enantiomers or glycolic acid that can be inserted in biochemical pathways [4].

To prolong the persistence time of very hydrophilic polymers, like alginate or hyaluronic acid, which are dissolved very fast in the humid environment of the peritoneum and washed away from the application side, the polymers usually have to be chemically or physically cross-linked in order to provide a sufficient long persistence time at the application site. Nevertheless these cross-linked polymer networks should still degrade after the peritoneal healing is ended. For example barrier films made of alginate that have been physically cross-linked with divalent cations like calcium degrade due to a slow exchange of the divalent cations by monovalent cations like sodium [4].

If the released polymer chains can be finally withdrawn from the body, also depends on the hydrodynamic radius of the free polymer chains. Molecules with a size of about 3-4 nm like for example albumin (3.6 nm) and larger would stay in the blood system instead of being withdrawn by renal filtration [26]. Accordingly larger water soluble polymers always have to contain certain cleavage sites, where the molecules can be broken down in order to be eliminated from the patient.

1.5.3 Adhesion

Most wound dressings firmly adhere to the skin because they are surrounded by sticky surfaces covered with glue or they can be fixed with the help of bandages or adhesive tape. Barrier films in the peritoneum cannot be fixed at their application site that easy. They have to be sutured to the tissue or be fixed with staples if they do not stick to the tissue themselves.

The use of mucoadhesive polymers here would allow an application without further fixation and the risk of adhesion formation.

Mucoadhesion can be defined as the ability to adhere to the mucus gel layer [27]. Mucoadhesive materials can be described as hydrophilic macromolecules that contain numerous hydrogen bond forming groups. The mechanism of mucoadhesion can be divided into two stages. The contact, where the wetting takes place and the consolidation state where the adhesive interactions are established [28]. Chitosan, sodium alginate and cellulose

derivatives belong to the so called “wet” adhesives. They can be activated by moistening due to capillary forces and adhere to many surfaces in a wet state. But these polymers can also overhydrate and form a slippery gel, which in consequence detaches from the application site [28]. Different mucoadhesive interactions can be achieved by the formation of differently strong bonds like ionic bonds, covalent bonds, hydrogen bonds, van-der-Waals bonds or hydrophobic bonds [27,29]. To improve the mucoadhesive properties of polymers like chitosan or alginate thiol groups can be introduced into the polymer chains, which later stabilize the adhesive by forming covalent disulfide bridges with components of the mucus. This can be achieved with the help of polymer analogue modification by coupling small bifunctional molecules like cysteine, thioglycolic acid or cysteamine to the existing polymer chain [27,28,30].

Due to its non-adhesive properties to skin defects, wound dressings prepared from alginate always have to be fixed with secondary dressings [3]. Regarding barrier devices, alginate is a good candidate due to its mucoadhesive properties, because it can adhere, due to the formation of hydrogen bonds, to the traumatized tissue without further fixation [8]. Therefore this polymer can be quite advantageous for both applications.

1.5.4 Uptake of wound exudates and bacteria

The uptake of wound exudates and bacteria is mainly beneficial for external wound dressings, because after incorporation and uptake of the bacteria the wound dressing can be exchanged with a fresh one [6].

In the peritoneal cavity, a polymer film that is capable to incorporate wound exudates like a wound dressing would also incorporate the surrounding peritoneal fluid until it is soaked completely. Furthermore, the wound exudate that is formed by a peritoneal defect commonly is anyway diluted with peritoneal fluid and washed away from the wound site. An efficient uptake of bacteria into a peritoneal adhesion barrier would furthermore be undesired, because they consequently cannot be removed from the peritoneal cavity. On the contrary, the bacteria would finally be released from the barrier again upon its ultimate degradation in the body.

1.6 Wound dressings and barrier films prepared from polymers

Many different wound dressings and peritoneal barrier films based on different natural and synthetic polymers are already available on the market and in clinical use.

One external wound dressing, Intracell®, for example is based on maltodextrin and is mainly available as powder for exuding wounds or formulated as gel containing 1 % ascorbic acid for drier wounds lacking the necessary liquid to bind the dry powder [31]. Usually when the powder comes in contact with the wound exudate it immediately forms a permeable, hydrophilic film that provides a moist environment to cover the wound. As an internal barrier for the prevention of peritoneal adhesions maltodextrin powder on the other hand would dissolve much too fast in the constantly produced peritoneal liquids and be eliminated from the wound area before the wound healing is finally completed. For an easy accessible skin defect the application as a dry powder that sticks to the wound surface and forms a gel with the uptake of wound exudate, is very promising and moreover allows adjusting the size of the protective hydrogel to the size of the defect. However for a peritoneal application, especially using the laparoscopic route of entry, a powder is obviously not applicable due to many technical reasons. Consequently a homogeneous application like on a skin defect is not possible, this is why dry polymer powders are not considered as alternative coatings for peritoneal wounds.

Further available and already marketed wound dressings are prepared from another polysaccharide, namely alginate, which is cross-linked and stabilized with bivalent calcium ions, like for example Algicell®, Algisite® or Curasorb® [1]. All these products are still highly absorptive and soft, which is beneficial for the effective treatment of exuding skin defects. Alginate dressings furthermore improve blood clotting and increase epithelization and the formation of granulation tissue, which finally enhances the closure of the wounds [19,31]. Other described properties, like alginate's erosion and hemostatic activity [1,31], could also be very beneficial for the preparation of peritoneal barrier films, however the most important material property of alginate being a good candidate for the preparation of barrier films are its mucoadhesive properties [8]. For the preparation of degradable barrier films, which only persist for several weeks and will be withdrawn from the wound after wound healing, alginate would also be ideal due to its reversible cross-linking possibility. With a defined amount of bivalent cations the cross-linking extent and therefore the resulting erosion time

of a prepared film can be easily adjusted and adapted to the speed of restoration of the peritoneum. Furthermore different techniques are already established to draw very thin and homogenous alginate films, which would provide the basis to improve the handling within the patient as well as the subsequent erosion time [32].

Other hydrocolloid dressings, like Comfeel®, Tegasorb® or Hydrocoll®, are made of carboxymethylcellulose, gelatin or pectins and also form soft gels by absorbing wound exudates. The fact, that hydrocolloid dressings adhere to moist as well as dry sites [19], make them not only promising as wound dressings but also for the preparation of barrier films. Studies moreover have shown that dressings prepared of carboxymethylcellulose could encapsulate large numbers of the bacteria Pseudomonas aeruginosa and Staphylococcus aureus [19], which both are bacteria known from infections of dermal wounds. After the uptake of bacteria, the wound dressing can be obviously exchanged and the bacteria can therefore be dislodged from the wound side. The uptake of bacteria into a peritoneal barrier film that degrades after several weeks in contrary is quite inadequate, since the bacteria would be released from the film again during the occurring erosion. Nevertheless carboxymethylcellulose is available as highly cross-linked Seprafilm® or Sepragel® for the preparation of barrier films in combination with hyaluronic acid as second polymer component. Beneficial for its use as barrier film is the finally occurring enzymatic degradation of the natural polymers, that takes place in the peritoneal cavity in about two to four weeks [18,33]. Hyaluronic acid itself is used as sodium salt or ferric salt in Sepracoat® and Intergel® as highly viscous liquid or partially cross-linked hydrogel. Due to the hydrophilicity and high water solubility elimination half-lifes of these devices are about 26 hours and 51 hours, respectively [33]. To enhance the overall persistence time at the application site thiolated hyaluronic acid was already chemically cross-linked with polyethylene glycol methacrylate following a michael addition reaction, in order to prepare an injectable, in situ forming hybrid hydrogel wound dressing with an enhanced dissolution stability [34]. Here, the ability of an easy chemical modification of hyaluronic acid to obtain thiolated hyaluronic acid derivatives with the help of carbodiimide chemistry is very promising. Accordingly thiolated hyaluronic acids can be cross-linked via stable but cleavable disulfide bonds to prolong the persistence time in the peritoneal cavity as barrier film without creating permanent implants. The crosslinking extend of the prepared films can be adjusted via the chosen substitution degree and consequently be used to adjust degradation times [35]. For the

preparation of thin films (100 µm), a solution of thiolated hyaluronic acid was dissolved in phosphate-buffered saline and poured into a petri dish after the adjustment of the pH. After drying the film was finally oxidized by immersion in H_2O_2 to further chemically link the individual polymer chains.

Even fully synthetic polymers are used to prepare in situ cross-linkable barrier devices, like SprayGel®, which is based on a hydrogel composed of branched polyethylene glycol NHS esters and PEG amines, which contain degradable bonds in order to allow elimination of the individual polyethylene glycol units within a degradation time of about 6 days [33]. The here applied PEG active esters however cross-link too fast in order to prepare evenly thick hydrogel films, which would be necessary for a prefabricated barrier system in form of a thin film.

Polyurethan foams or films like Lyofoam® or Tegaderm® [36,37] can also function as semipermeable wound dressing to cover hydrated alginate dressings. They allow the skin to breath because they are permeable for water and oxygen. Opsite™ a thin film made of polyurethane is semi-permeable, too and easily conforms to irregular contours like knees and does not need additional taping [19], its ability to adapt to uneven wound surfaces would also be quite beneficial for the use as a barrier film, too. However due to their non-degradability all these urethane based devices cannot be applied internally without being removed during a second surgical intervention.

Besides its non-degradability Gore-Tex Surgical Membrane is used as anti-adhesion barrier in pericardial surgery to replace the pericardium [38–40]. One advantage of Gore-Tex surgical membrane is that no adhesions are formed due to its very low porosity [40]. Furthermore the Gore-Tex Surgical membrane is antiadhesive to cells due to its hydrophobic character (see manufacturer information). The disadvantage of this membrane is that it has to be removed from the body with another surgery. Gore-Tex can also be used for a permanent gastrostomy tube as support conduit in open gastrostomy [41].

In order to combine the beneficial properties of different polymer compounds multilayered (multifunctional) devices have also been investigated, which furthermore enhance the possibilities to design devices for more sophisticated applications.

For initial clinical research a bilayered construct was prepared from silk and gelatin, two quite different proteins [42]. It consists of a non-adhesive layer prepared of wax-coated silk fibroin woven fabric to provide mechanical stability and a bioactive layer to enhance wound healing, which is a sponge made of sericin and glutaraldehyde-crosslinked silk fibroin/gelatin [42]. For this advanced wound dressing it is obviously beneficial when the dressing does not stick to the wound and can be removed without causing new damage to the new grown tissue. Fixation of the dressing on the wound must consequently be performed externally.

Another very promising approach for multifunctional devices are antimicrobial dressings that release an antiseptic agent at the wound surface, like Acticoat absorbent® made of calcium alginate releasing ionic silver [43]. Wound dressings made of chitosan have also been loaded with the procoagulant polyphosphate and the antimicrobial silver to prepare a hemostatic and antimicrobial wound dressing [44]. In current research a barrier prepared from chitosan alginate has been prepared via a new processing technique, electrospinning. In a rat model this device was quite effective in reducing the formation of tissue adhesions [45]. Gentamicin sulphate could be released from a hydrogel wound dressing prepared of acacia gum and carbopol, a cross-linked derivative of polyacrylic acid [46]. A controlled release of ibuprofen from an alginate based bilayer hydrocolloid film was also achieved by coating an upper layer with ibuprofen and combining it with a drug-free lower layer that functioned as membrane that ultimately controlled the release rate [47].

1.7 Summary and conclusions

From the above comparison of different polymers and essentially different prerequisites for the peritoneum in contrast to the cutaneous application, the most universally applicable polymers for both applications are certainly the degradable and easily hydrogel forming polymers, alginate and hyaluronic acid, which were consequently further investigated in this thesis.

Alginate itself is widely used as wound dressing due to its ability to take up large amounts of wound exudate, furthermore it is soft, hemostatic due to platelet activation [36,48] and biodegradable [1]. When it comes in contact with the wound exudate it becomes a viscous gel, which would also be beneficial for internal applications. Another advantage is the adjustability to different types of wounds and the fact, that it can be cut and perfectly fitted

to the wound's dimensions [36]. The released calcium ions from cross-linked alginate films can furthermore activate platelets [19] and macrophages, which is beneficial for the wound healing process [31]. Furthermore alginate dressings are able to bind bacteria and therefore decrease the infection of wounds. In research alginate is used for the preparation of barrier films due to its mucoadhesive properties. For oral formulations alginate of a pharmaceutical grade is sufficient, however, for parenteral applications, like the peritoneal application, it should be of ultrapure grade (NovaMatrix) with low levels of residual endotoxins.

The alternative polymer, hyaluronic acid, is also already available on the market in many different internal and some external wound dressing. Like alginate it fulfills most of the requirements for a peritoneal application. Being part of the natural human tissue and its ability to reduce scar formation makes it very promising to prepare barrier devices that reduce the formation of adhesions and can be withdrawn from or even incorporated in the human body. To prolong its degradation time, films prepared from hyaluronic acid can be cross-linked for example with disulfide cross-linking after preparation of thiolated hyaluronic acid via carbodiimide chemistry or other alternative cross-linking schemes.

2. Aim of this work

In this chapter the aim of the thesis is presented. Furthermore, it gives an overview and short summaries of the following chapters.

There are currently many different strategies for the prevention of the formation of postsurgical adhesions after peritoneal surgery in clinical application, but also under development. The most effective way to prevent the formation of adhesions is the spatial separation of the affected tissues after surgery. This approach can be done with liquids, gels or films [17], however, films are the most promising way of tissue separation because of their longer retention time in the peritoneum and the fact, that they stay in place, when sutured to the tissue. For the surgeon the applicability of the separation device is very important, since he or she decides based on the ease of application and the performance in different clinical situations which material will be applied. If only the route of application is concerned liquids and gels have the edge over films, because they can be easily applied by rinsing or spraying, which certainly reduces handling issues during application. To date most of the films that are commercially available like SurgiWrap® or Gore Tex® have to be sutured to the tissue in order to secure them at the application site. Hereby especially for the smooth PLA films the suturing step to the tissue could be moreover facilitated, if the films were slightly sticking to the tissue like iron-on patches before they are finally sewed on.

The general aim of this work was to prepare new films for adhesion prevention with several beneficial properties. Using hydrophilic alginate, films could be prepared that are mucoadhesive and have adjustable mechanical properties and erosion time due to physical cross-linking with divalent cations. Films prepared with alternative chemical cross-linking could be prepared by using hyaluronic acid that has previously been modified by introducing thiol groups. Another consequent aim was to improve the handling of PLA films by covering them with an adhesive layer, developed earlier. This adhesive layer should then fulfill several requirements for the application, which includes degradability and subsequent absorption as well as obviously good mucoadhesive properties.

Therefore this thesis deals with the establishment of film preparation methods of polymer films made of alginate and hyaluronic acid and the preparation of multilayers consisting of PLA, PLA-PEG-PLA and alginate. To obtain very thin, homogenously thick films with best suited properties and required degradation time the films were drawn on glass plates, cross-linked with different techniques and further low molecular weight additives like plasticizers were investigated.

Chapter 3 deals with the establishment of different cross-linking methods that were performed to investigate the best method for the preparation of thin alginate films with a homogenous distribution of cross-linking calcium [32]. Although till now no barrier device prepared of alginate is available on the market, many research groups have worked with it due to its promising properties.

The preparation by casting films into Teflon molds was replaced by the drawing of films on a glass plate, because this led to evenly thick and much larger films. As calcium source the hardly soluble calcium salts calcium citrate and dicalcium phosphate as well as a calcium-EDTA-complex were tested. There dicalcium phosphate showed the best properties, because it could be distributed in the alginate solution more homogenously than calcium citrate and the phosphate did not compete with the guluronic acid blocks for the calcium like EDTA did. For the acidic release of the calcium, GDL showed the best properties. Only glycerol showed the softening effect that was expected from the different investigated plasticizers. Therefore the best composition for a cross-linked alginate film is comprised of alginate, glycerol, water, dicalcium phosphate and GDL.

Due to the fact, that different types of alginate with varying amounts of guluronic acid and mannuronic acid can bind more or less calcium the composition should be perfected by finding out how much calcium can be effectively bound by the different alginates. **Chapter 4** deals with the assignment to find out, how much calcium is needed to cross-link an alginate film properly and achieve good mechanical properties and erosion time, but also to have binding sites left that are able to bind more calcium if necessary. Furthermore the differences in the cross-linking ability and the resulting properties of alginates with a high guluronic acid content and a low guluronic acid content were investigated. With the help of pretests like ITC measurements, viscosity measurements, rheological tests, turbidity measurements and a defined compression test it was found out how much calcium can be bound by the guluronic acid blocks of the alginates and how much calcium is necessary to cross-link the film properly. The film prepared regarding these results were tested mechanically. Furthermore an erosion test and a cytotoxicity test were performed.

Regarding peritoneal surgery not only the formation of adhesions is a major problem. Postsurgical infections, which also can enhance the formation of adhesions, are a problem, which can only be solved by sufficient antibiotic treatment. To minimize the side effects of

antibiotics, which often come along when they are given systemically, a controlled local application would be beneficial. Therefore the prepared films could be loaded with antibiotics to have a local release after implantation. **Chapter 5** deals with the preparation of thin polymer films prepared from alginate, a block copolymer made of PLA-PEG-PLA and PLA. These films were loaded with the two antibiotics vancomycin hydrochloride and gentamicin sulfate using different methods. Release studies and microbiological testing were performed with the prepared films to test their biological efficacy.

Beside alginate, hyaluronic acid was thought to be a suitable polymer for the preparation of mucoadhesive films. Like alginate, hyaluronic acid is a hydrophilic polymer, which would be dissolved in the humid environment of the peritoneum and therefore has to be cross-linked to increase the stability. **Chapter 6** deals with the preparation of films made of a hydrophilic polymer that has not been physically cross-linked by ionic interactions like the alginate films, but chemically cross-linked via disulfide bridges using thiofunctionalization of hyaluronic acid. Carbodiimide chemistry was performed to prepare thiolated hyaluronic acids with different molecular weights and substitution degrees to investigate the impact on cross-linking of prepared films. With the help of oxidizing additives and the adaption of the drying process the cross-linking of the films could be controlled.

After successful preparation of monolayers from alginate and hyaluronic acid, multilayers were prepared to investigate, if a layer of mucoadhesive alginate can improve the properties of a PLA film and also increase the mechanical stability of the hydrophilic polymer films. Due to the fact, that PLA is a lipophilic and alginate a hydrophilic polymer the combination could not be done by just drawing one layer on top of the other. To make the two layers stick to each other additives and surface modifications were tested in **Chapter 7**. The prepared multilayers were tested mechanically and a mucoadhesion test was performed to investigate, if the alginate layer improves the handling of the PLA film.

3. Alginate films cross-linked with different methods

Different cross-linking methods were established to investigate the best method for the preparation of thin alginate films with a homogenous distribution of cross-linking calcium.

Parts of this chapter were published in

Eva Esser, Joerg K. V. Tessmar

Preparation of well-defined calcium cross-linked alginate films for the prevention of surgical adhesions

Journal of Biomedical Research Part B: Applied Biomaterials

3.1 Introduction

Alginate is a linear block-copolymer produced in the stems or the leaves of marine brown algae and functions as structural component in the plant [49]. Furthermore it can also be produced via microbial fermentation [49–53]. The 1-4 linked uronic acids are arranged in homopolymeric blocks of β-D-mannuronic acid (M) or α-L-guluronic acid (G) and in MG-blocks containing both uronic acids [49,54]. Due to its biocompatibility and biodegradability as well as its non-toxic and non-immunogenic properties [55–57] alginate is widely used as an additive in the food industry as well as in the pharmaceutical industry [49]. With the addition of divalent cations like calcium, alginate can be cross-linked, because the guluronic acid blocks can complex bivalent cations and form a so called “egg-box” [58].

Alginates are provided in a broad range of products, therefore they can be used in many different applications. Alginic acid forms different salts with counter ions like sodium, magnesium or potassium. The gelling properties are highly influenced by the ratio of guluronic acid and mannuronic acid in the alginate chains. To prepare alginate films with adjustable mechanical properties and erosion time an adequate cross-linking is necessary. For this study three different sodium alginates Protanal® LF 10/ 60, its follower Protanal® LF 10/ 60 FT and Protanal® LF 10/ 60 LS (FMC BioPolymer) [59,60] were used to prepare thin films for the prevention of abdominal adhesions. These alginates are assigned for the preparation of wound healing dressings [61] and therefore ought to be suitable for the preparation of thin alginate films for adhesion prevention.

Its temperature independent [49] ability of forming a gel by a reversible cross-linking of the guluronic acid blocks with the help of bivalent cations like calcium [62] makes alginate a very promising polymer for the preparation of erodible polymer films. When the G-blocks in the alginate chains get in contact with calcium the guluronic acids can complex it and form a junction zone with other G-blocks by forming the so called “egg-box” [58]. For the preparation of different products of any shape made from cross-linked alginate many procedures for the internal gelation (Table 1) with a calcium release from a calcium source that has been mixed with the alginate solution and the external gelation (Table 1) with a calcium source that surrounds the prepared alginate product [61] have been established [63].

Table 1: Cross-linking of alginate

External calcium source	Internal calcium source
➔ calcium solution	➔ calcium salt, calcium complex
Important characteristics:	Important characteristics:
• fast gelation	• slow gelation
• calcium gradient	• defined calcium amount
• undefined calcium amount	
Examples:	Examples:
• alginate beads [64]	• scaffolds for tissue engineering [66]
• alginate inserts [65]	

The external gelation (Table 1) is used, when the gelation has to be very quick like for the formation of alginate beads, when an alginate solution is dripped into a solution of divalent cations [64] or when films are prepared and later immersed in a calcium solution [65]. However, the amount of calcium incorporated cannot be controlled and due to the fact that the calcium source is outside of the alginate product a gradient from the outer part to the center occurs. The internal gelation technique allows a slower gelation than the external gelation technique and therefore can be used, when alginate products with a defined shape are required, because the alginate can be cast into a mold before the cross-linking process starts [66]. For the internal gelation, it is very important to slow down the cross-linking process. This is mostly achieved by releasing the calcium slowly from a hardly soluble calcium salt (Table 1) like $CaCO_3$ or $CaSO_4$ [63,66,67] or by dissolving the calcium from a calcium complex like Ca-EDTA [68] or Ca-EGTA [69] by lowering the pH with a solution of GDL [67] or light-triggered dissolution of caged calcium [70]. Also, the counter ion of the used alginate has an impact on the cross-linking speed, since potassium-alginates cross-link faster than sodium-alginates [71].

Different film preparation techniques were investigated to obtain cross-linked alginate films with a homogenous distribution and a constant concentration of all used components like the alginate itself, calcium, cross-linking additives and plasticizers. External gelation of the prepared alginate film by dipping it into a solution containing calcium [63,65,72] or sprinkling the film with a calcium solution was not considered for the cross-linking of the alginate films, because these methods cannot guarantee a homogeneous distribution of a defined calcium amount in the film. Due to the fact that the calcium incorporated in the film is the main factor that influences the mechanical stability and degradation time of the alginate films it

was of utmost importance to investigate a preparation method that ensures a homogenous distribution of a defined amount of calcium in the film. For this purpose the alginate films were prepared with the help of the internal gelation technique.

3.2 Film preparation techniques

In general two film preparation procedures were investigated during the development of improved alginate films. In first trials alginate solutions were cast into molds (Figure 3). During further investigations drawing onto glass plates was used to provide larger homogenously thick film samples (Figure 4-6).

3.2.1 Cast films prepared with the inner gelation technique

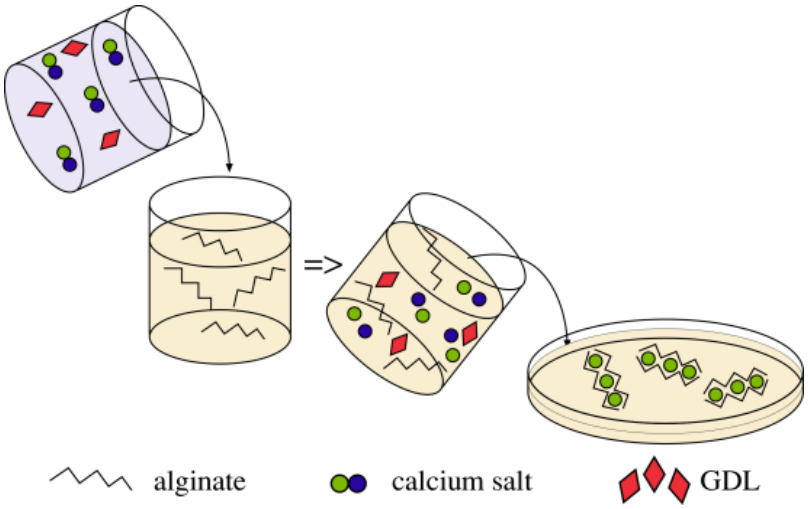


Figure 3: Inner gelation technique

For the inner gelation of cast films defined amounts of a hardly soluble calcium salt like calcium citrate and a pH controlling acid like a hydrolysable lactone, in this case gluconic acid δ-lactone (GDL), were weighed in solid form in a glass vial. After addition of water, the suspension was vortexed and mixed with a prepared alginate solution. The obtained mixture was cast into a Teflon® mold or a petri dish (Figure 3). Due to the hydrolysis of the lactone, the pH decreased and the calcium was released from its salt and could cross-link the alginate chains. After several hours of cross-linking time the film could be removed from the mold for further investigations [32].

3.2.2 Films drawn with a drawing apparatus

To obtain lager and evenly thick films, the alginate solution was drawn on a glass plate with the help of a commercial available drawing apparatus (ERICHSEN coatmaster 509 MC, Hemer, Germany). For the preparation of thin polymer films with the help of a drawing apparatus alginate was a very versatile polymer due to its non-Newtonian characteristics [54], because a shear thinning facilitated the flow of the alginate solution through the very thin gap of the drawing frame. The challenge of drawing cross-linked films with a homogenous cross-linking is, that the cross-linking process should be slow enough, that the alginate solution is able to be drawn, because it mustn't cross-link, before the drawing process is finished. After drawing the film, it should cross-link in a short time.

3.2.2.1 Drawn films with a hardly soluble calcium salt sprayed with acid

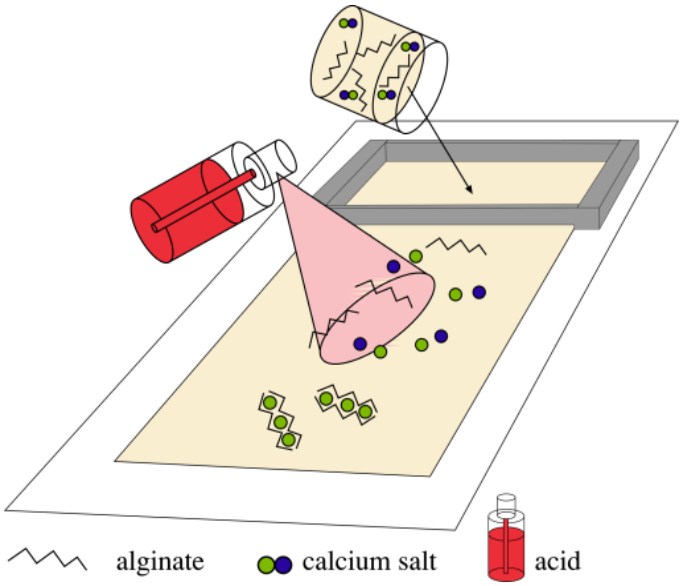


Figure 4: Inner gelation with sprayed acid

For this technique, hardly soluble calcium citrate was evenly dispersed in a viscous solution of alginate with the help of an ultra turrax. Then the alginate solution containing crystals was drawn on a glass plate with a gap clearance of 700 µm and a speed of 5 mm/ sec. To dissolve the calcium from its barely soluble calcium salt and initiate cross-linking, the freshly prepared, thin film was sprayed with a sufficient amount of lactic acid using a gas microsprayer (EcoSpray®, Labo Chimie France, Meyreuil, France) to obtain a visibly clear film (Figure 4). The obtained cross-linked film was subsequently air dried at 23 °C ±2 °C and 50 % ± 5 % relative humidity.

3.2.2.2 Drawn films with a calcium complex and a pH controlling acid

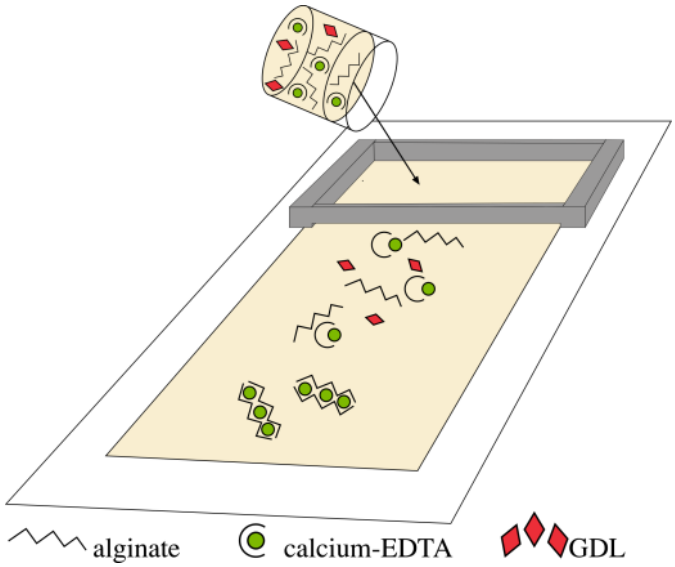


Figure 5: Inner gelation with calcium complex

To further improve the distribution of calcium, the barely soluble calcium citrate was replaced by the soluble, pH sensitive calcium-EDTA-complex (Figure 5) [68,73]. Due to the fact that a completely homogenous distribution of the lactic acid on the film was not possible by spraying, pH decreasing by hydrolysis of a lactone that was used in the casting procedure was tested. After mixing the lactone powder with the calcium-EDTA-complex containing alginate solution, the pH decreased and consequently released the calcium from its complex. The release of calcium from the complex was slowly enough to enable drawing of the film before the cross-linking started.

3.2.2.3 Drawn films with a hardly soluble calcium salt and a pH controlling acid

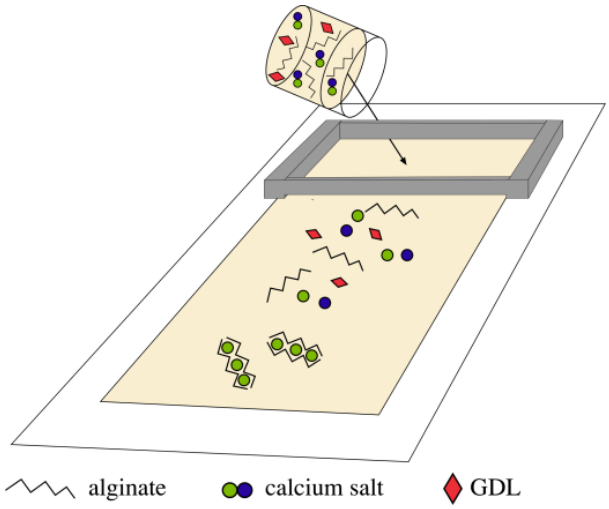


Figure 6: Inner gelation with calcium salt

To increase the cross-linking of the alginate films the calcium-EDTA-complex was replaced by dicalcium phosphate. Dicalcium phosphate was wetted with some droplets of water or a defined amount of glycerol, added to a prepared alginate solution and homogenized by stirring with a magnetic stirrer. Then GDL powder was added and after further homogenization the film was drawn with a gap clearance of 700 µm and a speed of 5 mm/ sec (Figure 6). To be able to decrease the pH and initiate cross-linking, the GDL needed water. Therefore, the cloudy film was stored in a closed chamber to slow down the drying process until the film became clear and could be air dried at 23 °C ±2 °C and 50 % ± 5 % relative humidity.

3.2.3 Identification of the optimal film preparation technique

The established film preparation techniques showed many differences regarding the homogeneous distribution of calcium in the film, the release kinetics of the calcium from the calcium source and the shape of the obtained film.

Films, drawn on a glass plate cross-linked with the help of $CaHPO_4$ and GDL showed the most promising results.

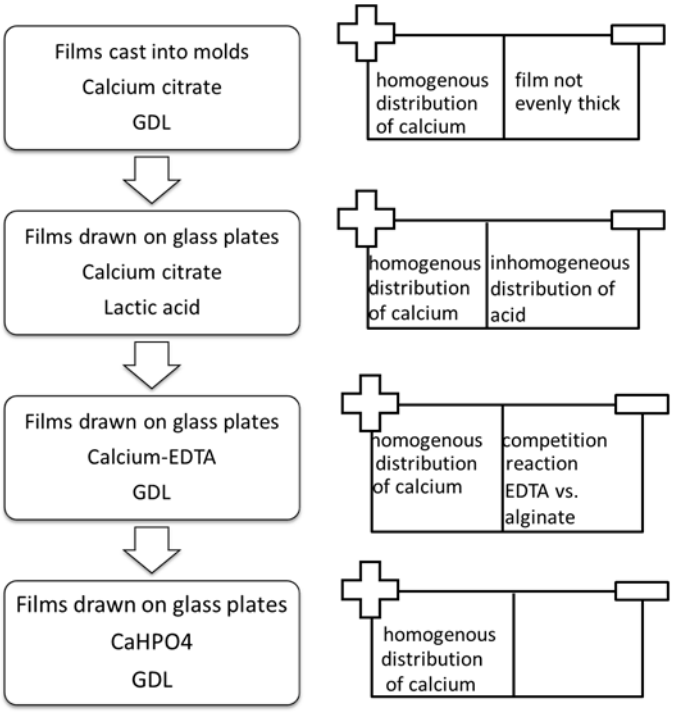


Figure 7: Film preparation techniques

To control the absolute calcium amount incorporated in the film, inner gelation techniques were performed instead of cross-linking the film after preparation by spraying it with or dipping it into calcium solutions. When alginate chains come in contact with free calcium ions, they cross-link immediately. To be able to cast the alginate solution into a mold or draw a film on a glass plate after addition of a defined amount of calcium it was necessary to slow down the speed of cross-linking. This could be achieved by incorporating hardly soluble calcium salts or calcium complexes in the film which slowly release the calcium and initiate cross-linking.

The first procedure tested was to cast films into molds. The obtained films were clear after drying, which indicated complete dissolution of the hardly soluble calcium citrate and the calcium could bind to the alginate chains and cross-link them. After drying, the films sticked to the molds and could not be removed easily. Furthermore, they were not evenly thick. In the edge region, the films were thicker than in the middle. To obtain evenly thick films, the film preparing procedure was optimized by drawing the films with a defined gap clearance onto glass plates. After drying at room temperature, the films could be cut and removed from the glass surface better than from the mold. The hardly soluble calcium salt calcium citrate required homogenization with an ultra turrax, because when the salt got in contact

with water or the alginate solution, it formed clusters and could not be distributed homogenously with a magnetic stirrer. This came along with some disadvantages because air was incorporated with the ultra turrax and the suspended calcium salt did not float in the alginate solution but sank to the ground instead. After drawing, the still wet film was sprinkled with lactic acid to release the calcium from its salt and initiate cross-linking. However it was quite difficult to ensure that a precise amount of lactic acid was sprayed evenly over the whole film. To overcome the problem of the inhomogeneous distribution of the calcium citrate, a soluble calcium-EDTA-complex was mixed with the alginate solution. Furthermore, the lactic acid was replaced by GDL that could be added in a defined amount to the alginate solution and be distributed homogenously before drawing the film. After drawing the film that was free of any solid compounds, the GDL hydrated and slowly decreased the pH of the alginate film which initiated the release of calcium from the complex that could than cross-link the alginate chains. But the EDTA was still in the film and competed with the guluronic acid blocks for the calcium ions. Therefore, the calcium release from a hardly soluble calcium salt was preferred. To avoid a cluster formation of the calcium salt, dicalcium phosphate was used instead of calcium citrate. The distribution in the alginate solution after wetting was easier and led to a more homogeneous distribution of calcium. Furthermore the smaller particles were less prone to sedimentation.

3.3 Optimizing the mechanical properties

3.3.1 Overcoming the brittleness of the prepared alginate films

When an alginate solution is drawn to a film and air dried, it becomes very brittle. To make the film more flexible and better to handle for the surgeon, plasticizers should be added. The plasticizer itself should be nontoxic, biocompatible, biodegradable or at least bio absorbable. A common plasticizer that fulfills all these requirements is glycerol [74–76]. Therefore, different glycerol concentrations calculated on the polymer weight were tested to adapt the mechanical properties of the prepared films. One disadvantage of glycerol is that it is a very small, hydrophilic molecule, which is washed out very fast in a humid environment like the abdomen. Although the emollient effect of the glycerol is not needed anymore in a humid environment due to the fact, that water itself has a softening effect, larger molecules, that are not washed out that fast like PEG 500 and PEG 2000 were tested, too. Another approach was, to link the plasticizer to the alginate and prevent the elution of the plasticizer.

Therefore, amino PEG 2000 synthesized according to the Mitsunobu reaction was linked to the alginate chains.

3.3.1.1 Films with different plasticizers without cross-linking

To be able to measure only the impact of plasticizer on the mechanical properties, films with glycerol and PEG 500 without cross-linking were prepared. For this purpose a defined amount of plasticizer (Table 2) calculated on the amount of alginate was added to a solution of alginate and dissolved to 25 g in water. The films were drawn on glass plates with a gap clearance of 700 µm and a speed of 5 mm/ sec. After drying at room temperature the roughly 25 µm thick films were cut into strips of 1 to 5 cm. 10 strips were tested as close as possible to the ASTM test standard for thin plastic sheeting.

Table 2: Plasticizer amount for non-cross-linked alginate films

Plasticizer amount [%] calculated on alginate	Plasticizer amount [g]
0	0
5	0.05
10	0.1
20	0.2
50	0.5
100	1

3.3.1.2 Films cross-linked with a hardly soluble calcium salt acidified with lactic acid

3 g of alginate and a defined amount of glycerol respectively PEG 2000 (Table 3) were dissolved to 50 g in water to obtain a 6 % (m/ V) alginate solution. To cross-link the film 750 mg of hardly soluble calcium citrate was suspended in 25 ml of the alginate solution and mixed with the help of an ultra turrax. After homogenization the suspension was sonicated to remove the air bubbles and drawn with a gap clearance of 700 µm and a speed of 5 mm/ sec on a glass plate. The films containing 1.5 g or 3 g of PEG 2000 did not have the required consistency to be drawn to a film and therefore were rejected. To dissolve the calcium from its hardly soluble salt, the still wet film was sprayed with lactic acid. The air dried films had a thickness of about 50-60 µm. 20 strips of 1 to 17.5 cm were tested as close as possible to the ASTM test standard.

Table 3: Components for films sprayed with lactic acid

Alginate[g]	Plasticizer [%]	Plasticizer [g]	Water [ml]
3	0	0	47
3	1	0.03	46.97
3	5	0.15	46.85
3	10	0.3	46.7
3	20	0.6	46.4
3	50	1.5	45.5
3	100	3	44

3.3.1.3 Films cross-linked via calcium release from a calcium-EDTA-complex

For the formation of the calcium-EDTA-complex, 6.09 g calcium chloride and 20.44 g Na-EDTA were dissolved in 175 ml water and the pH was adjusted to 7. For the alginate solution, 2 g alginate and a defined amount of the plasticizers glycerol and PEG 500 were dissolved to 25 g in water. After dissolution, 25 ml of the calcium-EDTA solution was given to the alginate solution and homogenized. Before drawing the film with a gap clearance of 700 µm and a speed of 5 mm/ sec, 500 mg GDL was added and homogenized. After the film was dried at ambient conditioning, it was cut into strips of 1 to 17.5 cm with a thickness of 30 to 40 µm and 20 strips were tested according to ASTM. The film containing 50 % PEG 500 could not be tested, because it was too brittle to be removed from the glass plate.

3.3.2 Water content of air dried alginate films

One of the main factors that influenced the mechanical properties of the prepared alginate films was the amount of plasticizer incorporated in the film. In this case not only plasticizers like glycerol or PEG should be mentioned. Also the softening effect of water should not be disregarded. Therefore it was very important to measure the water amount incorporated in the alginate films.

For a moisture measurement of alginate films containing glycerol as plasticizer, four films with 0 %, 10 %, 20 % and 50 % (w/ w) glycerol calculated on the alginate amount were drawn with a gap clearance of 700 µm and a speed of 5 mm/ sec. After drying at ambient conditions, the films were cut into squares of 25 cm^2 and weighed. Subsequently 3 pieces of

every film were stored in a cabinet drier for 15 minutes at 30 °C, 40 °C and 100 °C. After 15 minutes the films were taken out of the cabinet drier and weighed again to determine how much water the film had lost during the heating procedure.

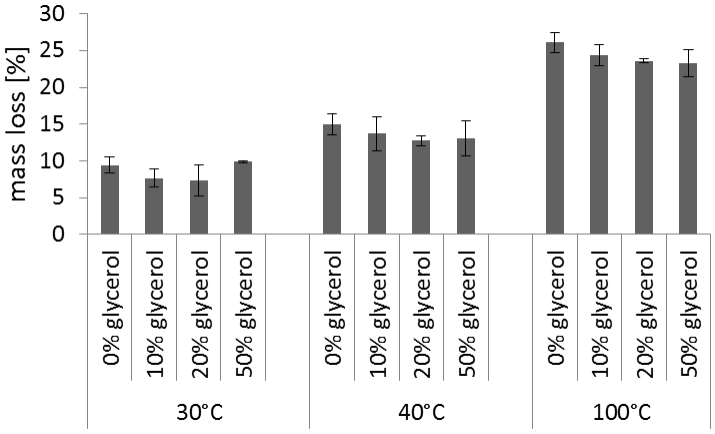


Figure 8: Mass loss of alginate films stored in a cabinet dryer for 15 minutes at different temperatures

Due to the fact that glycerol and PEG are not thermo stable and hydrolyze when they are heated, the results that were obtained by this method could be too high. Therefore a more gentle method was performed to determine the water content.

To further see the impact of cross-linking on the water content of air dried alginate films a cross-linked and a not cross-linked film was tested. 0.75 g alginate and 0.75 g glycerol were dissolved to 25 g in water and drawn with a gap clearance of 700 µm and a speed of 5 mm/ sec. To cross-link the film with the inner gelation technique, 0.6 g $CaHPO_4$ and 1.2 g GDL were added to the alginate solution before drawing. After drying at ambient conditions pieces of 25 cm^2 were cut out of the film and given into centrifuge tubes. After the initial weight was determined, the films were lyophilized for 24 hours. The not cross-linked film lost nearly 7 % of its weight and the cross-linked film lost nearly 6 %. After further 24 hours of lyophilization, the weight of the films remained constant. But with this method it is also not sure, if only the water evaporates, or if the plasticizer evaporates under these conditions, too.

Therefore, a Karl Fischer titration was performed to measure the residual water content of alginate films containing different amounts and types of plasticizer. The measurements were performed using a TitroLine® 7500 KF trace (SI Analytics, Mainz, Germany).

Alginate films without plasticizer and with physically addition of 10 %, 50 % or 100 % (w/w) glycerol, PEG 500 or PEG 2000 as plasticizer calculated on sodium alginate content and alginate films of the synthesized PEGalginates were drawn to films with a gap clearance of 700 µm and a speed of 5 mm/ sec and dried under the same conditions of temperature and humidity as before for 24 hours. Then the films were cut into strips of 1x5 cm. About 100 mg of the film strips were accurately weighed into glass vials and covered with 10.0 ml of dried methanol. The strips were pivoted with an orbital shaker Rotamax 120 (Heidolph Instruments GmbH & Co KG, Schwabach, Germany) at 100 rpm for 24 hours. After 24 hours, 1 ml of the methanol was taken and injected into the reaction medium of the Karl-Fischer titrator and titrated. The measurement was repeated 5 times. Pure dried methanol was used as control to subtract the blank water content.

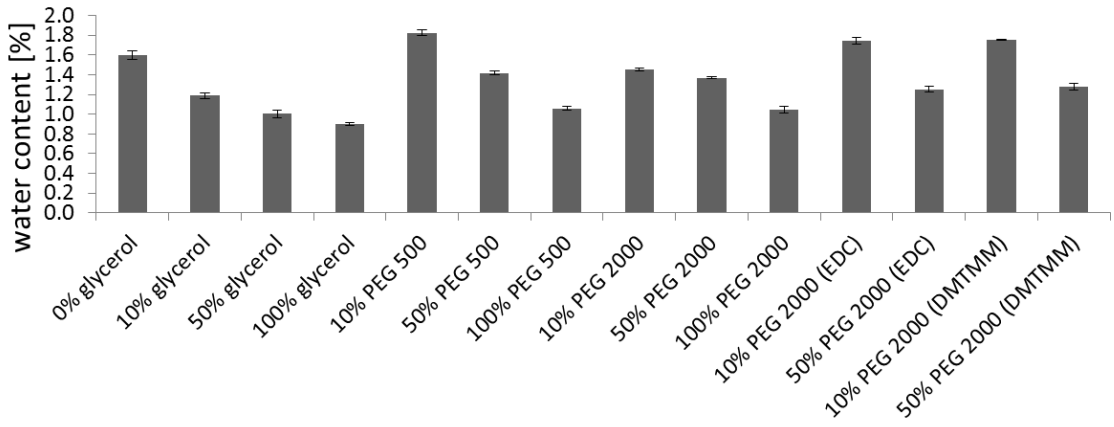


Figure 9: Moisture measurements of alginate films containing different amounts and types of plasticizer

The first attempt of measuring the water content of air dried alginate films by heating up the alginate films in a cabinet drier showed a mass loss of 6 % to 10% at 30 °C, 11 % to 17 % at 40 °C and 22 % to 27 % at 100 °C. This mass loss can be caused by the loss of water incorporated in the film, the loss of water that came from the hydrolysis of glycerol and the loss of other volatile substances that come out of the film by heating it up. As expected, the mass loss increased with higher temperatures of the cabinet drier. The more gentle method of lyophilization showed a mass loss of nearly 7 %. But with this method it could not be precluded that only water evaporated during the drying process. Therefore the common method of moisture determination via a Karl Fischer titration was performed. The moisture measurements of the films containing different amounts of plasticizer yielded a water content of 1 % to 1.8 %. With increasing amount of plasticizer the water amount in the film

decreased. With glycerol as plasticizer, the water amount in the film was lower than with PEG. The films with 10 % PEG 500 or 10 % AminoPEG seemed to have the highest water content. Comparing the first and the second method an inverse correlation between residual water content and amount of plasticizer became visible. Therefore it is necessary to find the right balance of softening effect by an added plasticizer and water.

With the help of the Mitsunobu reaction AminoPEG could be synthesized and subsequently linked to the alginate chains. PEG 2000 instead of PEG 500 was chosen for the preparation of AminoPEG to obtain a product that is in a solid state at room temperature. PEG was chosen instead of glycerol, because large amounts of the very small glycerol should have been linked to the alginate chains to achieve a softening effect. But this would come along with a loss of many carboxyl groups in the alginate chains which are essential for the cross-linking with bivalent cations like calcium. With both carboxyl activating agents a linkage of PEGamine to the alginate chains was possible. The wanted modification with 10 % was nearly achieved with 9.3 % and 8 %. With EDC more PEGamine could be linked to the alginate as with DMTMM.

3.3.3 Overcoming the loss of plasticizer

Hydrophilic plasticizers like glycerol and PEG can be easily washed out, when the films are implanted in the peritoneum. To retain the plasticizer in the alginate film PEG 2000 was linked to the alginate chains. PEG 2000 was chosen because it is a solid PEG at room temperature and therefore the resulting PEGalginate should be in a solid state, too. This would ease the handling for following film preparations. When the PEG is linked to the acids of the alginate chains, particularly the guluronic acids, these guluronic acids would no longer be available for the complexion of divalent ions and the resulting cross-linking of the alginate chains. Therefore a modification with 10 % and 50 % PEG calculated on alginate was required to obtain and alginate containing its plasticizer covalently linked, that can still be cross-linked with bivalent cations.

3.3.3.1 Preparation of mono AminoPEG (Mitsunobu reaction)

For the chemical linkage of PEG to alginate, AminoPEG was synthesized according to the Mitsunobu reaction. At first mPEG (20 g, 2000 g mol^{-1}, 10 mmol, 1 eq) was dissolved in toluene (200 ml) and dried by azeotropic distillation. The solvent was removed under

reduced pressure and the residue was dissolved in tetrahydrofuran (THF, 100 ml). Phthalimide (2.208 g, 147.13 g mol^{-1}, 15 mmol, 1.5 eq) and triphenlyphosphine (3.935 g, 262.29 g mol^{-1}, 15 mmol, 1.5 eq) were added. Diisopropyl azodicarboxylate (DIAD, 3 ml, 202.21 g mol^{-1}, 15 mmol, 1.5 eq) was dissolved in THF (30 ml) and was added to the reaction. Triphenylphosphine in combination with DIAD generated a phosphonium intermediate that activated the alcohol group of the PEG. The reaction mixture was stirred for 48 hours at room temperature. After the removal of the solvent, the raw product was dissolved in water, filtered and washed twice with diethyl ether. Afterwards the water was removed; the resulting product was dissolved in dichloromethane (DCM) and precipitated in diethyl ether (400 ml). The precipitate was collected by filtration, washed with cold diethyl ether and dried under vacuum. In the next step, PEG-phthalimide (20 g, 2129 g mol^{-1}, 9.4 mmol, 1 eq) was dissolved in ethanol. To remove the phthalimide, hydrazine hydrate (2875 µl, 59.05 g mol^{-1}, 50 mmol, 5 eq) was added subsequently and the reaction mixture was refluxed for 5 hours at 85 °C. After cooling to room temperature the solution was filtered and the pH was adjusted with hydrochloric acid to pH 2-3. After evaporation of the solvent, the residue was solved in water and the pH of the aqueous solution was adjusted with sodium hydroxide to pH 9-10. The raw product was extracted four times with DCM. The combined organic phases were dried over anhydrous magnesium sulfate ($MgSO_4$), and the solution was concentrated to 50 ml. The product was precipitated into cold diethyl ether, collected by filtration, washed with cold diethyl ether and dried under vacuum.

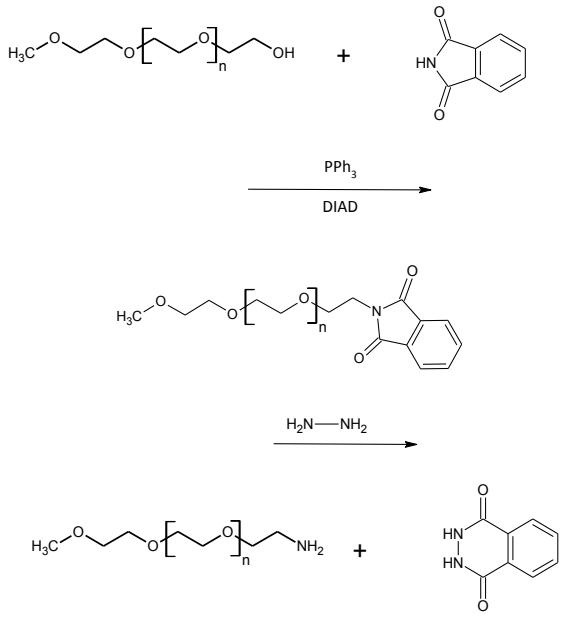


Figure 10: Synthesis of PEGamine with the help of the Mitsunobu reaction

3.3.3.2 Attachment of AminoPEG to alginate chains

In a further step, the resulting AminoPEG was linked to the alginate. Therefore alginate LF 10/ 60 (15 g) was dissolved in water (735 ml), while cooling to 0 °C. The carboxylic groups of the alginate were activated by addition of N- (3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 191.7 g mol^{-1}, 2 or 10 eq) or 4-(4.6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, 276.72 g mol^{-1}, 2 eq) and the pH was adjusted to 5 with hydrochloric acid. The cooled solution was stirred for two hours until AminoPEG (1.5 g or 7.5 g) was added and stirred at 0 °C over night. The reaction solution was dialyzed (MW cutoff 14000 Da) against water for 2 days and dried by lyophilization.

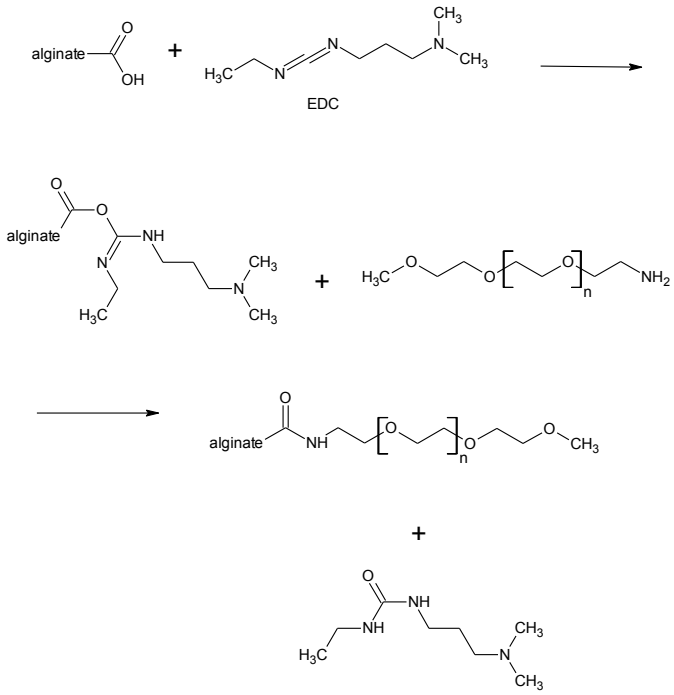
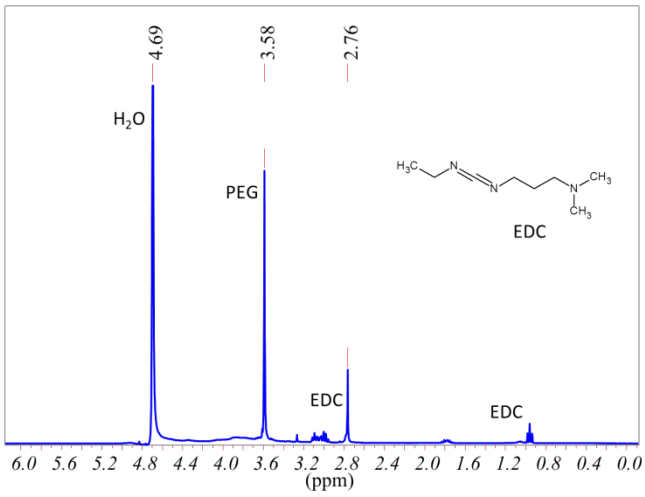


Figure 11: Linkage of PEGamine to the alginate by taking the example of activation with EDC

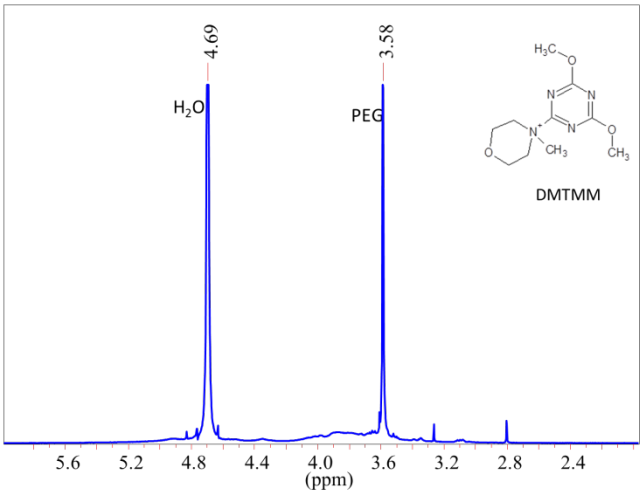
Table 4: Amounts of reaction components

AminoPEG	EDC	DMTMM
1.5 g	1.44 g (10 eq)	0.42 g (2 eq)
7.5 g	1.44 g (2 eq)	2.08 g (2 eq)

To examine if the linkage of aminoPEG to the alginate chains had been successful, ^{1}H-NMR measurements and GPC measurements were performed. The ^{1}H-NMR measurements were performed in D_2O with a Bruker Avance 300 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). For the GPC measurements EDC, DMTMM, PEG, AminoPEG and the prepared PEG alginates were dissolved in water in a concentration of 10 mg /ml. The GPC setup consisted of a system controller, a binary pump, an auto injector, a column oven at 30 °C, a UV-Vis detector (190 nm) and a refractive index detector (Shimadzu Corporation, Chromatographic & spectrophotometric instruments division, Kyoto, Japan). As stationary phase a PolySep-GFC-P Linear column (Phenomenex, Aschaffenburg, Germany) was chosen. For the mobile phase a phosphate buffer was prepared by dissolving 14.2 g dibasic sodium phosphate in 5 L of water. The pH of the buffer was adjusted to 6.5, before filtration with a sterile syringe filter from CORNING® (Corning Cable Systems GmbH + Co KG, Munich, Germany). The measurement was performed with a flow rate of 1 ml/ min. The resulting chromatograms from the UV-Vis detector measured at 190 nm were processed using the Class-VP software (Shimadzu).

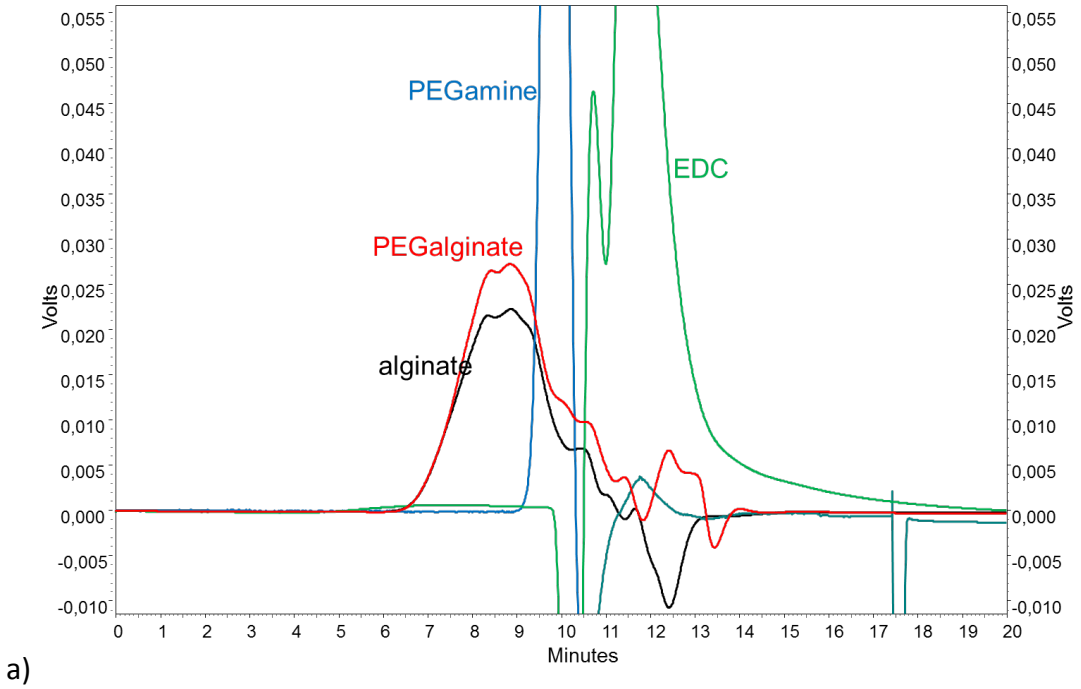


a)

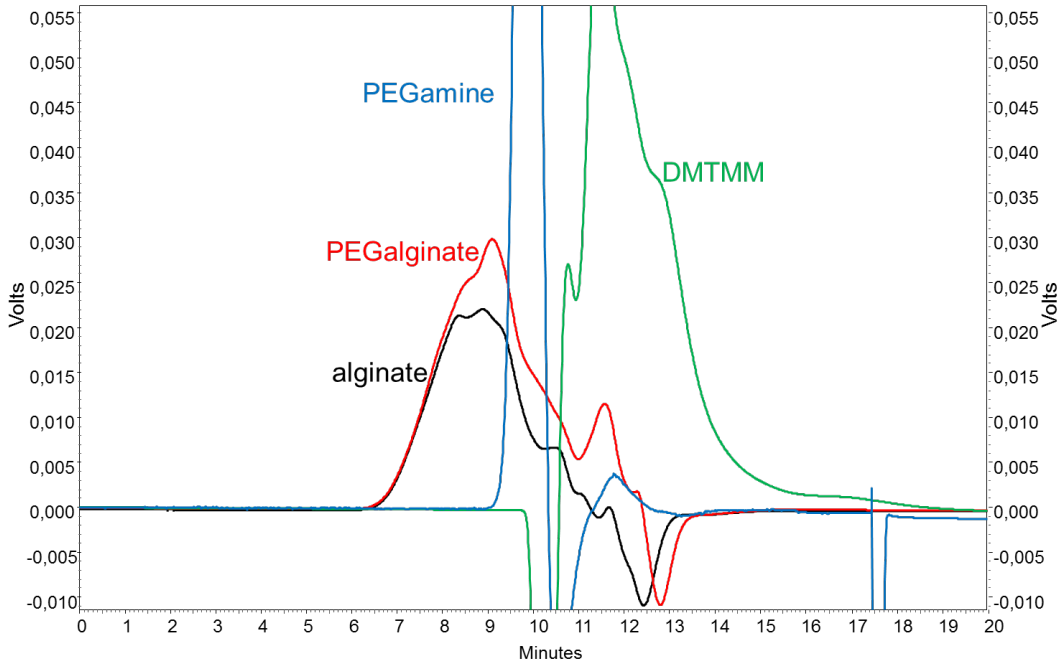


b)

Figure 12: ^{1}H-NMR spectra of PEGalginate synthesized with a) EDC and b) DMTMM



a)



b)

Figure 13: Chromatograms of PEGalginate and reaction components synthesized with a) EDC and b) DMTMM

With the help of NMR measurements, the PEG could be determined which indicated that it was linked successfully to the alginate chains, because during dialysis it would have been eluted otherwise. The GPC measurements also revealed a successful linkage of PEGamine to the alginate chains. Even the purification with the help of the dialysis was successful, because no residual PEGamine was detectable.

3.3.3.3 Determination of free PEG and attached PEG

To quantify how much free PEG that was washed out during dialysis, a PEG-iodine-assay was performed. An I_2/ KI-solution was prepared by dissolving 1 g I_2 and 2 g KI to 100 ml in water. For the calibration, a stock solution of 10 mg PEG in 100 ml water was prepared and diluted to concentrations ranging from 10 µg/ ml to 100 µg/ ml. To see if alginate would interfere under the testing conditions, an alginate solution of 10 mg alginate in 10 ml water was tested, too. 50 µl of the prepared solutions for the calibration curve or 50 µl of the dialysis solutions were added into Eppendorf cups and diluted with 950 µl water. After addition of 30 µl of the prepared I_2/ KI solution the mixture was vortexed. Subsequently 200 µl of these mixtures were added into a 96 well plate and the absorption was measured at 500 nm.

Table 5: PEG detection of free PEG after linkage with the help of EDC: Results of PEG-assay

Dialysis step	Water [l]	Absorption	PEG concentration [mg/ml]	Amount of PEG in water[mg]
1	9	0.0130	1.52	13.70
2	9	0.0325	3.81	34.26
3	9	0.0160	1.87	16.87
4	8	0.0205	2.40	19.21
5	8	0.0093	1.08	8.67
				92.70

The aim of this reaction was to link 10 % PEG to the alginate. 92.7 mg PEGamine were washed out during the dialysis (Table 5). Therefore nearly 1.4 g PEGamine was successfully linked to the alginate. This equals 9.33 % PEG. 1.4 g PEGamine with a molar mass of 2000 g/ mol linked to 15 g alginate with a molar mass of 194 g/ mol would correspond to a modification of every 110th acid group in the alginate.

Table 6: PEG detection of free PEG after linkage with the help of DMTMM: Results of PEG-assay

Dialysis step	Water [l]	absorption	PEG concentration [mg/ml]	Amount of PEG in water [mg]
1	9	0.0065	0.76	6.85
2	9	0.1478	17.30	155.74
3	9	0.0400	4.68	42.16
4	8	0.0378	4.42	35.37
5	8	0.0170	1.99	15.93
				256.06

With DMTMM 8 % PEGamine could be successfully linked to the alginate. 256.1 mg PEGamine were washed out during the dialysis. Therefore nearly, 1.2 g PEGamine was linked to the alginate. This means that every 130th acid group in the alginate was modified.

3.3.3.4 Films made of PEG-alginate

After lyophilization 1.5 g of the PEG-alginates containing 10 % PEG 2000 and 50 % PEG 2000 were dissolved to 12.5 g in water. Then 12.5 ml of the calcium-EDTA-complex solution was given to the alginate. The films were drawn with a gap clearance of 700 µm and a speed of 5 mm/ sec after addition of 500 mg GDL. The films could not be tested mechanically, because after air drying they were too brittle to be removed from the glass plate. Therefore the preparation was repeated without cross-linking. 1.5 g of the alginates was dissolved to 25 g in water. The films were drawn with a gap clearance of 700 µm and a speed of 5 mm/ sec. After drying in air the 25 µm thick films were cut into strips of 1 to 5 cm of which 20 were tested as close as possible to the ASTM test standard.

3.3.4 Mechanical evaluation

The mechanical measurements were performed at the chosen test conditions of 23 ± 2 °C and 50 ± 5 % relative humidity and were carried out either with the texture analyzer Instron® 5542 (Instron® Deutschland GmbH, Pfungstadt, Germany) or the texture analyzer Z010 AllroundLine Materials Testing Machine (Zwick GmbH & Co. KG, Ulm, Germany). Both texture analyzers were a constant rate-of-crosshead-movement type. As load indicator, they had a load cell with a capacity of 50 N or 100 N. Before the films were tested, the thickness

was measured with a microprocessor coating thickness gauge (MiniTest 600 or 650, ElektroPhysik Dr. Steingroever GmbH & Co. KG, Köln, Germany). The results of the mechanical tests were recorded and calculated by the software Bluehill® 2 version 2.16. (Instron® Deutschland GmbH, Pfungstadt, Germany) or testXpert® 2 version 3.1 (Zwick GmbH & Co. KG, Ulm, Germany).

The results of the mechanical testing were illustrated in box plots. The used box plots summarized the obtained data representing different statistical values within one graph. Due to the remaining variability of the thin polymer films with minor defects in the films or at the film edges, this kind of illustration was preferred. In the used type of box plot, the bottom boundary of the box indicates the 25th percentile of the obtained values, the line within the box marks the median, and the top boundary indicates the 75th percentile. Error bars above and below the box indicate the 90th and 10th percentiles. Further outliers are handled by only showing the 5th and 95th percentile as additional dots above and below the error bars (SigmaPlot, Systat Software GmbH, Erkrath, Germany).

In addition to the illustration, one and two way analysis of variance (ANOVA) were performed with SigmaPlot. In order to evaluate the statistical significance of the individual formulations, a post-hoc Tukey test was used as a pairwise multiple comparison procedure. The p-values are marked with *. ** indicates $p<0.001$, * indicates $p<0.05$.

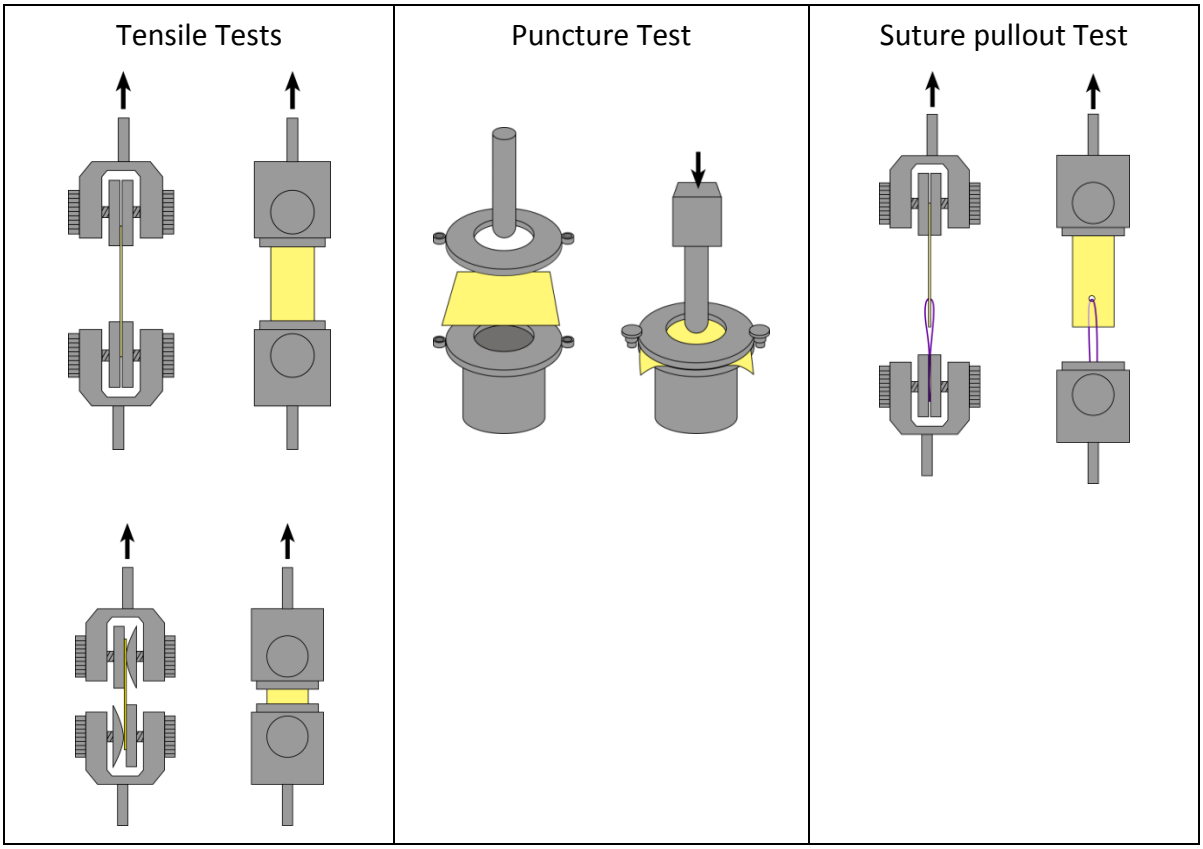


Figure 14: Setup for mechanical evaluation

3.3.4.1 Tensile test

For the tensile testing the air dried films were cut with a scalpel into strips of 1 to 5 cm respectively 1 to 17.5 cm. These strips were tested as close as possible to an American national standard of testing “Standard Test Method for Tensile Properties of Thin Plastic Sheeting D 882-02”. The 5 cm long strips were fixed with the grips in such a way that 3 cm of the film strips were between the grips and consequently stretched during the test. The 17.5 cm long films were fixed with12.5 cm between the grips. For the fixation two different kinds of grips were utilized, either two planar grips or one planar grip and one convex grip to avoid the formation of a breaking edge. The test was generally started with a slower grip separation of 10 mm/ min to stretch the clamped film samples. When a minimum load of 0.5 N was reached during the testing, the separation time of the grips increased to a speed of 12.5 mm/ min. Then the strips were stretched until the film ruptured. The rupture point was defined as the extension when the applied load suddenly decreased about 40 %.

3.3.4.2 Puncture Test

The setup of the puncture test was constituted of a film retainer consisting of a cylinder covered with a fixed ring, which was fixed at the bottom of the texture analyzer. In the center of the ring was a round hole with a diameter of 2.5 cm. This hole could be covered with the film that should be tested with the dimensions of 5 to 5 cm. To fix the film, a ring made of aluminum with a diameter of 7 cm and a hole with a diameter of 2.5 cm was fixed with screws on top of the lower ring in a way, that the film was clamped between the two rings. A piston with a diameter of 1 cm and a length of 5 cm was fixed at the upper arm of the texture analyzer. This piston was punched on top of the clamped film with a speed of 10 mm/ min [77] until the film ruptured. The recording started after a minimum load of 0.001 N was reached [77]. The maximum compressive load [N] and the elongation to puncture [%] [77] were recorded and calculated by the INSTRON® software Bluehill® 2 version 2.16..

3.3.4.3 Suture pullout test

For the suture pullout test specimens with the dimensions of 20 mm by 40 mm were cut from the film with a scalpel. The film was fixed in the upper clamp of the texture analyzer. A single strand of Vicryl® suture size 3-0 (Johnson & Johnson MEDICAL GmbH, Ethicon Deutschland, Norderstedt, Germany) was placed through the film sample at a distance of 15 mm from the end of the specimen and 10 mm of the side of the specimen. The two ends of the suture were fixed with the lower clamps. The two grips were separated with a speed of 55 mm/ min.

3.3.5 Results of mechanical testing

For the tensile testing of films containing different types and amounts of plasticizer 10 respectively 20 strips with a length of 5 or 17.5 cm and a width of 1 cm were tested as close as possible to the ASTM test standard, to see if plasticizers like glycerol or PEG have an effect on the mechanical properties of alginate films.

3.3.5.1 Effect of plasticizers on non-cross-linked alginate films

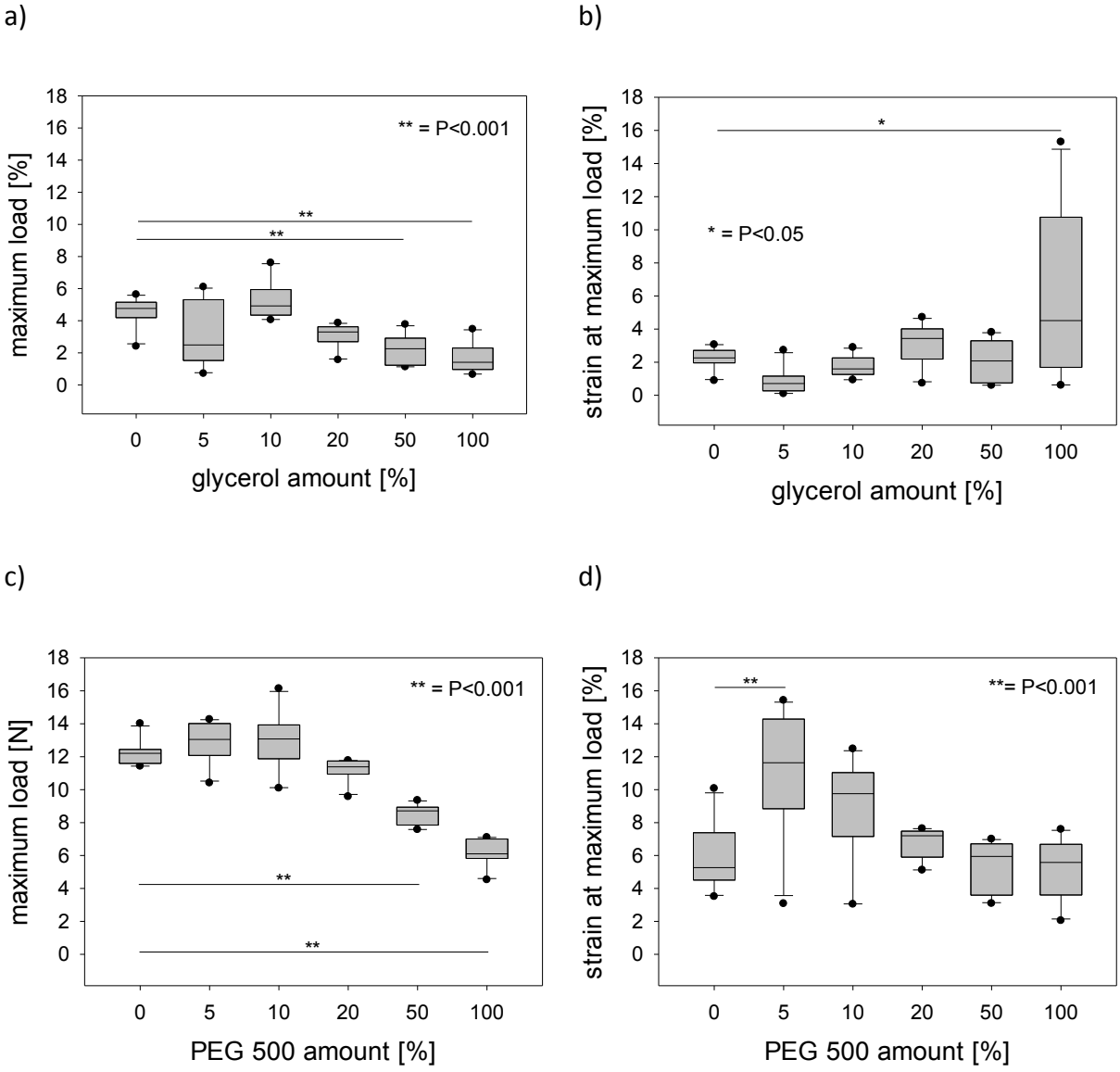


Figure 15: Mechanical testing of films containing different types and amounts of plasticizers without cross-linking. (a) Maximum load [N] of films containing glycerol as plasticizer. (b) Strain at maximum load [%] of films containing glycerol as plasticizer. (c) Maximum load [N] of films containing PEG 500 as plasticizer. (d) Strain at maximum load [%] of films containing PEG 500 as plasticizer. Statistical differences are only depicted in comparison to the 0 % plasticizer group, but were tested for all groups. Statistical differences ($p < 0.001$) are indicated with two asterisks **, ($p<0.05$) with one asterisks *.

The first mechanical trials were performed with films containing different amounts of the low molecular weight and liquid at room temperature plasticizers glycerol and PEG 500 in amounts of 0 % to 100 % relative to the dry alginate content. Both plasticizers showed a significant effect with the addition of 50 % or 100 % relative to the dry alginate content. As can be seen the two plasticizers did not show the same effect. Glycerol made the films softer. The maximum load decreased and the strain at maximum load increased. To the

contrary the films with PEG 500 became very brittle. The maximum load also decreased, but due to the fact, that the films were too brittle and did not withstand higher forces. With low amounts of 5 %or 10 % PEG the strain increased. But with higher amounts the films became very brittle and ruptured early.

3.3.5.2 Effect of plasticizers on films cross-linked via spraying with lactic acid

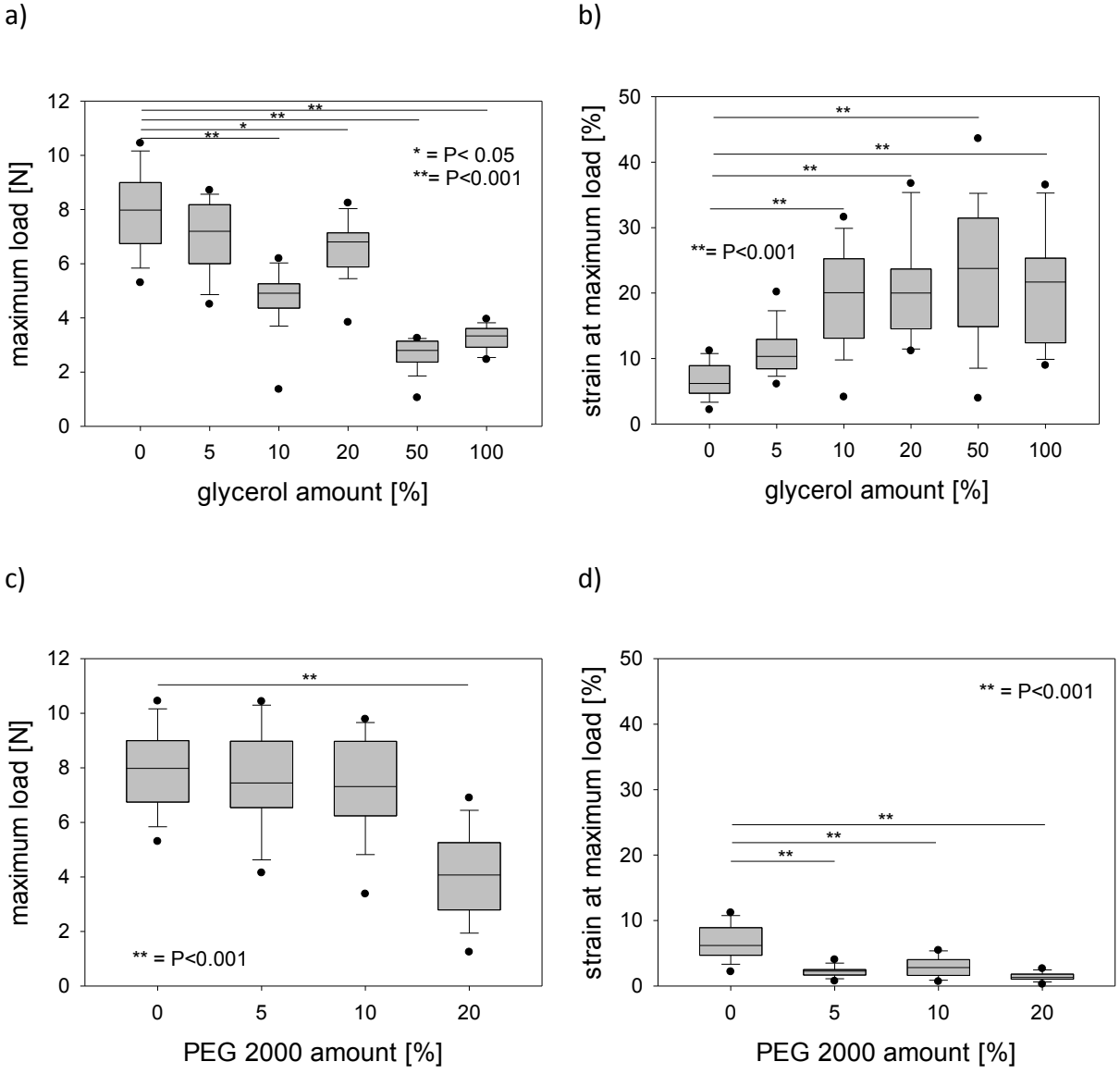


Figure 16: Mechanical testing of films containing different types and amounts of plasticizers cross-linked via calcium release from a hardly soluble calcium salt by spraying with lactic acid. (a) Maximum load [N] of films containing glycerol as plasticizer. (b) Strain at maximum load [%] of films containing glycerol as plasticizer. (c) Maximum load [N] of films containing PEG 2000 as plasticizer. (d) Strain at maximum load [%] of films containing PEG 2000 as plasticizer. Statistical differences are only depicted in comparison to the 0 % plasticizer group, but were tested for all groups. Statistical differences ($p < 0.001$) are indicated with two asterisks **, ($p<0.05$) with one asterisks *.

To improve the mechanical properties and erosion time of the films they were cross-linked with calcium. Two different cross-linking methods were tested. The films containing glycerol cross-linked with addition of calcium citrate and sprayed with lactic acid showed an increase of maximum load in comparison to the non-cross-linked films. Like the non-cross-linked films the maximum load decreased with higher amounts of glycerol and the films became more elastic. However with glycerol amounts higher than 10 % a plateau was reached and further addition of glycerol didn't show an increase of elasticity. Furthermore the films without glycerol showed a higher strain than the films without cross-linking. Besides the films sprayed with lactic acid were very sticky. Therefore the assumption occurs that the lactic acid has a softening effect on the alginate films, too. The addition of the solid PEG 2000 did not show a softening effect on the films. Mixtures with more 50 % or 100 % PEG 2000 could not be drawn at all to films and the films with less amounts of PEG 2000 became very stiff and brittle, too. The strain at maximum load decreased significantly after the addition of only 5 % PEG 2000. Whereas the maximum load was not affected until the addition of 20 % PEG 2000. The elasticity of the films containing PEG 2000 was even worse than the elasticity of the films containing PEG 500.

3.3.5.3 Effect of plasticizers on films cross-linked by EDTA-complexed calcium

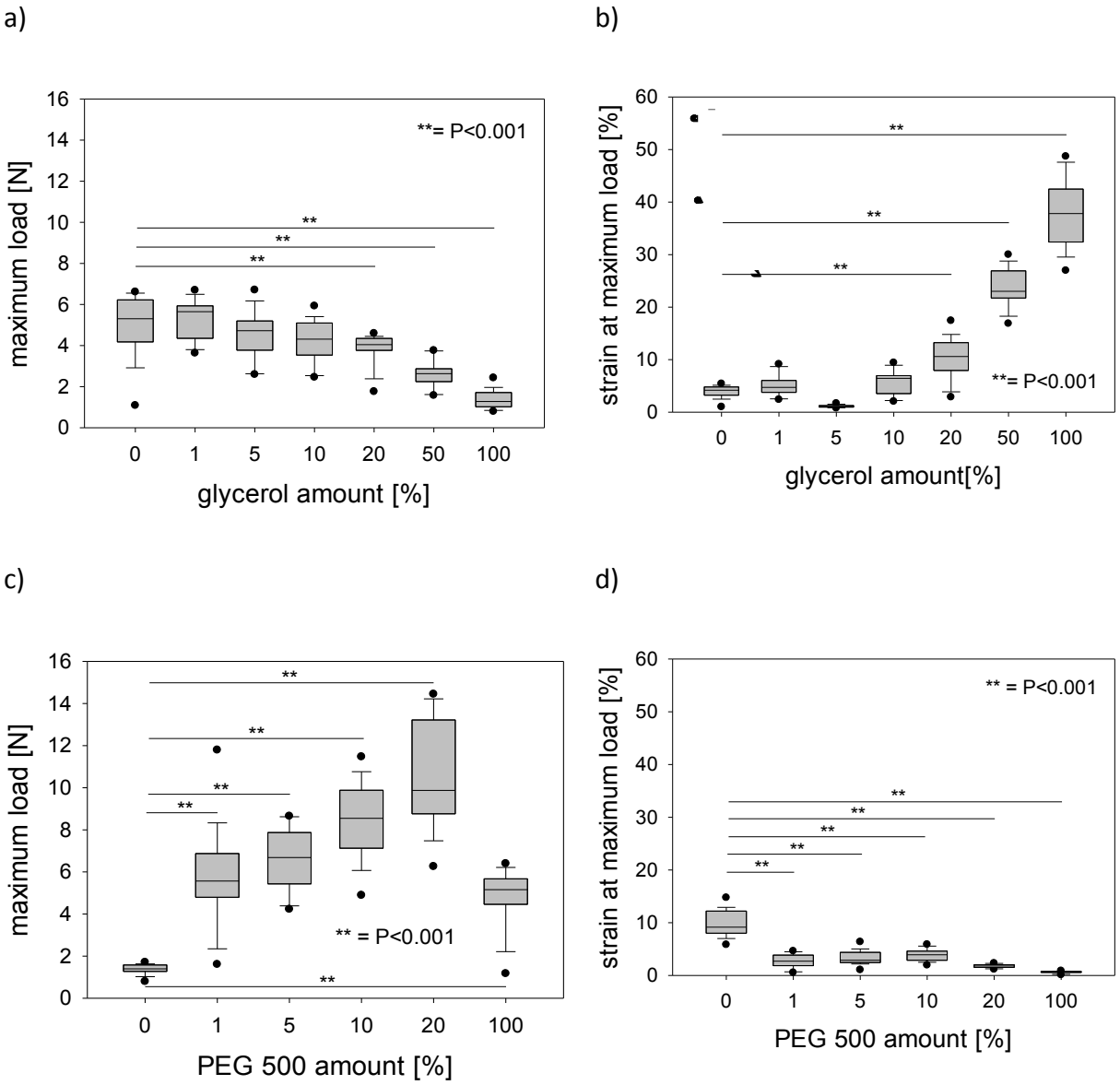


Figure 17: Mechanical testing of films containing different types and amounts of plasticizers cross-linked via calcium release from a calcium-EDTA-complex. (a) Maximum load [N] of films containing glycerol as plasticizer. (b) Strain at maximum load [%] of films containing glycerol as plasticizer. (c) Maximum load [N] of films containing PEG 500 as plasticizer. (d) Strain at maximum load [%] of films containing PEG 500 as plasticizer. Statistical differences are only depicted in comparison to the 0 % plasticizer group, but were tested for all groups. Statistical differences ($p < 0.001$) are indicated with two asterisks **, ($p<0.05$) with one asterisks *.

Figure 18: Film strips with increasing PEG 500 content cross-linked via the Ca-EDTA method.

To avoid the additional softening effect of the lactic acid, further films containing glycerol and PEG 500 as plasticizer were cross-linked by addition of a defined amount of a calcium-EDTA-complex and GDL. The strain at maximum load was strongly affected by the addition of glycerol. Increasing glycerol amounts led to a higher strain and consequently to a higher film elasticity. Up to the addition of 10 % glycerol the strain at maximum load stayed almost constant. Glycerol amounts larger than 20 % exhibited a significant drop in the maximum load and the films could only withstand very low tensile forces. Furthermore the cross-linked films could be stretched further than the films without cross-linking. The films containing 100 % glycerol for example could be stretched up to 45 % whereas the film without cross-linking containing 100 % glycerol could only be stretched up to 15 %. An increase in strain was also seen in comparison to the films sprayed with lactic acid. Additionally no plateau was visible which supports the theory of the softening effect of the lactic acid. With the addition of PEG 500 the films became very stiff and brittle. The appearance of the films changed too, because with increasing PEG 500 content the films became more opaque. With the addition of only 1 % PEG 500 a significant effect on the maximum load as well as on the strain at maximum load could be examined, both indicated very stiff and brittle films. With the addition of 100 % PEG 500 the maximum load immediately dropped down due to brittleness. Therefore physical addition of PEG to the alginate solution had not the wished softening effect on the alginate films.

3.3.5.4 Effect of linked AminoPEG 2000 as plasticizer

a) b)

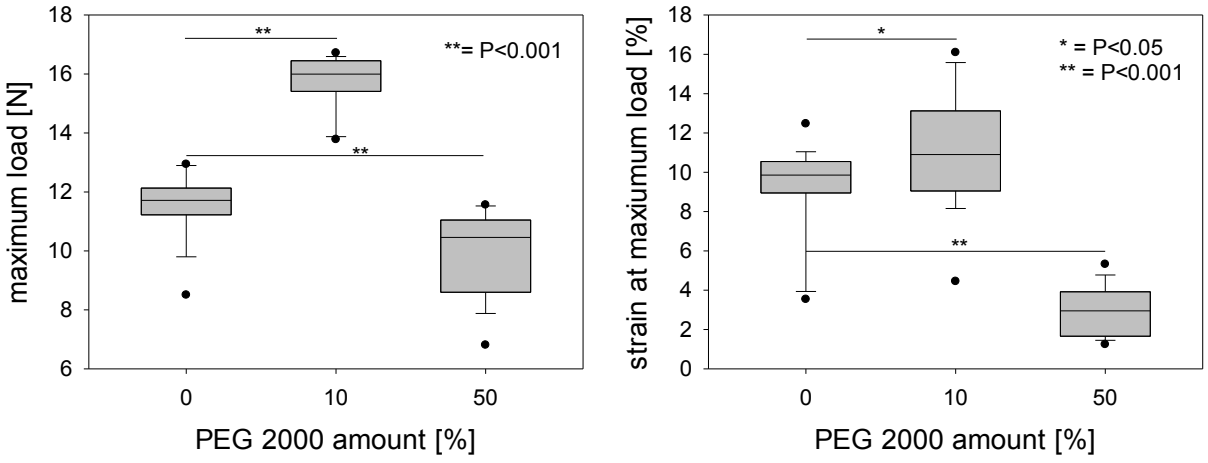


Figure 19: Mechanical testing of films containing PEG 2000 as plasticizer chemically linked to the alginate chains, without cross-linking. (a) Maximum load [N] (b) Strain at maximum load [%] Statistical differences are only depicted in comparison to the 0 % plasticizer group, but were tested for all groups. Statistical differences ($p < 0.001$) are indicated with two asterisks **, ($p<0.05$) with one asterisks *.

To avoid the washing out of the plasticizer from the alginate films PEG 2000 was linked chemically to the alginate chains. But this led to stiff and brittle films, too. The strain of the films containing 10 % PEG 2000 had a higher maximum load and a higher strain than the films with physically addition of PEG 2000. But this could be ascribed to the effect of the lactic acid. Tensile testing of these films cross-linked with the help of the calcium-EDTA-complex was not possible, because the films were too brittle to be removed from the glass plate.

3.4 Erosion and swelling of the alginate films

3.4.1 Erosion of alginate discs in buffer without buffer exchange (non-sink conditions)

To see the impact of calcium within the film as well as within the erosion medium, an erosion study with alginate film discs containing 0.6 mg respectively 1.2 mg calcium in Tris buffer without calcium and HEPES buffer with 2.5 mmol/ l calcium was performed. The films were prepared according to the inner gelation technique and cast into Teflon molds. Particularly one component containing 72 mg or 144 mg calcium citrate and 288 mg gluconolactone was suspended in 5.8 ml water by vortexing for 15 seconds and added to a second component containing 144 mg alginate and 144 mg glycerol dissolved in 14.4 ml water. After vortexing again for 15 seconds the mixture was poured into a rectangular Teflon dish of 72 cm^2 within two minutes. The films were allowed to dry at room temperature before cutting them into discs with a diameter of 2 cm with the help of a cork bore. The film discs were weighed and given into glass vials. After 10 ml of buffer have been added, the vials were closed and stored in a cabinet dryer at 37 °C. Every week 4 discs of each sample were taken out of the cabinet dryer. The dry weight was determined after lyophilization.

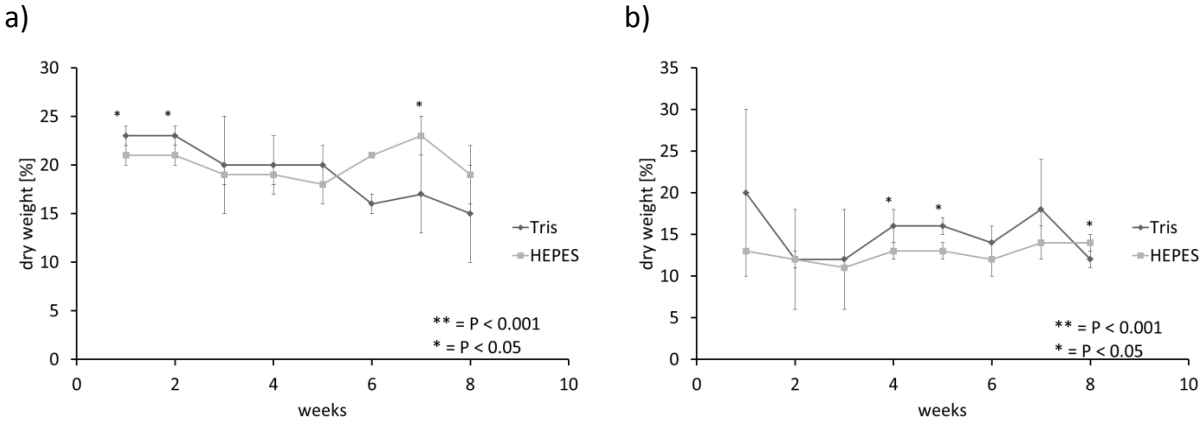


Figure 20: Erosion of cast alginate films with different calcium amounts stored in buffer with and without calcium. (a) Observed dry weights of alginate discs initially containing 0.6 mg calcium stored in Tris buffer and HEPES buffer. (b) Observed dry weights of alginate discs initially containing 1.2 mg calcium stored in Tris buffer and HEPES buffer.

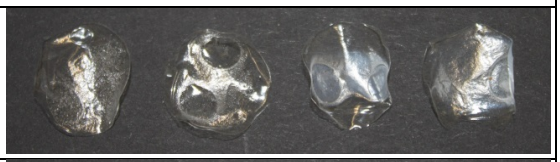
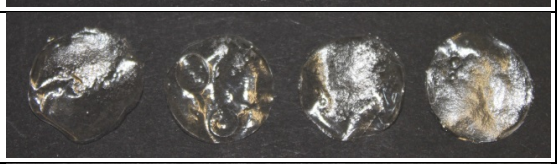
wk	discs containing 0.6 mg calcium stored in HEPES buffer	discs containing 1.2 mg calcium stored in HEPES buffer
1		
2		
3		
4		
5		
6		
7		
8		

Figure 21: Visual appearance of freeze dried alginate discs after storage in HEPES buffer

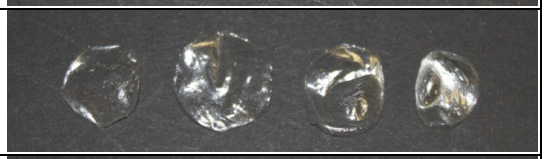
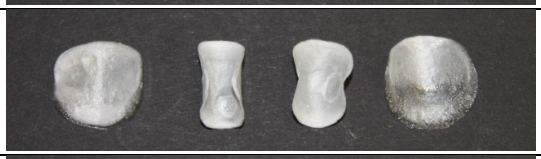
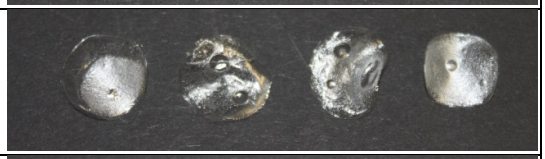
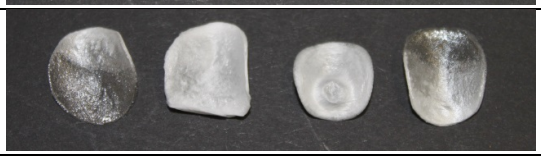
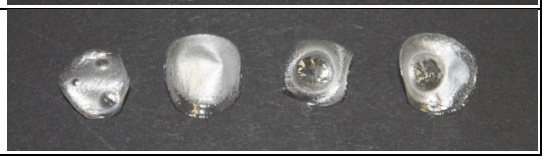
wk	discs containing 0.6 mg calcium stored in Tris buffer	discs containing 1.2 mg calcium stored in Tris buffer
1		
2		
3		
4		
5		
6		
7		
8		

Figure 22: Visual appearance of freeze dried alginate discs after storage in Tris buffer

3.4.2 Erosion of alginate in buffer with buffer exchange (sink-conditions)

To perform the erosion study at sink conditions for alginate as well as calcium, an erosion study with a weekly buffer exchange was performed. Therefore alginate discs containing different amounts of calcium and erosion buffers with different calcium concentrations at physiological levels were prepared. The films were produced according to the inner gelation technique and cast into Teflon molds, as described before. The alginate films without cross-linking were prepared without the second component and the alginate and glycerol amounts

were dissolved in 20.2 ml water. After 10 hours gelation time the films were cut into discs containing no calcium, 0.6 mg or 1.2 mg calcium with a diameter of 2 cm with the help of a cork bore. The discs were given into tared glass vials and the dry weight was determined after drying to constant water content. After addition of 10 ml of the respective buffers the closed vials were stored in a cabinet dryer at 37 °C. To investigate the film erosion and degradation four vials containing discs of each calcium amount incorporated and buffers of each calcium concentration were taken out of the cabinet dryer for further investigation. For GPC investigations one milliliter of the buffers was given into glass vials and stored at -20 °C. The rest of the buffer was decanted carefully and rejected. The remaining alginate discs were carefully washed with water by pouring the water into the vials, pivoting the vial and then pouring the water out of the vial again. Before determining the wet weight of the eroded alginate discs the glass vials were wiped with cotton swaps to remove the residual water. The remaining alginate discs were lyophilized and weighed again to determine the dry weight. To maintain sink conditions for the soluble alginate and calcium, a buffer exchange was performed for the remaining alginate discs by replacing the buffer with fresh buffer. After 8 weeks the erosion study was terminated because this was considered the maximum application time for the intended adhesion barrier. The resulting dry weights were normalized to the weight determined at 0 weeks which corresponds to the dry weight after 24 hours. The dry weight after 24 hours was chosen, to be able to neglect the mass loss of glycerol that was washed out of the films very quickly.

After 8 weeks, a GPC measurement of the collected erosion media was performed. The GPC setup consisted of a system controller, a binary pump, an auto injector, a column oven at 30 °C, a UV-Vis detector (190 nm) and a refractive index detector (Shimadzu Corporation, Chromatographic & spectrophotometric instruments division, Kyoto, Japan). As stationary phase a PolySep-GFC-P Linear column (Phenomenex, Aschaffenburg, Germany) was chosen. For the mobile phase a phosphate buffer was prepared by dissolving 14.2 g dibasic sodium phosphate in 5 L of water. The pH of the buffer was adjusted to 6.5, before filtration with a sterile syringe filter from CORNING® (Corning Cable Systems GmbH + Co KG, Munich, Germany). The measurement was performed with a flow rate of 1 ml/ min. The resulting chromatograms from the UV-Vis detector measured at 190 nm were processed using the Class-VP software (Shimadzu). The results from the RID calculated in relation to dextran

standards were given as M_w (weight average) and M_n (number average) and M_w/M_n (polydispersity index).

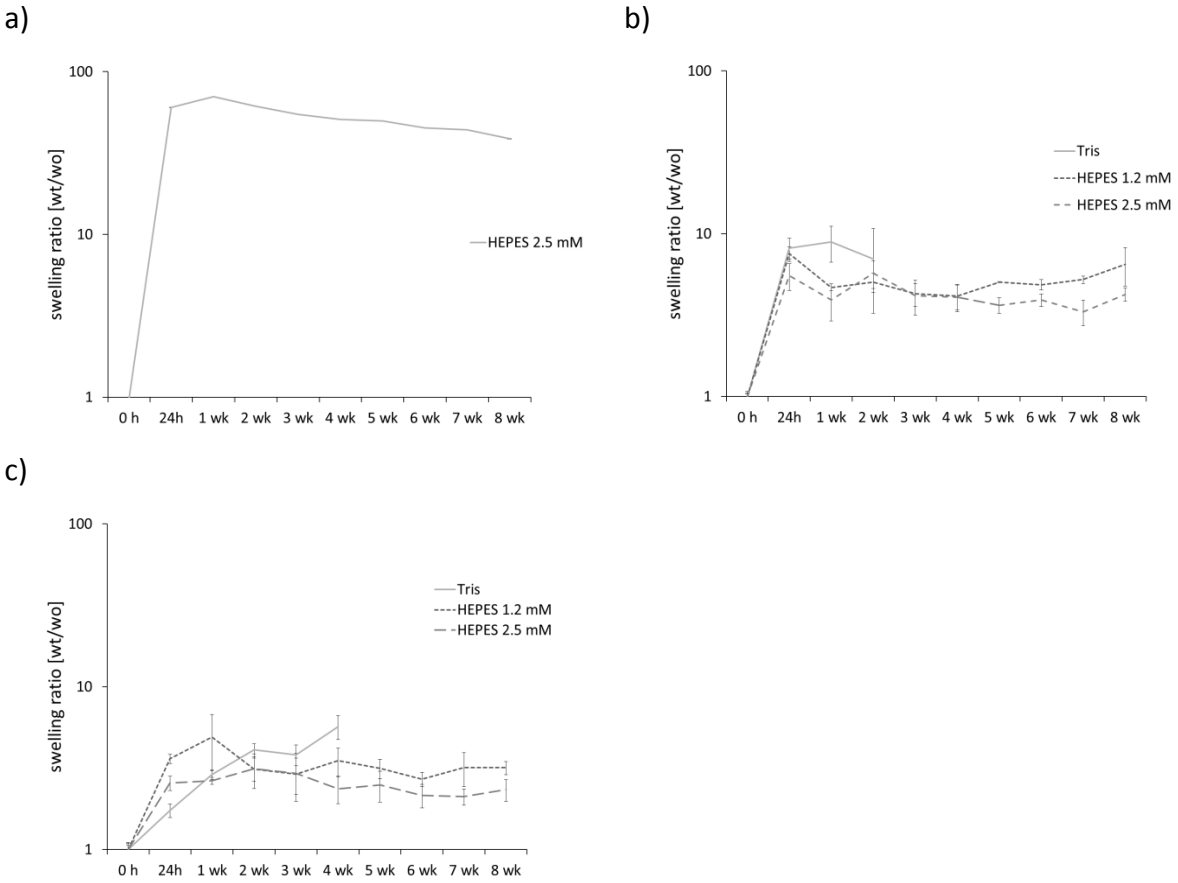


Figure 23: Swelling of alginate discs containing different amounts of calcium, stored buffer with different calcium concentrations. (a) Swelling ratio of alginate discs initially containing 0 mg calcium. (b) Swelling ratio of alginate discs initially containing 0.6 mg calcium. (c) Swelling ratio of alginate discs initially containing 1.2 mg calcium.

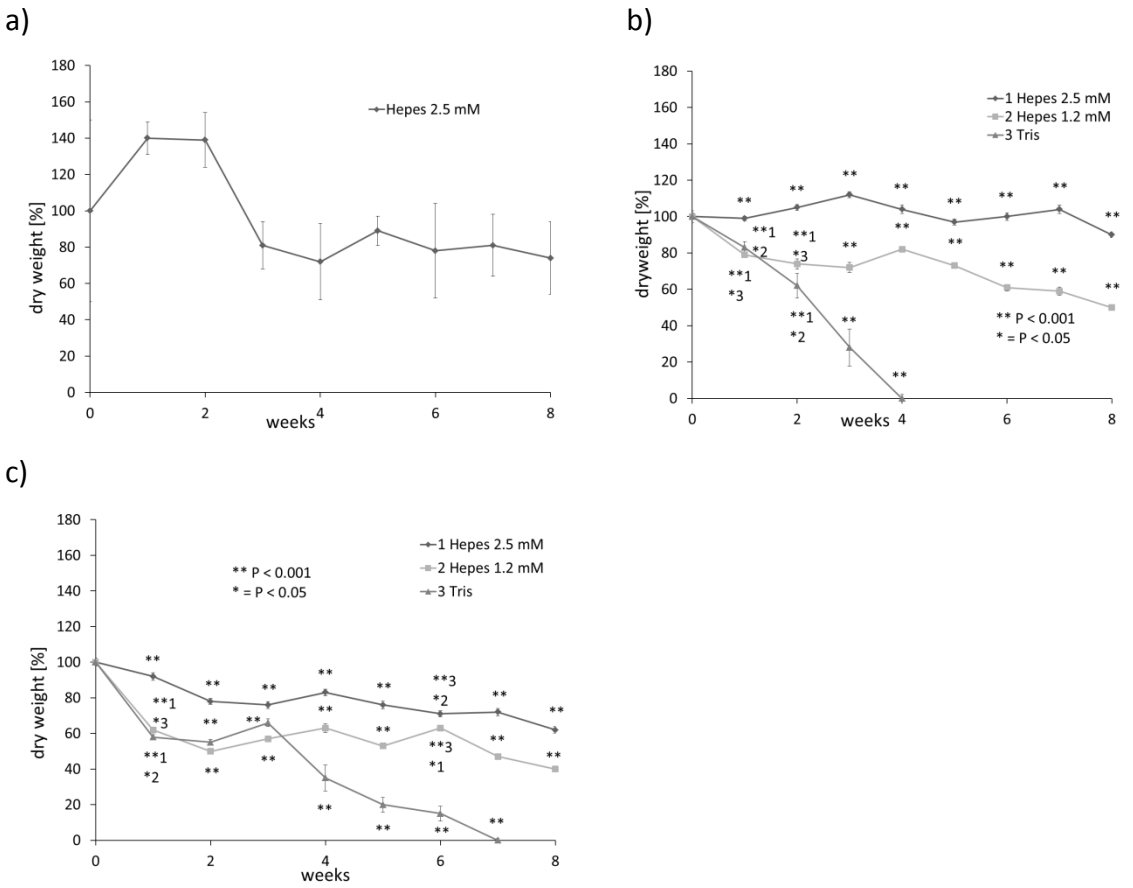


Figure 24: Erosion of cast alginate films with different amounts of calcium incorporated stored in buffers with different calcium concentrations. ** = P< 0.001, * = P< 0.05. The numbers behind the stars represent the different values obtained in the different buffers. 1: HEPES buffer with 2.5 mmol/ l calcium, 2: HEPES buffer with 1.2 mmol/ l calcium, 3: Tris buffer without calcium. The stars combined with the numbers indicate significant or not significant differences between the measurements at similar time points. (a) dry weights of alginate discs initially containing no calcium, (b) dry weights of alginate discs initially containing 0.6 mg calcium, (c) dry weights of alginate discs initially containing 1.2 mg calcium. Dry weights are normalized to the dry weight obtained after 24 h.

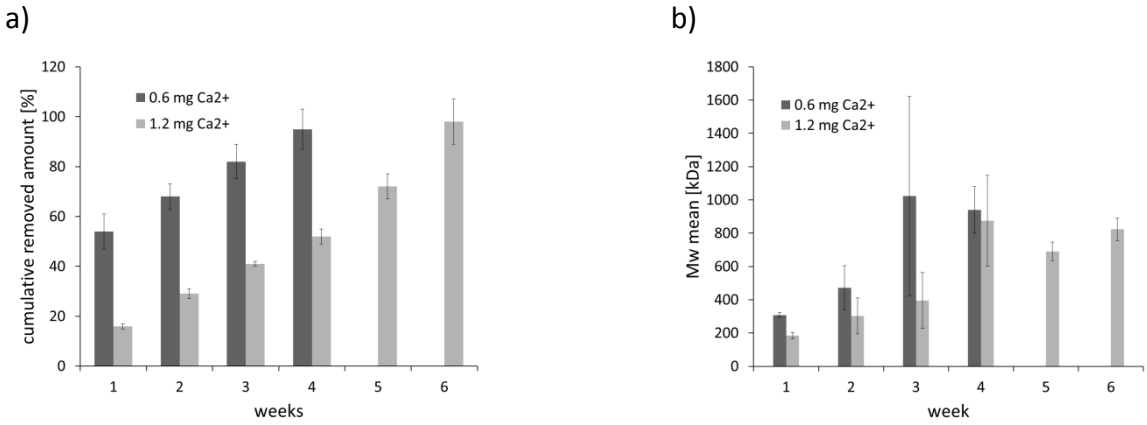


Figure 25: Investigation of the released alginate in the Tris buffer without calcium. (a) Cumulative released alginate amount determined with UV detection (190 nm). (Statistical differences were observed between all groups.) (b) Molecular weight of the released alginate chains compared to dextran standards. (No statistical differences were observed.)

wk	discs containing 1.2 mg calcium stored in Tris buffer	discs containing no calcium stored in HEPES buffer with 2.5 mmol/l calcium
1		
2		
3		
4		
5		
6		
7		
8		

Figure 26: Visual appearance of freeze dried alginate discs after storage in buffer

3.4.3 Influence of calcium on the erosion and swelling of alginate films

The erosion of alginate discs containing 0.6 mg respectively 1.2 mg calcium in buffers with different calcium concentrations under sink and non-sink conditions showed the important impact of calcium in the films as well as in the buffers on the erosion and swelling behavior of alginate films. The films tested without buffer exchange showed an enormous mass loss after the first week. This is due to the loss of the plasticizer glycerol. Glycerol is a very small

hydrophilic molecule and therefore could be easily washed out of the film. To avoid this effect for the erosion study with buffer exchange, samples were taken after 24 hours when the glycerol ought to be washed out and the other samples were normalized on the 24 hour samples. A film preparation without the plasticizer glycerol was not possible, because without plasticizer the thin alginate films were too brittle and splintered when the discs were punched out from the air dried films like it was done for the first erosion study. In the second erosion study the discs were punched out of the film after 10 hours of cross-linking, when they were still wet, because it facilitated the handling.

Without buffer exchange the films did not erode completely after 8 weeks. In the Tris buffer the discs containing 0.6 mg calcium showed a steadily mass loss over 8 weeks. In the HEPES buffer with calcium, they seemed to incorporate calcium from the buffer because after a slightly mass loss till week 5 they became heavier till week seven, until the mass decreased again. The discs containing 1.2 mg calcium had altogether a higher mass loss than the discs with less calcium. During the eight weeks the mass of the discs stored in HEPES buffer stayed more or less constant, whereas the films stored in the Tris buffer showed more ups and downs. The fact that the alginate discs, with the higher content of calcium, stored in HEPES-buffer had a higher mass loss than the disc with the lower content can be explained with the weight of the discs in the beginning. The discs with the lower content had a dry weight of about 20-30 mg. The discs with the higher content are thicker with a dry weight of about 35-50 mg. This is because of the stronger cross-linking with calcium ions and thus a higher concentration of polymer and additives in the discs. When the discs are thicker, they can lose more glycerol and water and therefore had a bigger loss of weight.

During the erosion study with buffer exchange the swelling of the films was monitored, too. The discs without calcium were only stable in HEPES buffer containing 2.5 mmol calcium because they were cross-linked with the uptake of calcium from the buffer. In the HEPES buffer with 1.2 mmol calcium and the Tris buffer without calcium the discs were completely dissolved within the first week of investigation. But in the HEPES buffer containing 2.5 mmol calcium the discs showed a swelling ratio of about 70 after 24 hours incubation time. The film discs containing 0.6 mg calcium were stable in both HEPES buffers containing calcium. In the Tris buffer without calcium the films were completely eroded after three weeks. It could be seen, that the incorporation of calcium in the film had a significant effect on the swelling

behavior, because the swelling ratio of the films containing 0.6 mg calcium was nearly 5 to 8 and the films containing 1.2 mg calcium had an increase of wet weight to about three or six times their starting weight. The impact of the calcium in the buffer could be observed in the trend that the discs stored in the calcium free Tris buffer had a higher swelling ratio than the discs stored in HEPES buffer with 1.2 mmol calcium and they had a higher swelling ratio than the discs stored in HEPES buffer with 2.5 mmol calcium. The alginate discs with the highest calcium amount of 1.2 mg calcium were also not stable in the calcium free Tris buffer and were completely eroded after five weeks.

The assumption that the discs without calcium stored in HEPES buffer with 2.5 mmol calcium incorporated calcium and therefore got cross-linked could be confirmed with the results of the erosion study, because instead of losing weight they even increased their weight during the first two weeks. In the beginning the discs became heavier up to 140 % until a rapid mass loss at week three to 80 % where they stayed nearly constant till week eight. The discs containing 0.6 mg calcium seemed to be stabilized in the HEPES buffer with 2.5 mmol calcium. They became slightly heavier in the first three weeks until they reached a steady state at week four after which no remarkable weight loss was observed within the eight weeks observation time. In the HEPES buffer with 1.2 mmol calcium they showed a small mass loss in the first week, followed by a steady state phase of three weeks until they lost half of their weight till week eight. In the Tris buffer without calcium they showed an almost linear mass loss until they were completely eroded after four weeks. The discs with the highest initial calcium content of 1.2 mg eroded in every buffer. During the first two weeks the weight loss was slightly greater than during the following week. The trend that the discs in the buffer with the higher calcium concentration eroded slower than the discs in the buffer with less or no calcium was also visible. Until week eight the weight of the film discs stored in the HEPES buffer containing 2.5 mmol calcium eroded to about 60 % of their weight after 24 hours. In the HEPES buffer containing 1.2 mmol calcium they eroded to about 40 %. The films were completely eroded after seven weeks in the Tris buffer containing no calcium.

The GPC investigation could only be made with the completely eroded film discs in Tris buffer, because in the other buffers the alginate concentration was under the quantification limit. The erosion of the alginate discs was more or less a release of alginate chains. Alginate

is a biopolymer produced by brown algae containing polymer chains with different length. When the calcium was washed out from the alginate discs, the positive charges of the calcium ions were missing to cross-link the negatively charged alginate chains. The shorter chains had less binding sites than the longer chains. Therefore they were washed out faster than the longer chains. When the whole discs eroded the longer chains were also detectable.

3.5 Biological evaluations of alginate films

3.5.1 Pretests

When the alginate films were cross-linked with the favored inner gelation technique, GDL was added to the alginate solution to decrease the pH. The decrease of the pH was necessary to dissolve the calcium from its hardly soluble calcium salt and initiate cross-linking. But unfortunately this decrease of pH might be a problem for the tissue that gets in contact with the alginate film.

Therefore a cytotoxicity test of the prepared alginate films was performed to see, if the GDL has to be removed from the film before application or if it would cause no cauterization on the application site.

To test, if the incorporated partial hydrolyzed GDL has an impact on the pH of the surrounding medium some pretests were performed. For these pretests films prepared according the inner gelation technique drawn on glass plates and films poured in a Teflon dish like described before were tested.

Table 7: Components of films prepared for pretests

components	amounts in drawn film [g]	amounts in poured film [g]
Alginate	0.75	0.144
glycerol	0.75	0.144
dicalcium	0.15	
calcium citrate		0.144
GDL	0.6	0.288

After air drying the films were cut into discs with a diameter of 2 cm. The discs were given into a well plate and covered with 3.14 ml (1 ml/ cm^2) DMEM (Dulbecco's modified eagle's medium). The phenol red which was added to the medium as pH indicator and is pink at neutral pH became yellow after addition to the film discs which indicated a pH decrease of

the medium. This result showed that the GDL incorporated in the film might be harmful to the tissue and therefore has to be washed out before application.

For a washing procedure three solutions with nearly neutral pH were chosen, PBS, 0.9 % sodium chloride solution and Ringer solution. Again the film discs were covered with 3 ml of these solutions for 5, 10, 15, 30 and 60 minutes to see how long the film has to be in contact with the solution to remove the incorporated GDL. After a defined incubation time the pH of the solution was measured.

Table 8: pH measurements of washed films discs

time [min]	0.9 % NaCl solution pH 7.1		Ringer solution pH 6.8		PBS buffer pH 7.4	
	poured film	drawn film	poured film	drawn film	poured film	drawn film
5	4.1	4.2	4.1	4.1	5.4	6.5
10	4.2	4.2	4.1	4.1	5.3	6.4
15	4.2	4.1	4.1	4.1	4.8	6.4
30	4.2	4.1	4.1	4.0	5.1	6.4
60	4.2	4.0	4.1	4.0	4.9	6.5

After the washing procedure, 3 ml of DMEM was added to the washed and the non-washed discs. With the non-washed discs the DMEM became yellow. With the washed discs it stayed pink.

3.5.2 MTT Assay

To further investigate the cytotoxicity of the alginate films a MTT assay was performed. The alginate films were prepared via the inner gelation technique and directly poured into sterile petri dishes with a size of 55 cm^2.

Table 9 : Components of alginate film for MTT assay

poured film	
alginate	0.110 g
glycerol	0.110 g
calcium citrate	0.110 g
GDL	0.220 g

During the drying process under laminar air flow to avoid a contamination with bacteria the film cross-linked and contracted to a size of about 40 cm^2. Therefore it was washed with 40 ml (1ml/ cm^2) 0.9 % sodium chloride solution for about 10 minutes to remove the GDL. After 10 minutes the sodium chloride solution was removed. Then 40 ml EMEM with 10 % FCS was added to the film to extract feasible cytotoxic components and the petri dish was given into an incubator for 24 hours at 37 °C and 5 % CO_2. For comparison a piece of 25 cm^2 SurgiWrap® was chosen and incubated with 25 ml EMEM with 10 % FCS as well. After incubation the medium was taken up with a syringe and sterile filtrated with a 28 mm syringe filter with a pore size of 0.2 µm (Corning Incorporated, Kaiserslautern, Germany) and later used for the culture of L929 mouse fibroblasts for toxicity testing. The MTT assay was performed according to ISO 10993-5 Part 5: Tests for in vitro cytotoxicity using commonly applied measurement conditions. The purple color of the formazan produced by reducing the tetrazolium dye MTT was quantified with a plate reader (Shimadzu CS 1903 PC) at 570 nm and 690 nm.

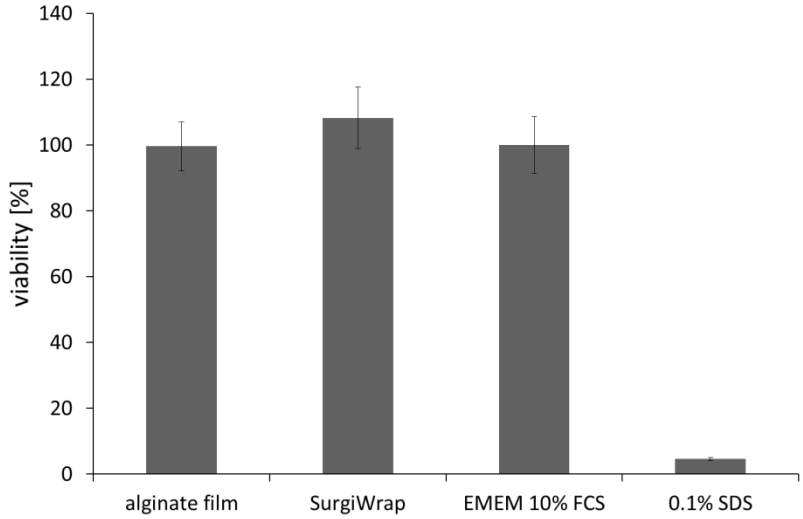


Figure 27: MTT assay of an alginate film prepared via the inner gelation technique and SurgiWrap® as comparison.

Both films, the alginate film as well as SurgiWrap® showed an activity larger than 95 %.

3.5.3 Cell adhesion

To test if cells would adhere on the antiadhesive alginate films a cell adhesion test was performed. Therefore films poured into Teflon dishes prepared according to the inner gelation technique were cut into discs with a diameter of 2 cm using a cork bore and subsequently cut in half to obtain semicircles. As control commercial available antiadhesive PLA foils (SurgiWrap®, Mast Biosurgery AG, Zürich, Switzerland) were cut into semicircles, too. These semicircles were given into 12 well plates made of polystyrene. Due to the fact that the film discs would float into the medium, they were fixed to the bottom with the help of a sterile glass annulus. Then about 100000 L929 mouse fibroblasts in EMEM containing 10 % FCS were given to the film semicircles and were incubated for 24 hours in an incubator at 37 °C and 5 % CO_2. After incubation the cells on and next to the film were carefully visualized using a phase contrast microscope (Leica DM IRB, Leica Microsystems Inc., Deerfield/ Illinois, USA) and then subsequently stained using SYTO-13® and fluorescent pictures were taken with a Zeiss Axiovert 200 microscope combined with a LSM 510 laser-scanning device (Zeiss, Oberkochen, Germany). The dye was excited using the 488 nm laser and detected using the long-pass filter (LP505) with a pinhole of 72 µm. Only the cells that were lying in the focal plane could be focused. The other cells appear out of focus. To test if the cells adhere to the films the wells were rinsed twice with PBS buffer to remove not adhering cells.

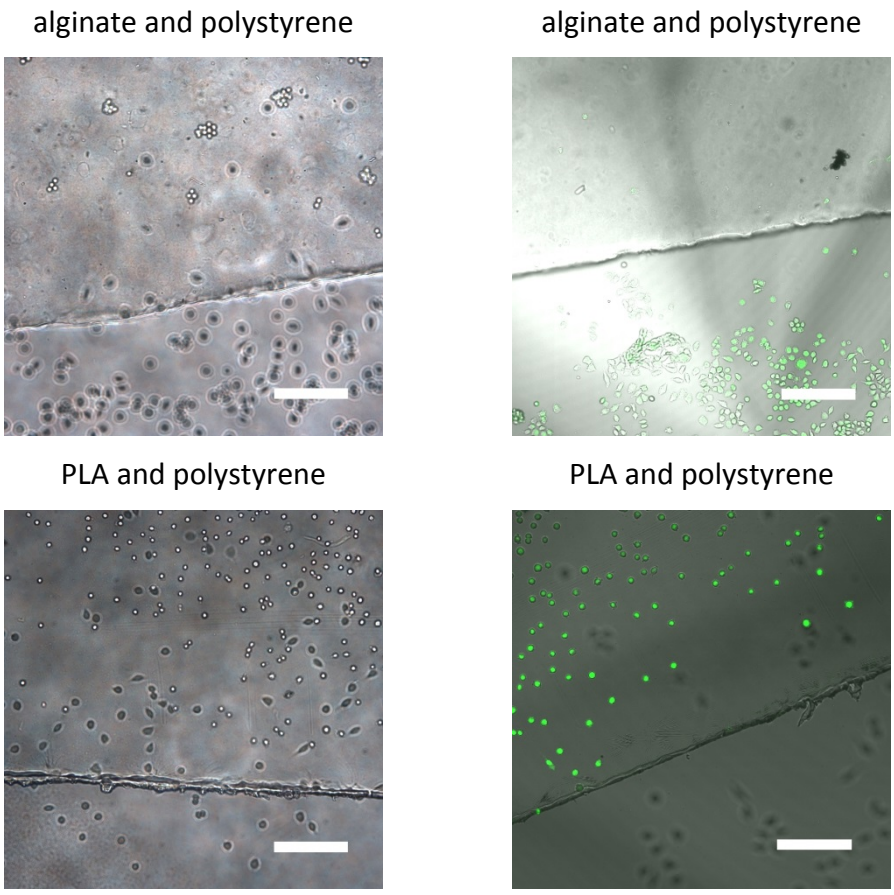


Figure 28: Images of adhesion behavior of L 929 mouse fibroblasts on polymer films made of alginate or PLA: (a) phase contrast image of cellular aggregates on the surface of an alginate film; (b) fluorescent image of L 929 mouse fibroblasts stained with SYTO-13® on polystyrene next to an alginate film; (c) phase contrast image of L 929 mouse fibroblasts on a PLA film; (d) fluorescent image of L 929 mouse fibroblasts stained with SYTO-13@ on a PLA film. Scale bar represents 200 µm in all images.

3.5.4 Compatibility of the prepared alginate films

The pretests of the alginate films prepared via the inner gelation test show, that the incorporated GDL has a great impact on the cytotoxicity of the films. Therefore a washing step has to be done before the surgeon can apply the film to the application site. This can be done after the surgeon has cut the film into the wished shape by giving the film into a sterile solution like 0.9 % sodium chloride, Ringer solution or PBS. The elution test showed that 5 minutes in one of these solutions would be sufficient, because further incubation did not further decrease the pH of the solution. To be on the safe side the film can also be incubated for ten minutes, like it was done before the MTT assay that showed with a cell viability of

95 %, that after 10 minutes incubation time the GDL was sufficiently removed from the alginate film and therefore the film did not show any cytotoxic effects in comparison to untreated medium as positive and 0.1 % SDS solution as negative control.

As expected the cell adhesion test showed, that cells do not adhere to the antiadhesive alginate films compared to the good adhesion of the slightly spread L929 mouse fibroblasts on the polystyrene surface of the well plate bottom. The cells on the films had a spherical shape and were forming cellular aggregates instead of spreading on the film therefore they were easily washed away during the staining process and washing procedure with PBS. The alginate film even showed better antiadhesive properties than the antiadhesive PLA foil on which the cells showed a minimal spreading and were still present after staining and washing, indeed less of them and in a rounded shape.

3.6 Summary and Conclusion

As expected the used alginates that are provided for the preparation of wound care products showed a good capability for the preparation of thin films. With the help of the inner gelation technique it was possible to draw evenly thick films with a homogenous distribution of a defined amount of calcium and other components used for the film preparation. All film preparation techniques resulted in films with a homogenous distribution of calcium. The release of calcium was slowly enough to be able to cast the film into Teflon molds respectively draw the films on glass plates. Some disadvantages could be seen by the use of calcium citrate or calcium EDTA as calcium sources, because for the sufficient homogenization of calcium citrate an ultra turrax was necessary which resulted in the formation of air bubbles, and EDTA competed with the guluronic acid blocks for the calcium ions which resulted in a weaker cross-linking of the alginate chains. Furthermore the unevenly sprinkling with lactic acid revealed a softening effect of the lactic acid itself. Therefore the use of GDL was preferable. Regarding the tested plasticizers glycerol showed the best softening properties, because both PEGs given to the film physically or linked to the alginate chains chemically did not show the wanted softening effect. Under physiological conditions with a free calcium concentration of 1.2 mmol/ l the films containing calcium were stable over the investigated time of 8 weeks. Using GDL as pH decreasing agent for the dissolution of calcium from its salt respectively complex came along with the preparation of

acidic films. Before application of the film to the traumatized tissue the film should be rinsed with PBS to remove the acidic GDL.

4. Optimization of alginate film preparation with predefined calcium amounts

To cross-link an alginate film properly and achieve good mechanical properties and erosion time, but also to have binding sites left that are able to bind more calcium if necessary, it is necessary to find out how much calcium can be bound by the used alginate. In this chapter different methods are presented for this purpose.

The main factor that influences the mechanical and mucoadhesive properties, swelling behavior and especially the degradation time of the alginate films in the peritoneum is the cross-linking extent and strength of the prepared films [8]. Therefore, it is of utmost importance to link and investigate the effect of the complexing capacity of the used alginate, which is correlated to the content of guluronic acid blocks, with the used calcium amount that is incorporated into the final films.

Many researches have been done to investigate the cross-linking process of alginate with bivalent cations. The complex resulting from the alginate chain, which binds calcium ions is often described with the so called “egg box model” [58] because the calcium ions are arranged between the guluronic acid blocks like eggs in an egg carton. Molecular modeling investigations have confirmed this thesis that is valid in the case of pure polyguluronates [78,79]. The influence of the guluronic acid content and the sequence of the uronic acids in alginates on the gelation behavior and the properties of the resulting gels was an issue in many publications [68,71,80,81]. Also the kind of bivalent cation is very important for the speed of the complex formation as well as the strength of the resulting complex [62,64,82,83].

Due to the fact that the mechanism of cross-linking is very well known it is possible to figure out how much calcium can be bound by the used alginate. For this attempt two different alginates with a high amount of guluronic acid (high G) and a low amount of guluronic acid (low G) were taken to see the impact of guluronic acid content on the cross-linking of the prepared alginate films and therefore the resulting properties like mechanical properties, erosion time and swelling behavior. The used high G alginate Protanal® LF 60/60 FT has a G/M (%) ratio of 60-70/30-40 and is an alginate extracted from the stem of the marina algae Laminaria hyperborean. The stem of the plant has to be more rigid than the leaf of the plant and therefore contains more guluronic acid (according to manufacture information). For the low G alginate Protanal® LF 10/60 LS was chosen which is extracted from Lessonia nigrescens and has a G/M (%) ratio of 35-45/55-65 [61].

The aim was to load the films with a defined amount of calcium. The film should be cross-linked properly, but not all binding sites should be occupied by calcium to obtain a film that is also able to bind more calcium from the surrounding medium after implantation if needed.

4.1 Pretests before film preparation with “optimized” calcium amount

4.1.1 ITC measurements for the evaluation of the calcium binding capacity of the used alginate

In highly diluted solutions of alginate mixed step by step with very small amounts of calcium it is possible to measure the interactions between the guluronic acid blocks of the alginate chains with the bivalent calcium during the cross-linking process. The interactions can be measured with an isothermal titration calorimeter [84] that measures the heat of binding of the interactions between the calcium ions and the guluronic acid blocks. With the resulting thermo grams the amount of calcium that can be bound by the guluronic acid blocks can be calculated.

In this experiment the interactions of the bivalent calcium ions and the guluronic acid blocks of alginate in solution were measured. The enthalpies of binding were measured with a VP-ITC MicroCalorimeter (MicroCal, Northampton, Massachusetts, USA). More precisely a 1.5 mM alginate solution of an alginate with a high guluronic acid content and a 7.5 mM calcium chloride solution were prepared in water and degassed under gentle stirring before the measurement. The sample cell of the calorimeter was filled with 1.436 ml of the alginate solution, tempered at 25 °C and stirred at 300 rpm during the experiment. The syringe of the calorimeter was filled with 300 µl of the prepared calcium chloride solution. During the experiment 30 injections of 10 µl calcium chloride solution over 10 seconds with 2 minutes time interval between the injections were performed.

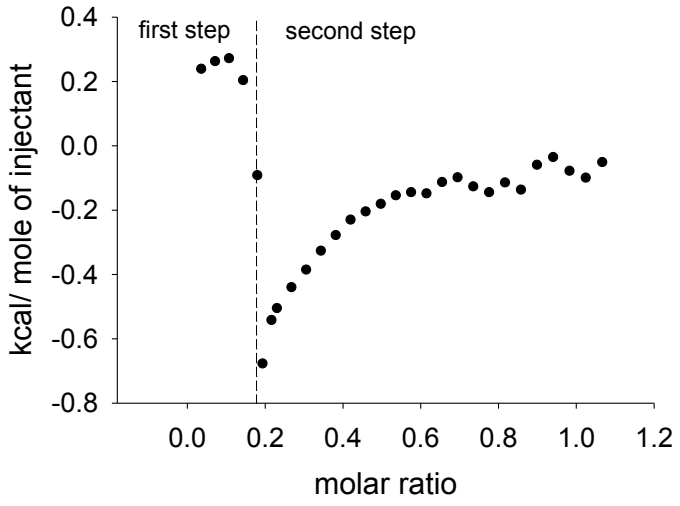


Figure 29: ITC thermogram of 1.5 mM high G alginate solution titrated with a 7.5 mM $CaCl_2$ solution

The resulting heat flow versus time profile was fitted with Microsoft Excel. It showed the expected multiple step binding behavior that is described in literature [84]. In the beginning of the measurement the formation of mono-complexes between calcium ions and the guluronic acids in the alginate chains occurs. Therefore the heat flow showed a slight increase than dropped within a few titration steps until a steadily increase that ended in a plateau when all binding partners have reacted. The turning point of the titration appeared in the second step of the titration curve at a molar ratio of 0.3. In the second step the mono-complexes pair and form the so called egg-box dimers, which came along with a very strong exothermicity.

For the cross-linking of the alginate films it was postulated, that the amount of calcium incorporated in the film should be enough to cross-link the film properly for a resisting time of 8 weeks in the peritoneum after implantation, but it should not be too high resulting in heterogeneous gels with lower mechanical properties [82]. Additionally a further uptake of calcium from the surrounding should be possible. Therefore it was decided that half of the guluronic acid blocks should be involved in the cross-linking.

For this purpose the turning point was taken to calculate the calcium amount that was necessary to cross-link the alginate film properly. For the preparation of 25 ml of a 3 % alginate solution 0.75 g alginate powder were needed. Divided through the molecular mass of alginic acid with 194.14 g/ mol the resulted 0.00386 mol could be multiplied with the molar ratio of 0.3 resulted from the ITC measurement. The resulting 0.00116 mol multiplied with the molecular weight of $CaHPO_4$ of 136.06 g/mol led to a weighed portion of 0.16 g $CaHPO_4$ that should be given to 25 ml of the alginate solution. Therefore 0.6 % $CaHPO_4$ was needed to cross-link half of the guluronic acid blocks from a 3 % high G alginate solution. The resulted 0.6 % of $CaHPO_4$ was used to cross-link the high G alginate and the low G alginate, too, to be able to compare the behavior of both alginates with the same cross-linking conditions.

4.1.2 Solution viscosity for appropriate film drawing

For the drawing of very thin films with the help of a drawing apparatus, the gap clearance of the frame, the drawing speed and the viscosity respectively concentration of the polymer solution are the factors that have to be regarded.

Previous film preparations (see Chapter 3) with the drawing apparatus revealed that a 3 % solution of the high G alginate drawn with a gap clearance of 700 µm led to the favored 20 µm thick films.

Therefore, the viscosities of 6 concentrations of the low G alginate, which had a lower viscosity than the solution prepared from high G alginate, were tested to find the right amount of low G alginate that should be used to obtain the same viscosity as a 3 % solution of high G alginate. Rheological tests were performed with 1.26 ml of a 3 %, 3.5 %, 4 %, 4.5 % and 5 % alginate solution, at 20 °C, with a 40 mm steel plate and a gap of 1000 µm. After equilibration for 2 minutes, the continuous ramp step started with a shear rate of 1/s from 0.1 to 500. The duration of the measurement was 5 minutes. After a delay time of 10 seconds, the peak hold step started with a hold shear rate of 1/s at 500 for 1 minute. The continuous ramp step started after a delay time of 10 seconds with a shear rate of 1/s from 500 to 0.1. This took 5 minutes.

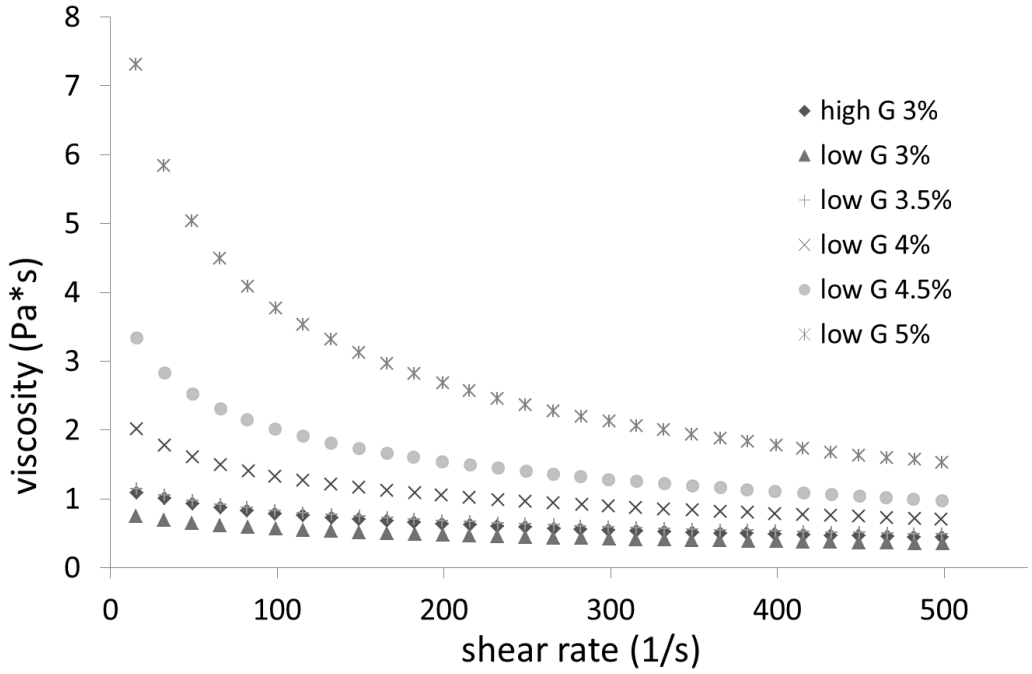


Figure 30: Viscosity measurements of high G and low G alginates

Viscosity measurements showed that a 3.5 % solution of the low G alginate nearly showed the same viscosity as the 3 % high G alginate solution. Therefore a 3.5 % solution was chosen for the preparation of low G alginate films.

4.1.3 Gelation time of alginate after addition of calcium salt and acidic agent

As mentioned before the inner gelation technique was preferred to prepare thin, cross-linked alginate films. This technique enabled a homogenous distribution of calcium in the film, which is of utmost importance. When the GDL powder comes in contact with the water of the alginate solution it hydrolyses to gluconic acid and therefore decreases the pH of the alginate solution which leads to a release of the calcium ions from the hardly soluble calcium salt.

The homogenous mixture of the GDL with the calcium salt containing alginate solution and the drawing process itself takes some time. Therefore it is important to know how much time passes until the alginate solution is cross-linked and cannot be drawn to a film anymore. Therefore rheological tests were performed to observe the cross-linking speed.

According to the results from the ITC measurements, 0.6 % $CaHPO_4$ and four times GDL that ought to be enough to obtain a clear film were added to a 3 % (m/V) solution of high G alginate 1.26 ml of the freshly prepared mixture was inserted into the rheometer with the help of a syringe and was measured with a 40 mm steel plate and a 1000 µm gap at 20 °C. The measurement was performed with a time sweep method. The controlled variable was the torque (micro N m) of 10 with a frequency (Hz) of 1.

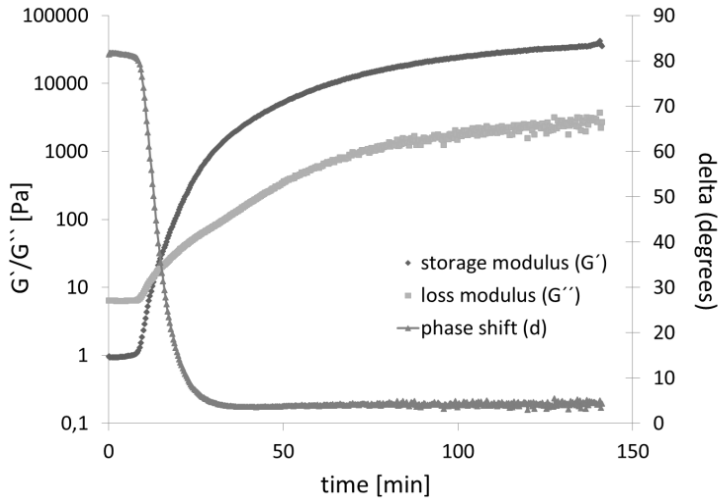


Figure 31: Rheogram of high G alginate cross-linked with 0.6% $CaHPO_4$ and 2.4% GDL

The dynamic mechanical analyses of three mixtures prepared as described were performed to determine the elastic (G`) and viscous (G``) modulus. Figure 31 shows the time sweep

curve of a 3 % high G alginate solution after addition of 0.6 % $CaHPO_4$ and 2.4 % GDL. The time dependent changes in the storage modulus (G`) during the cross-linking process could be observed. After a period of 9.3 minutes the G` value started to increase rapidly with time. After 40 minutes the curve of G` becomes parallel to that of G`` over a wide frequency range. The gel point was determined at 13.6 minutes were the two curves cross. With the other two mixtures this point was visible at 24.3 minutes and 9.5 minutes. A maximum storage modulus of 40 kPa was reached at 140 minutes. This measurement showed, that after mixing the components the film should be drawn within more or less ten minutes, which is far enough time for the drawing process.

4.1.4 Time needed for the complete dissolution of the calcium salt

A solution of the used high G and low G alginate of pharmaceutical grade in water is clear and has a brownish color. When the hardly soluble $CaHPO_4$ is suspended in the alginate solution a cloudy suspension is obtained. After the addition of GDL the subsequent decrease of the pH leads to the dissolution of the calcium from its hardly soluble salt and step by step the cloudy suspension becomes a clear gel. To measure the time it takes to obtain a clear gel, a 3 % high G alginate solution with 3 % glycerol and a 3.5 % low G alginate solution with 3.5 % glycerol were prepared. After addition of 0.6 % $CaHPO_4$ and 2.4 % GDL the mixtures were given into precision cells made of Quartz (SUPRASIL®, Hellma Analytics, Müllheim, Germany) and measured at 500 nm with a spectrophotometer (Uvicon 941, Kontron Instruments, Basel, Switzerland). Clear, pure alginate solutions were measured as references, too.

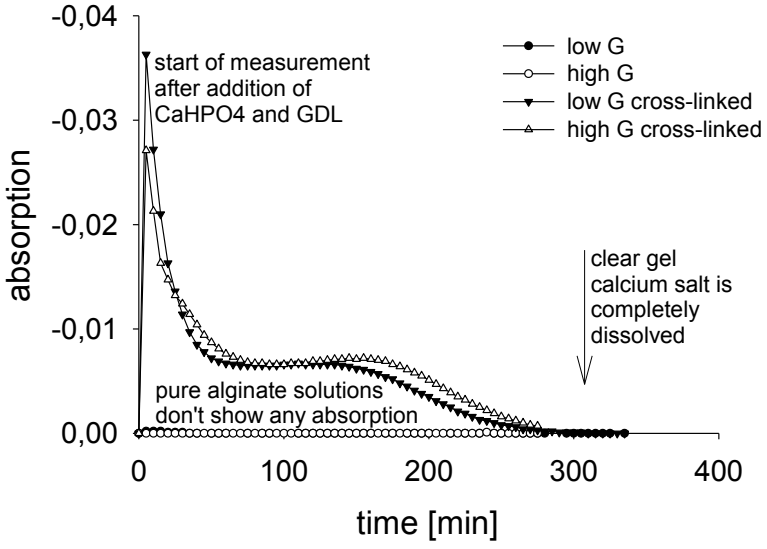


Figure 32: Turbidity measurements of high G and low G alginates after addition of $CaHPO_4$ and GDL

The pure alginate solutions measured at 500 nm showed no absorption at all. The suspensions with $CaHPO_4$ and GDL showed an absorption that decreased very quickly after the starting of the measurement. Than after approximately 50 minutes a plateau was reached until the absorption further decreased after 180 minutes. The mixtures were as clear as the pure alginate solutions after 310 minutes. The fast decrease in the beginning that slowed down time by time can be explained with the immediately occurring gel formation. Time by time the alginate solution became a gel that worked like a barrier and slowed down the diffusion of the dissolved GDL through the viscous gel and therefore the dissolution of the calcium from its salt slowed down.

4.1.5 Differences in gel strength of high G and low G alginate

To further characterize the difference in cross-linking between the high G and the low G alginate a compression test was performed to test the strength of the gel during the cross-linking process and the resulting gel strength that could be reached.

0.75 g high G alginate and 0.75 g glycerol were dissolved in water. For the low G alginate, 0.88 g alginate and 0.88 g glycerol were dissolved. After addition of 0.15 g respectively 0.6 % $CaHPO_4$ and 0.6 g GDL, 200 µl of the mixture were given in each well of a 96 well plate. Every 30 minutes a piston with a diameter of 4 mm was pushed into the gel with a speed of

0.5 mm/sec. After a preload of 0.01 N was reached, the piston was pushed for 2 mm into the gel. The stress [Pa] was calculated from the maximum force [N] that was reached.

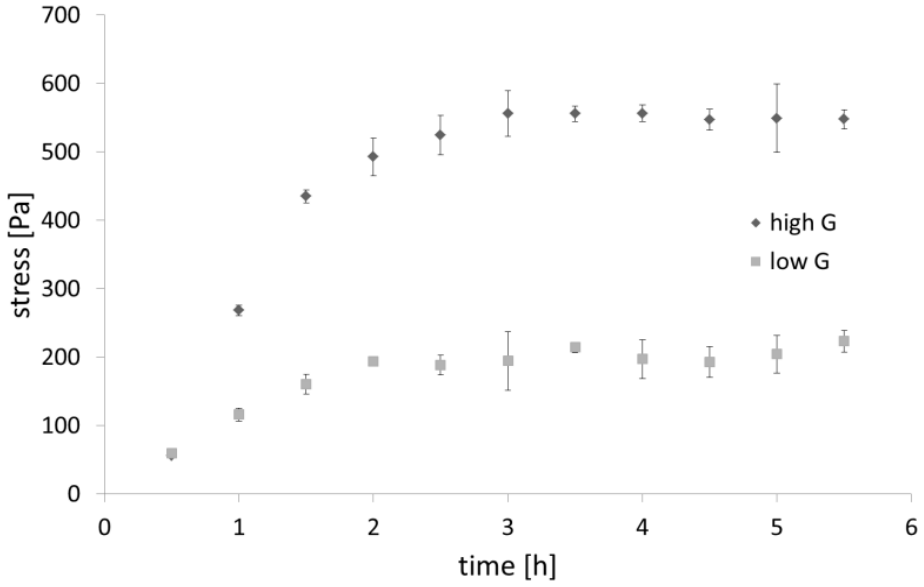


Figure 33: Compression of high G and low G alginate during the cross-linking process

The compression test showed that the cross-linking of high G alginate led to stronger gels with a stress of about 550 Pa than that of the low G alginate with a stress of about 200 Pa. Furthermore, the plateau of the low G alginate was reached after 2 hours because no more G blocks were left to be cross-linked. The high G alginate reached the plateau nearly one hour later.

4.2 Film preparation

After the general procedure of film preparation with the help of the inner gelation technique was established, the amount of the two main components that influence the mechanical properties of the alginate films, more precisely the amounts of calcium in the form of $CaHPO_4$ and the plasticizer glycerol were varied to investigate the impact of this components on the mechanical properties of the film.

4.2.1 Films prepared for the erosion study

For the erosion study 3 g of high G alginate or 3.5 g of low G alginate were dissolved to 100 g in water. 0.6 g $CaHPO_4$ was suspended in 3 g or 3.5 g glycerol to obtain identical amounts of glycerol compared to alginate in the solutions. After the hardly soluble salt was completely wetted with the glycerol the alginate solution was added. The suspension was stirred

manually with a spatula to avoid formation of air bubbles. After homogenization 2.4 g of GDL in solid form was added and carefully folded in the suspension. The cloudy suspension was drawn within 3 minutes, using a drawing apparatus (ERICHSEN coatmaster 509 MC, Hemer, Germany) with a gap clearance of 700 µm and a drawing speed of 5 mm/sec, in order to avoid premature cross-linking. The obtained film was given into a closed box for several hours to slow down the drying speed. The clear film was taken out of the box to dry at room temperature until further investigation.

4.2.2 Films prepared for the tensile tests

For the tensile tests the impact of calcium or plasticizer was investigated. Therefore films with different amounts of these components were prepared. The preparation procedure was the same as for the films prepared for the erosion study. For the films prepared with different amounts of calcium 0 g, 0.3 g, 0.6 g, and 1.2 g $CaHPO_4$ were wetted with 3 g respectively 3.5 g glycerol. For the films prepared with different amounts of glycerol 10 %, 20 %, 50 % and 100 % of the 3 g respectively 3.5 g alginate was given to 0.6 g $CaHPO_4$. Afterwards the alginate solution and the GDL were added and the films were drawn like described before.

4.2.3 Films prepared for the puncture test and the suture pullout test

The films were made in another order. 3 g of the high G respectively 3.5 g of the low G alginate and a defined amount of glycerol (50 % and 100 % calculated on the alginate) were dissolved in water to obtain 100 ml of the alginate solution. A further improvement has been done by suspending 0.6 g $CaHPO_4$ in several droplets of water to wet the salt and inhibit cluster formation, because suspending in water led to a faster homogenization than suspending in a defined amount of glycerol. Subsequently this suspension was added to the alginate solution and stirred at maximum speed with a magnetic stirrer. Then 2.4 g of GDL were added to this suspension and stirred at maximum speed again. The film was drawn as described before.

4.2.4 Films with different thicknesses

To test the impact of the film thickness on the tensile properties of the films, the films made of high G alginate prepared according to the films for the puncture test were drawn with frames with different gap clearances of 500 µm, 700 µm, 1000 µm and 1500 µm.

4.2.5 Films for the evaluation of the adhesiveness

Films for the evaluation of the adhesiveness were prepared according to the films for the puncture test with 10 %, 20 %, 50 % and 100 % glycerol calculated on the alginate amount.

4.2.6 Films for the evaluation in a wet state

The films that should also be tested after storage in water were prepared like the films for the erosion study. To be able to see the impact of calcium on the mechanical stability of the films in a wet state, films were prepared with 0.6 % $CaHPO_4$. Additionally half of the amount and a doubled amount of 0.3 % and 1.2 % $CaHPO_4$ were incorporated in the film to investigate whether there is a difference in the stability of the film.

4.3 Preparation with optimized film preparation technique

With the performed pretests it was possible to find a good composition of alginate, $CaHPO_4$ and GDL to prepare alginate films with desired properties. The ITC measurements were the first step of the pretests to see how much calcium the alginate can bind without being overloaded or having less junction zones to be cross-linked properly. After a suitable amount of calcium has been calculated from the ITC data it was necessary to see how much GDL is needed to dissolve the incorporated calcium. For sure a large excess of GDL would have resulted in clear films. However a very high content of GDL would lead to a gel formation by lowering the pH of the alginate under the pKs of the uronic acids even without addition of cross-linking cations like calcium. Before implantation it would take long time to get rid of the residual GDL and obtain films that can be transplanted. Furthermore the gelation time would decrease by increasing amounts of GDL and a film preparation with the help of a film drawing apparatus would be impossible due to a too fast cross-linking. Therefore rheological tests and turbidity measurements were performed to find the right amount of GDL that would lead to clear films with completely dissolved calcium in a timeframe where mixing of the components and drawing of the whole mixture onto a glass plate to a homogenous film would be possible. A prospect on the mechanical properties of alginate films prepared of alginates with a different content of guluronic acid could successfully be conducted with the compression test, because it revealed the impact of the guluronic acid content on the cross-linked gel in resulting in gels with a higher compressive load of the alginate with the larger amount of guluronic acid residues.

With the optimized amounts of alginate, $CaHPO_4$ and GDL, homogenously thick films could be prepared. The properties of the prepared films could be varied with the addition of different amounts of the plasticizer glycerol, by using frames with different gap clearances or by addition of different amounts of calcium. After drawing all prepared films were stored in a closed box to obtain a humid environment in order to slow down the drying speed of the film that was necessary to dissolve the calcium from its hardly soluble salt.

4.4 Mechanical evaluation

The different testing methods were conducted like described earlier. Due to the fact that 10 % and 20 % glycerol showed no positive effect during the tensile testing, the puncture test and the suture pullout test were just performed with films containing 50 % and 100 % glycerol.

4.4.1 Tensile tests

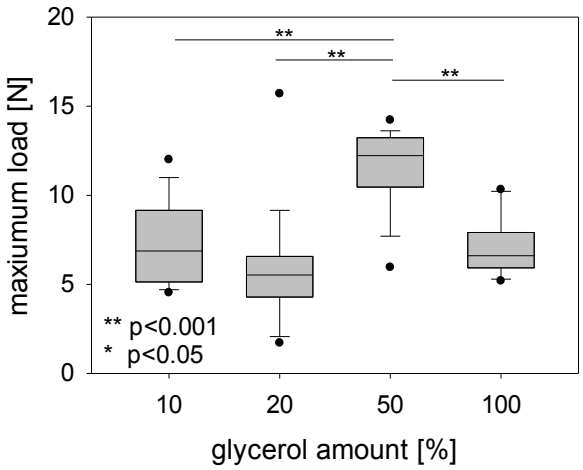
4.4.1.1 Films prepared of low G and high G alginate containing different amounts of plasticizer

For the tensile test 20 strips were tested as described before.

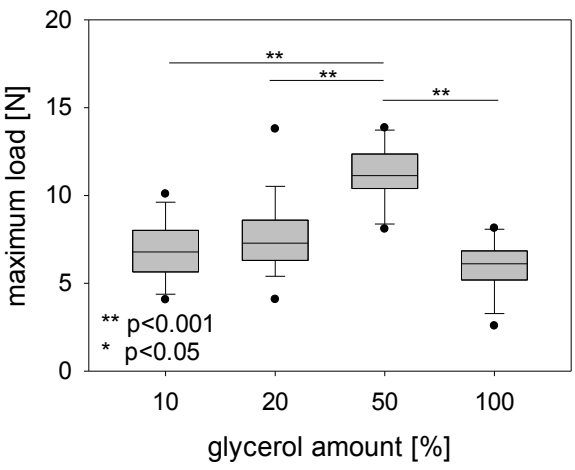
High G

Low G

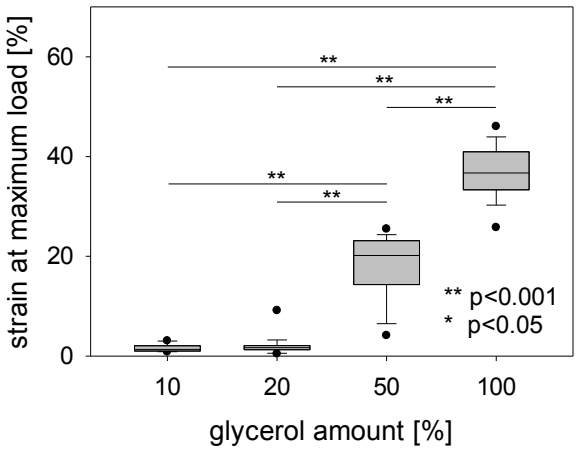
a)



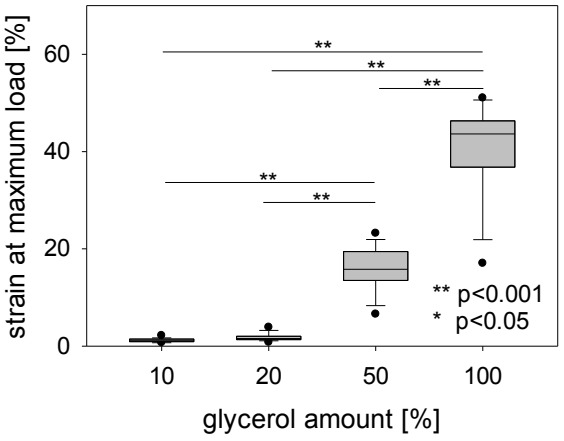
b)



c)



d)



e) f)

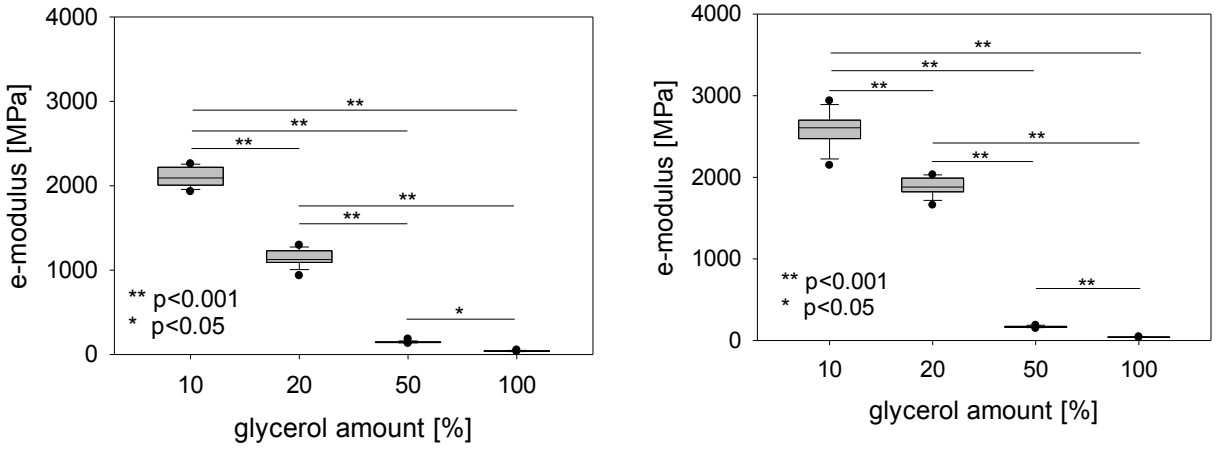


Figure 34: Tensile test of films made of high G and low G alginate containing different amounts of plasticizer. ** equates $p < 0.001$, * equates $p < 0.05$. a), b) maximum load [N]; c), d) strain at maximum load [%]; e), f) elastic modulus of high G and low G alginate films.

The maximum load was estimated to decrease from 10 % glycerol to 100 % glycerol, because the film becomes softer. This effect was only visible from 50 % glycerol to 100 % glycerol. The films with 10 % and 20 % glycerol could only withstand very low forces, because they were very brittle and therefore ruptured early. The maximum load of the low G alginate films behaved in a similar fashion to that of high G alginate.

The strain at maximum load was also affected by the glycerol concentrations used. With increasing amounts of glycerol the films became more elastic. From 20 % to 50 % and up to 100 % a significant effect on the strain was measurable. The strain for both alginates was also very similar. Only the films containing 100 % glycerol had a significant difference ($p=0.011$). The high G alginate had a strain of round about 37 % whereas the low G alginate had a strain of more or less 41 %.

Due to the fact that the films containing 10 % and 20 % glycerol were brittle and very stiff, the elastic modulus was very high. With higher glycerol amounts the elasticity became higher and therefore the elastic modulus decreased significantly.

The low G alginate films containing 10 % and 20 % glycerol had a significantly higher elastic modulus ($p<0.001$) than the high G alginate films. With little amounts of plasticizer, the cross-linking was the main factor that influenced the mechanical properties of the alginate films.

4.4.1.2 Films prepared of low G and high G alginate containing different amounts of calcium

20 strips were tested as described before.

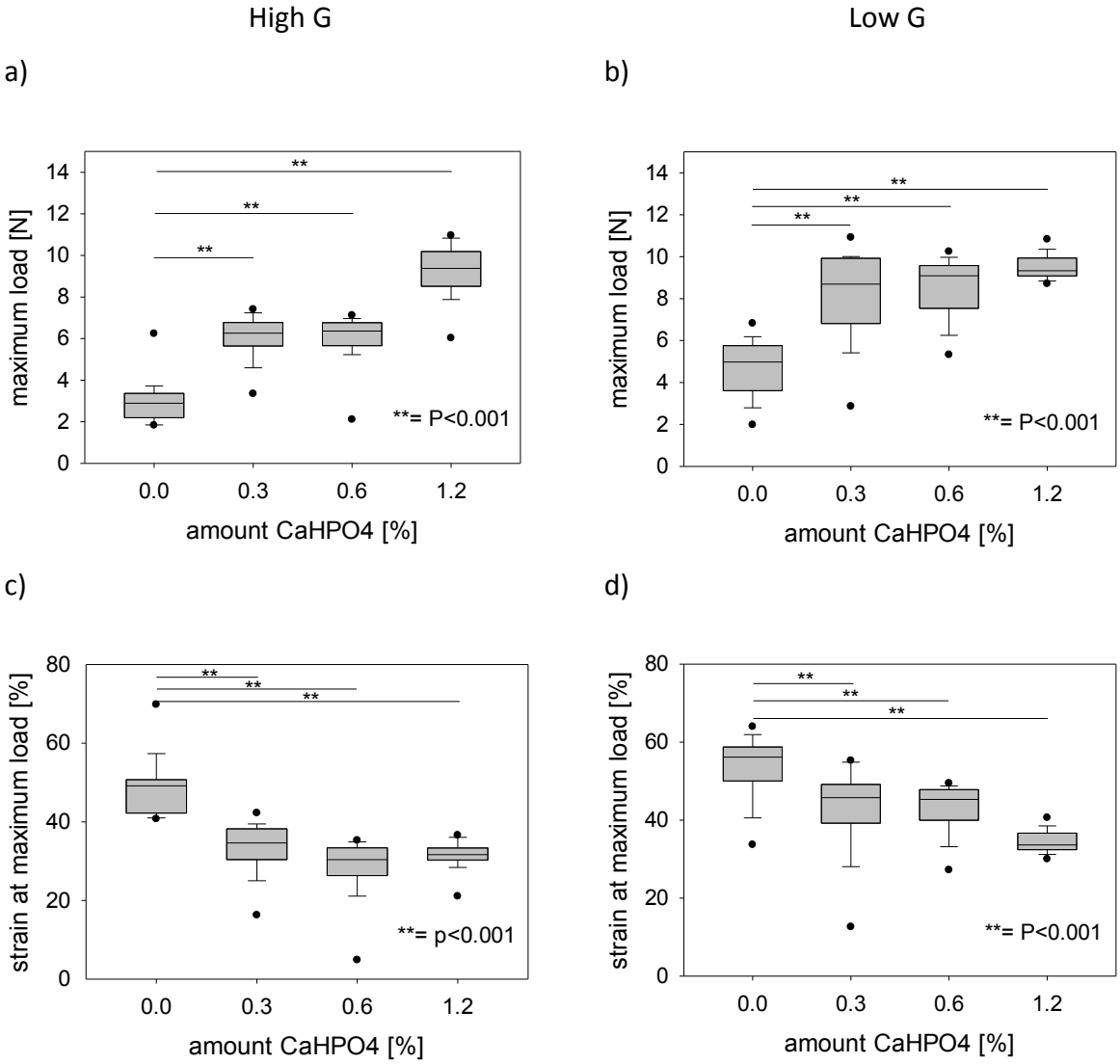


Figure 35: Tensile test of films made of high G and low G alginate containing different amounts of $CaHPO_4$. ** equates $p < 0.001$. a), b) maximum load [N] of high G and low G alginate films. c), d) strain at maximum load [%] of high G and low G alginate films.

Based on the ITC data an amount of 0.6 g $CaHPO_4$ per 100 ml alginate solution should cross-link half of the guluronic acid blocks in a film made of high G alginate. To see the impact of cross-linking on the mechanical properties of the film also half of the amount and the doubled amount were given to the alginate films. Even the smaller amount led to a significant increase of the maximum load [N] and a significant decrease in the strain at maximum load [%] for the high G alginate films as well as for the low G alginate films. A further addition of $CaHPO_4$ to 0.6 g per 100 ml did not show an effect. In case of the high G

alginate a significant difference in terms of the maximum load [N] between the addition of 0.6 g and 1.2 g $CaHPO_4$ could be measured. The addition of 0.6 g or 1.2 g $CaHPO_4$ to the low G alginate did not show a significant difference (P=0.208). It might be possible, that the G blocks were already saturated with calcium so further uptake and respectively a further cross-linking was not possible. In case of the strain at maximum load [N] no significant differences could be seen between the addition of 0.3 g or 0.6 g $CaHPO_4$ (P=0.08s) and 0.6 g or 1.2 g (P=0.440) to the high G alginate film. For the low G alginate film the addition of 0.6 g $CaHPO_4$ or the addition of 1.2 g $CaHPO_4$ showed a significant difference (P=0.002) in the strain at maximum load [%]. When oversaturated with calcium the film was more brittle and broke earlier which led to a decrease of the strain.

4.4.1.3 Tensile properties of cross-linked high G films after wetting

When the alginate films get implanted in the peritoneum, they will swell and lose stability due to the uptake of water. To see the impact of water uptake on the mechanical stability, 10 strips of high G alginate films containing 0.3 g, 0.6 g or 1.2 g $CaHPO_4$ were tested in a dry state and after 30 minutes incubation in water.

a) b)

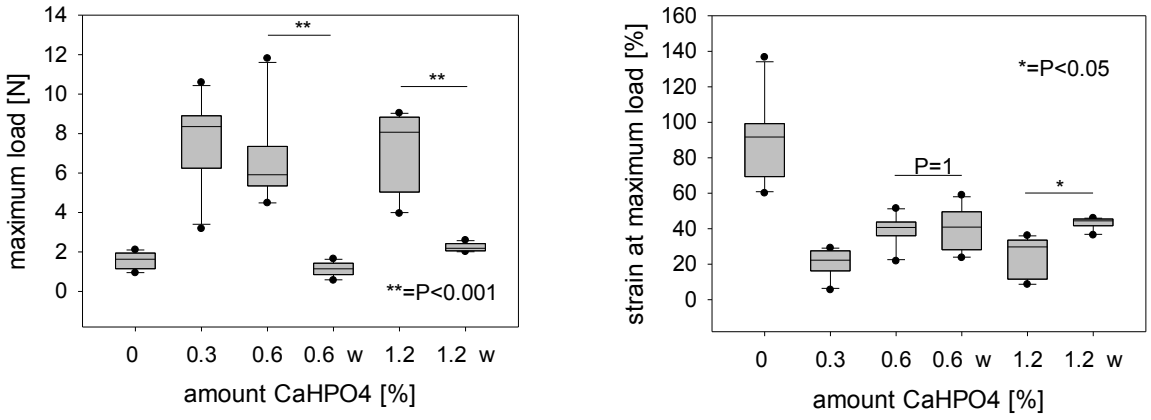


Figure 36: Tensile test of films made of high G alginate containing different amounts of calcium dry and wet. ** equates p < 0.001, * equates p < 0.05. a) Maximum load [N] of high G alginate films dry and wet. b) Strain at maximum load [%] of high G alginate films dry and wet.

The films containing 0.3 g $CaHPO_4$ could not be tested in a wet state because they were swollen too much. As expected the films containing 0.6 g and 1.2 g $CaHPO_4$ showed a significant decrease in the maximum load [N] after incubation in water. Due to the plasticizing effect of water the strain at maximum load [%] should increase with the uptake

of water, but in the swollen state the films could not withstand high forces and ruptured very early.

4.4.1.4 Tensile test of films with different thicknesses

The high G films cross linked with 0.6 g $CaHPO_4$ per 100 ml alginate solution drawn with different gap clearances had a thickness of 20, 25, 50 and 90 µm. 20 strips were tested.

a) b)

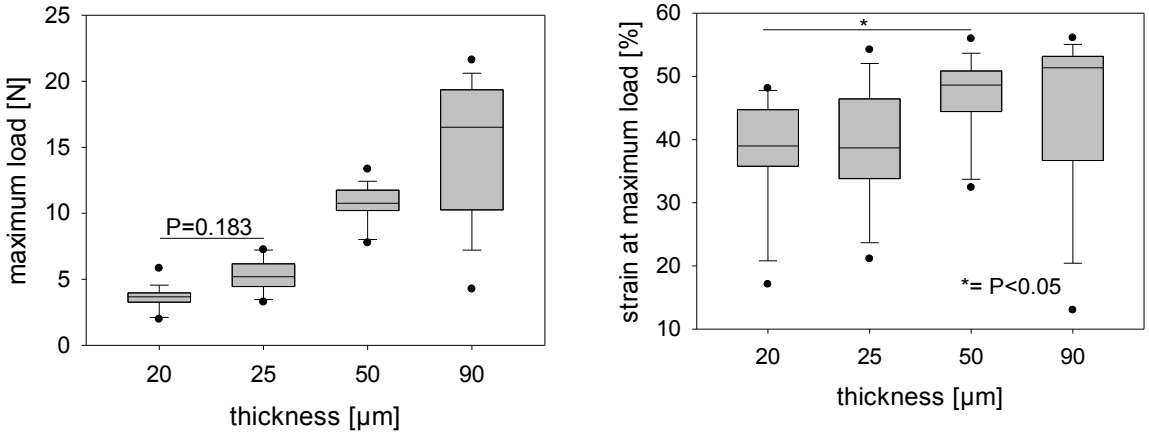


Figure 37: Tensile test of films made of high G alginate containing 0.6 g CaHPO4 cast with different gap clearances, * equates p < 0.05. a) Maximum load [N] of high G alginate films. b) Strain at maximum load [%] of high G alginate films.

Concerning the maximum load [N] only the films with the thicknesses of 20 µm and 25 µm showed no significant differences. All other films showed a significant difference (P<0.001). The thicker the film was the higher was the maximum load [N]. The film thickness did not affect the strain of the films in the same way it affected the maximum load. Only the films with a thickness of 20 µm and 50 µm had a significant difference in strain (P<0.05).

4.4.2 Puncture Test

To investigate, if the polymer film can resist forces when it is for example wrapped around the intestinal and chymus gets through it, or if the patient coughs, a puncture test was performed. The technical setup of the test used is described in chapter 3.

a) b)

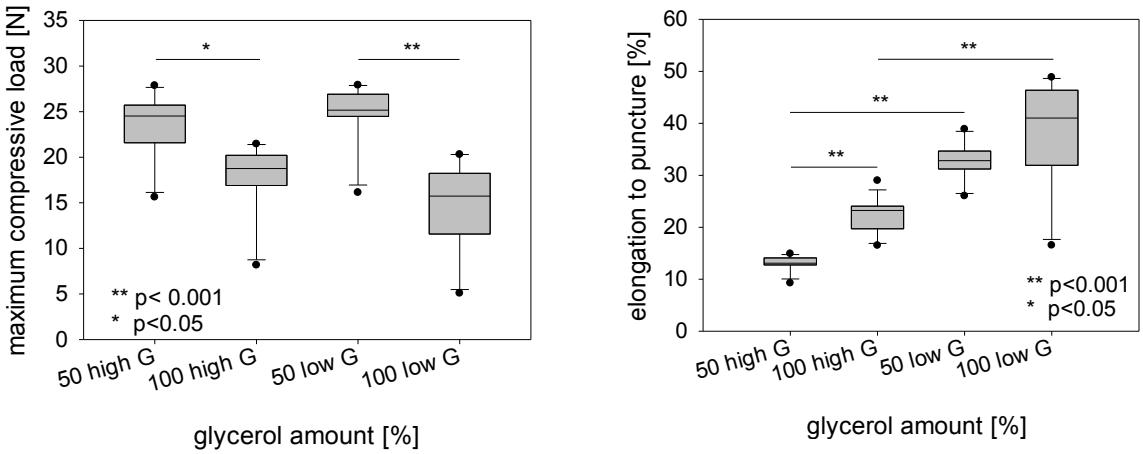


Figure 38: Puncture test of high G and low G alginate films containing different amounts of glycerol as plasticizer

In terms of the maximum compressive load, the results were as expected. With a higher glycerol amount the load decreased. There were no significant differences in the maximum load between the two alginate types, whereas the elongation to puncture showed a significant effect of the chosen alginate type.

The more glycerol was in the film, the more the film could be elongated until it ruptured. The elongation of the films made of low G alginate was much higher than the elongation of the high G alginate. This effect could be explained with the stronger cross-linking of the high G alginate films and the resulting decrease in elasticity.

4.4.3 Suture pullout test

A film made from cross-linked alginate that incorporates water after implantation and time by time becomes a viscous gel will never be sutured to a tissue alone. The mucoadhesive properties of the alginate can be helpful to suture a bilayer made of alginate as an adhesive layer and another polymer. Therefore suture pullout tests were performed to see if the alginate can also stabilize a bilayer when it is sutured to tissue.

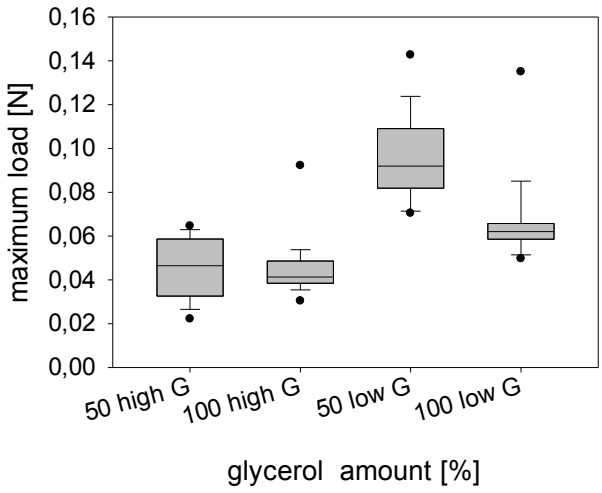


Figure 39: Maximum load measured during suture pullout test

For the high G alginate no significant effect could be measured with the addition of 50 % or 100 % glycerol calculated on the alginate amount. Regarding the results from the tensile test a higher load for the high G alginate was expected. But the results showed that the low G alginate had a higher maximum load and furthermore an effect of different amounts of plasticizer could be measured. The more glycerol was in the film the less strength was needed to pull the suture through it. It might be possible, that the alginate containing less G blocks could form a denser network due to slower cross-linking than the high G alginate that could lead to a higher maximum load. But both films could not resist loads higher than 0.15 N. A further characterization of multilayers will be done in chapter 7.

4.4.4 Mucoadhesion tests

The mucoadhesive properties of alginate and modified alginate for example alginate-cysteine conjugates [85] can be beneficial for several applications like for example mucoadhesive tablets that adhere to the intestine or mucoadhesive buccal films [86]. Several approaches have been done to test the mucoadhesive properties of alginate and other

polymers, like tensile strength measurements with tablets prepared from these polymers and tensile testing after attachment to a porcine mucosa [85,87] or to a mucin gel prepared of mucin from porcine stomach type II (Sigma- Aldrich) [88]. Ellypsometry to test the adsorption to silica surfaces [86,87] or rheological measurement that measure the increase of viscosity of dispersions prepared of the respective polymer and mucin [86,89] were also performed. To date no test standard for the testing of the adhesive properties of polymer films to peritoneal tissue exist.

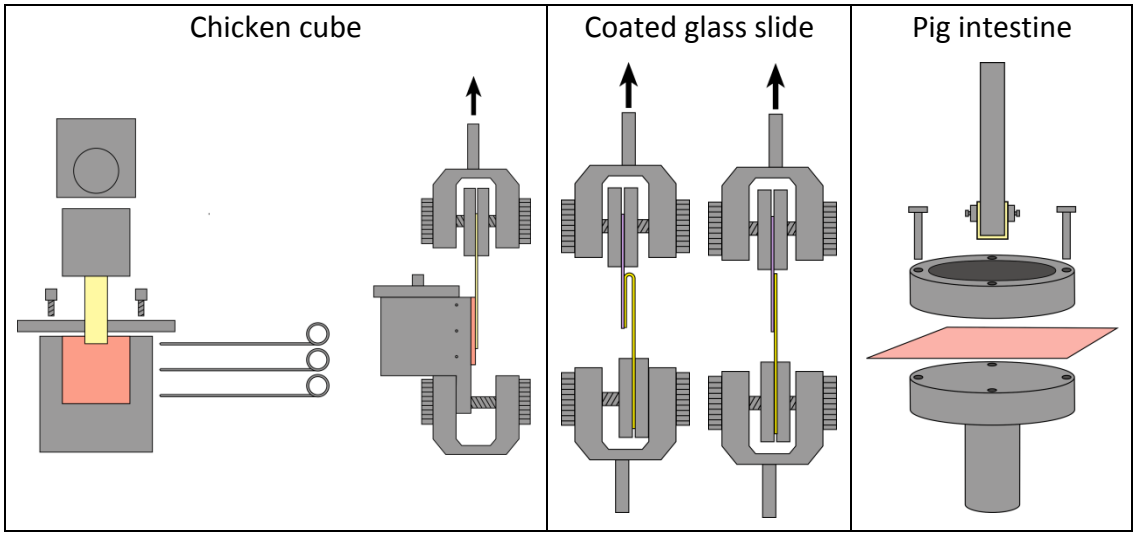


Figure 40: Setup for adhesion tests

To test the adhesion of the alginate films to tissue several approaches have been done. One trial was to test the adhesive force by attaching alginate film strips on chicken breast. On that account cubes of chicken breast with an edge length of 3 cm were fixed with a special holder at the lower part of the texture analyzer. A 1 to 5 cm alginate film strip was fixed in the upper grip of the texture analyzer and 2 cm of the film were attached to the chicken breast. The upper grip was lifted with a tension rate of 10 mm/min. The strips adhered very well to the chicken breast. Due to the fact, that the adherence depended on the surface and moistness of the chicken breast, the test was not reproducible. Therefore other surfaces were necessary.

To obtain reproducible and homogenous surfaces object slides were chosen. For the investigation of the adherence to different surfaces with different features the object slides were coated with Sigmacote® to obtain a more lipophilic surface and with (3-Aminopropyl)trimethoxysilane to obtain a surface with positive charges. The treated surface of the object slides was 2.5 to 5.5 cm. At first the object slides were cleaned in an ultrasound

bath. Afterwards the slides were soaked for 5 minutes in acetone followed by 5 minutes in deionized water and at last 5 minutes in isopropanol. After air drying three slides were left as they were. Three samples were coated with Sigmacote® by rinsing them with Sigmacote® followed by a washing step with deionized water. For the aminosilanization three slides were etched with piranha solution (H_2O_2: H_2SO_4, 2:1) for 30 minutes and rinsed with deionized water until the pH neutralized. After drying with compressed air they were placed over night in a desiccator with 100 µl of (3-Aminoproply)trimethoxysilane in a watch glass at around 100 mbar.

To test the hydrophilicity of the surfaces contact angle measurements with a Contact Angle System (OCA 20, Data Physics, Filderstadt, Germany) were performed. 5 droplets of 100 µl were given on different locations of the glass slides with a rate of 1 µl/sec. The contact angles were obtained with an ellipse fitting.

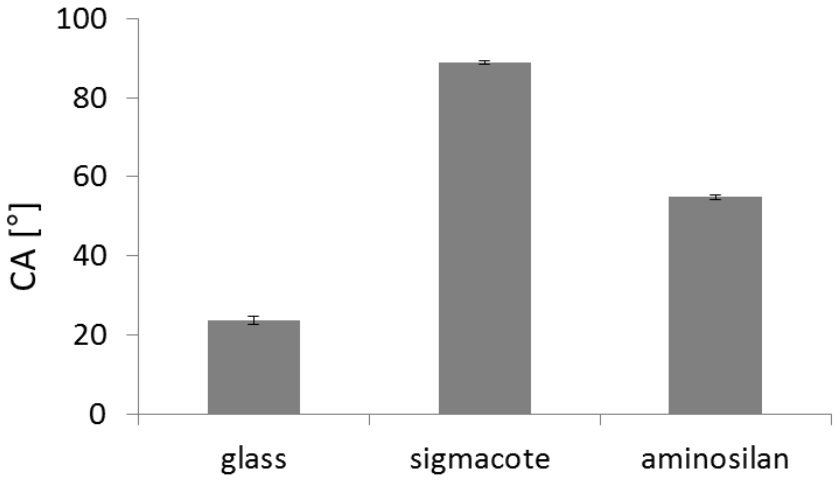


Figure 41: Contact angles of 100µl drops on different surfaces of object slides

For the adhesion measurement the glass slide was fixed in the upper grip of the texture analyzer in a way that the treated part was hanging under the grip. A film strip with the dimensions of 2 to 15 cm was attached from the bottom up on the glass slide forming a loop. The loose part of the film strip was fixed in the lower grip that had a distance of 8 cm to the upper grip. The test was started with a preload rate of 10 mm/min until a preload value of 0.0001 N was reached. Then the test was performed with a test speed of 152.4 mm/min according to the Standard Test Method for Peel or Stripping Strength of Adhesive Bonds ASTM D903-98.

The peel test with monolayers of alginate was not possible, because the film stuck to itself and made a peeling from the glass slide impossible.

Therefore the film strips were cut into half and the 7.5 cm long strips were stuck to the glass slide in a way that 2 cm were hanging loose from the glass slide and could be fixed with the lower clamp. Then the test was started with the same preload value and speed as before.

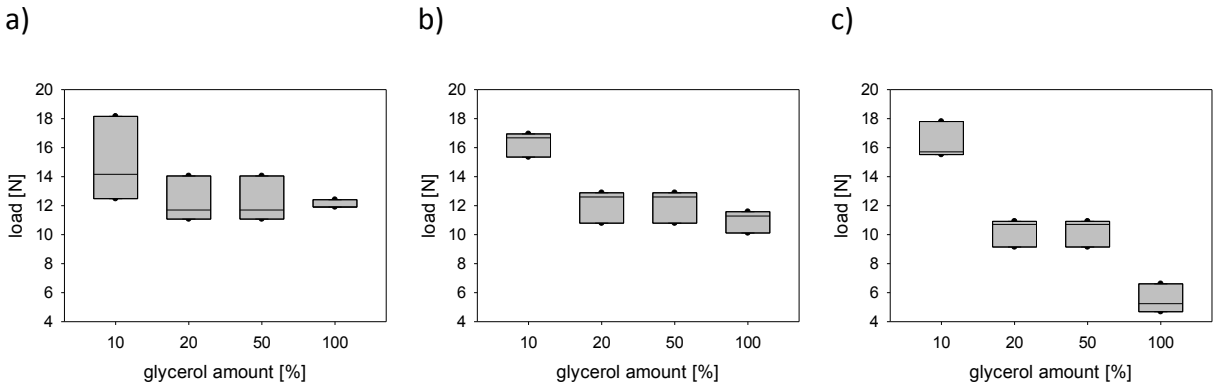


Figure 42: Films containing different amounts of glycerol from 10 % to 100% (w/w) calculated to the alginate amount stuck to glass slide and tested. (a) Untreated glass slide; (b) glass slide covered with aminosilan, (c) glass slide covered with Sigmacote®

This test setup did not show representable results for adhesion because the film strips ruptured instead of detaching from the glass slides. Therefore the recorded loads where the maximum loads recorded until the film ruptured. These loads showed the same trend from a higher load with low glycerol amounts till a lower load with higher glycerol amounts. The film strips attached to the untreated glass slides and the aminosilanized glass slides nearly ruptured at the same load. The strips attached to the glass slide coated with sigmacote® showed a lower load. Although the film ruptured and did not glide from the glass slide a partial slip from the glass slide would explain these results.

To test the adhesion of the prepared alginate films on pig intestine a piece of small intestine stored in PBS containing 5 ml/100ml Pen Strep from a 8 week old, 18 kg, male domestic pig was opened by cutting one side lengthwise and fixed with the inner side down on a platform with the help of double faced adhesive tape. A strip of cross-linked alginate film with the dimensions of 5 to 1 cm was fixed to the bottom of a piston with the help of two clamps. The resulting locating surface had the dimension of one square centimeter.

Before each measurement the piece of small intestine was wetted with some droplets of PBS with Pen Strep to obtain the same humid test conditions for every specimen. In a first step the piston was lowered to the intestine with a speed of 10 mm/min and pushed onto the intestine layer until a load of 0.1 N was measured. The load of 0.1 N was held for 30 seconds. Subsequently the piston was raised from the intestine with a speed of 0.5 mm/sec until the film was separated from the intestine and the load was recorded.

5 film strips were tested this way, as comparison 5 strips were tested without intestine and pressed on the metal surface of the setup.

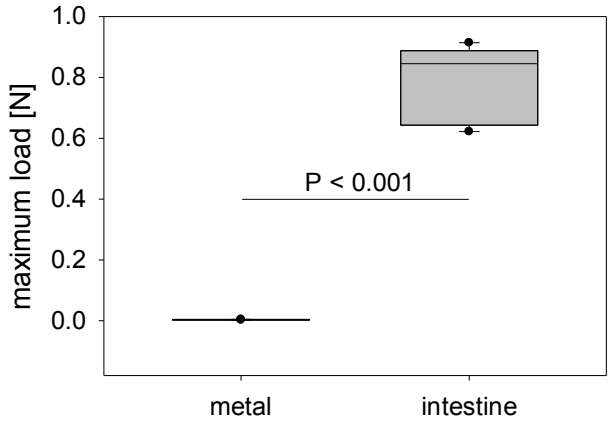


Figure 43: Adhesion test of alginate films on metal and pig intestine

The alginate film strips adhered to the pig intestine and did not rupture during the testing. The load recorded was about 0.8 N.

4.5 Erosion in different buffers

For the erosion study, buffers were used, which had different pH values. pH 7.4 of normal body fluid and pH 5.5 to simulate the lower pH in inflamed tissue. These pH values were chosen to investigate if there is a difference of erosion in normal or inflamed tissue. Due to the fact, that calcium also plays a very important role in the erosion time of cross-linked alginate films, the buffers contained 3 different concentrations of calcium, 0 mM, 1.2 mM and 2.5 mM. The 1.2 mM simulates the concentration of unbound calcium in body fluids. 2.5 mM simulates the calcium concentration of unbound and bound calcium in the body that is very constant because of the calcium homeostasis.

The amount of cross-linker was expected to be the main factor for the film stability.

For the subsequent erosion study, the alginate films were cut into discs with 2 cm diameter and stored in the buffers, to see the impact of pH and the calcium within the erosion medium.

After the film discs were cut from the films with the help of a cork bore, they were weighed to determine the starting weight. Then 10 ml of the respective buffers were added to the alginate discs and the closed vials were put on an orbital shaker (Unimax 2010, Heidolph Instruments GmbH & Co. KG , Schwabach, Germany), shaked with 100 rpm while stored in a cabinet dryer at 37 °C to investigate the film erosion and degradation.

The first sample was taken after 24 hours, than the sampling took place every week, 4 discs of each buffer were removed from the cabinet dryer and the buffer was decanted carefully. The remaining alginate discs were carefully washed several times with deionized water by pouring the water into the glass vials, pivoting the vial and then pouring the water out of the vial again. The residual water was carefully removed using cotton swabs, and then the vials were immediately weighed to determine the wet weight of the swollen discs. The remaining discs were then lyophilized overnight and weighed again to determine the remaining dry weight of the residual films. For the remaining disc samples, a buffer exchange was performed every week by replacing the buffer with fresh buffer in order to maintain sink conditions for soluble alginate and calcium. The last sample of the erosion study was obtained after 8 weeks, which was considered the maximum application time for the intended anti-adhesion barrier.

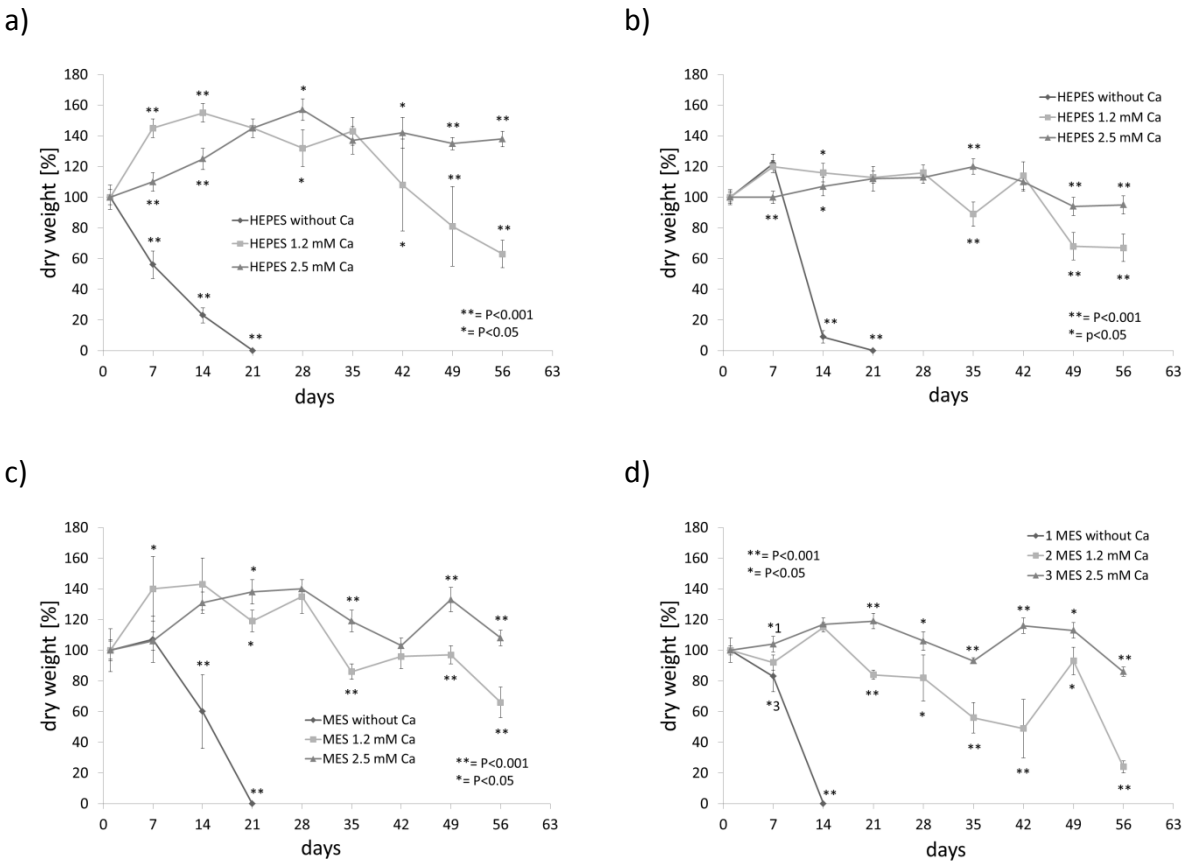


Figure 44: Erosion of alginate films stored in buffers with different calcium concentrations and different pH values. ** = P< 0.001, * = P< 0.05. The numbers behind the stars represent the different values obtained in the different buffers. 1: Buffer without calcium, 2: Buffer with 1.2 mM calcium, 3: Buffer with 2.5 mM calcium. The stars combined with the numbers indicate significant or not significant differences between the measurements at similar time points. (a) dry weights of high G alginate discs at pH 7.4, (b) dry weights of low G alginate discs at pH 7.4 (c) dry weights of high G alginate discs at pH 5.5 d) dry weights of low G alginate discs at pH 5.5. Dry weights are normalized to the dry weight obtained after 24 h.

In HEPES buffer without calcium, the high G as well as the low G alginate discs eroded completely. The high G alginate discs were degraded after 3 weeks, while the low G alginate discs had a mass increase in the first week due to the swelling and accompanied uptake of salts from the buffer. Then the discs eroded completely until week 3. In the HEPES buffers containing calcium, both alginates did not erode completely during the time of investigation. In the buffer containing 1.2 mM Ca^{2+}, the high G alginate discs had a mass increase up to 155 % after two weeks and stayed constant with a variation of 10 % until week 5. Then the discs eroded to 60 % at week 8. A higher and continuous mass increase to about 160 % was visible in the buffer containing 2.5 mM calcium until week 4. After a mass loss to 140 % at week 5, the dry weight stayed nearly constant until week 8.

After one week there was just a slight mass increase of the low G alginate discs in the HEPES buffer containing 2.5 mM calcium to 120 % visible and stayed constant until week 5. Then the weight decreased to 95 % at week 8. In the HEPES buffer containing 1.2 mM calcium, the mass increased after one week was nearly the same as in the buffer without calcium. The mass stayed nearly constant till week 6 with an intermediate decrease to 90 % at week 5 and eroded to 70 % by week 8.

In the buffer without calcium the high G as well as the low G alginate discs eroded completely. Whereas the low G alginate discs were eroded to 10 % by week two and were completely eroded after three weeks, the discs were completely eroded after two weeks in MES buffer. The mass increase of the high G alginate discs in both calcium concentrations was about 140 %, 10 % lower than in HEPES buffer and the discs eroded to 110 % in MES buffer containing 2.5 mM calcium and to 70 % in the buffer with 1.2 mM calcium. The mass increase of the low G alginate discs was 5 % lower than the increase in HEPES buffer to 115 %. They eroded to 85 % in the MES buffer with the higher calcium concentration and to 25 % in the MES buffer with the lower calcium concentration. This indicates a difference of 10 % and 40 % to the erosion in HEPES buffer.

4.5.1 Swelling

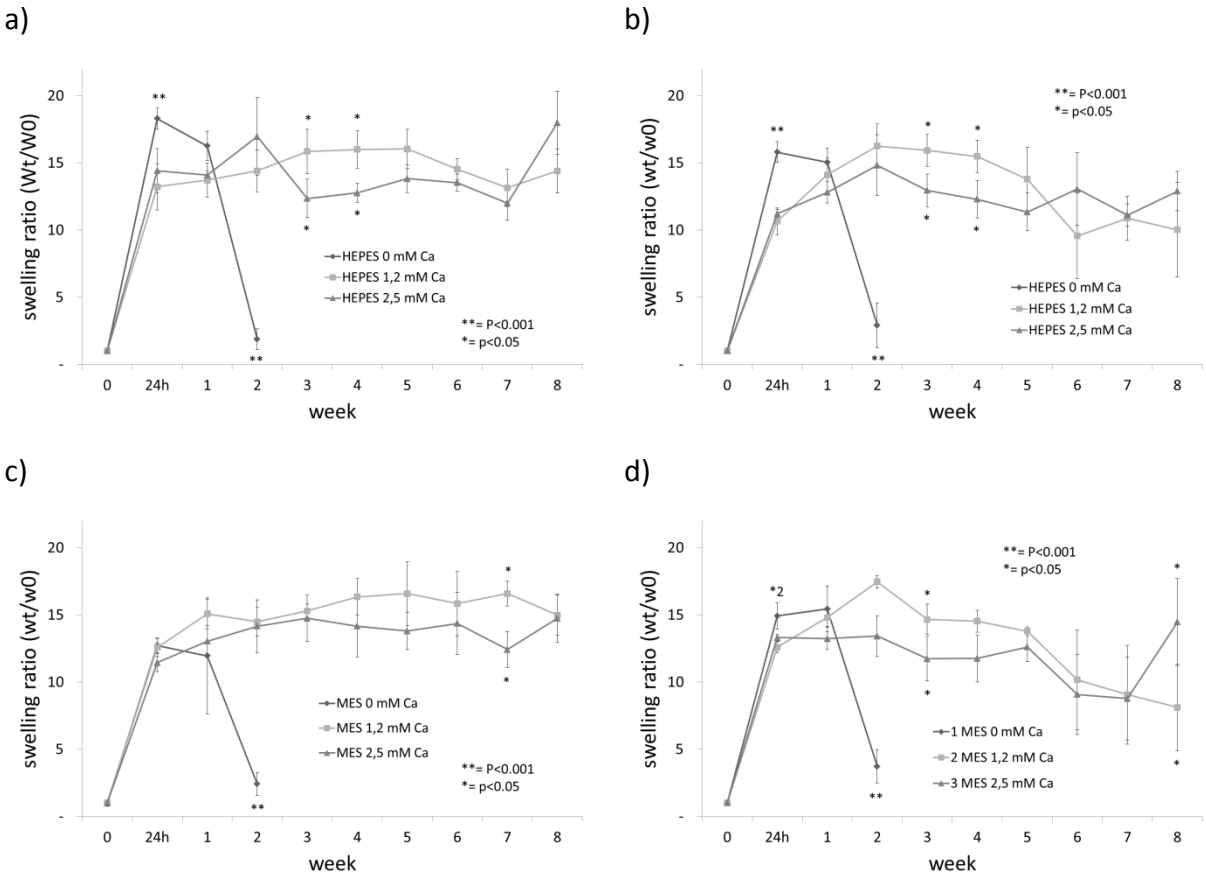


Figure 45: Swelling of alginate films stored in buffers with different calcium concentrations and different pH values. ** = P< 0.001, * = P< 0.05. The numbers behind the stars represent the different values obtained in the different buffers. 1: Buffer without calcium, 2: Buffer with 1.2 mmol/l calcium, 3: Buffer with 2.5 mmol/l calcium. The stars combined with the numbers indicate significant or not significant differences between the measurements at similar time points. (a) swelling ratio of high G alginate discs at pH 7.4, (b) swelling ratio of low G alginate discs at pH 7.4 (c) swelling ratio of high G alginate discs at pH 5.5 d) swelling ratio of low G alginate discs at pH 5.5. Dry weights are normalized to the dry weight obtained after 24 h.

In the HEPES buffer without calcium the high G alginate discs had a swelling ratio of 18 and the low G alginate discs a swelling ratio of 16 after incubation for 24 hours. In the MES buffer the swelling ratio was lower with 13 and 15. In the buffers with calcium the swelling ratio after 24 hours varied from 11 to 14. After 24 hours the swelling of the alginate discs was nearly completed.

4.5.2 Homogeneity of calcium distribution and swelling

To test the homogenous distribution of calcium in an alginate film that is drawn over a distance of 40 cm a swelling study was performed. When the calcium is distributed homogenously all over the film, the cross-linking should be the same and therefore the film should swell equally.

Three discs of a high G alginate film with a diameter of 2 cm were cut from the beginning, the middle and the end of the film. These discs were weighed and incubated in HEPES buffer containing 1.2 mmol calcium. Every hour the wet weight of the alginate discs was determined.

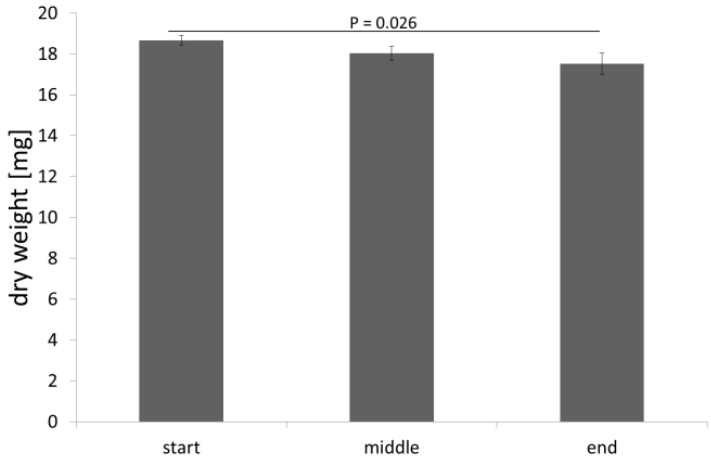


Figure 46: Dry weight of alginate discs cut from the beginning, the middle and the end of a drawn alginate film

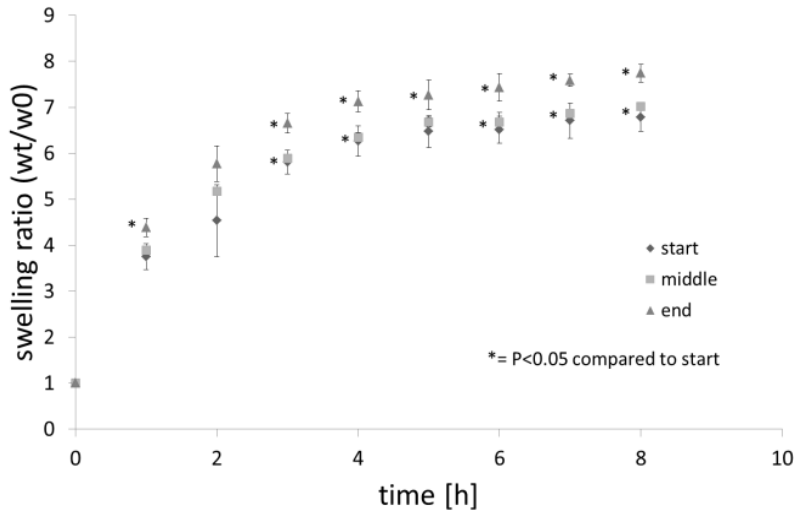


Figure 47: Swelling of alginate discs from the beginning, the middle and the end of a drawn alginate film

The swelling study revealed that the alginate discs taken from the beginning of the film were heavier and had a lower swelling ratio than the alginate discs taken from the end of the film.

4.5.3 Erosion behavior regarding guluronic acid content, pH and calcium concentration

The erosion study reveals the high impact of calcium in the erosion medium on the erosion of alginate discs. The higher the calcium concentration in the buffer was the lower was the erosion of the alginate discs. The content of guluronic acid in the alginate and therefore the degree of cross-linking respectively the property of binding more calcium had also a great effect on the erosion behavior. For the high G alginate discs the pH of 7.4 respectively 5.5 showed a negligible impact on the erosion time. The low G alginate discs eroded faster in the buffer with the lower pH.

Due to the fact, that some G blocks in the high G alginate had the capacity to complex more calcium, the alginate discs became heavier in the beginning because of the uptake of calcium and buffer salts in the film. The low G alginate discs did not have this capacity.

The lower pH of 5.5 made no great difference in the erosion behavior of the high G alginate discs.

The swelling of the discs in the different buffers confirms the results from the erosion study. The lower the calcium concentration in the buffer was the higher was the swelling of the alginate discs due to lower cross-linking. During the observation over eight weeks no big changes in the swelling ratio could be measured. It nearly stayed constant or decreased due to the contemporaneous erosion of the alginate discs.

The film discs cut from the beginning of the alginate film were heavier and swelled less than discs from the other parts of the film. This could be explained by the sinking of the suspended $CaHPO_4$ particles during the drawing process. When the calcium was sinking to the ground in the beginning of the drawing process, less calcium remains for the rest of the film. Although the difference is statistically significant $P<0.05$ the consequence for the resulting mechanical properties as well as erosion time is negligible.

4.6 Cytotoxicity tests of the prepared alginate films

The alginate films were tested with the elution method according to DIN EN ISO 10993-5, -12 in an accredited laboratory. After the film was humidified in PBS buffer to avoid an uptake of DMEM during the extraction, the film was extracted with 1 ml DMEM per 0.1 g film for 48 hours at 37 °C and 5 % CO_2. For the positive control, discs made of Vekoplan KT PVC plates (König GmbH, Wendelstein, Germany) which contained cytotoxic zinc were extracted with DMEM, too. The negative control DMEM was also stored under the same conditions. After 48 hours the extract and two serial dilutions to 50 % and 25 % were used to cultivate L929 CC1 mice fibroblasts (American type culture collection, Rockeville, Maryland, USA). After further 48 hours, a WST test was performed and the cells were counted with the help of a cell counter type CASY 1 (Roche GmbH, Mannheim, Germany).

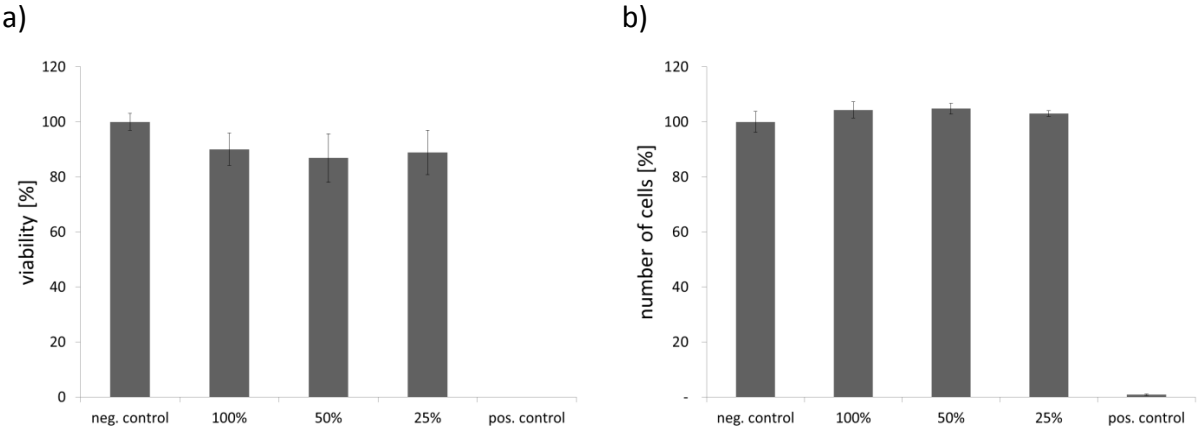


Figure 48: Viability and number of cells of mice fibroblasts

The results of the WST test show a metabolic activity of more than 80 %. The cell proliferation even shows a result of more than 100 % compared with the negative control. Therefore it can be concluded, that the alginate film is not toxic to cells. The slightly decrease of the metabolic activity might be explained with the pH degrease of the medium during the extraction of the GDL containing alginate film.

4.7 Discussion

After the amounts of the components which resulted from the pretests were drawn to films to obtain films with suitable properties it was obvious that the results of these measurements could not be transferred one to one to the procedure of film preparation. Although the gel discs obtained after the rheological measurements and the gels obtained after the turbidity measurements and the compression test where clear and revealed a complete dissolution of the hardly soluble calcium salt, the suspension drawn to a thin film led to brittle turbid films with visible crystals of the calcium salt that has not been dissolved completely. This could be explained with the faster drying speed that came from the bigger surface of the mixture drawn to a thin film. The suspension in the measuring gap of the rheometer kept its humidity during the measurement and therefore led to clear films. The gels in the precision cell as well as in the wells of the well plate also dried very slowly and therefore allowed the complete dissolution of the hardly soluble calcium salt. Nevertheless the amounts of the components obtained from the pretests were used to prepare very thin alginate films with the help of the film drawing apparatus. To overcome the problem of an incomplete dissolution of the hardly soluble calcium salt, the drying speed of the drawn film was prolonged by giving the glass plate with the thin film into a closed box. After several hours the still wet but clear film could be taken out of the box and dried at room temperature to a clear film. The pretest only revealed the amounts of calcium salt and GDL that should be given to the alginate solution to achieve a suitable cross-linking. To prepare films with good mechanical properties besides the cross-linking also the addition of plasticizer is important, because without plasticizer a very thin air dried alginate film is very brittle. The mechanical evaluation revealed that the addition of 50 % glycerol calculated on the alginate amount would lead to films with good mechanical properties. Different test setups were investigated to find an appropriate method to test the mucoadhesive properties of the prepared alginate films on peritoneal tissue. The established method of the detachment from alginate films strips from a wetted pig intestine showed reproducible results. The erosion study revealed that both films prepared from high G and low G alginate would be stable for 8 weeks if calcium is available in the environment. It could be seen, that the film prepared from high G alginate was still able to incorporate more calcium from the buffer. The pH of the erosion buffer had no influence on the erosion time. The cytotoxicity

test implies that a wetting with PBS before implantation would remove the acidic and harmful GDL.

4.8 Summary and Conclusion

In previous investigations alginate films cast into Teflon molds or drawn on glass plates were cross-linked with the help of different calcium salts and different cross-linking techniques (Chapter 3). These films contained about 0.11 g to 0.21 g calcium ions per gram alginate powder (Table 10). The films cast into Teflon molds containing two different amounts of calcium to investigate the impact of incorporated calcium on the erosion time contained 0.5 g or 1 g calcium citrate tetrahydrate (Table 10). The erosion study with these films revealed, that even with less calcium the films were cross-linked properly and eroded time by time instead of being dissolved rapidly.

Table 10: Calcium amounts used for cross-linking before optimization

Calcium source	Calcium salt [g] / g alginate powder	→Equal amount of Ca^{2+} [g] / g alginate powder
Calcium citrate tetrahydrate $C_{12}H_{10}Ca_3O_{14}$ 4 H_2O (Mr 570.51 g mol^{-1})	0.5 1	0.11 0.21
Calcium-EDTA →calcium chloride $CaCl_2$ (Mr 110.98 g mol^{-1})	0.435	0.16

The pretests performed (Chapter 4) for the investigation of an appropriate amount of calcium for cross-linking showed, that the drawn films prepared with different cross-linking techniques (Chapter 3) were saturated with calcium.

Table 11: Calcium amounts used for cross-linking after optimization

Calcium source	Calcium salt [g] / g alginate powder	→Equal amount of Ca^{2+} [g] / g alginate powder
Calcium phosphate $CaHPO_4$ (Mr 136.06 g mol^{-1})	0.2	0.06

The pretests showed that an amount of 0.06 g Ca^{2+} per gram alginate powder (Table 11) is able to cross-link half of the guluronic acid blocks in an alginate film and lead to films with appropriate mechanical properties and erosion time.

Regarding the guluronic acid content in the alginate film the compression test revealed that alginate gels prepared of alginate with high guluronic acid content resulted in stronger gels than alginate gels prepared from alginate with less guluronic acid.

The tensile tests showed no significant differences regarding the maximum load and the tensile strain. The elastic modulus of the film prepared of low G alginate was higher than the elastic modulus of the films prepared of the high G alginate film which might come from a higher amount of calcium that could not be bound due to the lack of free guluronic acid.

The elongation of the films also revealed a difference between the two alginates. The films made of low G alginate had a much higher elongation than the films prepared of the high G alginate. This effect could be explained with the stronger cross-linking of the high G alginate films and the resulting decrease in elasticity.

It can be concluded, that the cross-linking of alginate films and as a consequence thereof the mechanical properties and erosion time can be influenced by different things. More precisely the choice of the alginate regarding the content of guluronic acid, the calcium source and the release of calcium from its calcium source, the calcium amount used for cross-linking and the addition of plasticizer.

It is important to have a suitable balance between all the components used for the film preparation to obtain films that are cross-linked good enough to withstand forces during surgery, but be flexible enough to enable movement of the organs and tissue. With the addition of plasticizer the film can be softened but with higher amounts glycerol the film becomes very sticky and is harder to handle.

5. Drug release from thin polymer films

This Chapter deals with the preparation of thin polymer films prepared from alginate, a block copolymer made of PLA PEG PLA and PLA. These films were loaded with the two antibiotics vancomycin hydrochloride and gentamicin sulfate using different methods. Release studies and microbiological testing were performed with the prepared films to test their biological efficacy.

5.1 Introduction

Inflammations due to bacterial infections are a quite common side effect after abdominal surgery or trauma [90,91] and can in consequence strongly promote the formation of abdominal adhesions. The trauma associated infections of the abdomen are mainly treated systemically with high doses of antibiotics [92,93] and accordingly come along with many undesired side effects at other organs and tissues [94].

A delivery device for a directed local application providing controlled release of antibiotics in much lower doses would minimize these side effects and lead to a higher concentration of the antibiotic directly at the infected wound site [94,95]. For this purpose soft and flexible drug delivery systems made of hydrogels like poly (ethylene glycol), poly (ethylene oxide), cellulose and collagen are already successfully used for the delivery of for example gentamicin sulphate, cefotaxim or vancomycin [96–99]. However, for the preparation of a delivery device made of a degradable natural hydrogel like alginate or hyaluronic acid [98,100] many key points have to be addressed and carefully investigated, like the gel biocompatibility, mechanical properties, degradation behavior, sterilization procedures, as well as the resulting material solubility due to occurring erosion or hydrolysis [101].

Cross-linked alginate layers loaded with vancomycin have already been used as coating for porous scaffolds to achieve a slower sustained drug release rate of vancomycin at a local site to inhibit infection after surgery [102], as alginate beads in an injectable anti-infection tissue-engineered construct for the treatment of chronic osteomyelitis due to the difficulty of antibiotics to penetrate into the local sites when given systemically [103]. Vancomycin itself is also commonly used for the coating of polypropylene meshes used for the repair of hernias to inhibit infections caused by bacteria [104]. The aminoglycoside gentamicin on the other side is also a very commonly used antibiotic for the local treatment after trauma or surgery like in collagen sponges [96], collagen sheets or PMMA beads to prevent implant-related infections [105], electrospun polylactide based fibers for wound dressings with modified release characteristics [106], PLA films that can be attached to the surface of an implant [107], cylindrical larger PLA implants to provide local bactericidal drug concentrations [108] as well as non resorbable polyvinylidenfluoride meshes for hernia repair with local drug release [109].

Vancomycin hydrochloride is a glycopeptide antibiotic for hard to treat cases of multiple drug resistant bacteria and is highly effective against gram positive bacteria like staphylococci and enterococci. Due to the fact that it is not absorbed when taken orally it is normally given parenterally, which explains the restricted application as a drug of last resort. Moreover, when given at high therapeutic doses it is ototoxic, which is why plasma concentrations have to be carefully monitored. Gentamicin sulphate on the other side is a wide-spectrum aminoglycoside antibiotic, which is highly effective against enterobacteria and staphylococci. Orally it is only given for local treatment of the intestine because it is also not resorbed due to its highly charged character. For other indications outside the intestine it also has to be applied parenterally in order to reach the side of action. Similar to vancomycin it is also ototoxic and nephrotoxic when it is given in high doses without careful management of the blood plasma levels [110]. A strictly local application of lower drug amounts instead of the systemic application of high doses consequently would strongly minimize the risk of toxic side effects and lead to improved therapy of the patient [94].

The aim of this study was to investigate the impact on the observable controlled drug release from differently composed thin polymer films by the choice of suitable drug substances, different hydrophilic or lipophilic polymer carriers as well as by different film preparation techniques. Two high molecular weight, hydrophilic and positively charged antibiotics, vancomycin hydrochloride and gentamicin sulphate were chosen as active drug components. Two positively charged drugs were used because of the consideration that an ionic interaction with the negatively charged alginate might be possible and consequently lead to a more prolonged drug release [111]. To furthermore vary the release of the hydrophilic drugs from the hydrophilic alginate, a more lipophilic PLA PEG PLA and a lipophilic PLA [107] were investigated in comparison, in order to see the potential impact of the hydrophilicity of the used polymer on the drug release. Due to the fact, that the hydrophilic drugs could not be dissolved in the organic solvents, which is necessary for the film preparation of the PLA PEG PLA films and PLA films, two different methods for the homogenous distribution of the drug in the film were investigated. When applying the suspension method, the accordingly micronized hydrophilic drugs were merely suspended in the polymer solution before the film preparation. For the cosolvens method, in contrast, the drugs were dissolved in a suitable cosolvens, namely methanol, before addition of a drug solution to the prepared polymer solution.

5.2 Drug loading of thin polymer films

5.2.1 Alginate films

To ensure that ionic interactions between the negatively charged alginate and the positively charged drugs are possible, the alginate films were prepared according to the previously described inner gelation technique (Chapter 4). By cross-linking the alginate film with a half maximum amount of calcium that can be bound by the guluronic and mannuronic acid blocks, enough acid blocks remain for the binding of the positively charged drug. More precisely, 0.75 g of high G alginate and 0.75 g glycerol were dissolved to 25 g in water. 100 mg vancomycin hydrochloride (Vancomycin CP 500 mg, Hikma Pharma GmbH, Gräfelfing, Germany) was added and dissolved in the aqueous polymer solution. To subsequently cross-link the film with the help of the inner gelation technique, 0.15 g $CaHPO_4$ suspended in some droplets of water and 0.6 g GDL powder were added. After homogenization the film was drawn on a glass plate with a gap clearance of 700 µm and a speed of 5 mm/ sec. To slow down the drying speed the film was stored in a closed box until it was completely clear. Subsequently it was taken out of the box and was allowed to dry at room temperature.

When gentamicin was given to the alginate solution, it did not dissolve but formed clusters instead. To load the films with gentamicin (Caesar & Loretz GmbH, Hilden, Germany) a gentamicin-complex with alginate was prepared before the drug loaded film preparation. More precisely, to a solution of 1.44 g gentamicin sulphate and 1.5 g glycerol in 47 g water, step by step 1.5 g high G alginate were added and stirred with a magnetic stirrer at maximum speed to obtain a homogeneous distribution. With the addition of alginate a coprecipitation of the two components but not an increase of the solution viscosity due to the addition of alginate could be observed. For a more homogenous distribution of the formed complex, the suspension was homogenized with the help of an ultra turrax and subsequently isolated from solution by filtration with the help of a Büchner funnel followed by washing steps with water. After subsequent lyophilisation the precipitate was milled with a CryoMill (Retsch GmbH, Haan, Germany) while cooled with liquid nitrogen to increase the brittleness of the drug-polymer precipitate. The obtained powder could subsequently be used for the drug loading of the alginate films without further precipitation or interaction with the added alginate. Therefore, 0.75 g glycerol was dissolved in 22.75 ml water. 250 mg

of the gentamicin-alginate-precipitate was added, while the solution was stirred at maximum speed with a magnetic stirrer. The final suspension was further homogenized with the help of an ultra turrax. Then 0.75 g high G alginate was added step by step under stirring at maximum speed to obtain a homogeneous distribution of the gentamicin-alginate-precipitate in the alginate solution. Subsequently this mixture was again homogenized with an ultra turrax again. For the cross-linking of the alginate chains, 0.15 g $CaHPO_4$ suspended in some droplets of water and 0.6 g GDL powder were added. After further homogenization the film was drawn on a glass plate with a gap clearance of 700 µm and a speed of 5 mm/ sec. The film was stored in a closed box to provide sufficient time for the dissolution of the calcium salt.

5.2.2 PLA PEG PLA and PLA films

For the drug loading of the films, prepared of the organic soluble polymers PLA PEG PLA, a lipophilic triblock copolymer of poly(lactic acid) and poly(ethylene glycol) [HWL73, PLA35kDa-PEG10kDa-PLA35kDa (poly(lactide)-b-poly(ethylene glycol)-b-poly(lactide))), Polymaterials, Kaufbeuren, Germany] as well as PLA [PLA (Resomer® LR708, Poly (L-lactide-co-D,L-lactide; 70:30), Boehringer Ingelheim, Ingelheim am Rhein, Germany)] , with the hydrophilic antibiotics two different manufacturing methods were performed to obtain films with a differently homogenous distribution of the drug.

Applying the “suspension method” the hydrophilic drugs were just suspended in the lipophilic polymer solution. For the PLA PEG PLA films, 1.5 g polymer was dissolved in 3 ml DCM. 100 mg vancomycin hydrochloride, respectively 100 mg gentamicin sulphate, was directly added after the addition of DCM. After the PLA PEG PLA was completely dissolved, the suspension was drawn on a glass plate coated with Sigmacote® with a gap clearance of 120 µm and a speed of 5 mm/ sec.

To further improve the even distribution of the drug, the films were additionally prepared with the “cosolvens method”. More precisely the antibiotics were dissolved in a suitable cosolvens, namely methanol before addition to the also prepared polymer solution in DCM.

The films of the higher molecular weight PLA were also loaded with the “suspension method” as well as the “cosolvens method”. For this purpose 1.2 g PLA were dissolved in 21.5 ml respectively 20.5 ml DCM when additional cosolvens was added. 100 mg of the

antibiotic were directly given to the mixture and suspended in DCM or dissolved in 1 ml methanol before addition to the polymer solution. After homogenization the films were drawn on a glass plate coated with Sigmacote® with a gap clearance of 700 µm and a speed of 5 mm/ sec.

5.2.3 Observations during the film preparation

After addition of vancomycin hydrochloride to the alginate solution, the solution became cloudy which might indicate a slight interaction between the positively charged drug and the negatively charged polymer. Upon further stirring the solution became clear again.

The drug loading with the up to triply charged gentamicin sulphate was much more complicated due to an immediate complex formation with the alginate. A drug loading just by adding the powder would have resulted in an inhomogeneous distribution of gentamicin in the film, because after the simple addition of gentamicin to the alginate solution precipitates were formed and the remaining alginate solution became visibly runnier, disallowing the correct drawing of the polymer films. Since longer stirring could also not dissolve the obtained drug-polymer precipitates, a complex of gentamicin and alginate was prepared separately and the final alginate solution was subsequently loaded with this complex.

Using the suspension method the drug was added to the mixture before the polymer was dissolved, because the even distribution in the low viscous DCM with the help of a magnetic stirrer was easier to obtain than later in a viscous polymer solution. While continuously stirring for several hours the polymer dissolved and the resulting drug suspension became more viscous.

The dry films prepared with the “suspension method” had a visibly rougher surface than the films prepared with the “cosolvens method”. The “cosolvens method” led to generally smaller drug crystals, with 65 °C methanol having a higher boiling point than DCM with 40 °C. After drawing the film on the glass plate, the DCM with a boiling point of 39.6 °C evaporated faster than the methanol with a boiling point of 64.7 °C. Therefore the polymer solution became more viscous and prevented a coagulation of the drug crystals that crystalized in the film with the subsequent evaporation of the methanol.

5.2.4 Investigation of the prepared polymer films

After drying at room temperature the thickness of all obtained films was measured with a microprocessor coating thickness gauge (MiniTest 650, ElektroPhysik Dr. Steingroever GmbH & Co. KG, Köln, Germany).

Table 12: Thickness of polymer films with vancomycin hydrochloride

polymer	placebo	drug loading method		
		dissolution	cosolvens method	suspension method
alginate	31-32	28-36		
PLA PEG PLA	22-23		17-24	19-24
PLA	18-22		18-20	21-25

Table 13: Thickness of polymer films with gentamicin sulfate

polymer	placebo	drug loading method		
		dissolution	cosolvens method	suspension method
alginate	31-32	34-37		
PLA PEG PLA	22-23		19-24	24-27
PLA	18-22		19-23	19-25

To visualize the size and distribution of the drug crystals or the precipitates of the drugs in the polymer films, light microscopic images with phase contrast were taken (Axio Imager.M1, Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Furthermore SEM images were obtained using a Digital Scanning Microscope DSM 940 (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) at an accelerating voltage of 5 kV after gold sputtering with an EMITECH K550 sputter coater (Quorum Technologies, West Sussex, United Kingdom) to investigate, if the formed crystals were generally covered by a thin polymer layer.

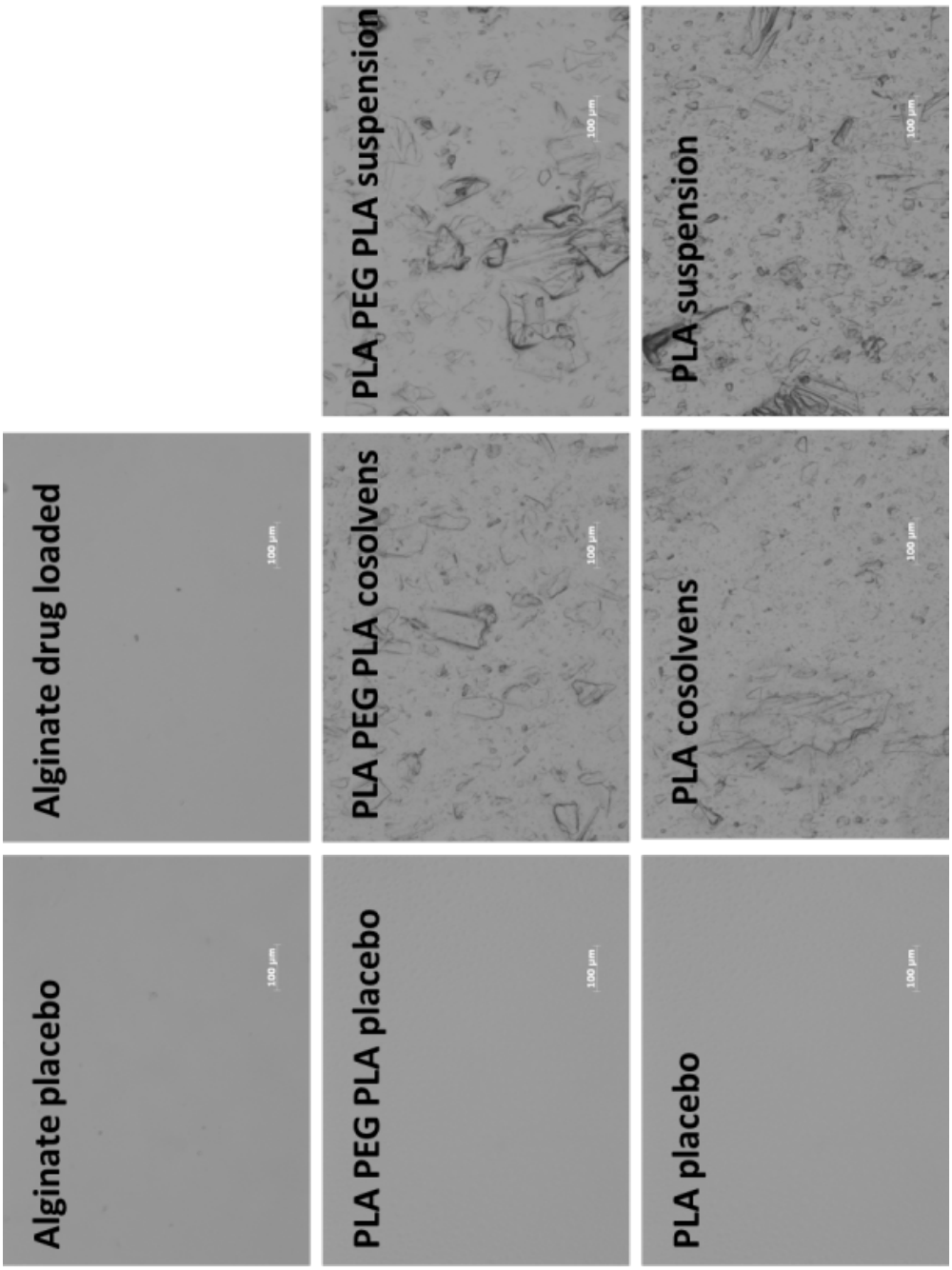


Figure 49: Light microscopic images of the vancomycin hydrochloride loaded polymer films

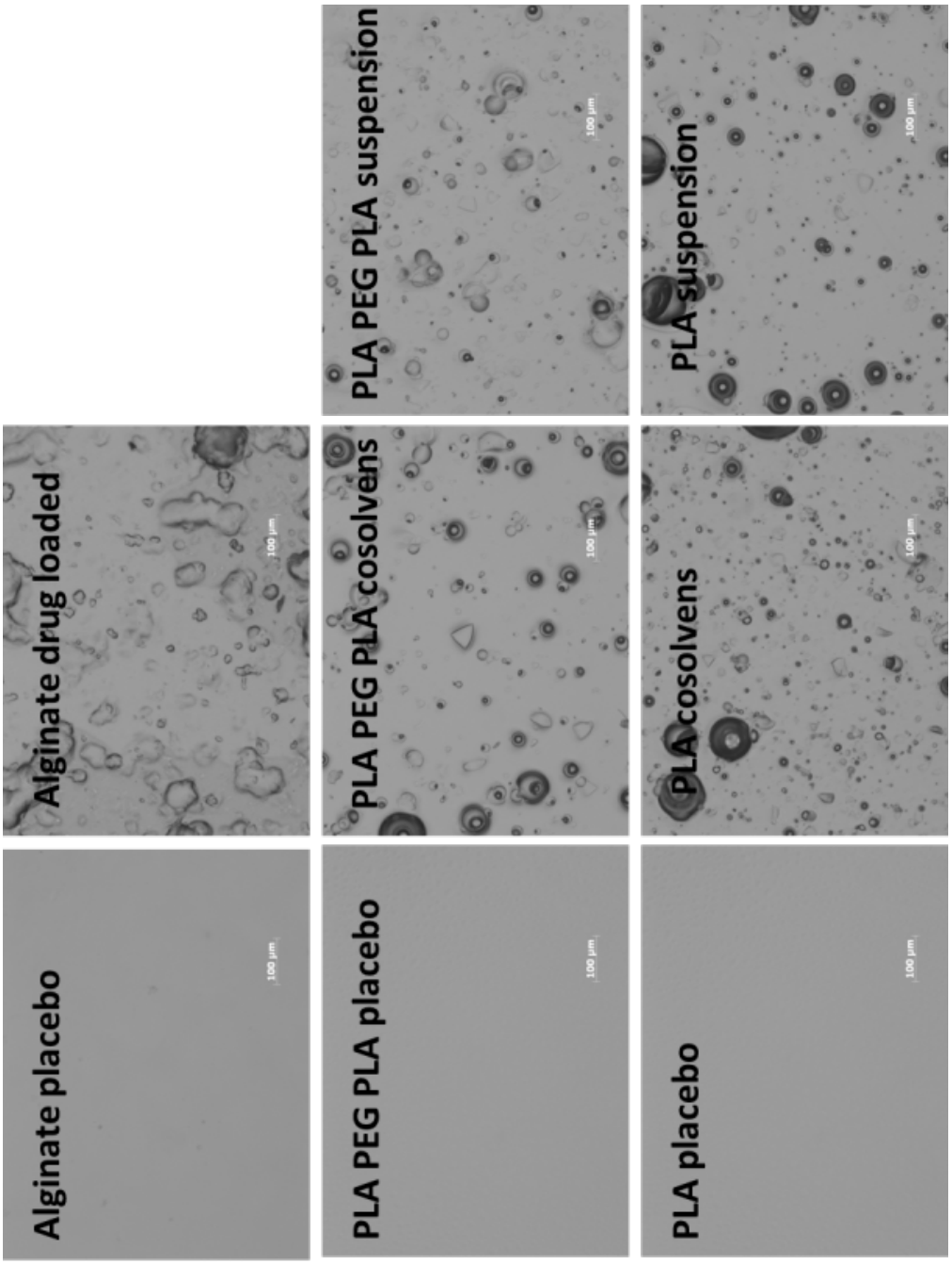


Figure 50: Light microscopic images of the gentamicin sulfate loaded polymer films

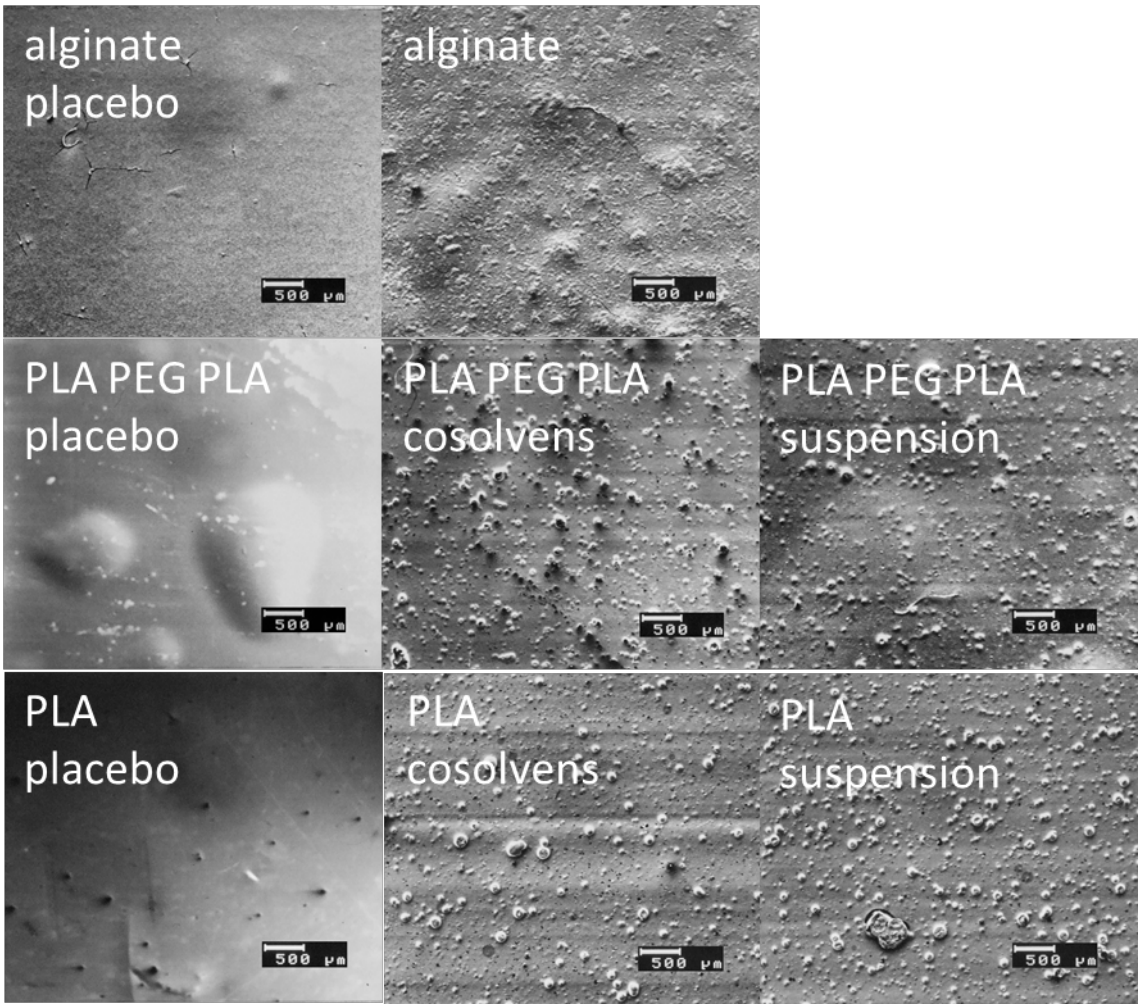


Figure 51: SEM images of vancomycin hydrochloride loaded polymer films

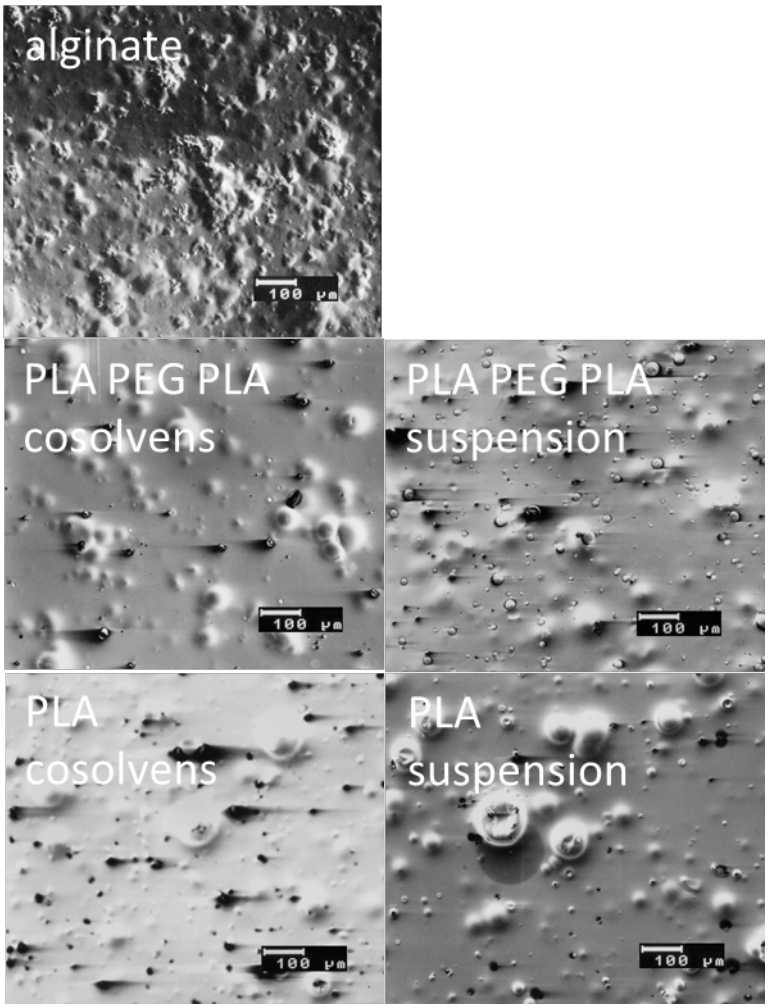


Figure 52: SEM images of gentamicin sulphate loaded polymer films

5.3 Release studies

The release studies were performed with round film discs with a diameter of 2 cm. The discs were incubated in glass vials with 10 ml HEPES buffer containing 1.2 mmol/l calcium and stored at 37 °C with 100 rpm shaking. At predetermined time intervals 1 ml buffer was removed for investigation and replaced with fresh buffer. The absorption of vancomycin could be measured with a UV-VIS plate reader (Tecan infinite® 200, Tecan Group Ldt., Männedorf, Switzerland) at an absorption wavelength of 280 nm. Due to the fact, that gentamicin is not detectable with an UV-VIS detector because of its low absorption maximum; further derivatisation with phthaldialdehyde was performed. For this purpose 0.2 g o-phthaldialdehyde were dissolved in 1 ml methanol. 19 ml of a 0.4 M boric buffer with pH 10.4 and 0.4 ml 2-mercaptoethanol were added. The pH of the solution was adjusted with potassium hydroxide solution to pH 10.4. This solution was diluted with buffer 1 to 9 for further use. Before the measurement, 80 µl of the release sample were mixed with 48 µl methanol and 72 µl of the prepared derivatization solution. The resulting absorption was measured after 15 minutes incubation using the same UV-VIS plate reader at a wavelength of 332 nm.

At the end of the release study the whole buffer was removed from the vials and the remaining polymer discs were lyophilized for further analysis. After lyophilization the discs respectively disc fragments of the organic soluble polymer (PLA PEG PLA or PLA) were dissolved in 2 ml 1 N NaOH over night at 37 °C. Before measuring the absorption of the residual drug amount the solutions were neutralized with 1.85 ml HCl and diluted with 6.15 ml HEPES buffer.

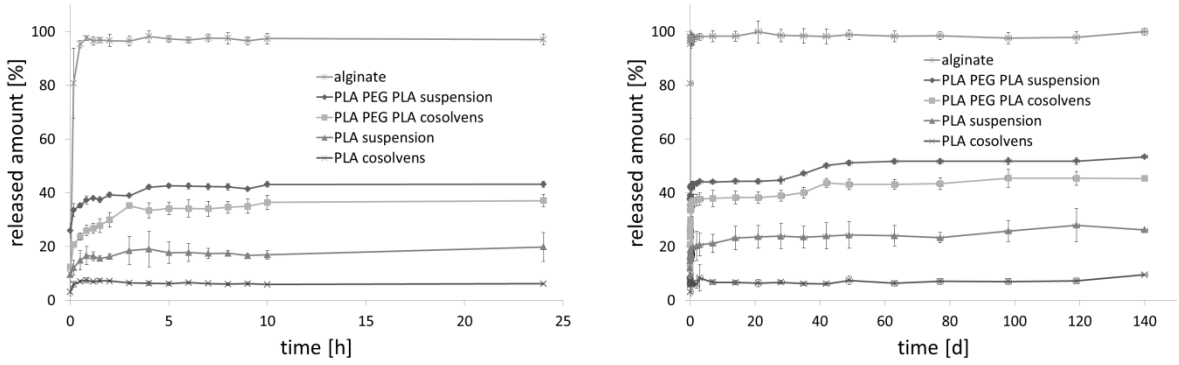


Figure 53: Release profiles of vancomycin hydrochloride (short and long term release)

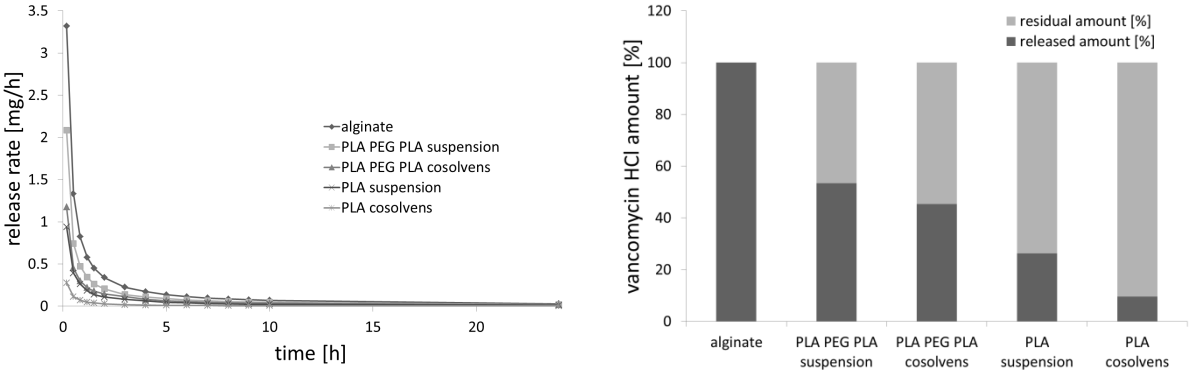


Figure 54: Release rates and overall released amount of vancomycin hydrochloride after 24 hours and 140 days

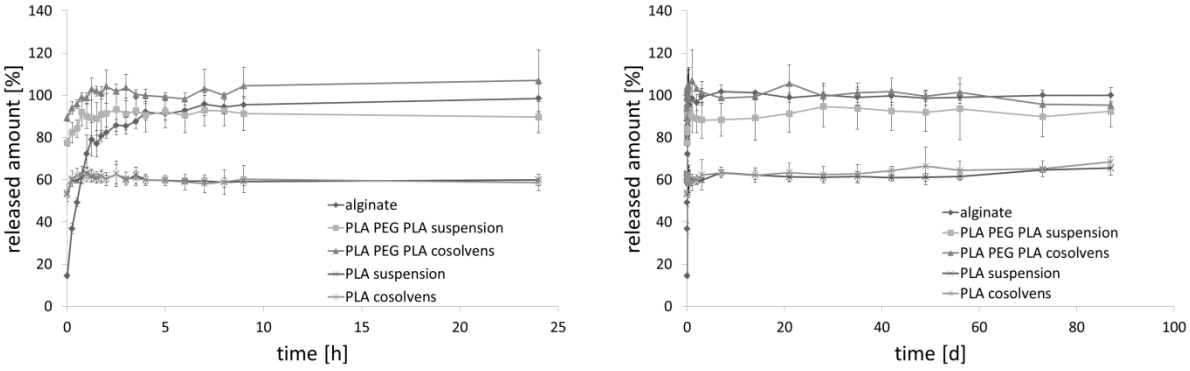


Figure 55: Release profiles of gentamicin sulfate (short and long term release)

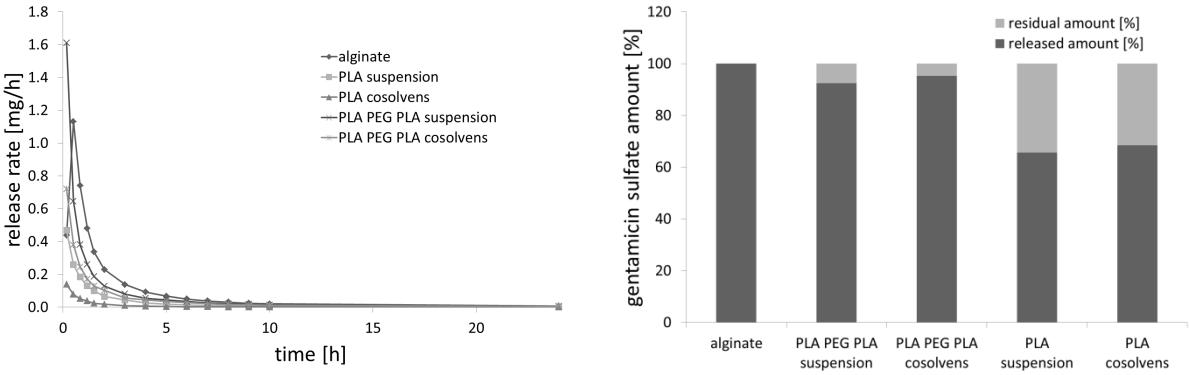


Figure 56: Release rates and overall released amount of gentamicin sulfate after 24 hours and 90 days.

5.4 Water uptake of the different polymer films

The main factor that influences the drug release from a polymer matrix is the uptake of water and the resulting possibility to dissolve and liberate the drug from the matrix. Therefore the swelling of the different polymer films was investigated for several days. Three discs with a diameter of 2 cm made from polymer films without drug were stored in HEPES buffer containing 1.2 mmol/l calcium. Every day the wet weight (wt) of the polymer film discs was determined to calculate the swelling ratio (wt/w0) of the polymer film by dividing the wet weight through the initial weight (w0).

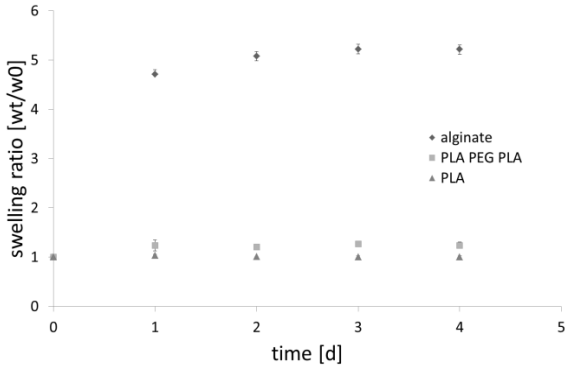


Figure 57: Swelling ratio of the different polymer films stored in buffer.

5.5 Biological activity of released drug tested via microbiological testing

To test the biological activity of the drug loaded polymer films microbiological tests were performed using different bacteria inoculated petri dishes. For these testing typical bacteria that may lead to an infection after peritoneal surgery were chosen, including escherichia coli, staphylococcus aureus and staphylococcus epidermidis.

The microbiological studies of the drug loaded polymer films were performed using a modified agar diffusion test. Three drug loaded film discs with a diameter of 1 cm were put on top of a filter paper on an agar plate inoculated with the respective bacteria, as control a film disc without the investigated drugs was also placed onto the plate. After incubation for 24 hours at 37 °C the zones of inhibition around the polymer discs were measured. With the help of the additional filter paper for stabilizing the polymer films, particularly the alginate films, the discs were replaced onto a freshly inoculated agar plate with the same stem of

bacteria. This procedure was repeated daily until no inhibition zone was detectable any more.

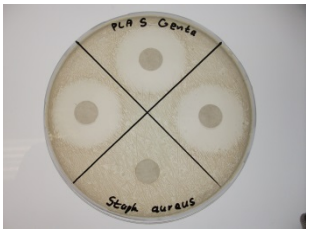
	Vancomycin hydrochloride	Gentamicin sulfate
Alginate		
PLA PEG PLA Cosolvens		
PLA PEG PLA Suspension		
PLA Cosolvens		
PLA Suspension		

Figure 58: Zones of inhibition after 24 hours of incubation on agar plates inoculated with staphylococcus aureus

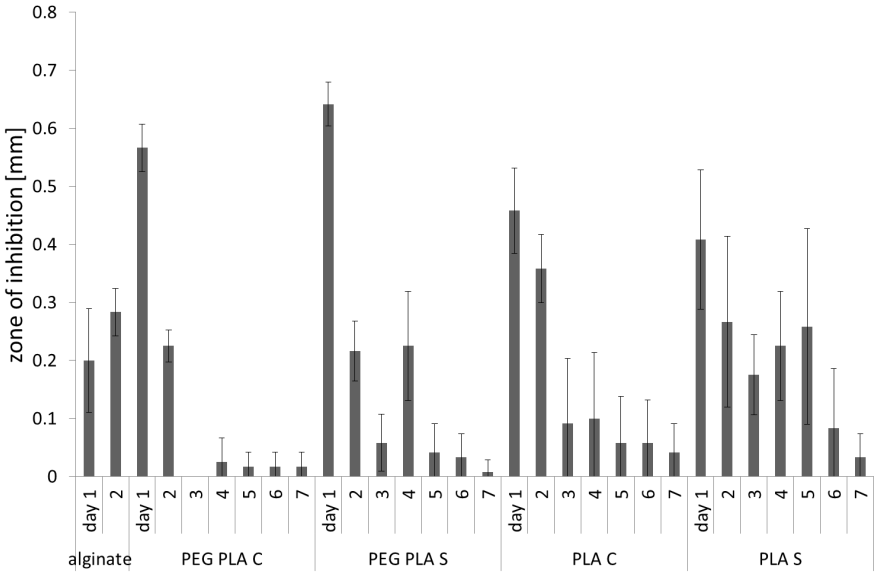


Figure 59: Zones of inhibition gentamicin-sulfate loaded polymer film discs on agar plates inoculated with escherichia coli

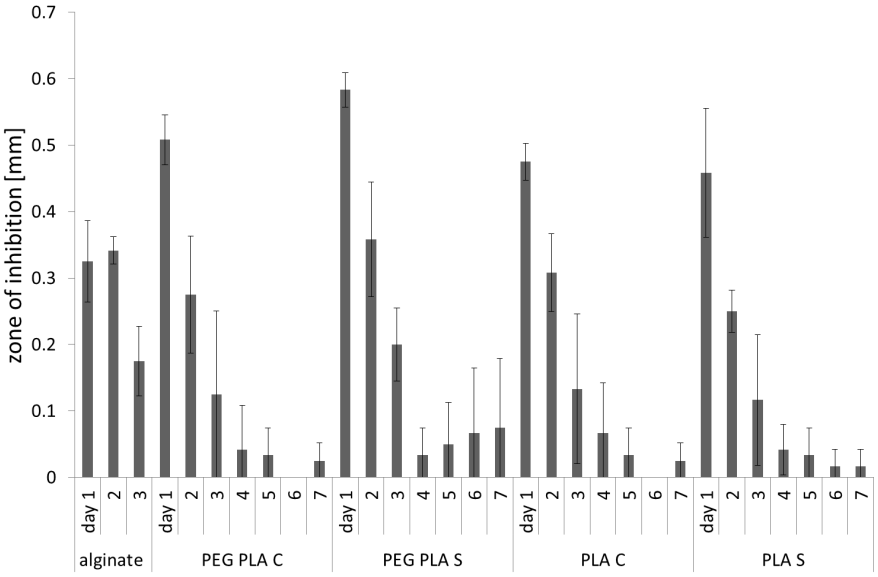


Figure 60: Zones of inhibition gentamicin-sulfate loaded polymer film discs on agar plates inoculated with staphylococcus epidermidis

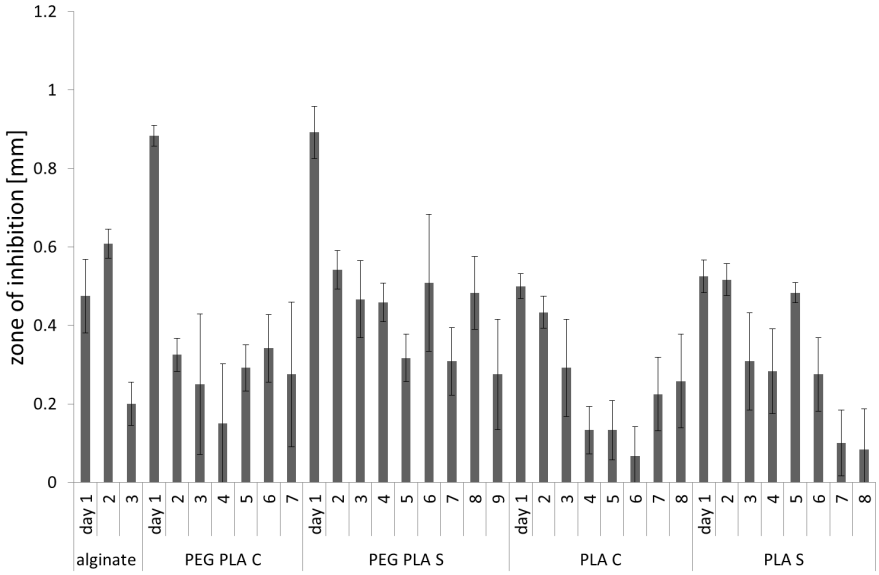


Figure 61: Zones of inhibition gentamicin-sulfate loaded polymer film discs on agar plates inoculated with staphylococcus aureus

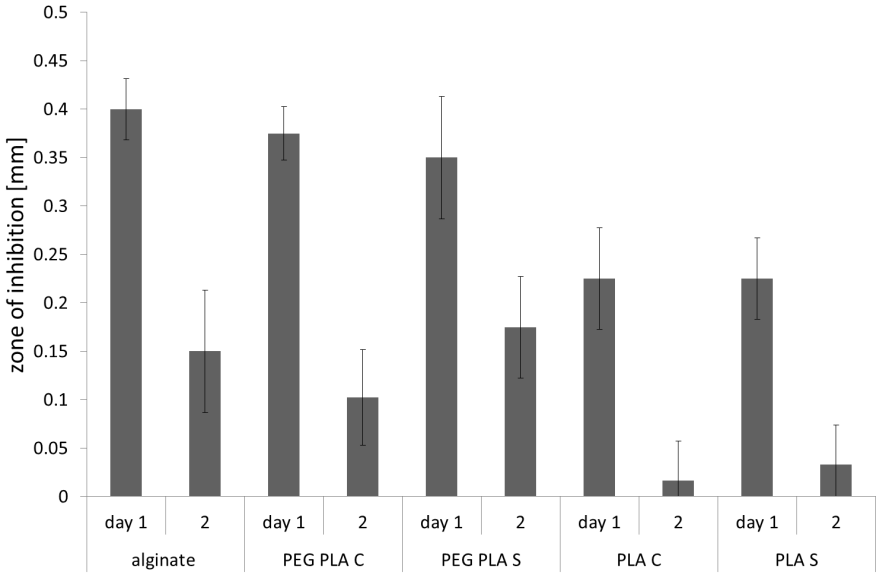


Figure 62: Zones of inhibition vancomycin-hydrochloride loaded polymer film discs on agar plates inoculated with staphylococcus aureus

5.6 Results and Discussion

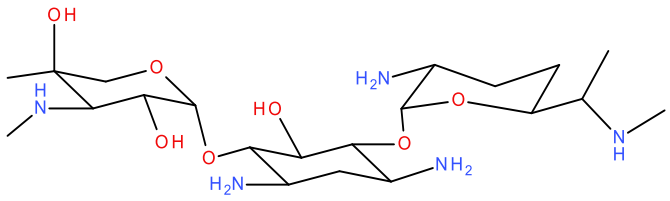
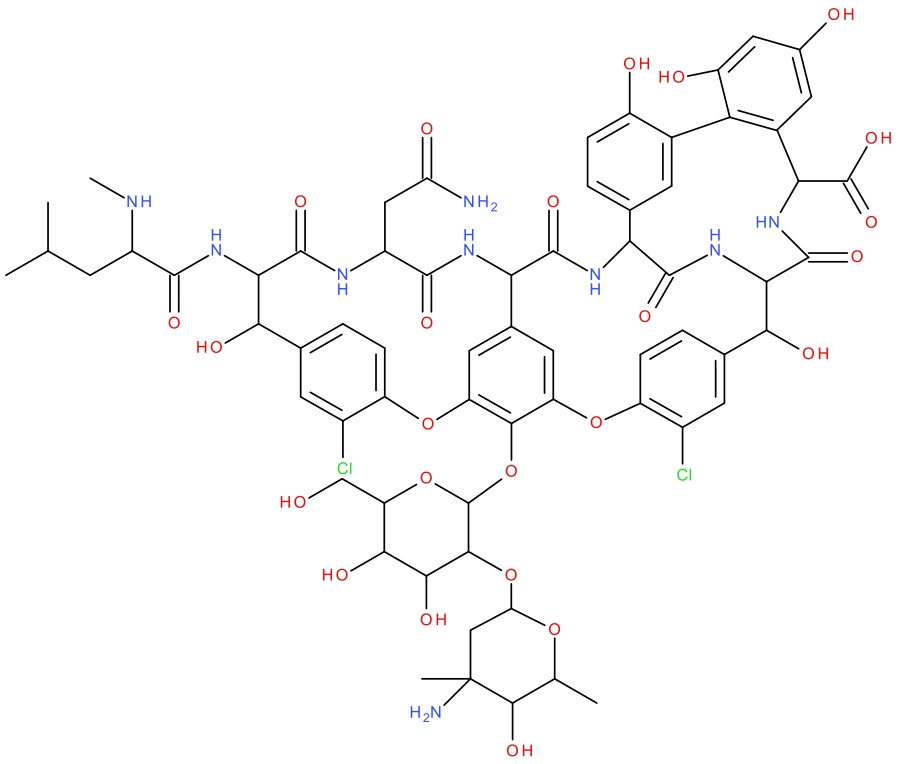
Gentamicin	
Vancomicin	

Figure 63: Molecular structures of the used drug components

In the hydrophilic alginate film the hydrophilic antibiotic vancomycin hydrochloride was completely dissolved during the film preparation (figures 49 + 51). The microscopic image showed no crystals of the drug in the alginate film. However the SEM images showed some particles on top of the alginate film which might be complexes of vancomycin and alginate.

The alginate films loaded with gentamicin showed the precipitates of gentamicin and alginate in the microscopic image as well as in the SEM image, because these bigger solid pieces were also reaching out of the films.

Regarding the lipophilic films, the hydrophilic drugs were not dissolved in the polymer. The cosolvens method (c) led to a more homogenous distribution of the drug with smaller crystals than the suspension method (s) (figures 51 + 52). The microscopic images revealed

the distribution of the drug in the different polymer films after drug loading with different methods (figures 49 + 50). In the films made of PLA PEG PLA and PLA crystals of the drug were visible in the microscopic images as well as in the SEM images. In the microscopic images a difference between the drug loading with the suspension method and the cosolvens method could be observed (figures 49 + 50). With the cosolvens method the drug was initially dissolved in methanol before addition to the polymer solution and again slowly crystalized during the drying process after the film was drawn on a glass plate. This consequently led to the formation of much smaller crystals. The SEM images showed that the vancomycin crystals in the lipophilic films were more edgy than the gentamicin crystals and seem to stick out of the film, while the surface of the lipophilic polymer films loaded with gentamicin seem to be smoother with rounder crystals (figures 51 + 52).

The release of vancomycin hydrochloride exhibited a large burst release from the hydrophilic alginate film, releasing 100 % of the drug load within the first 24 hours (figure 53). The lipophilic polymers however showed a much smaller initial drug release, because only the drug crystals that reached out of the polymer films got dissolved in the release medium (figure 53). Due to the limited amounts of water, which could permeate into the lipophilic films, only a partial release of vancomycin hydrochloride could be achieved during the time of investigation (figure 54). The films made of the slightly more hydrophilic PLA PEG PLA showed a release of 50 % after 140 days for the film loaded with the suspension method and 45 % for the film loaded with the cosolvens method. This trend was observed in all cases, since the cosolvens method led to slightly smaller crystals that did not reach out of the polymer film and therefore were not dissolved (figure 54).

For the additionally investigated long term release, the PLA PEG PLA films partially started to fragment after about 40 days, which was observed visually and additionally accompanied with a slight increase of the released drug amount (figure 53). This observation can explain the creation of new surfaces and accordingly the exposing of new drug crystals, which now get in contact with the release medium and subsequently get dissolved. Till the end of investigation these obtained fragments almost stayed in place and since the film did not further dissolve a complete drug release was also not obtained for this polymer. As expected, the most lipophilic PLA film showed the lowest overall drug release. For the film prepared via the suspension method only 26 % of the vancomycin hydrochloride were

released and from the films prepared via the cosolvens method only about 10 % (figure 54) during the time of investigation. For the PLA film the same trend was observed, that the films prepared with the suspension method led to bigger crystals showing a higher absolute released amount than the film prepared via the cosolvens method. However, since the higher molecular weight PLA films did not fragment during the investigation time, no further drug release could be observed after the initially released drug most likely being in contact with the release medium.

With the gentamicin-alginate complex a slightly slower release from the hydrophilic alginate film was measured especially on the first time point due to the ionic interactions that slowed down the release (figures 55+56). Despite the possible ionic interactions and the initial complex formation, 100 % of the gentamicin was released from the hydrophilic alginate film (figures 55+56). The PLA PEG PLA film showed also a very high drug release of 92 % and 95 % (figures 55 + 56). But from all these films the drug release can be considered as immediate burst release. The PLA PEG PLA suspension film had released 92 % of the gentamicin after 4 hours and the PLA PEG PLA cosolvens film even had a much a faster release of about 2 hours (figure 56). Different from the films loaded with vancomycin hydrochloride in this case the drug release from the films prepared with the cosolvens method had a slightly higher release than the film prepared with the suspension method (figure 55). This tendency could be observed with the PLA films, too. The PLA suspension film had with a release of 65 % also a slightly lower release than the PLA cosolvens film with a release of 68 % (figure 55). Both films also showed a burst release and reached 60 % after 2 hours.

Furthermore the lipophilic character, the molecular weight and size of the drug itself may play an important role in the drug release. Vancomycin is much larger and more lipophilic than gentamicin (figure 63). On one hand, this can explain the stronger ionic interactions between the smaller but higher protonated gentamicin, which can consequently interact stronger with the guluronic acid blocks. On the other hand, it also explains the much better solubility of vancomycin in the lipophilic PLA and the high residual drug amount after the investigated dissolution time.

The release rate of vancomycin hydrochloride which was higher than the release rate of gentamicin sulfate could be explained with the existence of some sharp crystals that reached out of the film and were dissolved in the beginning of the release study (figures 54 + 56).

Furthermore the ionic interaction between the negatively charged alginate and the positively charged gentamicin (figure 55), that could not be seen for vancomycin could explain these results.

As expected, the swelling ratio or the water uptake of the hydrophilic alginate film was much higher than for the PLA PEG PLA and PLA films, which are not soluble in hydrophilic solvents (figure 57). Due to the copolymerized water soluble PEG, the swelling ratio of the slightly more hydrophilic PLA PEG PLA was higher than for PLA. Nevertheless while the alginate films increase their wet weight more than 4.2 times after 4 days, the water uptake of the PLA PEG PLA film was only about 30 % of its initial weight and for the pure PLA film nearly no swelling could be observed with in the 4 days investigation period. This will most likely only start to occur once hydrophilic acid functions are created upon degradation the material. These values correlate well with the released amounts of vancomycin hydrochloride, where the lower water uptake of the films also resulted in the lower absolute release of the drug component.

The stepwise investigated microbiological evaluation of the antibiotics loaded polymer films showed that the gentamicin sulfate loaded alginate films were only biologically active against the tested bacteria for the first 2 days of the study. The lipophilic films however were active for 7 days and in the case of staphylococcus aureus even for 8 or 9 days. Here the observed zones of inhibition of the PLA PEG PLA films were generally larger than for the PLA film, which also indicates a slightly enhanced release from the slightly more hydrophilic polymer. The smaller inhibition zones of the alginate films on the other hand can be attributed to the significantly stronger retention of the charged alginate in the much dryer agar than the buffer during the release studies. Regarding the zones of inhibition of the PLA PEG PLA films it can be observed, that the films prepared via the suspension method had lager zones of inhibition than the films prepared via the cosolvens method. The vancomycin hydrochloride showed due to its spectrum of efficacy only zones of inhibition for staphylococcus aureus. Due to the fact, that on the agar plates only small amounts of water were available for the drug release from the polymer films, the drug release and consequently the biological activity was much lower than during the release study in buffer. For a long term study of biological activity it furthermore has to be kept in mind that for

vancomycin larger amounts of the drug will stay in the lipophilic films, which will be released at a later time point once the films break apart.

5.7 Summary and Conclusion

In current clinical practice the initial dose of gentamicin is 1.5 – 2 mg/kg/day is given without regarding the renal function. Then the single dose and the interval have to be adjusted to the renal function [110]. Vancomicin is generally given with a dose of 2 g per day, also using a systemic application. This finally also has to be adjusted to the function of the patient's kidneys [110]. The ototoxic and nephrotoxic side effects of these antibiotics can obviously be weakened by applying drug loaded films directly to the wound site instead of giving the antibiotic systemically. This is why release kinetics were evaluated for differently prepared film based release systems.

Using different polymers with different hydrophobicity the drug release of the hydrophilic antibiotics vancomycin hydrochloride and gentamicin sulfate could be influenced and controlled to a certain extend also from very thin polymer carriers of about 20 µm thickness. In the case of gentamicin sulfate the positive charge of the drug and the negative charge of the alginate led to only a slightly slower drug release due to ionic interactions, but still 100 % was released from the polymer films. The hydrophilic properties of the chosen polymer also played a very important role for the release of a hydrophilic drug into a hydrophilic release medium. This effect was very distinctive for the release of vancomycin hydrochloride. With the hydrophilic alginate a burst release of almost 100 % was observed. With the more lipophilic PLA PEG PLA the drug release decreased to more or less 50 % and from the lipophilic PLA film only 30 % respectively 7 % could be released from the polymer film depending on the preparation technique. Furthermore it could be seen that a fragmentation of the polymer films accompanied with the exposure of new drug crystals can lead to a further progressing drug release, which would also be an issue during an in-vivo application in a moving animal or patient.

Consequently a combination of the differently composed materials or layers could help for the preparation of films resulting in different release kinetics. Even the preparation method regarding the suspension method and the cosolvens method strongly influenced the size and distribution of the drug crystals and therefore the later observed drug release. Using the

suspension method a faster release can be obtained with bigger drug crystals, which reach out of the film more effectively and therefore can be dissolved significantly faster. The ionic interactions between the positively charged drug and the negatively charged alginate were more distinct for gentamicin than for vancomycin, which is most likely attributed to the higher charging possibility and the multivalent ionic binding to the polymer.

Regarding the release studies and the microbiological testing an effective way for the prevention of bacterial growth after surgery would be a more sophisticated combination of differently composed polymer films. However a more in-vivo like evaluation also seems to be necessary in order to better predict the resulting release profiles. For the time directly after surgery a burst release from an alginate film and subsequently a more continuous release for several days from another polymer layer like PLA PEG PLA would probably be most likely best effective to provide sufficient levels of antibiotic drugs. Furthermore a combination with a lipophilic film loaded with an antibiotic drug like triclosan, which is known to be soluble in lipophilic polymers [112], could serve as a promising alternative for long term applications.

6. Thiolated hyaluronic acid

This chapter deals with the preparation of films made of hyaluronic acid cross-linked chemically via disulfide bridges using thiofunctionalization.

6.1 Introduction

Hyaluronic acid is a very well established biopolymer used for the prevention of post-surgical adhesions. It is a linear polysaccharide consisting of the uronic acid D-glucuronic acid and the amino sugar N-acetyl-D-glucosamine, arranged in alternating units linked by β1-3 and β1-4 linkages [113]. There are several products available on the marked which contain hyaluronic acid as a fluid [114] such as Sepracoat® made of sodium hyaluronate, a gel like Intergel® [115] made of ferric hyaluronate where the carboxylate groups of the hyaluronic acid are cross-linked due to the chelation with ferric ions (Fe^{3+}), INCERT®-S [33,116] made of chemically cross-linked hyaluronic acid, respectively Hyalobarrier an auto-cross-linked viscous gel [117,118]. Also films prepared of hyaluronic acid alone or in combination with other polymers like carboxymethylcellulose [119] in Seprafilm® [120,121] where the two polymers have been cross-linked with the help of the activating agent N-(3-Dimethyl-aminopropyl)-N`-ethylcarbodiimide hydrochloride (EDC) (see package insert) or Sepragel® [33,122,123] are very promising.

To prevent the formation of peritoneal adhesions effectively it is necessary to have an appropriate separation of the injured tissue during the time of healing. Hyaluronic acid without chemical modification is a very hydrophilic polymer that, given on top of a wound in the peritoneum would be dissolved and washed away faster than the wound would take to heal. To prolong the persistence time of the hyaluronic acid an appropriate modification via cross-linking by chemical means for example with glutaraldehyde [124] or newly introduced disulfide bonds using carbodiimide chemistry [35] has to be performed.

The aim of this work was to prepare thin films made of cross-linked hyaluronic acid that can be applied to the wounded tissue as a cross-linked film that is evenly thick and has a homogenous distribution of cross-linking over the whole film. The formation of a hemiacetal using glutaraldehyde was not considered for the cross-linking because glutaraldehyde is toxic [124] and further purification steps would be necessary before the modified hyaluronic acid could be implanted. Therefore the non-toxic cross-linking with disulfide bonds was chosen. In literature the preparation of thiolated hyaluronic acid is described as a very well established, controllable procedure [125]. Using carbodiimide chemistry, disulfide containing dihydrazides can be linked to the hyaluronic acid chain [35]. By controlling the substitution degree, the cross-linking extent of the films prepared from the thiolated hyaluronic acid can

be controlled. After film preparation the free thiol groups of the cleaved dihydrazides can cross-link by air oxidation to disulfides. The cross-linking extent of the prepared film can be influenced by addition of oxidizing agents like H_2O_2 or alloxan [126]. In previous investigations films have been prepared by dissolving the thiolated hyaluronic acid in buffer and pouring it into a petri dish. After drying the films were further oxidized with H_2O_2 [35]. To obtain very thin and homogenously thick films the films of this study were accurately drawn onto a glass plate with the help of a drawing apparatus using a frame with a defined gap clearance. To adapt the viscosity of the solution made for the film preparation, mechanical properties, the swelling behaviors as well as the erosion time of the films hyaluronic acids with different chain length were utilized. Furthermore the hyaluronic acids were modified with different degrees of substitution to influence the degree of cross-linking in the prepared films.

6.2 Synthesis of thiolated hyaluronic acid

Thiol-modified hyaluronic acids with a high substitution degree (high SD) and a low substitution degree (low SD) were prepared using standard carbodiimide-chemistry [35]. To obtain thiolated hyaluronic acids with different molecular masses a hyaluronic acid sodium salt with a molecular mass of 1.36 MDa (HA, BaccaraRose, Alpen, Germany) was used as received or the polysaccharide was partially hydrolyzed prior to thiol modification.

For the hydrolysis 40 g of the 1.36 MDa hyaluronic acid (native hyaluronic acid) were dissolved in 4 liters deionized water acidified with hydrochloric acid to pH 0.5 and stirred for 24 hours at 37 °C with a magnetic stirrer at 250-300 rpm. After 24 hours the solution was neutralized to pH 7 with 1 N NaOH, purified from hydrolyzed breakdown products via dialysis using a Mw cutoff of 3.5 kDa and subsequently lyophilized. To determine the molecular weight of the resulting hydrolyzed hyaluronic acid a GPC setup consisting of a Viscotek TDAmax SEC/GPC-System and SEC-MALS 20 Detector (Malvern Instruments, Herrenberg, Germany) was used. As stationary phase two ViscoGEL A6000M columns (Malvern Instruments) were chosen. The mobile phase was water with 0.01 mol/ l $NaNO_3$. The measurement was performed with a flow rate of 0.7 ml/ min at 35 °C. GPC measurements revealed a weight average molecular mass of approximately 143 kDa after hydrolysis (hydrolyzed hyaluronic acid).

Table 14: hydrolyzed hyaluronic acid: Weighted amounts [g] auf substances in the reaction mixture and calculated amounts [%] based on molecular weights of monomers

	g	mmol	EQ	g	mmol	EQ
HA	5	12.46	1	5	12.46	1
DTP	5.95	49.93	4.01	1.04	8.73	0.70
EDC	4.8	25.04	2.01	0.84	4.38	0.35
DTT	25	162.07	13.01	5	32.41	2.60

Table 15: Native hyaluronic acid: Weighted amounts [g] auf substances in the reaction mixture and calculated amounts [%] based on molecular weights of monomers

	g	mmol	EQ	g	mmol	EQ
HA	10	24.92	1	10	24.92	1
DTP	4.89	41.04	1.65	2.45	20.56	0.83
EDC	6.56	34.22	1.37	3.28	17.11	0.69
DTT	9.5	61.59	2.47	4.75	30.79	1.24

For the first synthesis the amounts used by Shu et al [35] were taken to obtain a hyaluronic acid with a hydrolyzed and a degree of substitution of 100 %. In this trial the hyaluronic acid was activated with a double molar excess of EDC. DTP although having two binding sites also was added in twice the necessary amounts. To see, if a large excess is necessary for this reaction a second approach with reduced amounts was performed in order to obtain a degree of substitution of more or less 25 %. For the native hyaluronic acid the amounts were adapted to obtain extrapolated degrees of substitution of 100 % and 50 %.

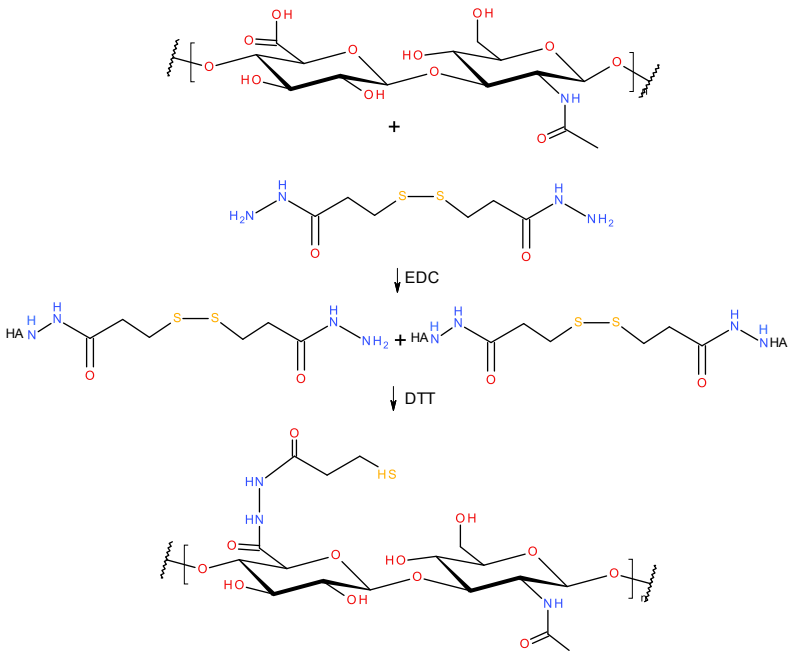


Figure 64: Thiolation of HA

Hyaluronic acid was activated with water soluble N-(3-Dimethyl-aminopropyl)-N`-ethylcarbodiimide hydrochloride (EDC, Merck Schuchard OHG, Hohenbrunn, Germany) at pH 4 to be able to react with the added Dithiopropionic acid dihydrazide (DTP, Arkè Organics, Fornacette Pl, Italy). After stirring for four hours at room temperature, the reaction was stopped by neutralization. At pH 8 the disulfide bonds of the hydrazide were cleaved with the help of 1,4-Dithiothreitol (DTT, BioChemica, AppliChem GmbH Darmstadt, Germany). After 24 hours the pH was again lowered to 3 to prevent the reformation of disulfide bonds during dialysis. The reaction mixture was subsequently dialyzed against water of pH 3-4 using a membrane with a Mw cutoff of 3.5 kDa. After dialysis the thiolated hyaluronic acid was centrifuged and the supernatant was subsequently lyophilized and finally stored in an oxygen free atmosphere to prevent the oxidation and cross-linking of the thiolated hyaluronic acid before further use.

6.3 Characterization of thiolated hyaluronic acid

6.3.1 NMR measurements

^{1}H NMR spectroscopy (300 MHz) of the synthesized polymers was performed in D_2O with a Fourier 300 NMR (Bruker Corporation, Billerica, MA, USA).

6.3.2 Ellmans assay

To quantify the thiol groups in the thiolated hyaluronic acid an Ellmans assay was performed. For this purpose an Ellmans reagent solution was freshly prepared by dissolving 4 mg Ellmans reagent ((5,5`- dithiobis-(2-nitrobenzoic acid)) in 1 ml of 40 mM PBS. To generate a standard curve a 0.2 mM cysteine stock solution in water was prepared and serially diluted from 0.2- 0.04 mM. Defined amounts of thiolated hyaluronic acid were weighted in oxygen free atmosphere. Directly after exposure to air they were dissolved in water. For the measurement 1250 µl 40 mM PBS were given into an Eppendorf cup. 125 µl of the sample solution were added and mixed with 25 µl of the freshly prepared Ellmans reagent solution. After 15 minutes incubation time at room temperature the absorbance was measured at 412 nm. The absorbance versus cysteine concentration was plotted for each of the standards and the thiol content was determined by comparison to the standard curve.

6.3.3 Elementary analysis

Elementary analysis was performed with a vario MICRO cube system (Elementar Analysensysteme GmbH, Hanau, Germany).

6.3.4 Oscillation Rheology - Time Sweep

The formation of disulfide bonds of the thiolated hyaluronic acid needs oxidizing agents and therefore can take place in air due to the available oxygen. During the measurement with a rheometer no air and therefore no oxygen from the air is available for this purpose. Nevertheless the speed of cross-linking of the as prepared polymer solutions was determined using oscillation rheology to get an idea of the cross-linking speed of the material, which is of utmost importance for the later film drawing procedure on glass plates. The thiolated hyaluronic acid, weighed in the oxygen free atmosphere of storage, was subsequently dissolved in regular phosphate buffer containing oxygen. After adjusting the

pH to 7.4, the HA-SH solutions were tested with a 50 mm plate geometry and a 1000 µm gap at 20 °C (MCR 301 Rheometer, Anton Paar GmbH, Graz, Austria). Oscillation frequency was set to 1 Hz with an applied strain of 10 %. The evolution of storage and loss modulus was followed with time their crossing was considered as gel point of this setup and was calculated with the help of the RHEOPLUS software.

6.3.5 Confined compression test

To be able to monitor the cross-linking in direct contact with air or other oxidizing conditions, a modified gel compression test was established. 5 % solutions of the hydrolyzed thiolated hyaluronic acids were prepared in PBS. The pH was adjusted to 7.4 before 200 µl/ well were poured into each well of a 96 well plate. Every hour a piston with a diameter of 4 mm was pushed 2 mm into the gel with a speed of 0.5 mm/ sec (according to Bloom test Ph. Eur. 6.0) after a preload of 0.01 N was reached. If the gel was not cross-linked and the preload did not reach 0.01 N, the test was stopped by reaching the soft end.

To test, if oxidizing agents like alloxan or hydrogen peroxide (HOOH) would speed up the cross-linking the test was repeated with 3 % solutions of the native hyaluronic acids in PBS. After the pH was adjusted to 7.4, 150 µl/ well of the HA-SH solution were poured into the wells of a 96 well plate. To enhance the cross-linking 20 µl of 3 % H_2O_2 solution respectively 20 µl of a freshly prepared 1 M alloxan solution was given on top of the hyaluronic acid solution. The compression test was started, without further mixing of the oxidizing agents with the hyaluronic acid solution. Every day a Piston with a diameter of 4 mm was pushed into the gels with a speed of 0.5 mm/ sec after a preload of 0.01 N was reached.

6.3.6 State of knowledge after characterization

During the reaction steps of the synthesis of thiolated hyaluronic acid characteristic changes of the reaction mixture could be observed. After addition of the dihydrazide (DTP) the reaction mixture became very viscous, which indicated that the reaction was progressing. Due to the fact that the dihydrazide can attach to the hyaluronic acid with both hydrazide ends, an immediate gel formation can also occur. Stirring with a magnetic stirrer to obtain a homogenously mixed polymer solution was consequently no longer possible. Therefore the mixture was stirred by hand from time to time. But this could not guarantee a complete reaction of both sides of the dihydrazide, because steric interactions might lead to the

reaction of only one side of the dihydrazide and the other side will get lost after cleavage and dialysis steps. After the addition of DTT the mixture became liquid again due to the cleavage of the disulfide bonds in the dihydrazide. Only the bound part of the dihydrazide, which was linked to the hyaluronic acid will be able to form disulfide bonds with the other remaining thiols after film preparation. For the native hyaluronic acid a formation of much stronger gels was expected. Therefore the reaction mixture was stirred with the help of a sealed precision glass stirrer directly from the beginning. In this case the steric interactions were higher than with the hydrolyzed hyaluronic acid, which led to a higher loss of dihydrazide residues during dialysis.

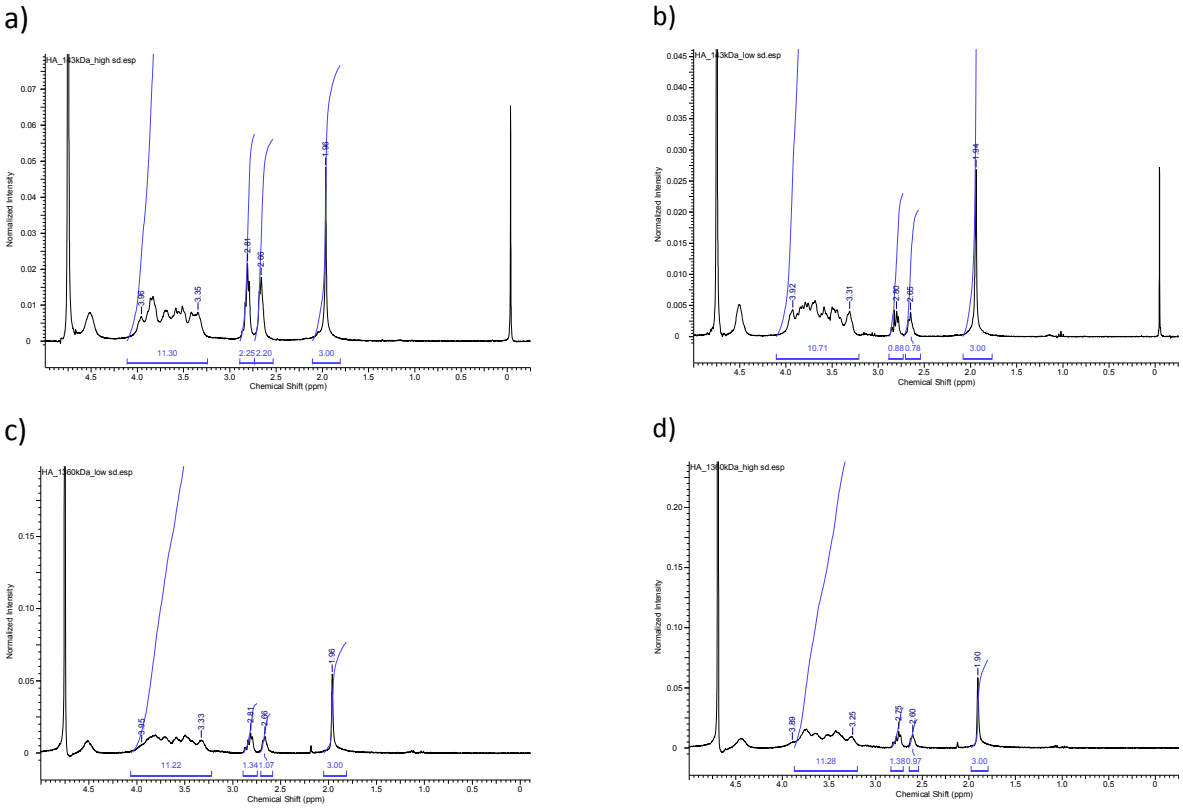


Figure 65: a) hydrolyzed HA high SD, b) hydrolyzed HA low SD, c) native HA high SD, d) native HA low SD

The degree of substitution was calculated by dividing the average of the peak areas of the methylene bridge from the hydrazide (δ 1-1.6 ppm) through the methyl resonance (δ 2.6-2.8 ppm) of the acetamino moiety of the N-acetyl-D-glucosamine residue which was used as an internal reference.

Table 16: Degree of substitution of thiolated hyaluronic acids

Thiolated hyaluronic acid	Degree of substitution [%]
hydrolyzedHA high SD	74
hydrolyzed HA low SD	27.5
native HA high SD	40
native HA low SD	39

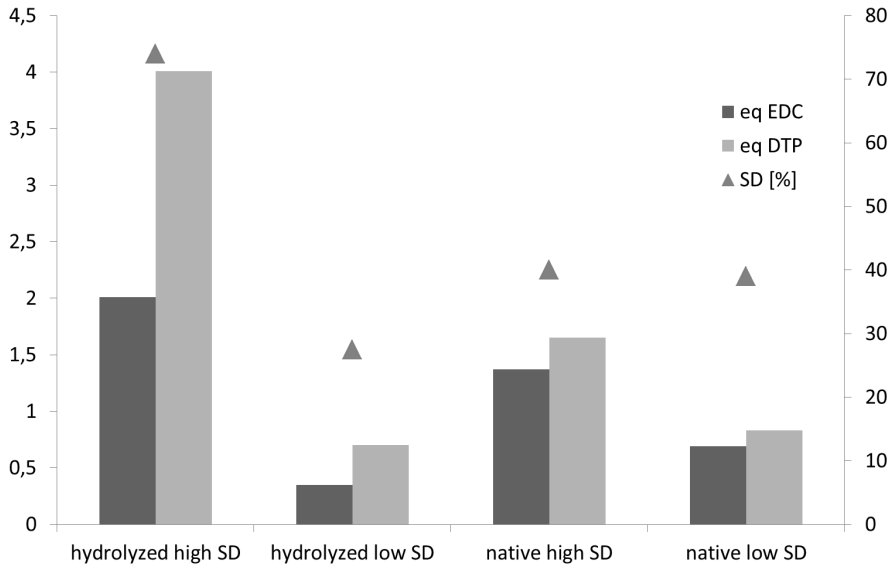


Figure 66: Achieved substitution degree in relation to reaction components

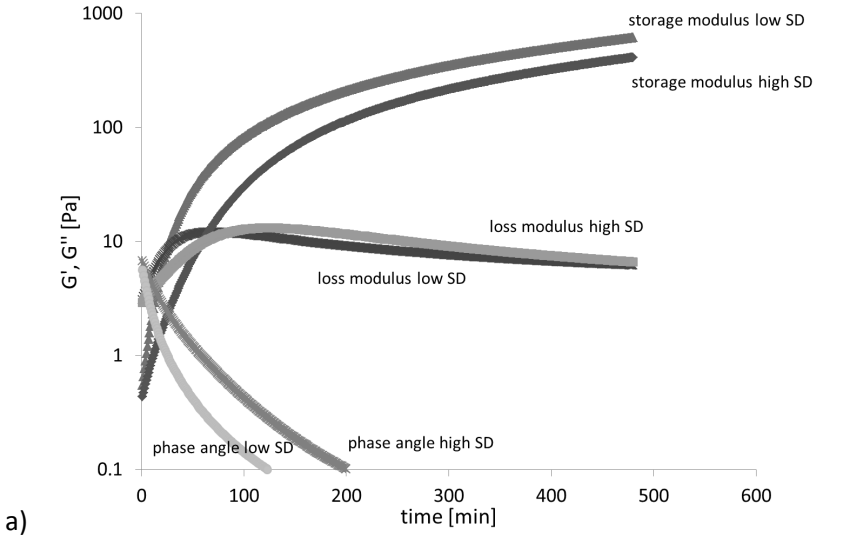
Regarding the NMR spectra for the hydrolyzed hyaluronic acid even with the two folded excess of water soluble carbodiimide only 74 % the substitution degree of the expected 100 % was not obtained for the high degree of substitution (figure 66). For the planned low SD HA with 0.35 equivalents of water soluble carbodiimide a substitution degree as expected was almost obtained (27.5%) (figure 66). The NMR spectra of the native hyaluronic acid showed nearly the same degree of substitution for the high and the low degree of substitution (39% and 40%) regardless of the chosen equivalents of the activating reagent. The first peak of the methylene bridge showed a higher peak area than the second peak, which can be explained with an overlay of the peak area of DTT that was not extracted completely by dialysis. The reaction of the hyaluronic acid with DTP led as expected in all experiments to the formation of very strong gels, but the expected degrees of substitution were not achieved. The high viscosity most likely slowed down the reaction kinetics; this is why the mixtures should have been stirred longer to ensure a complete reaction of the components.

The observed thiol amounts with the Ellmans assay (data not shown) were also significantly higher than the theoretically calculated thiol amounts. DTT that has not been successfully washed out during the dialysis step might be the cause of these results.

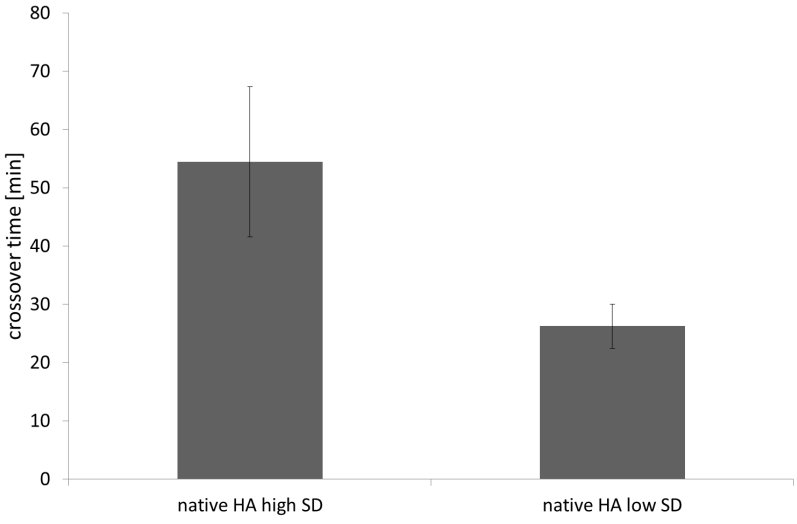
Table 17: Results of elementary analysis of the prepared thiolated hyaluronic acids

	C %	H %	N %	S %
HA	37.85	5.56	3.17	
HA hydrolyzed	34.63	6.09	2.78	
Thiolated HA (theoretical amount 100% modification)	42.41	5.65	8.73	6.66
Hydrolyzed HA high SD	37.24	5.88	7.16	5.21
Hydrolyzed HA low SD	40.22	6.08	4.99	2.58
Native HA high SD	27.74	4.2	3.92	1.94
Native HA low SD	28.24	4.28	4.08	2.03

The elementary analysis verified the successful incorporation of the thiol compound. The two thiolated native hyaluronic acids had nearly the same sulfur content. For the hydrolyzed hyaluronic acids the elementary analysis revealed a nearly twice as high sulfur content for the high substitution degree compared to the low substitution degree. The results of carbon and hydrogen showed that the hygroscopic hyaluronic acid contained small amounts of water.



a)



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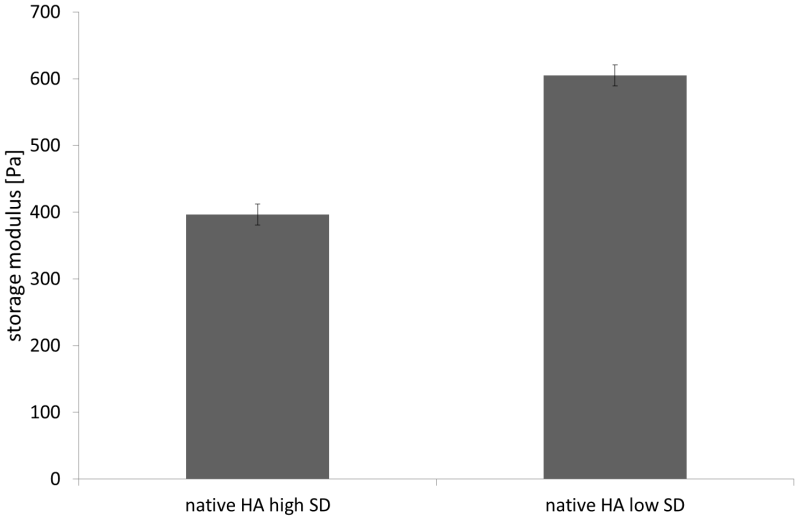


c)

Figure 67: a) Time sweep measurements of native HA with low and high degree of substitution b) crossover time [min], c) storage modulus [Pa] after 8 hours measurement time

Table 18: Cross-linking time of HASH

	Cross over time [h]
Hydrolyzed HA high SD	0.18
Hydrolyzed HA low SD	4.45
Native HA high SD	0.92
Native HA low SD	0.41

The rheological measurements revealed contrary results for the native and the hydrolyzed hyaluronic acids. The hydrolyzed hyaluronic acids showed the expected results of a faster cross-linking of the thiolated hyaluronic acid with the high SD than of the thiolated hyaluronic acid with the low SD. Surprisingly the results of the native hyaluronic acid showed a difference, even when the NMR measurements and the elementary analysis implied a similar degree of substitution. Furthermore they showed the opposite results to the hydrolyzed hyaluronic acid. The thiolated hyaluronic acid that should be lower substituted regarding the reaction mixture showed a faster cross-linking and led to stronger gels, which was attributed to the lower probability to cross-link with itself. Therefore these results showed a difference between the native hyaluronic acids which the NMR analysis and the elementary analysis did not show.

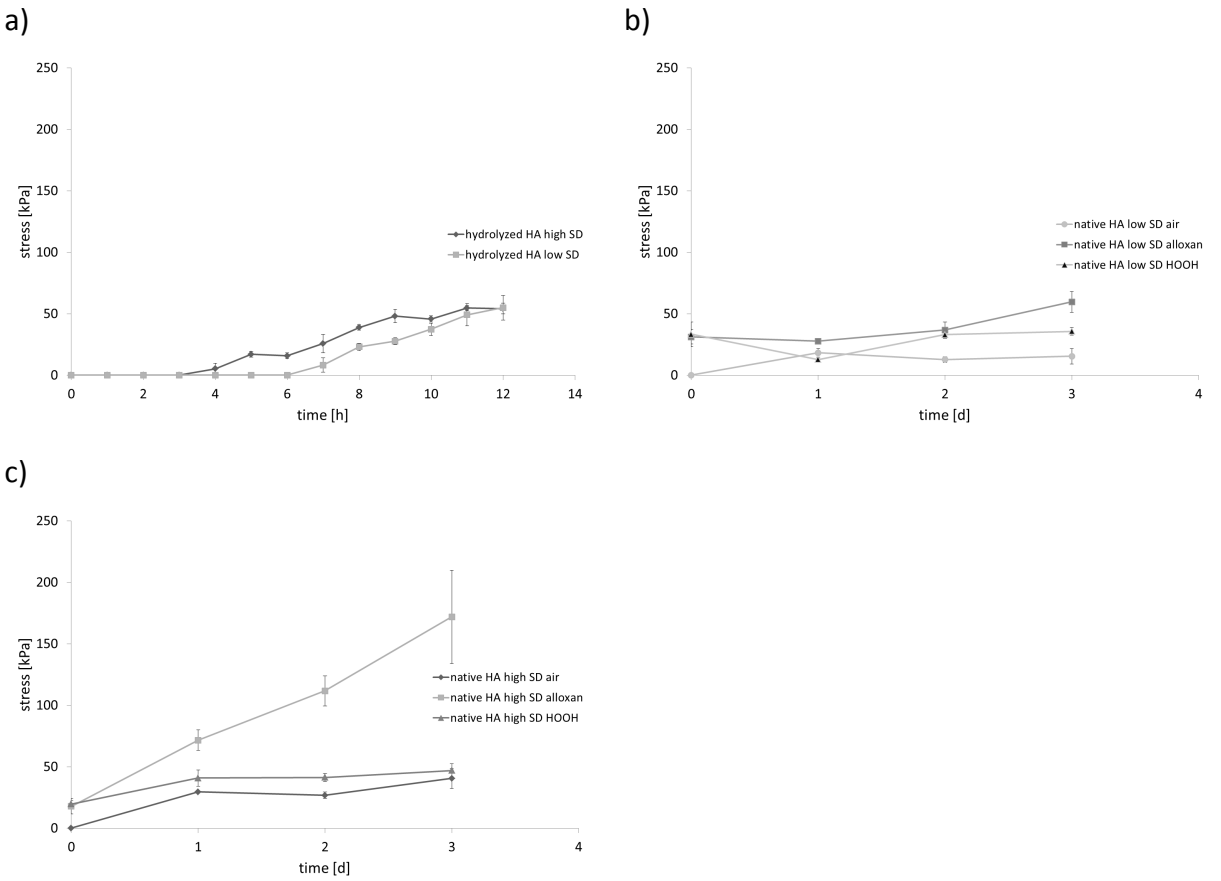


Figure 68: Confined compression test thiolated hyaluronic acid a) hydrolyzed HA high SD and low SD in PBS b) native HA low SD in PBS with oxidizing agents c) native HA high SD in PBS with oxidizing agents

With the help of the compression test the cross-linking in air could be monitored. The measurement of the hydrolyzed hyaluronic acid showed that hyaluronic acid with a high degree of substitution cross-linked faster than the hyaluronic acid with the low degree of substitution. This could be seen, because the compressive load of the high substituted hyaluronic acid started to rise after 4 hours exposure to oxygen, the compressive load of the low substituted hyaluronic acid 3 hours later. In the end, both hyaluronic acid gels reached the same stress of nearly 54 kPa. The addition of H_2O_2 or alloxan demonstrated that oxidizing agents can further increase the gel strength compared to air oxidation alone indicating a diffusion limited oxidation reaction. With the native hyaluronic acid and the low SD only with alloxan a stress of 60 kPa could be measured. With H_2O_2 it was about 36 kPa and in air about 15 kPa. This can conclude that a longer polymer chain does not automatically lead to higher compressive forces. With the addition of alloxan the stress of the native hyaluronic acid with high degree of substitution became three times higher than with air. With H_2O_2 47 kPa and in air 41 kPa were reached.

6.4 Film preparation

The pretests that have been conducted with the different hyaluronic acids have shown that there are many things that can influence the cross-linking of thiolated hyaluronic acid. To investigate these influences on the film preparation itself and the mechanical properties of the prepared films, different films prepared from different hyaluronic acids and cross-linked via different methods have been prepared.

More precisely the influence of molecular weight, substitution degree, pH of the hyaluronic acid solution, addition of cross-linking agents and drying speed have been investigated.

For the film drawing with a drawing apparatus on glass plates the viscosity of the polymer solution plays an important role. The solution should flow through the gap of the frame and form a film that keeps its shape until drying. To find a suitable concentration for the film preparation, films without cross-linking have been prepared. Furthermore these films were used to evaluate the mechanical properties of films prepared from unmodified hydrolyzed hyaluronic acid. More precisely 4 solutions in water with the concentrations of 3 %, 4 %, 5 % and 6 % were drawn on a glass plate with a gap clearance of 700 µm and a speed of 5 mm/ sec.

To investigate the influence of the polymer analogue modification of the hyaluronic acid on the film preparation and the mechanical properties of the resulting films, films of thiolated hyaluronic acid have been prepared. In this case the influence of molecular weight of the hyaluronic acid and the substitution degree have been investigated.

6.4.1 Hydrolyzed hyaluronic acid with high substitution degree in PBS

0.5 g of the thiolated hyaluronic acid was dissolved in 10 ml PBS. After the pH was adjusted to 7.5 the solution was still too runny to be drawn to a film and was therefore stirred several hours exposed to air before it was drawn on a glass plate with a gap clearance of 250 µm and a speed of 5 mm/ sec.

6.4.2 Hydrolyzed hyaluronic acid with low and high substitution degree in PBS

For the hyaluronic acid with the low substitution degree a 10 % solution was prepared, because a 5 % solution was too runny to be drawn on the glass plate because it directly contracted after drawing. 500 mg of the thiolated hyaluronic acid were dissolved in PBS and

the pH was adjusted to 7.53 with 1 N NaOH. After several hours stirring the film was drawn with a gap clearance of 250 µm and a speed of 5 mm/ sec.

To be able to compare the films prepared from thiolated hydrolyzed hyaluronic acid another film from the hyaluronic acid with the high substitution degree was prepared. Therefore 500 mg of the polymer were dissolved in PBS and the pH of the solution was adjusted to 7.39 with 1 N NaOH. The film was drawn with a gap clearance of 250 µm and a speed of 5 mm/ sec and dried at room temperature.

A 10 % solution of unmodified hyaluronic acid in PBS was drawn after adjusting the pH to 7.49, too, to be able to compare films of unmodified and thiolated hyaluronic acid.

To enhance the cross-linking of the films made from the thiolated hyaluronic acid further films were prepared. 500 mg of the hyaluronic acid with the high substitution degree respectively the low substitution degree were dissolved in PBS. The pH was adjusted to 7.4 with 1 N NaOH. Both films were drawn with a gap clearance of 250 µm and a speed of 5 mm/ sec. Then the films were stored in a closed box for 72 hours to keep them wet, because this ought to increase the cross-linking time. After 72 hours the films were taken out of the box and dried at room temperature before subsequently mechanical investigation.

6.4.3 Native hyaluronic acid with low and high substitution degree in PBS

For the films prepared from the high Mw HA 2 % solutions were drawn. More precisely 200 mg of the high SD and the low SD HA were dissolved in 10 ml PBS. The pH was adjusted to 7.4 by addition of 1 N NaOH. Both films were drawn on a glass plate with a gap clearance of 1000 µm and a speed of 5 mm/ sec. These films were produced three times. One film was directly dried at room temperature, and the others were stored in a closed box for 72 hours respectively 7 days before they were allowed to dry at room temperature, too.

The films drawn on glass plates became opaque after drying, which was accredited to buffer salts that precipitate in the film. With the objective of obtaining clear films thiolated hyaluronic acid was dissolved in water instead of PBS. For this purpose 200 mg of the hyaluronic acids were dissolved in 10 ml water and drawn with a gap clearance of 1000 µm

and a speed of 5 mm/ sec. The films directly dried at room temperature or were stored in a plastic box for 72 hours respectively 7 days before drying at room temperature.
To increase the cross-linking of the prepared films they were sprinkled with a 3 % solution of H_2O_2 before storing in a closed box.

6.4.4 Observations during and after film preparation

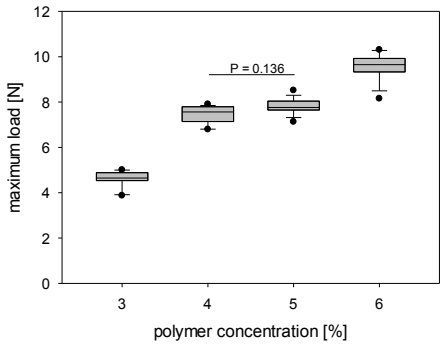
The first film drawing tests performed with the unmodified hyaluronic acid could not be transferred to the preparation of films from substituted hyaluronic acid, because they behaved in a different manner. With the modified hyaluronic acid it was possible to draw films with a lower gap clearance than with the unsubstituted hyaluronic acid. The films without cross-linking had a thickness of 14-15 µm, 15-16 µm, 19-21 µm and 24-27 µm. For the film drawing on glass plates a higher concentration of the hyaluronic acid with the low substitution degree was needed than from the hyaluronic acid with the high substitution degree. The films prepared from the substituted hydrolyzed hyaluronic had a thickness off 9-16 µm. The subsequent bursting of the film made of the hyaluronic acid with the high substitution degree after storage in a closed box for 72 hours showed that a tension in the film occurred during the drying process that could be explained with a stronger cross-linking compared to the films that dried directly after drawing. The films prepared from the native hyaluronic acid had a thickness of 16-20 µm prepared in PBS and 7-11 µm prepared in water. All cross-linked films prepared in PBS buffer became opaque after drying. To obtain clear films the thiolated hyaluronic acid was also dissolved in water because the opaque appearance of the film was thought to come from the buffer salts. But the films prepared with water became opaque, too. A further cross-linking with H_2O_2 was not possible, because the film ruptured during the drying process due to the increased cross-linking.

6.5 Mechanical evaluation

6.5.1 Hydrolyzed hyaluronic acid without modification

To test the mechanical properties of the hyaluronic acid films a tensile test as described before (Chapter 3) was performed. From each film 15 strips were tested.

a)



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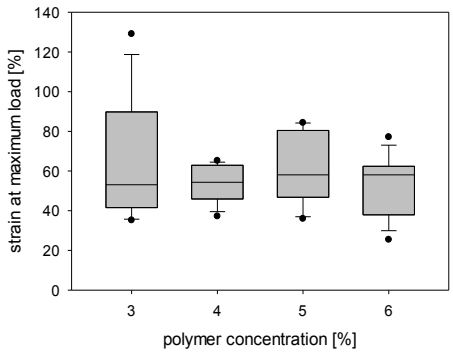

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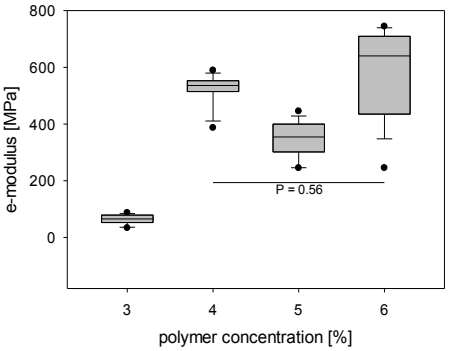


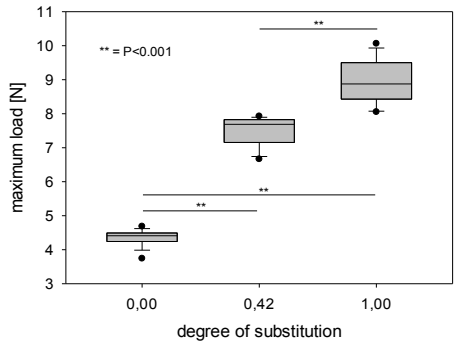
Figure 69: Tensile test of films made of hydrolyzed hyaluronic acid in different polymer concentrations. a) maximum load [N].b) strain at maximum load [%] c) elastic modulus

The higher the polymer concentration was the thicker was the resulting polymer film. This affected the maximum load and the elastic modulus of the films. With increasing polymer concentration the maximum load [N] increased. Except the films made of 4 % and 5 % hyaluronic acid the films showed a significant difference ($P<0.001$). The strain at maximum load [%] was not influenced by the polymer concentration. The films did not show a significant difference. With increasing polymer concentration the elastic modulus increased too. The film prepared from the 4 % solution showed a higher elastic modulus than expected and therefore was not significantly different from the film prepared from the 6 % solution. The other films showed a significant difference ($P<0.001$) among themselves.

6.5.2 Hydrolyzed hyaluronic acids with different substitution degrees in PBS

To see the impact of the substitution degree on the mechanical properties of the films made of thiolated hyaluronic acid 15 strips of the films made from the hydrolyzed hyaluronic acid without substitution and with high and low substitution degree were tested.

a)



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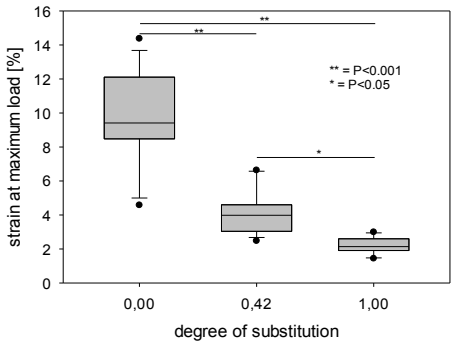

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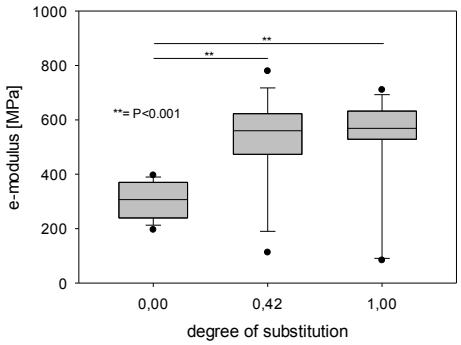
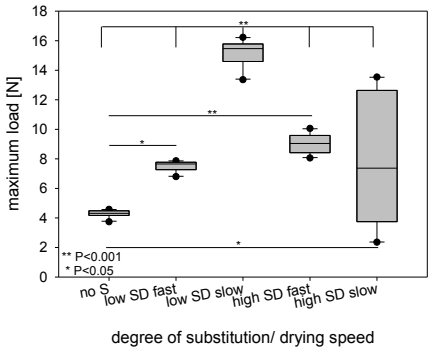


Figure 70: Tensile test of films made of hydrolyzed hyaluronic acid with different degrees of substitution ** equates $p < 0.001$, * equates $p<0.05$ a) maximum load [N] b) strain at maximum load [%] c) elastic modulus [MPa]

As expected the maximum load [N] increased and the strain at maximum load [%] decreased with increasing substitution degree and showed a significant difference between the polymer films. The elastic modulus [MPa] increased, too. But in this case no significant difference between the thiolated hyaluronic acids was measurable.

6.5.3 Hydrolyzed hyaluronic acid with longer cross-linking time in PBS

a)



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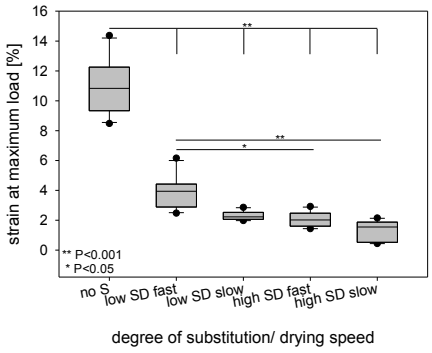

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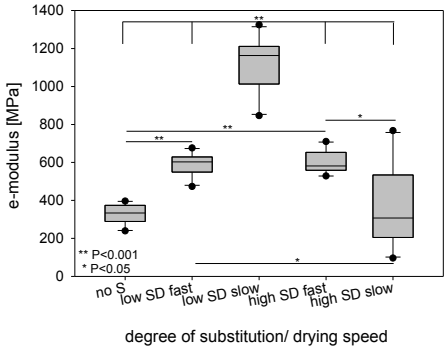
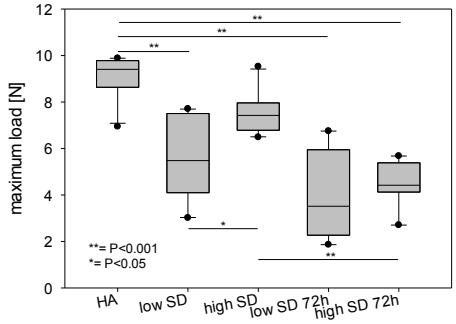


Figure 71: Tensile test of films made of hydrolyzed hyaluronic acid with different degrees of substitution ** equates p < 0.001, * equates p<0.05 a) maximum load [N] b) strain at maximum load [%] c) elastic modulus [MPa]

The tensile tests showed a significant difference between the tested hyaluronic acids. With higher substitution degree, the strain at maximum load [%] decreased and the maximum load [N] as well as the elastic modulus [MPa] increased as expected. A slower drying further enhanced these effects. However long cross-linking of the film prepared from the hyaluronic acid with the high substitution degree didn't show the expected positive effect on the mechanical properties. The film ruptured during the drying process and became very stiff and brittle due to the strong cross-linking and therefore showed very inhomogeneous results. The maximum load [N] and the elastic modulus [MPa] were expected to be higher than for the film that died directly after drawing. But during the tensile test the film strips ruptured very early and therefore led to a lower maximum load [N] and a lower elastic modulus [MPa]. These results show that an improved cross-linking of the films did not lead to improved mechanical properties.

6.5.4 Native hyaluronic acid with longer cross-linking time in PBS

a)



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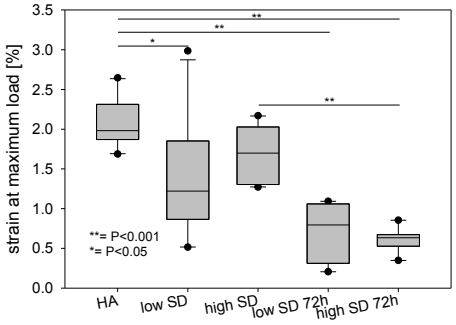

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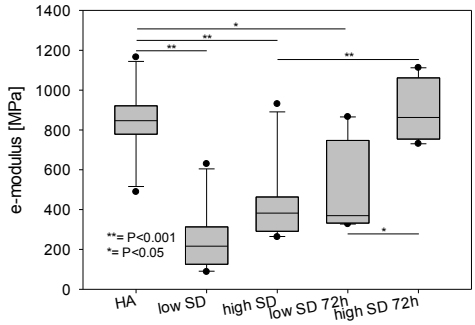


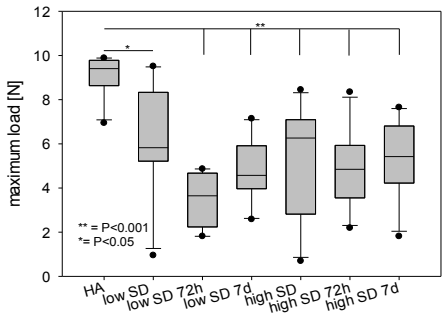
Figure 72: Tensile test of films made of native hyaluronic acid with different degrees of substitution ** equates p < 0.001, * equates p<0.05 a) maximum load [N] b) strain at maximum load [%] c) elastic modulus [MPa]

The films stored in the box became very stiff and brittle due to the stronger cross-linking. Therefore, from the film with the low substitution degree only 4 strips could be tested and from the film with the high substitution degree 9 strips could be tested. From the other films ten strips were tested. For the native hyaluronic acid the cross-linking affected the mechanical properties in a negative way. In comparison to the films without cross-linking the maximum load decreased about 2 to 6 N. The strain decreased too from about 2 % for not cross-linked hyaluronic acid to about 1.7 % for high substituted fast drying hyaluronic acid and even to 0.6 % for the high substituted hyaluronic acid stored in the plastic box. Regarding the elastic modulus the measurement showed an increase with higher substitution and longer cross-linking time. But the not cross-linked hyaluronic acid with an elastic modulus of 840 MPa was only a little bit lower than the highest elastic modulus of the hyaluronic acid with the high substitution degree and the longer cross-linking time and an elastic modulus of 890 MPa.

The tensile tests showed no significant differences between the low substituted and the high substituted native hyaluronic acid concerning the maximum load, the strain at maximum load [%] and the elastic modulus [MPa]. But the film that should have a lower degree of substitution became more brittle than the film that should have a higher degree of substitution. This could be due to less cross-linking with itself.

6.5.5 Native hyaluronic acid with longer cross-linking time in water

a)



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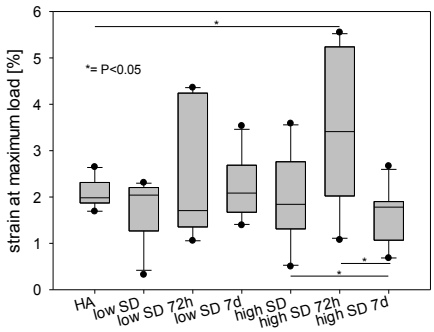

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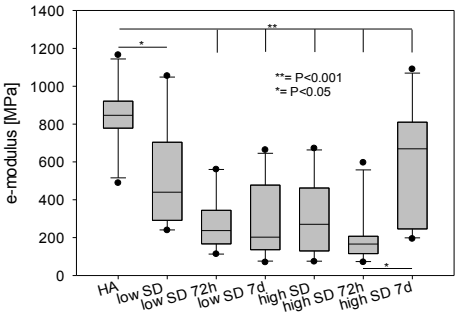

Figure 73: Tensile test of films made of native hyaluronic acid with different degrees of substitution prepared in water ** equates p < 0.001, * equates p<0.05 a) maximum load [N] b) strain at maximum load [%] c) elastic modulus [MPa]

The tensile tests of the films prepared of thiolated native hyaluronic acid in water showed no significant differences between the degree of substitution and the drying speed. Due to brittleness only seven strips of the low substituted hyaluronic acid stored for 72 hours in the plastic box could be tested. For the other films 10 strips were tested.

6.6 Film swelling

The films prepared of the different thiolated hyaluronic acids and cross-linked for different time intervals in air were die-cut to discs with a diameter of 1 cm. These discs were stored in 1 ml of PBS respectively phosphate buffer without sodium chloride. Every hour the discs were removed from the buffer, dry plotted and weighted to investigate the water uptake of the films.

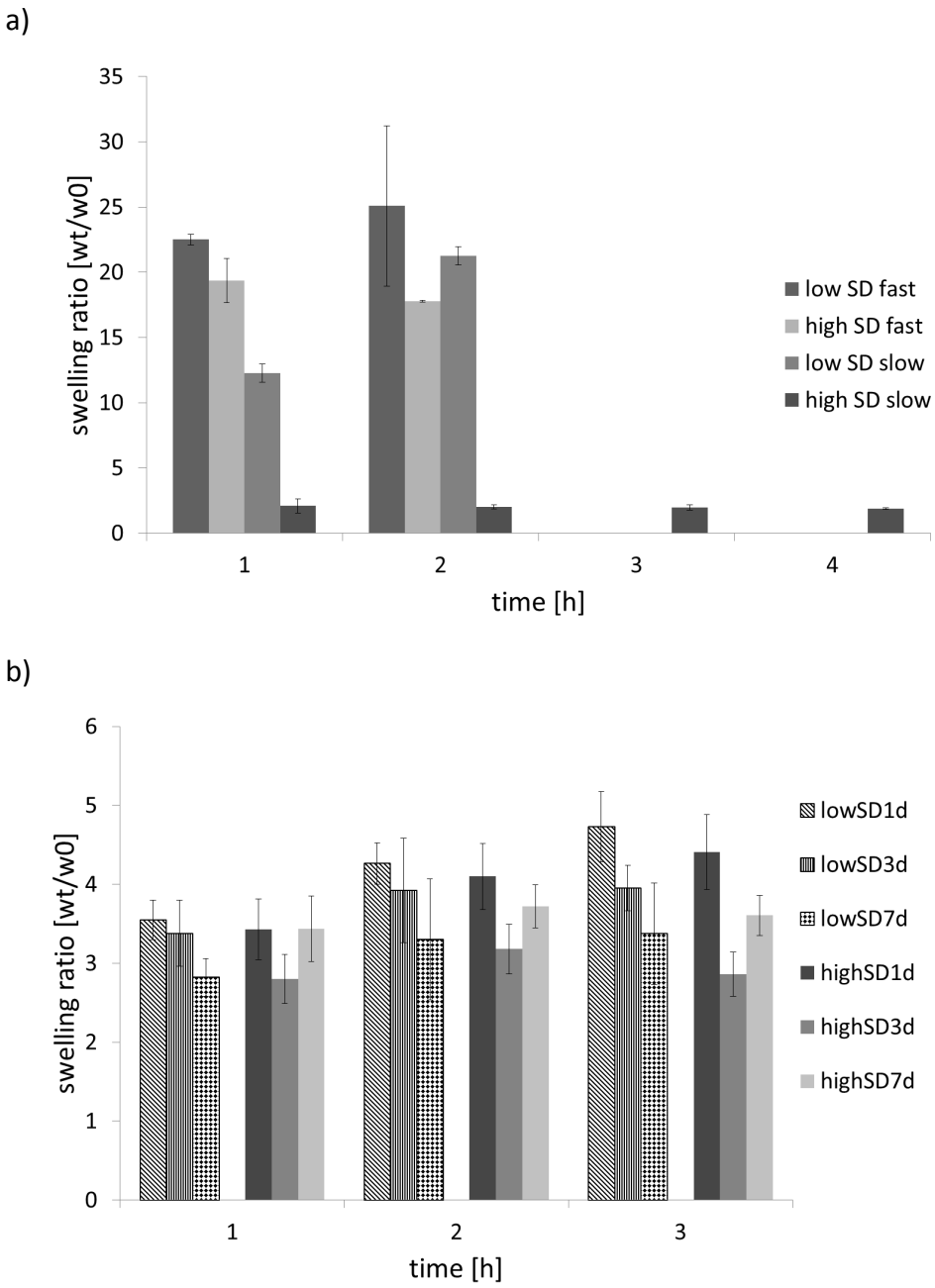

Figure 74: Swelling of films prepared from thiolated hyaluronic acid a) swelling of films from thiolated hydrolyzed HA stored in PBS b) swelling of films prepared from thiolated native HA stored in phosphate buffer.

The films prepared from the thiolated hydrolyzed hyaluronic acid that dried directly after drawing were not stable in PBS and swelled very fast until they were completely dissolved. The high SD slowly dried film was the most stable. The films prepared from the thiolated native hyaluronic acid in general showed less swelling than the films prepared from the hydrolyzed hyaluronic acid. This can be attributed to the length of the polymer chains. The longer the polymer chain was the denser was the cross-linking and the less was the swelling. For the low SD films it could be observed, that the swelling decreased with increasing cross-linking time. For the high SD films this could not be observed due to a higher swelling of the films cross-linked for 7 days. All films became very brittle during the swelling study, even at low substitution degrees.

All the films that did not dissolve during the swelling determination became very brittle. Therefore, the swelling study was stopped after 3 respectively 4 days, because the fragments could not be weighted anymore.

The films prepared in water without adjustment of the pH were not stable during the swelling study and were completely dissolved after 1 hour. This shows that the adjustment of the pH is necessary to enable the thiolated hyaluronic acid to form disulfide bridges and form films that are stable in buffer.

For the film preparation with PBS the pH of the solution was adjusted to a neutral pH. With a pH higher than 8 the cross-linking was too fast to be able to cast a film.

6.7 Summary and Conclusion

Carbodiimide chemistry was successfully applied to prepare thiolated hyaluronic acid, which could be subsequently cross-linked using different oxidizing agents. NMR studies and elementary analysis revealed different degrees of substitution. Only with a large excess of educts the expected 100 % substitution was achieved. For the native hyaluronic acid longer reaction times would be necessary for a complete reaction, because during the reaction the components formed a viscous gel and inhibited a good mixing. Longer stirring time and therefore a longer reaction time might have led to further thiol modification. Additionally it must be concluded that a mere extrapolation of the degree of functionalization based on the applied reagents is not easily possible due to the increased viscosity of the native hyaluronic acid and therefore even bigger effects on the viscosity and reaction effectivity.

Regarding the analysis after synthesis the Ellmans assay detected more thiol groups as possible due to the degree of substitution (results not shown). DTT linked to the polymer chain or not properly washed out during the hydrolysis could lead to these too high results.

With oscillation rheology the gel point of the differently thiolated hyaluronic acids could be determined despite the limited amount of oxygen present in the buffer that was used for the gel preparation. Surprisingly the lower substituted native hyaluronic acid showed a faster cross-linking and led to stronger gels, which was attributed to the lower probability to cross-link with itself. In order to measure cross-linking in air and with other oxidizing agents an alternative compression method was established, which demonstrated that oxidizing agents line H_2O_2 or alloxan can further increase the extent of cross-linking as well as the gel strength compared to air oxidation alone indicating a diffusion limited oxidation reaction.

Using a film drawing apparatus very thin homogenously thick films could be obtained. To increase the cross-linking of the films they were stored in a closed box to slow down the drying of the films and increase the time of cross-linking that was only possible in a humid environment. Furthermore the films were sprinkled with H_2O_2. But the increase of cross-linking with H_2O_2 led to the rupture of the film. The films prepared with buffer as well as the films prepared with water became opaque therefore this phenomenon could not be ascribed to salts from the buffer that crystallized in the dried film.

In order to obtain films that have the required mechanical properties it was necessary to adjust the substitution degree of the hyaluronic acid as well as the drying speed of the thin polymer films. Further addition of oxidizing agents helped to increase the extent of cross-linking, but the degree of cross-linking should be not to strong, because the film might rupture very easy. Appropriate countermeasures have to be undertaken to decrease brittleness, like addition of plasticizers to improve the surgical handling of the films.

The swelling determination showed that it was possible to achieve an adequate cross-linking of thin films prepared of thiolated hyaluronic acid. It was necessary to slow down the drying speed and to adjust the pH, because only the films prepared with PBS at pH 7.4 were cross-linked and therefore stable in buffer. With increasing cross-linking time the films became more stable. It can be concluded that a stable film can be prepared by dissolving the thiolated hyaluronic acid in buffer, adjusting the pH to a pH optimum and cross-linking the film by keeping it wet in presence of oxygen from air.

7. Multilayer

After successful preparation of monolayers from alginate and hyaluronic acid, multilayers were prepared to investigate, if a layer of mucoadhesive alginate can improve the properties of a PLA film and if the formation of a multilayer can also increase the mechanical stability of the hydrophilic polymer films.

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M. Kessler*, E. Esser*, J. Groll, J. Tessmar

Bilateral PLA/ alginate membranes for the prevention of postsurgical adhesions

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*equally contributing authors

7.1 Introduction

For wound dressings as well as for adhesion barrier films it can be beneficial to combine polymers by the preparation of multilayers to obtain a product that comprises the good advantages of the used polymers. Wound dressings can be easily fixed onto a wound by sticky parts of the wound dressing itself or they can be additionally bandaged, because after wound healing the wound dressings are removed from the wound. For peritoneal barrier films this opportunity of a simple fixation and removal does not exist. They have to be fixed in the peritoneum with the help of sutures [127] or staples [128] if they do not adhere to the wound site themselves and for a removal another surgery is necessary if the film and the fixation material are not biodegradable [38]. For wound dressings it is favorable that they do not adhere directly on the damaged tissue. This aim can be accomplished when the dressing contains a lipophilic non-adhesive layer for example a wax-coated silk fibroin woven fabric [42] that prevents the wound of dehydration and can be removed from the wound without causing further trauma. Barrier films however are normally not removed but withdrawn from the body by degradation. For several barrier devices polymers have been mixed before the preparation of the barrier device like hyaluronic acid or polyvinyl alcohol with carboxymethylcellulose [121,129]. Other barrier devices combine polymers in a multi layered structure [119] or in form of a polymer mesh coated with another polymer [130]. A sticky barrier film that stays in place by itself till the peritoneal healing is finished and that degrades would be the ideal device. For this purpose an adhesive layer of mucoadhesive respectively bioadhesive polymers like alginate or carboxymethylcellulose would be beneficial [8,127]. Other bi-layers combine anti adhesive films with a layer that should promote wound healing for example a film made of PLA PEG PLA combined with a layer of collagen or hyaluronic acid which are known for their effectiveness in wound healing [128]. With the preparation of antimicrobial dressings made of polymers like alginate loaded with silver, polyhexamethyl biguanide, gentamycin, minocycline or chlorhexidine gluconate [6,19,43] it was possible to combine the antimicrobial action of the drug component with the beneficial property of the polymer like providing a moist environment which are both beneficial for wound healing. Furthermore bilayers can also be prepared to control the drug release from different layers. For this aim one layer can be loaded or impregnated with a drug and another layer can act as a rate-controlling membrane [47]. The aim of this work was to combine the appropriate mechanical properties and erosion time of a film made from PLA with the mucoadhesive

properties of alginate. The alginate layer should fix the PLA film on the wound area without slipping away until the surgeon has sutured it onto the right place.

7.2 Materials and methods

PLA (Resomer® LR708, Poly (L-lactide-co-D,L-lactide; 70:30) was purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany) and linear PLA PEG PLA (HWL73 PLA35kDa-PEG10kDa-PLA35kDa (poly(lactide)-b-poly(ethylene glycol)-b-poly(lactide))) was purchased from Polymaterials (Kaufbeuren, Germany). Alginate PROTANAL® LF 10/60 FT with a guluronic acid content of 60-70 % was a generous gift from FMCBioPolymer (Sandvika, Norway). Glycerin 87 % was obtained from AppliChem (Darmstadt, Germany). Water Blue from Fluka, Sigmacote®, D-(+)-Gluconic acid δ-lactone and dibasic calcium phosphate were obtained from Sigma-Aldrich (Steinheim, Germany). Pen Strep gibco® with 10000 units/ml Penicillin and 10000 µg/ml Streptomycin was purchased from Life Technologies Corporation (Grand Island, NY, USA). Poly(ethyleneimine) (PEI) and Dichloromethane were analytical grade.

7.3 Preparation of Multilayers using different cohesion promoters

When a solution of hydrophilic alginate is drawn on a lipophilic PLA film it contracts on the film instead of spreading on it, because of the chemically different lipophilic, smooth surface of the PLA film. After drying, the two layers can be easily separated. To overcome this problem and make the two layers stick to each different additives were given to the PLA layer respectively alginate layer that functioned as cohesion promotors. With the addition of positively charged PEI to the PLA layer, it becomes more hydrophilic and the negatively charged alginate can bind to the positively charged PLA layer. Alternatively, glycerol can be added to alginate films respectively alginate layer as plasticizer to make the film less brittle and therefore easier to handle for the surgeon (see chapter 3 and 4). For the preparation of bilayers the cohesion between the two layers is important. With the addition of more glycerol, the film can be made stickier [131]. Therefore glycerol was added in a higher concentration than when it was used as plasticizer.

7.3.1 Preparation of films using PEI as cohesion promoter

To see, if it makes a difference, the two layers were drawn in different orders. Either the PLA layer was drawn first and the alginate layer was drawn over the PLA layer (PLAPEI_A) or the alginate layer was drawn first (A_PLAPEI). For the PLAPEI_A film the PLA layer was prepared of 1.8 g PLA and 0.036 g PEI were dissolved in 43 ml DCM and drawn on a glass plate with a film drawing apparatus (ERICHSEN coatmaster 509 MC) using a gap clearance of 250 µm and a speed of 5 mm/ sec. The alginate layer consisted of 4 g alginate and 0.4 g glycerol dissolved in 95.6 ml water. This layer was drawn with a gap clearance of 700 µm and a speed of 5 mm/ sec. The PLA layer was drawn on a glass slide coated with Sigmacote® to facilitate the detachment of the film after drying. The alginate layer was drawn on an uncoated glass slide. The A_PLAPEI film was prepared by drawing the PLA layer on top of the alginate layer. Before the second layer was drawn on the first layer the first layer was allowed to dry for 24 hours at room temperature. For the mechanical evaluation both films were also drawn as single layers for comparison. After drying the films were cut in the wished shape and peeled from the glass slides. The PLA film had a thickness of 5 µm and the alginate film a thickness of 15 µm. The bilayers had a thickness of 20 µm.

7.3.2 Application of glycerol to enhance film cohesion

7.3.2.1 Bilayers using non-crosslinked alginate layers

To increase the stickiness of the alginate layer, an increased amount of glycerol was added to the alginate solution. Films were prepared by dissolving 1.8 g PLA in 43 ml DCM and drawing them on a glass slide coated with Sigmacote® with a gap clearance of 250 µm and a speed of 5 mm/ sec. After drying for 24 hours, a second layer consisting of 4 g alginate and 4 g glycerol dissolved in 92 ml water was drawn on the PLA layer with a speed of 5 mm/ sec. To evaluate the impact of the thickness of the alginate layer on the mechanical properties of the bilayer the alginate layers were drawn with different gap clearances of 120 µm, 250 µm, 500 µm, 700 µm, 1000 µm and 1500 µm. After drying, the bilayers had a thickness of 10 µm, 15 µm, 25 µm, 40 µm, 45 µm and 55 µm.

For a puncture test and a suture pullout test bilayers were prepared as described before but without the gap clearance of 1000 µm resulting in bilayers with a thickness of 5 µm, 12 µm, 50 µm and 62 µm.

7.3.2.2 Bilayers using cross-linked alginate layers

3.6 g PLA were dissolved in 86 ml DCM and drawn on a coated glass slide with a gap clearance of 250 µm and a speed of 5 mm/ sec. After drying for 24 hours, the alginate layer containing 2 g alginate and 2 g glycerol dissolved in 46 ml water was drawn on top of the PLA layer with a gap clearance of 700 µm and a speed of 5 mm/ sec. Just before casting the alginate layer, 0.4 g $CaHPO_4$ suspended in some droplets of water and 1.6 g GDL were added to the alginate solution to cross-link the alginate layer after the drawing process.

7.3.3 Observations during multilayer preparation

The preparation of bilayers made from one layer of lipophilic PLA and one layer of hydrophilic alginate could not be done by simply drawing the two layers above each other. To make the PLA layer more attractive for the hydrophilic and negatively charged alginate, PEI was added which integrated positive charges in the PLA layer and also made it more hydrophilic. To avoid the shrinkage of the alginate layer after drawing it onto the PLA film, a higher concentrated solution was prepared. But with a higher concentration the drawing of the film became more difficult. Therefore the solution with the lower alginate amount was made stickier by addition of the plasticizer glycerol. Further film preparations showed that the addition of glycerol was sufficient to enhance the cohesion of the two layers. The addition of PEI therefore is not necessary. Previous investigations showed that cross-linking would enhance the stability of the alginate layer and decrease the erosion time. Therefore a bilayer of a PLA layer and a cross-linked alginate layer was successfully prepared.

7.3.4 Mechanical evaluation of bilayers

For the surgeon, who wants to apply the adhesive film to the wounded tissue the mechanical properties of the film are very important. When the surgeon takes the film out of the packaging, cuts it into the wished shape and sutures it to the tissue, the film must not rupture. To investigate, which forces the bilayers can withstand mechanical tests like a tensile test and a suture pullout test were performed.

7.3.4.1 Tensile test of bilayers made of PLA and PEI with alginate

To test the mechanical properties of the single layers and the bilayers, a tensile test like described before was performed with 20 strips.

a) b)

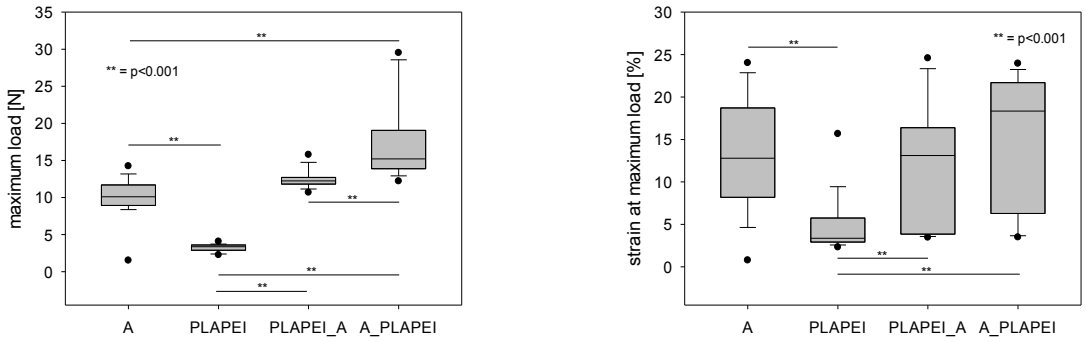


Figure 75: Tensile test of monolayers and bilayers (a) Maximum load [N] (b) Strain at maximum load [%] Statistical differences ($p< 0.001$) are indicated with two asterisks ** or ($p<0.05$) with one asterisks *.

The film made of a single layer of PLA with PEI showed the lowest maximum load [N] and the lowest strain at maximum load [%]. The alginate films showed a twice as high maximum load [N] and the strain at maximum load was even higher. The film strips of the bilayer did not separate while cutting them into strips, which indicated, that the addition of PEI to the PLA layer and glycerol to the alginate layer enhanced the coherence of the two layers. The maximum load [N] as well as the strain at maximum load [%] of the bilayers was significantly ($P<0.001$) higher than that of the PLA film. The maximum load [N] showed only a significant difference for the alginate film in comparison to the alginate film covered with a PLA layer. The other results showed no significant differences of the alginate film and the bilayers. During the tensile testing of the bilayers the PLA layer ruptured earlier than the alginate layer, which could explain the similarity in the mechanical properties of the bilayers and the alginate film, because in the end only the mechanical properties of the alginate layer were tested, until it ruptured, too.

7.3.4.2 Tensile tests of bilayers made of PLA and alginate with glycerol

10 strips of the prepared bilayers where tested as described before (Chapter 3).

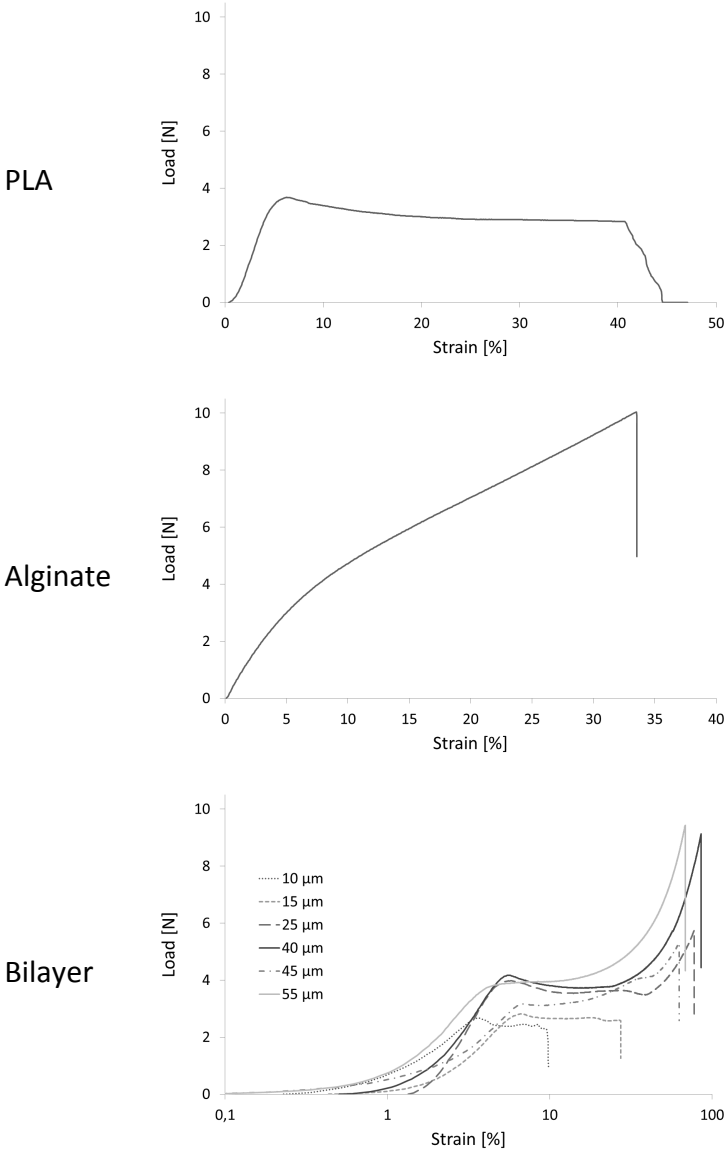


Figure 76: Load strain curves of monolayers and bilayers

The first graph shows a typical example of a load strain curve from a PLA film with the elastic part in the beginning until the maximum load was reached and the plastic deformation that started when the maximum load was reached and ended when the film ruptured where the strain at maximum load could be recorded. The load strain curve of the alginate film had only a very small elastic part in the beginning and the plastic deformation started very early while the load [N] rose constantly until the film ruptured. The load at strain curves showed

that the curves of the bilayers were more alike the curves of the alginate film the thicker the alginate layer was.

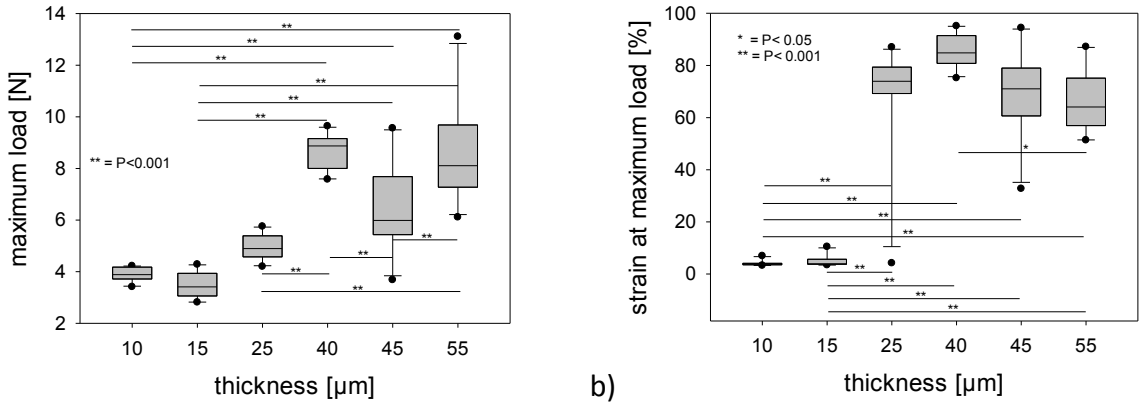


Figure 77: Tensile test of bilayers containing an alginate layer with different thicknesses (a) Maximum load [N] (b) Strain at maximum load [%] Statistical differences (p< 0.001) are indicated with two asterisks ** or (p<0.05) with one asterisks *.

The thicker the alginate layer was the higher was the maximum load [N] that was reached until the film ruptured. While the strain at maximum load [%] for the films with a thickness of 10 µm or 15 µm was very low, at the thickness of 25 µm a remarkable increase in strain was observed. The film with a thickness of 40 µm even showed the highest strain together with the highest maximum load. Moreover it was observed, that at this thickness the shapes of the load/strain curves changed and became more similar to curves of a pure alginate film, indicating the dominating properties of alginate films.

7.3.4.3 Puncture test of bilayers made of a PLA film covered with a layer of alginate

The puncture test was performed with 5 pieces of the bilayers like described before. As comparison a PLA film with a thickness of 5 µm and the commercially available PLA film Surgiwrap® with a thickness of 50 µm were tested, too.

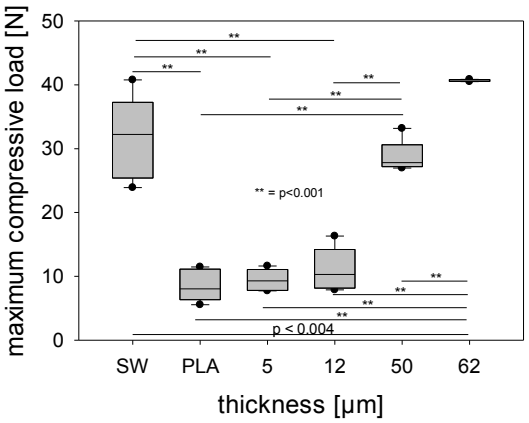


Figure 78: Puncture test of monolayers and of bilayers containing an alginate layer with different thicknesses. Statistical differences (p< 0.001) are indicated with two asterisks **

Surgiwrap® had a higher maximum compressive load [N] than the PLA film and the very thin bilayers with a thickness of 5 µm and 12 µm. The bilayers with a thickness of 50 µm respectively 62 µm had the same maximum compressive load [N] like Surgiwrap®. The thicker the alginate layer was, the higher was the maximum compressive load [N] that was needed to push through the film.

7.3.4.4 Suture pullout test of bilayers made of a PLA film covered with a layer of alginate

For the suture pullout test 5 specimens were tested as described before. As comparison a PLA film with a thickness of 5 µm and the commercially available PLA film Surgiwrap® with a thickness of 50 µm were tested, too.

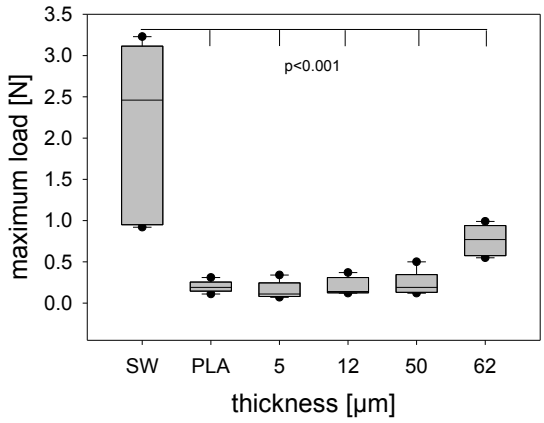


Figure 79: Suture pullout test of monolayers and of bilayers containing an alginate layer with different thicknesses.

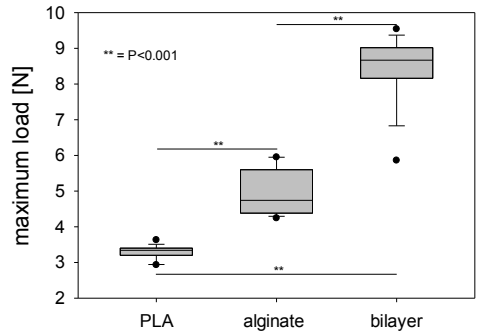
With the suture pullout test only the maximum loads reached with Surgiwrap® that was used as comparison significant different results from the other films could be measured. The load of Surgiwrap® was much higher than the load of the other films which indicated that it would withstand higher forces when sutured to the tissue.

7.3.4.5 Mechanical evaluation of bilayers made of PLA film covered with cross-linked alginate

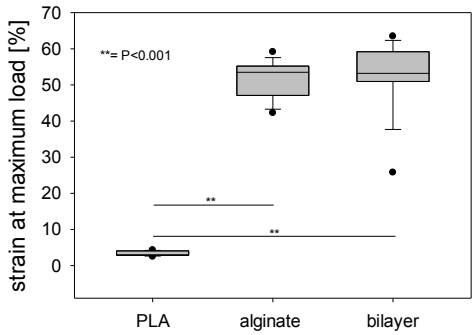
For the mechanical evaluation 15 specimens were tested for every setup. The tests were performed like described before.

Tensile Test

a)



b)



c)

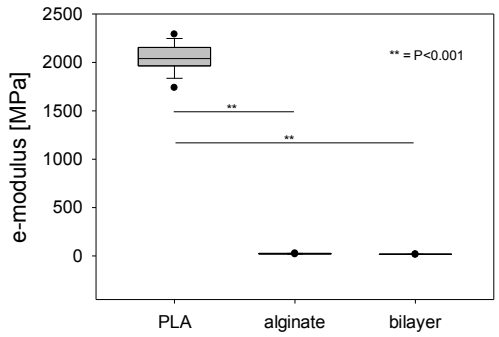


Figure 80: Tensile test of monolayers and bilayers (a) Maximum load [N] (b) Strain at maximum load [%] (c) Elastic modulus [MPa]. Statistical differences (p< 0.001) are indicated with two asterisks **

The tensile tests revealed a significant difference in the maximum load between the three films. The very thin PLA film only resisted very low forces and ruptured at a load of about 3 N. The alginate layer could withstand higher forces of about 5 N and the bilayer was even higher with about 8.5 N. Due to the fact that the PLA layer ruptured earlier than the alginate layer during the tensile testing, the strain of the alginate film and the bilayer were similar

with about 55 %. The PLA film only had a strain of about 5 %. As expected the elastic modulus of the PLA film with 2000 MPa was much higher than the elastic modulus of the alginate film and the bilayer of about 20 MPa.

Puncture test

a) b)

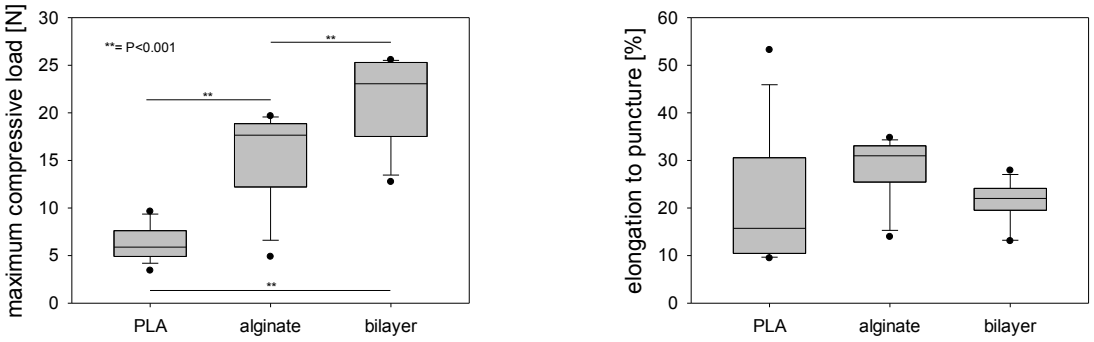


Figure 81: Puncture test of monolayers and bilayer. Statistical differences (p< 0.001) are indicated with two asterisks **

The puncture test showed the advantage of a bilayer, too. The bilayer showed a significant higher maximum compressive load [N] than the monolayers. The elongation showed no significant differences.

Suture pullout test

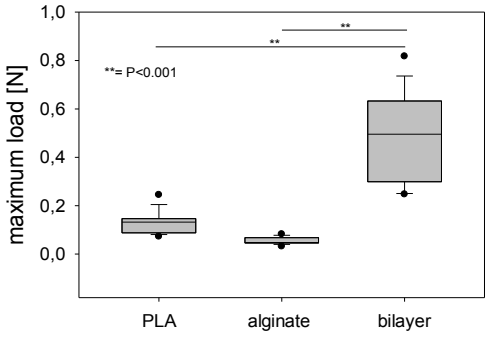


Figure 82: Suture pullout test of monolayers and the bilayer. Statistical differences (p< 0.001) are indicated with two asterisks **

The suture pullout test showed the beneficial effect of the formation of a bilayer. The bilayer could withstand significantly higher forces than the monolayers. This should ease the fixation with sutures.

7.3.5 Stability test of bilayers

Even if PLA is known to degrade very slowly, it degrades when stored in a humid environment. The alginate layer containing plasticizer always contained residual moisture and never dried completely under the conditions the bilayers were prepared. To see the impact of moisture on the PLA layer, a stability test was performed.

0.9 g PLA were dissolved in 21.5 ml DCM and drawn with a gap clearance of 250 µm and a speed of 5 mm/ sec. After 24 h the PLA layer was covered with a second layer consisting of 2 g alginate and 2 g glycerol dissolved in 46 ml water drawn with a gap clearance of 700 µm and speed of 5 mm/ sec. After drying at room temperature the film was cut into pieces of 5x5 cm, sealed in foil and stored at room temperature. Every second week one film was taken out of the foil, cut into 5 strips of 1 x 5 cm and a tensile test was performed. Subsequently the PLA layers was peeled from the alginate layer and stored at -20 °C for further GPC investigation.

After 18 weeks, the ruptured PLA strips were dissolved in 2 ml chloroform and filtrated before GPC investigation. The GPC setup consisted of a system controller, a binary pump, an auto injector, a column oven at 40 °C and a refractive index detector (Shimadzu Corporation, Chromatographic & spectrophotometric instruments division, Kyoto, Japan). As stationary phase a Phenogel 5 u Linear, 300 x 7.8 mm (Phenomenex, Aschaffenburg, Germany) was chosen. The mobile phase was helium purged chloroform. The measurement was performed with a flow rate of 1 ml/ min. The results from the RID calculated in relation to polystyrene standards were given as M_w (weight average) and M_n (number average) and M_w/M_n (polydispersity index).

a) b)

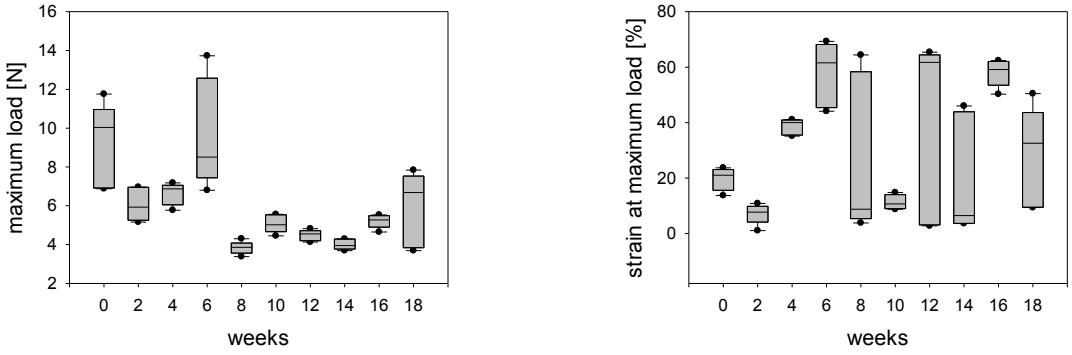


Figure 83: Tensile test of bilayer strips after storage for several weeks

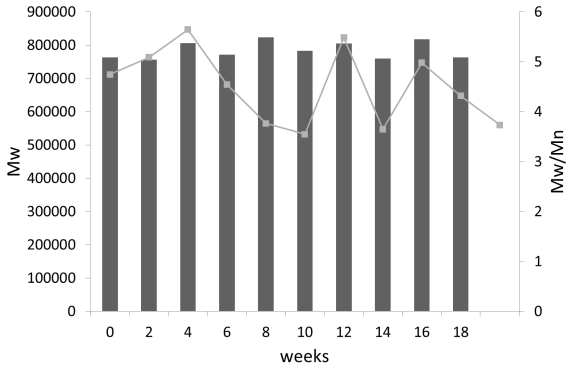


Figure 84: GPC data of PLA layer after storage for several weeks

The tensile test showed that the maximum load [N] decreased over time whereas the strain at maximum load [%] increased. This indicated a softening effect of water respectively glycerol on the PLA layer. The measurements showed results with high standard deviations. On the contrary the GPC data revealed that the moisture in the alginate film did not affect the PLA over the eight weeks.

7.3.6 Discussion

The mechanical tests revealed that an alginate layer is able to further stabilize the investigated thin PLA layer. Regarding the films prepared with PEI as cohesion promoter, the individual PLA layer could only withstand forces of 5 N and had a strain of 5 % whereas the individual alginate layer could withstand forces of 10 N with a strain of 15 %. The combined bilayer as expected could withstand higher forces of 15 N and the observed strain was only little higher with 15 % to 20%.

Regarding the optimal thickness of the supporting alginate layer, it could be seen, that there is a high increase in the carried maximum load from 25 µm to 40 µm, but the investigated films with a thickness of 40 µm and more had more or less the same strain at maximum load of 70 %. Therefore it was concluded, that a thickness of about 40 µm and more is sufficient to provide stable films with good mechanical properties for an application without rupturing.

During the tensile tests it was furthermore observed, that the PLA layer ruptured earlier than the alginate layer, indicating that the added alginate layer can stabilize the PLA layer, but this stabilization is also only limited. The surgeon should still handle the fragile films with care and be cautious, that the PLA layer does not rupture while suturing the bilayer to the application site. The additionally investigated cross-linking of the alginate layer showed no edge over the non-cross-linked alginate, only the maximum strain of the bilayer with cross-linking was slightly decreased due an increased stiffness. Instead the cross-linked layer could even be separated from the PLA layer much easier than the layer without cross-linking, indicating limited benefit of the cross-linking for the cohesion.

Furthermore it could be observed, that after incubation in water respectively buffer, the soaked alginate films always could be easily separated from the PLA layer, which can be attributed to an occurring hydration of the gel layer and accordingly the breakdown of the linking capillary forces.

With the also investigated chemical stability test it could be seen, that the storage of the PLA layer in a humid environment due to the residual water content in the alginate layer did not significantly affect the molecular weight of the lipophilic polymer PLA. However with time the mechanical properties of the bilayer became more inconsistent, with lower maximum forces and the tendency to higher strains at maximum load. Furthermore the performed measurements indicate that the mechanical properties of the investigated films became more and more inhomogeneous, indicating the need for effective sealing and packaging of the polymers to provide constant humidity and only limited mechanical stress to the stored film samples.

7.4 Preparation of Multilayers using increased surface roughness[132]

Although the addition of PEI to the PLA layer respectively the addition of glycerol to the alginate layer significantly improved the cohesion of the two layers, peeling one layer from the other still could easily separate them. To enhance the adhesion of the alginate solution respectively alginate film on the PLA layer, the surface of the PLA layer was modified. The PLA layer itself has a very smooth surface and therefore the alginate film contracted after it has been drawn on the PLA film. On a rough surface the alginate solution would be able penetrate into the rough structure and increase the adhesion. To obtain a rough, porous surface, a mesh was spun on top of the PLA film. The mesh did not only make the surface rough but also gave the alginate a structure it could penetrate into. The mesh that was spun onto the polymer film was prepared of two polymers. PLA and PLA PEG PLA. PLA was chosen to ease the cohesion of the mesh to the film, because they were both prepared from the same polymer. But a PLA mesh is to lipophilic to absorb the hydrophilic alginate solution. To make the alginate solution sink into the mesh, it was also prepared from PLA PEG PLA.

7.4.1 Preparation of the multilayer

7.4.1.1 First layer → PLA film

To obtain a film with a thickness of 20 µm 1.1 g PLA were dissolved in 20 ml DCM. The solution was drawn on a glass plate coated with Sigmacote® with a gap clearance of 700 µm and a speed of 5 mm/ sec.

7.4.1.2 Second layer → electro spun mesh

Solution electrospinning was carried out with the respective polymer solutions containing PLA respectively PLA PEG PLA prepared in a solvent mixture of acetone and DMSO (90:10 V/ V). The solution, placed in a syringe, was fed through a needle having an inner diameter of 0.4 mm with a syringe pump at a feeding rate of 0.5 ml/ h. A high voltage power supply was attached to the needle and fibers were fabricated at an applied voltage of 12 kV. The electrospun fibers were collected on a rotating drum that was covered with the film made of PLA, which was wrapped around it. The drum was positioned at a distance of 15 cm from the needle tip.

7.4.1.3 Third layer → alginate

For the third layer, consisting of alginate, 3 % and 5 % solutions were prepared. For the 3 % solution 450 mg alginate and 450 mg glycerol were dissolved in 14.1 ml water. The 5 % solution consisted of 750 mg alginate and 750 mg glycerol in 13.5 ml water. To be able to visualize and distinguish between the different layers, the alginate solutions were stained with Water Blue before they were drawn. After the alginate was completely dissolved the solutions were drawn on top of the mesh with a gap clearance of 700 µm and a speed of 5 mm/ sec. Then the films were allowed to dry over night at room temperature before further investigation.

Table 19 : Thicknesses of monolayers

monolayer	thickness [µm]
PLA	14-16
alginate 3%	14-16
alginate 5%	20-22

Table 20: Thicknesses of bilayers

alginate [%]	thickness [µm]
3	45-48
5	41-44

Table 21: Thicknesses of trilayers

mesh component	alginate [%]	thickness [µm]
PLA PEG PLA	3	53-57
PLA	3	56-61
PLA PEG PLA	5	53-57
PLA	5	58-69

7.4.2 Contact angle measurements

To get an idea of the hydrophilicity respectively the probability of the hydrophilic alginate solution to spread on the lipophilic film respectively mesh contact angle measurements were performed. The measurements were performed at room temperature with a contact angle system (OCA 20, Data Physics, Filderstadt, Germany). The measurements were performed in triplicate. A 3 µl water droplet was set on different locations of the surface of the mesh respectively film with a dosing rate of 1.0 µl/ s. The contact angle was measured directly after the droplet got in contact with the sample and was assessed via an ellipse fitting.

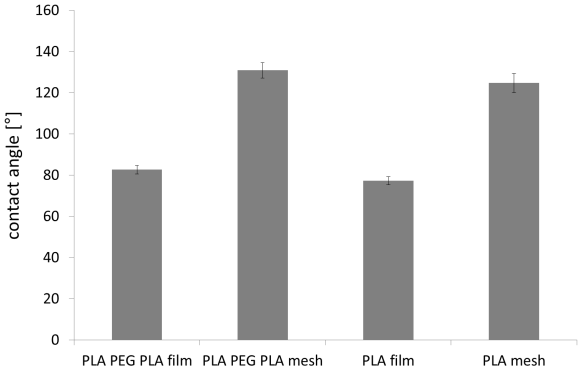


Figure 85: Contact angle measurements of films and meshes

The contact angle measurements revealed that the meshes hat a higher contact angle than the films and therefore are more hydrophobic.

7.4.3 Visual characterization of the prepared Trilayers

7.4.3.1 SEM images

SEM images were taken with a Digital Scanning Microscope (DSM940, Carl Zeiss, Oberkochen, Germany) at an accelerating voltage of 5 kV. The samples were pretreated by gold sputtering (EMITECH K550 sputter coater, Quorum Technologies, West Sussex, UK).

7.4.3.2 Light microscopic images

Light microscopic images were taken with a stereomicroscope (Zeiss Discovery V20, Carl Zeiss, Oberkochen, Germany).

7.4.4 Mechanical evaluation

7.4.4.1 Tensile test

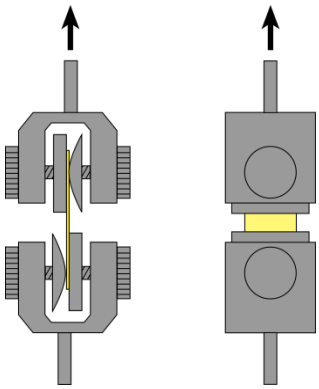


Figure 86: Tensile test

For the tensile testing the trilayers were cut with a scalpel into strips of 1 to 5 cm. 5 of these strips were tested as close as possible to an American national standard of testing “Standard Test Method for Tensile Properties of Thin Plastic Sheeting D 882-02” like described before.

7.4.4.2 T-peel test

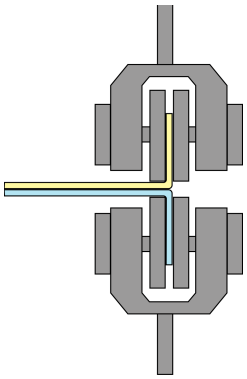


Figure 87: T-peel test

To measure the cohesion of the layers a t-peel test (basic principle from ASTM D 1876-08) was performed. 5 film strips with the dimensions of 1 cm to 5 cm were separated at one end for a length of 1 cm. The PLA layer respectively the PLA layer with the mesh was fixed in the upper grip of the texture analyzer and the alginate layer respectively alginate layer with mesh was fixed in the lower grip that had a distance of 5 mm to the upper grip. The two layers were subsequently separated with a speed of 10 mm/ min until a preload of 0.01 N was reached. Then the testing speed was increased to 250 mm/ min. During the test the load [N] was recorded. The average load was taken from 5 mm to 60 mm movement of the upper grip. The test ended when the layers were completely separated.

7.4.4.3 Mucoadhesion test

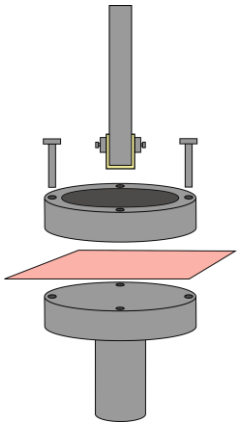


Figure 88: Mucoadhesion test

To test the mucoadhesive properties of the alginate layer a mucoadhesion test was performed with a texture analyzer (Zwick Z010, Zwick GmbH & Co. KG, Ulm, Germany). For this purpose a piece of small intestine stored in PBS containing 5 ml/100 ml Pen Strep from a 8 week old, 18 kg, male domestic pig was opened by cutting one side lengthwise and fixed with the inner side down on a platform with the help of double faced adhesive tape. A strip of the trilayer with the dimensions of 5 cm to 1 cm was fixed to the bottom of a piston with the help of two clamps. The resulting locating surface had the dimension of one square centimeter.

Before each measurement the piece of small intestine was wetted with some droplets of PBS with Pen Strep to obtain the same test conditions for every specimen. In a first step the piston was lowered to the intestine with a speed of 10 mm/ min and pushed into the intestine until a load of 0.1 N was measured. The load of 0.1 N was held for 30 seconds. Subsequently the piston was raised from the intestine with a speed of 0.5 mm/ sec until the film was separated from the intestine and the load was recorded. The adhesive strength based on the area of 1 cm^3 could be calculated.

7.5 Results

7.5.1 Images

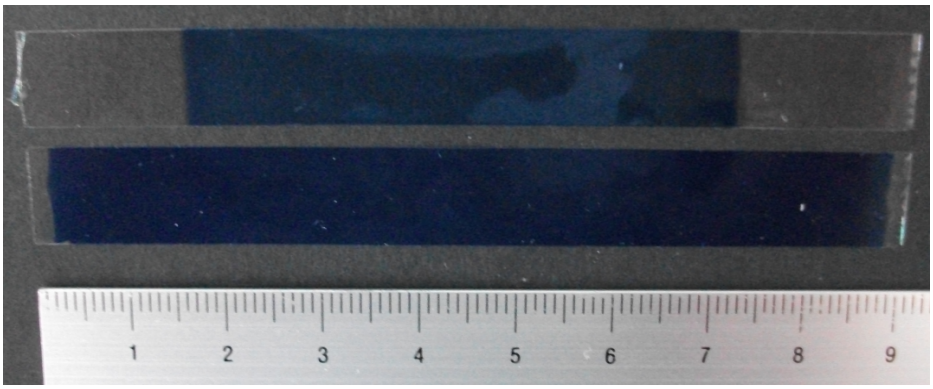


Figure 89: Cross section of bilayers made from LR708 covered with 3% respectively 5% alginate

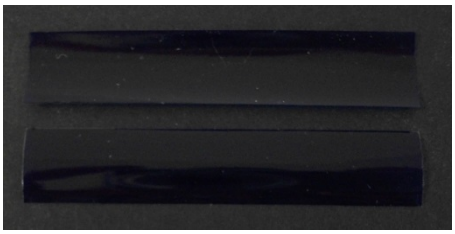
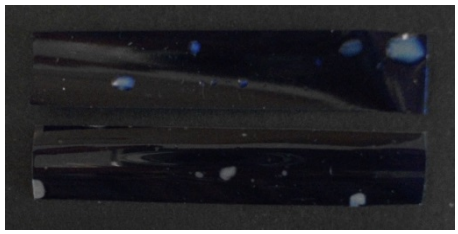
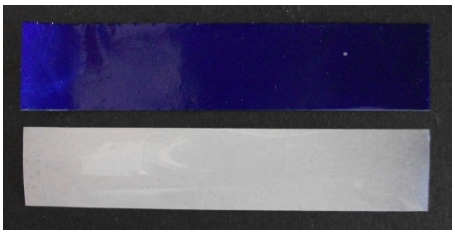
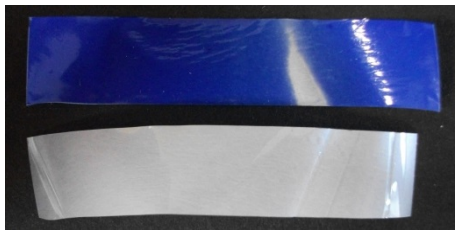
mesh	3 % alginate	5 % alginate
PLA PEG PLA		
PLA		

Figure 90: Images of strips made from the bilayer from the top and the bottom

The images of strips from the top and from the bottom of the trilayers reveal the penetration of the stained alginate solution into the mesh. The film made of PLA is transparent and colorless. The mesh spun on top of the film has a whitish appearance. The more lipophilic the polymer of the mesh, the less did the stained alginate solution sink into the mesh. Therefore with the mesh prepared from PLA no alginate solution could be seen at all, because it was just on top of the mesh without penetrating it. With the mesh made of PLA PEG PLA the bottom was blue, too, because the alginate solution did sink into the mesh.

7.5.1.1 SEM images

PLA PEG PLA mesh PLA mesh

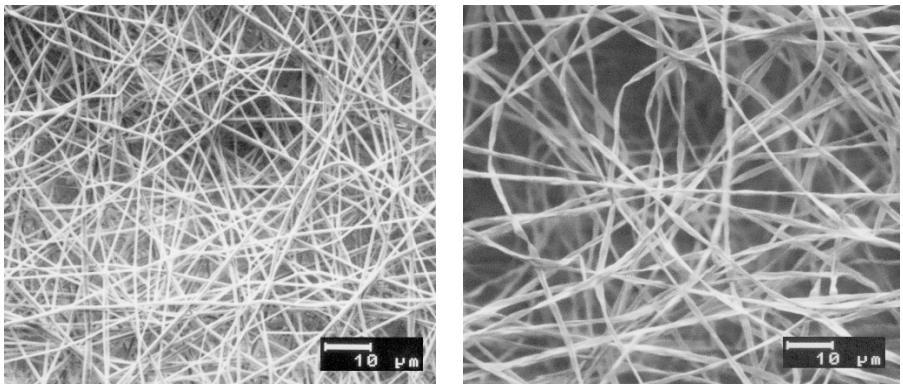


Figure 91: SEM images of meshes

The SEM images revealed the obtained fiber morphology of the electrospun meshes. Electrospinning of PLA PEG PLA resulted in cylindrical shaped thin fibers, while electrospinning of the PLA solution led to ribbon like slightly thicker fibers.

7.5.1.2 Light microscopic images

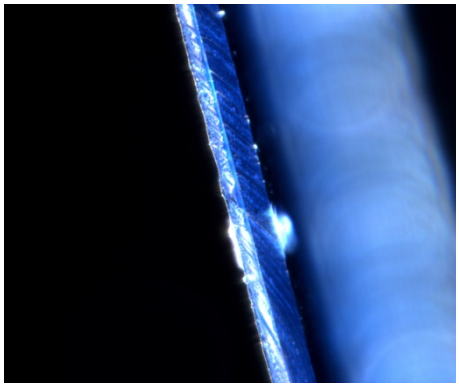
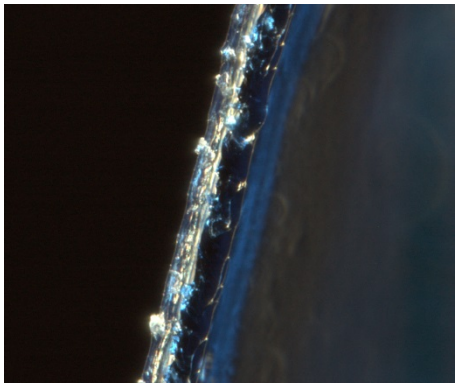
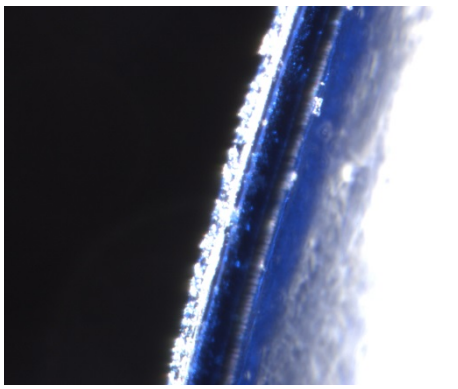
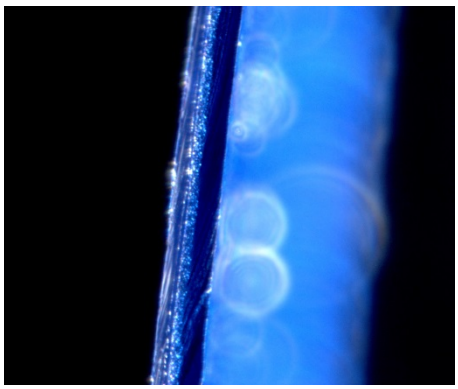
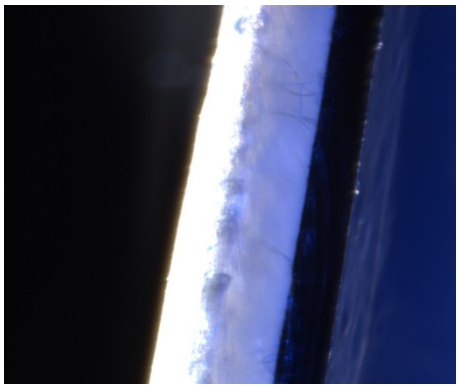
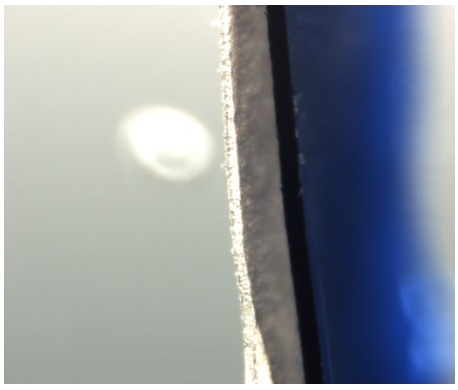
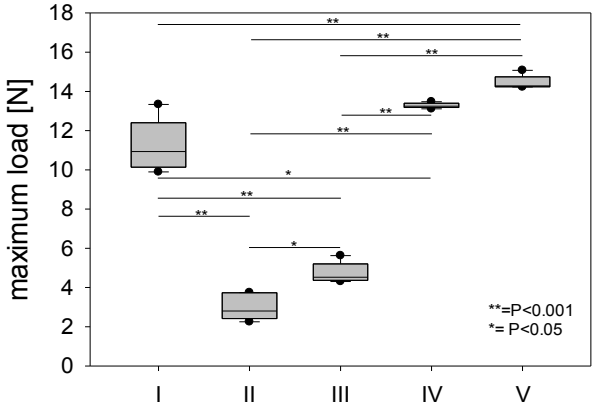
	3% alginate	5% alginate
PLA film		
PLA film PLA PEG PLA mesh		
PLA film PLA mesh		

Figure 92: Light microscopic images of multilayers

In the light microscopic images show the different layers of the prepared films. The images of the bilayer show the PLA layer and the alginate layer that are bound to each other. Images of the trilayers show the difference between the PLA PEG PLA mesh and the PLA mesh. The alginate layer sank into the more hydrophilic PLA PEG PLA mesh and did not sink into the PLA mesh but was covering it completely.

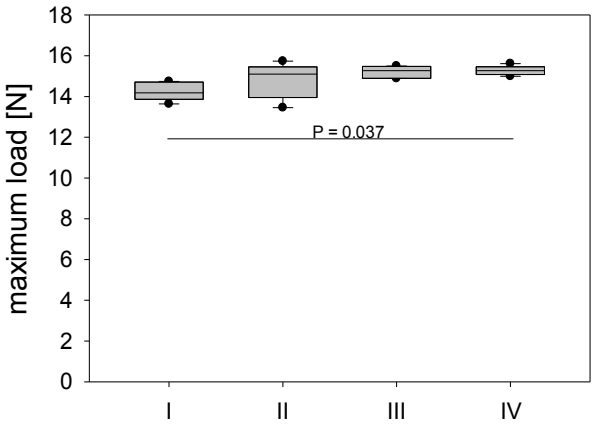
7.5.1.3 Tensile test

a)



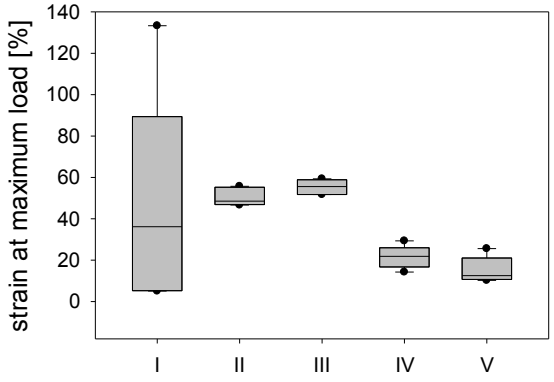
I PLA film
II 3% alginate film
III 5 % alginate film
IV PLA film covered with 3 % alginate
V PLA film covered with 5 % alginate

b)



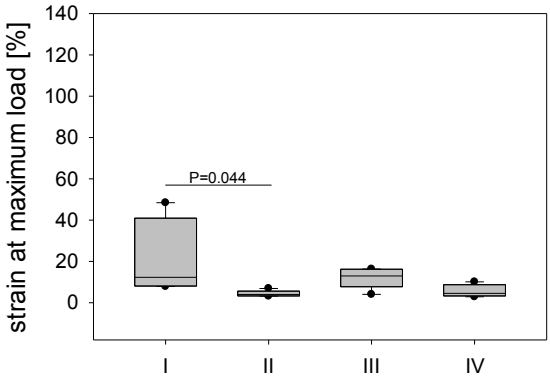
I PLA films with PLA PEG PLA mesh covered with 3 % alginate
II PLA film with PLA mesh covered with 3 % alginate
III PLA film with PLA PEG PLA mesh covered with 5 % alginate
IV PLA film with PLA mesh covered with 5 % alginate

c)



I PLA film
II 3% alginate film
III 5 % alginate film
IV PLA film covered with 3 % alginate
V PLA film covered with 5 % alginate

d)

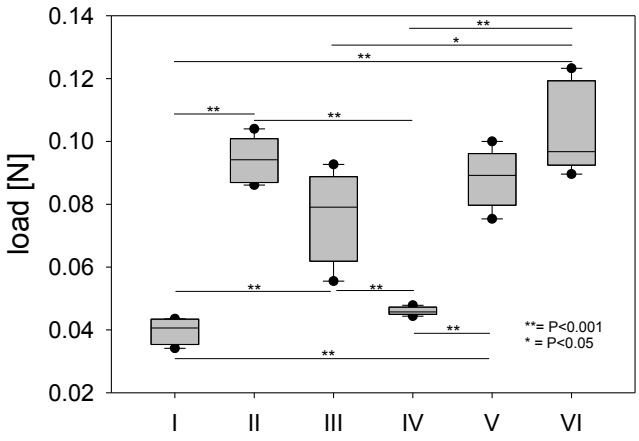


I PLA films with PLA PEG PLA mesh covered with 3 % alginate
II PLA film with PLA mesh covered with 3 % alginate
III PLA film with PLA PEG PLA mesh covered with 5 % alginate
IV PLA film with PLA mesh covered with 5 % alginate

Figure 93: Tensile test of trilayers. First layer is always made of LR708. Polymers for the mesh and alginate solution are as indicated. * equates $p < 0.05$. a) maximum load [N] of mono and bilayers b) maximum load [N] of trilayers c) strain at maximum load [%]of mono and bilayers d) strain at maximum load [%]of trilayers

The PLA film with about 11 N had a higher maximum load than the alginate films with about 3 N and 5 N. The bilayers as well as the trilayers had a maximum load of about 14 N whereas the maximum load of the trilayers was only a little bit higher than the maximum load of the bilayers. The individual PLA film had the highest strain with a quite high variability, followed by the two elastic alginate films of about 50 %. The combined bilayers and trilayers then had the lowest strain of 20 % and less. Generally, this showed that the trilayers can withstand higher forces, but the monolayers can be stretched more than the combined bilayers and the monolayers.

7.5.1.4 T-peel test



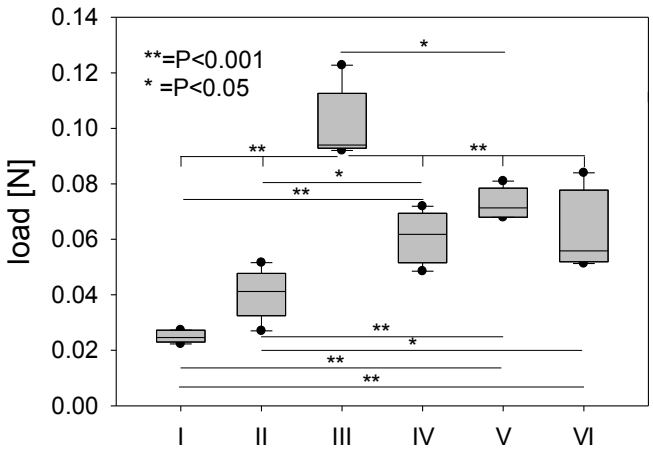
I PLA film covered with 3 % alginate
II PLA film with PLA PEG PLA mesh covered with 3 % alginate
III PLA film with PLA mesh covered with 3 % alginate
IV PLA film covered with 5 % alginate
V PLA film with PLA PEG PLA mesh covered with 5 % alginate
VI PLA film with PLA mesh covered with 5 % alginate

Figure 94: T-peel test of bilayers and trilayers ** equates p<0.001, * equates p < 0.05

The subsequently performed T-peel test showed that the mesh increased the adhesive forces of the PLA film and the alginate film. The bilayers could be separated with a maximum force of about 0.04 N. The films prepared from the 5 % alginate solution thereby sticked slightly better to the PLA film than the film from the 3 % alginate solution. With the structured meshes the load increased significantly to about 0.9 N. During the measurements in could be observed, that the alginate layer was separated from the PLA film with the PLA mesh still adhering to the PLA film. In case of the trilayers with the PLA PEG PLA meshes the mesh together with the alginate layer was removed from the PLA film. The trilayers with the

3 % alginate solution showed a higher adhesive force with the PLA PEG PLA meshes than with the PLA meshes, whereas the trilayers with the 5 % alginate solution had a higher force with the PLA mesh.

7.5.1.5 Mucoadhesion test



I piston
II PLA film
III PLA films with PLA PEG PLA mesh covered with 3 % alginate
IV PLA film with PLA mesh covered with 3 % alginate
V PLA film with PLA PEG PLA mesh covered with 5 % alginate
VI PLA film with PLA mesh covered with 5 % alginate

Figure 95: Mucoadhesion test of the alginate layer from trilayers. ** equates p<0.001, * equates p < 0.05.

The piston without a film and the individual PLA layer showed significantly lower adhesive forces to the pig intestine than all alginate layers of the different trilayers. The trilayers prepared with a PLA PEG PLA mesh and the 3 % solution of alginate generally had the highest average adhesive force of about 0.1 [N].

7.6 Discussion

In contrast to the earlier investigated bilayers, for trilayers it was necessary to use a PLA layer with a higher thickness, because it had to be cut and peeled from the class-plate alone before the second layer, the mesh was added on top of the PLA film using the electro spinning process. Therefore, the absolute values of the mechanical strengths are not comparable.

Similar to the bilayers prepared with cohesion promoters an additive effect regarding the mechanical properties, maximum load and strain at maximum load, could be observed with trilayers prepared with different meshes as cohesion promoter (Figure 93 a + b). As expected the PLA layer alone and the alginate layers alone could only withstand lower forces than the combined trilayers. However, the mesh used as cohesion promoter did not massively affect the tensile properties of the trilayer, indicating only a limited mechanical stability to withstand stretching of the mesh layer. The maximum load as well as the strain at maximum load was nearly in the same range as the respective values of the bilayers. But with the help of the meshes the cohesion between the alginate and the PLA layer could be improved significantly. The T-peel test showed, that higher forces were necessary to separate the layers connected via a mesh than the two layers without a mesh connected via the cohesion promoter glycerol investigated in the bi-layered constructs. Finally this established interdigitation between the two different polymer layers firstly allowed applying a mucoadhesion test without the physical separation of the layers during the test procedure. Accordingly, the PLA film covered with all types of mucoadhesive alginate layers showed a significantly higher adhesion to tissue than the PLA layer alone, indicating a quite promising connection of the different polymer layers.

7.7 Conclusion

With the help of small molecular weight additives like PEI or glycerol it was possible to make the two polymer layers of lipophilic PLA and hydrophilic alginate adhere to each other. The combined bilayers could already withstand higher forces than the monolayers, due to an additive effect of the mechanical properties of the individual monolayers. Testing several thicknesses of the alginate layer it could be seen that with a thickness of about 40 µm an

optimum is reached and that thicker layers show no further improvements regarding the maximum load and the tensile strain.

Due to the still observed separation of the layers upon incorporation in buffers, further stabilization was achieved with the help of mediating polymer meshes and the formation of trilayered constructs. This combination allowed the successful attachment to wet tissue surfaces without an observed separation of the individual film layers. Accordingly, a surgeon would be able to stick the multilayer to the tissue after cutting it into the desired shape and subsequently suture it to the tissue without disturbing slipping at the site of application. Due to the temporary mucoadhesion of the non-cross-linked alginate this additional fixation would still be necessary.

8. Summary and Outlook

Chapter 8 and 9 give a summary of the whole thesis in English as well as in German. Furthermore an outlook is given.

8.1 Summary

Current clinically used lipophilic anti-adhesive films, like SurgiWrap®, have to be fixed to the affected tissue using sutures due to their limited adhesiveness and only degrade very slowly within months and years due to the hydrophobicity and the high molecular weight of the used PLA, which effectively inhibits the uptake of water. The aim of this work was to prepare films that have a much faster degradation pathway due to a hydrogel nature and ideally adhere to the tissue by themselves. Therefore, films of different hydrophilic polymers were prepared, which were thought to provide faster degradation and subsequent elimination as well as good adhesive properties. One investigated polymer was the very well established alginate, which has shown its good characteristics to support skin healing already in commercial wound dressings. However, due to its hydrophilicity and good water solubility, a film consisting of alginate alone would swell and dissolve very fast in the moist environment of the peritoneal cavity. Therefore several cross-linking techniques were established and investigated to obtain thin films with suitable mechanical properties as well as erosion time. As alternative polymer with beneficial properties for wound healing applications hyaluronic acid was chosen, since it is an essential component of the physiological extracellular matrix of many tissues. Whereas a labile physical cross-linking was preferred for the alginate films, films prepared of hyaluronic acid were cross-linked chemically using an easy degradable disulfide linkage to provide sufficient dissolution stability. Due to the obtained limited mechanical stability of the hydrophilic alginate film, it was also investigated in different combinations with anti-adhesive PLA layers to prepare a bifacial film for adhesion prevention after peritoneal surgery. The resulting layered constructs accordingly would be mucoadhesive on one side and anti-adhesive on the PLA side allowing to keep affected tissues glidingly separated and eventually allow a more convenient application by the surgeon.

To obtain a homogeneous distribution of the alginate cross-linking calcium within the film, calcium was added to the polymer solution in form of hardly soluble calcium salts or as stable calcium complex to avoid an uncontrolled and rapid gelation. After casting thin films into a perti dish or with the help of a drawing apparatus onto a glass plate (**Chapter 3**) the calcium could be released well-controlled by lowering the pH with addition of acids, like

lactic acid or a slowly hydrolyzing lactone, like GDL. This process subsequently led to the formation of visibly clear films of accurate thickness and defined cross-linking density.

Initially the process was established using lactic acid solutions, which were sprayed on the turbid films to dissolve the calcium salts. Despite the observed clearing of the films, the added lactic acid also acted as softener, which was revealed during the subsequent mechanical testing of the dry films. The technically different spraying onto films was therefore replaced with a slowly hydrolyzing lactone added to the drawing solution. As further alternative calcium source, the pH-dependent calcium complex with EDTA was also investigated, but here the remaining EDTA acid in the films impeded the cross-linking leading to a fast dissolution in neutral buffer solutions. Accordingly, the better dispersible dibasic calcium phosphate was chosen as calcium source together with gluconic acid δ-lactone as acid component, which lead to homogenous films with adjustable mechanical properties as well as erosion time.

As the preparation of thin films using the drawing apparatus led to very brittle air-dried films, this problem had to be overcome by the addition of suitable plasticizers, like hydrophilic glycerol or polyethylene glycol. Since the small glycerol is most likely withdrawn from the film in the peritoneal cavity very rapidly, the immobilization of the larger PEG was also investigated via chemical linkage to the alginate chains. The physical as well as the chemical addition of PEG did not lead to the desired softening effect, because in both cases PEG tended to crystalize in the dry films. Instead only glycerol with an optimal concentration of 10 % was applied as plasticizer for further film preparation, which was even independent of the minimal residual amounts of water retained by different amounts of glycerol.

The obtained films were investigated regarding their mechanical properties (tensile, puncture and suture pullout tests) and regarding their degradation time in calcium containing buffer solutions over eight weeks. All these investigations of differently composed films and preparation techniques proved that thin alginate films with adjustable mechanical properties and erosion times could be prepared, mainly controlled by the chosen calcium amount and the appropriate content of plasticizer.

Based on these results it was concluded that the actually incorporated calcium amounts mainly determined the properties of the obtained films. For this reason two alginates with

different guluronic acid contents were chosen to investigate the impact of the polymer on the cross-linking process and the resulting film properties (**Chapter 4**). In order to use accurate calcium amounts the calcium binding capacity of the used polymers was verified using isothermal titration calorimetry and accordingly defined amounts of calcium were used for the film preparation. Additional rheological tests, turbidity measurements and a mechanical test of the freshly formed gels further verified the dependence of the cross-linking efficiency on the present calcium amounts as well as the presence of guluronic acid blocks. Finally also the obtained films prepared from the different alginates were tested mechanically and a degradation study in buffers with different calcium amounts was performed. These results showed that the mechanical properties as well as degradation time can also be significantly influenced by the choice of alginate with respect to its guluronic acid content and the used calcium amounts incorporated in the films. The higher the cross-linking extent the longer was the observed degradation time. The mechanical tests furthermore showed, that the properties of the films could not steadily be improved with increasing cross-linking extent, but instead reached a maximum and became even worse, because the resulting films became too stiff and brittle, e.g. for surgical handling. This furthermore highlights the important necessity to determine the actual binding capacity of each used alginate.

To additionally investigate the drug release from thin polymer films two different positively charged drugs, gentamicin sulfate and vancomycin hydrochloride, were incorporated in films prepared from hydrophilic alginate, lipophilic PLA and PLA PEG PLA consisting of hydrophilic and lipophilic polymer blocks (**Chapter 5**). To be able to prepare films from the lipophilic polymers with a homogenous distribution of the hydrophilic drugs two loading procedures were established. With the suspension method, the hydrophilic drug was merely suspended in the polymer solution in form of a dry powder leading to quite big as received drug crystals. With the cosolvens method, the hydrophilic drug was initially dissolved in the cosolvens methanol before it was added to the lipophilic polymer solution, which generally led to the formation of much smaller drug crystals in the dry polymer films.

During the drug loading of the hydrophilic alginate films with the charged drugs, especially gentamicin showed strong ionic interactions with alginate leading to the formation of stable complexes. Therefore a drug-polymer-complex was prepared via precipitation and

subsequently added to the drawn films. The studied drug release for all films was performed with discs in HEPES buffer containing a defined amount of calcium in order to sufficiently stabilize the otherwise dissolving alginate film during the investigation time of the release.

The observed release profiles demonstrated that the release of a hydrophilic drug could be strongly influenced by the polymer used for film preparation and the method of drug loading. The more hydrophilic polymer films also led to a higher released amount of the hydrophilic drug. Even the different methods of drug loading showed a detectable effect, indicating that bigger crystals prepared with the suspension method led to a higher absolute drug release in the investigated time, because the bigger drug crystals reaching out of the thin polymer film could be dissolved faster and more effective than small crystals buried in the lipophilic polymers.

However it was also shown that the expected ionic interactions between the negatively charged polymer alginate and the positively charged gentamicin did not significantly affect the release kinetics, leading only to a minimally delayed drug release, but instead 100 % released amounts from the hydrophilic alginate films, which was generally not achieved for the lipophilic PLA based polymers.

With the help of additional microbiological tests the efficacy of the films against the bacteria escherichia coli, staphylococcus aureus and staphylococcus epidermidis could be demonstrated. Accordingly films were prepared, which release biologically effective drug compounds and provide antibiotic activity that correlates well with the drug amounts detected during the release study.

For the finally investigated cross-linking of hyaluronic acid, a chemical cross-linking was chosen due to the less effective interaction with divalent ions. To cross-link the films properly, hyaluronic acid was thiolated using carbodiimide chemistry with a thiol bearing hydrazide (**Chapter 6**). The cross-linking extent of the prepared films was investigated using hyaluronic acids of two different molecular weights and different degrees of substitution. As expected, the substitution degree could be strongly influenced by the used amounts of activating carbodiimide. However it could also be demonstrated, that steric interactions of the high molecular weight polymer strongly influenced the resulting substitution with thiol. Especially for the high molecular weight polymer the observed strong gel formation during

synthesis lead to less effective conjugation and therefor unpredictable and less effective substitution, probably even with different local distribution along the polymer chain.

During the subsequent film preparation, the influence of the drying speed and the pH of the polymer solutions on the cross-linking extend were investigated. In order to form disulfide bridges, the thiolated hyaluronic acid had to be dissolved in appropriate buffers or be neutralized before drawing on the glass plate. Furthermore the still hydrated films needed oxidizing agents like oxygen and retention of the humidity in order to allow effective oxidation and initiation of cross-linking throughout the films. Fast drying of the films led to insufficient cross-linking and rapid dissolution of the obtained dried polymer films.

In order to enhance the cross-linking process, thiolated hyaluronic acids were furthermore oxidized with alloxan and H_2O_2 and investigated using rheological measurements and a confined compression test. The mechanical evaluation of the obtained films finally demonstrated that similar to alginate the higher cross-linking extend led to more brittle films, indicating the need for additional plasticizers. Also the swelling of the films demonstrated a clear dependence on the achieved cross-linking of the hyaluronic acid. Accordingly further improvements are necessary before these pure hyaluronic acid films can be used in a clinical setting.

The preparation of bifacial films of hydrophilic and lipophilic polymers comes along with several aspects that have to be regarded. Besides the different solubilities of the polymers the wettability and spreading of the layers had to be optimized (**Chapter7**). Furthermore due to the significantly different properties of the used polymers, the two layers did not stick to each other after drying and an easy procedure to enhance cohesion had to be identified. To overcome these problems different additives like PEI and glycerol were investigated as cohesion promoters. PEI was added to the lipophilic PLA layer, to introduce positive charges and enhance spreading of the subsequently drawn hydrophilic alginate layer. Furthermore the negatively charged alginate could interact with the positive charges of the PEI in the PLA layer, which might additionally enhance the cohesion. Glycerol in significantly higher amounts than as plasticizer (Chapter 3 and 4) was also supplied to the alginate layer in order to make it stickier and increase the cohesion to the PLA layer. That already allowed the preparation of bilayers and made the two layers stick to each other initially. Unfortunately the two layers of all bilayers could be easily separated after drying, further incubation of the

films in buffer even showed, that the cohesion promoters are only effective in the air dried films, resulting in immediate delamination in buffer. Accordingly these bifacial films are only beneficial for the immediate handling of the films during surgical application, since the mucoadhesive film will only stay temporarily on the PLA layer.

Another investigated possibility to enhance cohesion of the two film layers was the modification with the help of porous polymer meshes. The PLA layer was accordingly covered with an electrospun mesh consisting of lipophilic PLA or the more hydrophilic PLA PEG PLA. Although contact angle measurements revealed, that the meshes had a higher contact angle than the pure PLA film, the alginate solution drawn onto the PLA film covered with the PLA PEG PLA mesh penetrated into the mesh and made the two layers stick to each other much stronger than for the investigated bilayers. In contrast the alginate solution did not penetrate into the more lipophilic PLA mesh, indicating also an effect of the used polymer for electrospinning. Subsequently performed T-peel tests revealed that the separation of the two layers connected with the mesh needed higher forces than the separation of the two layers without the mesh. Accordingly, the finally performed mucoadhesion test demonstrated that the created layered device was stable enough to show adhesion to tissue without separation of the alginate layer from the PLA layer. Layered constructs of different polymers may therefore be ideal new devices to provide mucoadhesion and anti-adhesive properties in one system.

8.2 Conclusion and Outlook

With the help of different chemical and physical cross-linking techniques it was possible to prepare polymer films of the hydrophilic polymers alginate and hyaluronic acid with adjustable mechanical properties and erosion time. The achieved variability is highly valuable to adjust the prepared films for the treatment of different wound sites and applications.

By successfully combining two different polymer layers, a bifacial film with a mucoadhesive hydrophilic layer and a smooth lipophilic layer could be prepared, which might be useful as new anti-adhesive device with potentially easier fixation at the application site. However, this needs to be verified and later probably improved using hydrophilic polymers, which form permanent covalent linkages to the damaged peritoneum.

The established at least bilayered films might furthermore be promising to combine different polymers and drug loading techniques to achieve devices with multiple release steps. This is highly intriguing to provide for example an initial burst release followed by a slower release over the time in order to initially fight infections and later support wound healing using the appropriate drug substances. Accordingly designed sophisticated systems would need to be investigated in-vitro and subsequently tested in appropriate animal models.

9. Zusammenfassung und Ausblick

9.1 Zusammenfassung

Zurzeit müssen lipophile antiadhäsive Filme, wie SurgiWrap®, an betroffenes Gewebe angenäht werden, da sie nicht unmittelbar am Gewebe anhaften. Des Weiteren bauen sich diese Filme aus PLA aufgrund ihres hydrophoben Charakters und der dadurch gehinderten Wasseraufnahme im Körper sehr langsam ab, was Monate bis Jahre dauern kann. Ziel dieser Arbeit war die Herstellung von Filmen, welche sich aufgrund ihres hydrophilen Charakters schneller abbauen und sich idealerweise am Gewebe anheften. Um dies zu erreichen wurden Filme aus unterschiedlichen hydrophilen Polymeren hergestellt, welche einen schnelleren Abbau und daher eine schnellere Elimination, sowie auch die Fähigkeit zur Gewebeanhaftung aufwiesen. Ein dafür eingesetztes Polymer war das schon klinisch etablierte Alginat, welches seine guten Eigenschaften bei der Unterstützung der Wundheilung in kommerziell erwerblichen Wundauflagen gezeigt hat. Allerdings würde sich ein Film, welcher nur aus Alginat besteht aufgrund seiner Hydrophilie und guten Wasserlöslichkeit in der feuchten Umgebung des Peritoneums relativ schnell auflösen. Daher wurden mehrere Quervernetzungsmethoden etabliert und untersucht, um dünne Filme mit geeigneten mechanischen Eigenschaften und Abbauzeiten zu erhalten. Als weiteres Polymer mit guten Eigenschaften für die Wundheilung wurde Hyaluronsäure gewählt, da sie ein essentieller Bestandteil der physiologischen Extrazellulärmatrix vieler Gewebe ist. Wohingegen eine leichte physikalische Quervernetzung für die Alginatfilme bevorzugt wurde, wurden die Filme aus Hyaluronsäure chemisch mit reduktiv spaltbaren Disulfidbrücken quervernetzt, um eine ausreichende Stabilität gegenüber einer schnellen Auflösung zu gewährleisten. Aufgrund der nur limitierten mechanischen Stabilität der hydrophilen Alginatfilme wurden diese außerdem mit einer antiadhäsiven PLA Schicht kombiniert, um einen zweiseitigen Film für die Vermeidung von Adhäsionen nach peritonealen Operationen herzustellen. Die resultierenden mehrlagigen Konstrukte weisen eine mukoadhäsive Schicht auf der einen Seite und eine antiadhäsive Schicht auf der PLA Seite auf, welche das Gleiten des beeinträchtigten Gewebes gewährleisten und ebenso die Handhabung durch den Chirurgen erleichtern soll.

Um eine homogene Verteilung des Alginat-vernetzenden Calciums im Film zu erhalten, wurde das Calcium in Form eines schwer löslichen Calciumsalzes oder eines stabilen

Calciumkomplexes zu der Polymerlösung hinzugegeben, um eine unkontrollierte schnelle Quervernetzung zu vermeiden. Nachdem dünne Filme in Petrischalen gegossen oder mit Hilfe eines Filmziehgerätes auf Glasplatten gezogen wurden (**Kapitel 3**) konnte das Calcium kontrolliert freigesetzt werden, indem der pH-Wert durch Zugabe von Säuren, wie Milchsäure oder des langsam hydrolysierenden Laktons GDL, herabgesetzt wurde. Mit diesem Prozess konnten transparente, klare Filme mit einer definierten Schichtdicke und Quervernetzungsdichte hergestellt werden.

Zunächst wurde der Prozess mit Milchsäure etabliert, welche auf die trüben Filme gesprüht wurde, um das Calciumsalz aufzulösen. Neben der zu beobachtenden Klärung der Filme wirkte die Milchsäure aber zusätzlich auch als Weichmacher, was anschließende mechanische Testungen der getrockneten Filme zeigten. Das undefinierte Besprühen der Filme wurde daher durch ein langsam hydrolysierendes Lakton ersetzt, welches zu der auszuziehenden Lösung gegeben wurde. Eine weitere alternative Calciumquelle, der pH sensitive Calcium-EDTA-Komplex wurde auch untersucht, in dem Fall verhinderte das im Film verbleibende EDTA die effiziente Quervernetzung, was zu einer zügigen Auflösung in neutralem Puffer führte. Demzufolge wurde das besser dispergierbare Calcium-hydrogenphosphat als bevorzugte Calciumquelle in Kombination mit GDL als saure Komponente verwendet, was zu homogenen Filmen mit einstellbaren mechanischen Eigenschaften sowie variablen Abbauzeiten führte.

Da die Herstellung von dünnen Filmen mit Hilfe eines Filmziehgeräts zu sehr brüchigen luftgetrockneten Filmen führte, mussten Weichmacher, wie Glycerin oder Polyethylenglykol, hinzugegeben werden, um dieses Problem zu beheben. Weil das kleine Glycerin in der Peritonealhöhle wahrscheinlich recht schnell aus dem Film herausgelöst wird, wurde eine Fixierung des größeren PEGs über eine chemische Bindung an die Alginatkette untersucht. Die physikalische aber auch die chemische Zugabe von PEG führte jedoch nicht zu dem erwünschten weichmachenden Effekt, da in beiden Fällen das PEG dazu neigte in den getrockneten Filmen auszukristallisieren. Stattdessen wurde nur Glycerin mit einer optimalen Konzentration von 10 % als Weichmacher für weitere Filmherstellungen verwendet, was unabhängig von dem zurückgehaltenem Wasser war, welches von unterschiedlichen Mengen Glycerin im Film zusätzlich gebunden wurde.

Die erhaltenen Filme wurden bezüglich ihrer mechanischen Eigenschaften (Zug-, Durchstoß- und Fadenausreißtest) und ihrer Abbaubarkeit in Calcium haltigen Pufferlösungen über einen Zeitraum von acht Wochen untersucht. All diese Untersuchungen von Filmen mit unterschiedlicher Zusammensetzung und unterschiedlichen Herstellungsmethoden zeigten, dass Filme mit einstellbaren mechanischen Eigenschaften und Abbauzeiten hergestellt werden konnten. Dies wurde hauptsächlich von der gewählten Calciummenge und dem entsprechenden Gehalt an Glycerin kontrolliert.

Basierend auf diesen Ergebnissen konnte man schlussfolgern, dass die tatsächlich vorhandene Menge Calcium hauptsächlich verantwortlich ist für die Eigenschaften der resultierenden Filme. Aus diesem Grund wurden zwei Alginate mit unterschiedlichem Gehalt an Guluronsäure gewählt, um auch den Einfluss des Polymers auf den Quervernetzungsprozess und die erhaltenen Filmeigenschaften zu untersuchen (**Kapitel 4**). Um eine möglichst exakte Menge an Calcium zu benutzen, wurde die Calciumbindungskapazität der benutzen Polymere mit Hilfe der isothermen Titrationskalorimetrie überprüft und entsprechende Calciummengen für die Filmherstellung benutzt. Zusätzlich durchgeführte rheologische Tests, Trübungsmessungen und mechanische Tests der frisch hergestellten Gele zeigten die Abhängigkeit der Quervernetzungseffektivität sowohl von der vorhandenen Calciummenge als auch der Häufigkeit der Guluronsäureblöcke. Schlussendlich wurden auch die aus den unterschiedlichen Alginaten hergestellten Filme mechanisch getestet und eine Abbaustudie in Puffern mit unterschiedlichen Calciummengen durchgeführt. Diese Untersuchungen zeigten, dass sowohl die mechanischen Eigenschaften als auch die Abbauzeit stark von der Wahl des Alginates mit seinem Guluronsäuregehalt und der eingearbeiteten Calciummenge beeinflusst werden können. Je höher der Quervernetzungsgrad, desto länger war die beobachtete Abbauzeit. Die mechanischen Tests zeigten außerdem, dass die Eigenschaften der Filme nicht stetig mit einer Erhöhung des Quervernetzungsgrads verbessert werden konnten. Es wurde im Gegenteil ein Maximum beobachtet, nach welchem die Filme schlechter wurden, da die Filme dann steif und brüchig wurden, was für die chirurgische Handhabung nicht geeignet wäre. Dies unterstreicht die Wichtigkeit der Untersuchung der Bindungskapazität jedes verwendeten Alginats für weitere Filmherstellungen.

Um zusätzlich die Arzneistofffreisetzung aus dünnen Polymerfilmen zu untersuchen, wurden zwei positiv geladene Arzneistoffe, Gentamicinsulfat und Vancomycin-hydrochlorid in Filme eingearbeitet, welche aus hydrophilem Alginat, lipophilem PLA und PLA PEG PLA bestehend aus hydrophilen und lipophilen Polymerblöcken hergestellt wurden (**Kapitel 5**). Um Filme aus den lipophilen Polymeren mit einer homogenen Verteilung der hydrophilen Arzneistoffe herstellen zu können, wurden zudem zwei Beladungsmethoden etabliert. Mit der Suspensionsmethode wurde der Arzneistoff in Form eines trockenen Pulvers lediglich in der Polymerlösung suspendiert was zu recht großen Arzneistoffkristallen im Film führte. Mit der Kosolvensmethode wurde der hydrophile Arzneistoff zunächst im Kosolvens Methanol gelöst, bevor er zur Polymerlösung gegeben wurde, was generell zur Bildung von viel kleineren Kristallen im trockenen Polymerfilm führte.

Während der Arzneistoffbeladung der hydrophilen Alginatfilme mit den geladenen Arzneistoffen, zeigte insbesondere Gentamicin eine starke ionische Wechselwirkung mit dem Alginat, was zur Bildung eines stabilen Komplexes führte. Daher wurde mit einer Präzipitation ein Arzneistoff-Polymer-Komplex hergestellt, mit welchem anschließend die Filme beladen und gezogen werden konnten. Die resultierende Arzneistofffreisetzung aus allen Filmen wurde mit Scheiben in HEPES Puffer untersucht, welcher eine definierte Menge an Calcium enthielt, um den Alginatfilm über die Zeit der Untersuchung der Arzneistofffreisetzung zu stabilisieren, da dieser sind sonst aufgelöst hätte.

Die beobachteten Freisetzungsprofile zeigten, dass die Freisetzung eines hydrophilen Arzneistoffs sowohl stark vom für die Filmherstellung verwendeten Polymer als auch von der Methode der Arzneistoffbeladung beeinflusst werden kann. Die hydrophileren Polymerfilme führten so zu einer deutlich höheren Freisetzung des hydrophilen Arzneistoffs. Auch die unterschiedlichen Methoden der Arzneistoffbeladungen zeigten einen nachweisbaren Effekt. Größere Kristalle, hergestellt mit der Suspensionsmethode führten zu einer höheren Freisetzung im untersuchten Zeitraum, da die größeren Kristalle aus dem dünnen Film herausschauten und schneller aufgelöst werden konnten als kleine Kristalle, welche im lipophilen Polymer eingebettet waren.

Jedoch konnte auch gezeigt werden, dass die erwarteten ionischen Interaktionen zwischen dem negativ geladenen Alginat und dem positiv geladenen Gentamicin die Freisetzungskinetik nicht signifikant beeinflussten. Es kam lediglich zu einer minimalen

initialen Verzögerung der Arzneistofffreisetzung, dann aber zu einer vollständigen Freisetzung aus den hydrophilen Alginatfilmen, was jedoch generell nicht mit den lipophilen Polymeren beobachtet wurde.

Mit Hilfe zusätzlich durchgeführter mikrobiologischer Tests konnte die Effektivität der Filme gegen Bakterien wie Escherichia Coli, Staphylococcus Aureus und Stapylococcus Epidermidis gezeigt werden. Entsprechend wurden Filme hergestellt, welche biologisch aktive Arzneistoffkomponenten freisetzten und eine antibiotische Aktivität boten, welche mit den Arzneistoffmengen, welche während der Freisetzungsversuche gefunden wurden korrelierten.

Für die weiterhin untersuchte Quervernetzung der Hyaluronsäure wurde eine chemische Quervernetzung gewählt, was aufgrund der weniger effektiven Interaktion mit bivalenten Ionen notwendig war. Um die Filme ausreichend zu vernetzten, wurde die Hyaluronsäure mit einem Thiol haltigen Hydrazid mit Hilfe der Carbodiimid-Chemie thiofunktionalisiert (**Kapitel 6**). Um den Quervernetzungsgrad der hergestellten Filme zu untersuchen, wurden zwei Hyaluronsäuren mit unterschiedlichen Molekulargewichten und unterschiedlichen Substitutionsgraden benutzt. Wie erwartet konnte der Substitutionsgrad stark von den eingesetzten Mengen an aktivierendem Carbodiimid beeinflusst werden. Jedoch konnte auch gezeigt werden, dass sterische Interaktionen der hochmolekularen Polymere die resultierende Substitution mit Thiolen stark beeinflussten. Speziell für das Polymer mit dem hohen Molekulargewicht konnte während der Synthese so eine starke Gelbildung beobachtet werden, was zu einer wenig effektiven Konjugation und somit weniger effektiven Substitutionen führte. Wahrscheinlich führte das sogar zu einer unterschiedlichen lokalen Verteilung der Thiolfunktionen entlang der Polymerkette.

Während der anschließenden Filmherstellung wurde der Einfluss der Trocknungs-geschwindigkeit und des pH-Wertes der Polymerlösung auf den Quervernetzungsgrad untersucht. Um Disulfidbrücken auszubilden, musste die thiofunktionalisierte Hyaluronsäure in einem angemessenen Puffer gelöst und neutralisiert werden, bevor sie auf einer Glasplatte ausgezogen werden konnte. Außerdem benötigten die hydratisierten Filme oxidierende Substanzen wie Sauerstoff und eine gewisse Restfeuchtigkeit, um eine effektive Oxidation und Initiation der Quervernetzung im Film zu gewährleisten. Eine zu schnelle

Trocknung der Filme führte zu einer nicht ausreichenden Quervernetzung und somit zu einer vorzeitigen Auflösung der erhaltenen trockenen Polymerfilme.

Um den Quervernetzungsprozess zu verbessern wurde die thiofunktionalisierte Hyaluronsäure mit Alloxan oder H_2O_2 oxidiert und mit Hilfe von rheologischen Messungen und eines Kompressionstests untersucht. Die mechanischen Tests der erhaltenen Filme zeigten, dass ähnlich zu den Alginatfilmen eine höhere Quervernetzung zu brüchigeren Filmen führte, was zeigt, dass ein zusätzlicher Weichmacher benötigt wird. Auch die Quellung der Filme zeigte eine deutliche Abhängigkeit von der erreichten Quervernetzung der Hyaluronsäure. Daher sind weitere Verbesserungen notwendig damit die puren Hyaluronsäurefilme eingesetzt werden können.

Die Herstellung von zweiseitigen Filmen aus einem hydrophilen und einem lipophilen Polymer geht einher mit mehreren Aspekten, welche in Betracht gezogen werden müssen. Neben der unterschiedlichen Löslichkeit der Polymere, muss die Benetzbarkeit und die Spreitung der Schichten optimiert werden (**Kapitel 7**). Aufgrund der signifikant unterschiedlichen Eigenschaften der Polymere, haften die zwei Lagen nach Trocknung nicht aneinander. Daher musste eine einfache Prozedur entwickelt werden um die Anhaftung der Lagen zu verbessern. Um dieses Problem zu beheben wurden unterschiedliche Zusätze wie Polyethylenimin (PEI) und Glycerin eingesetzt um die Haftung zu verbessern. PEI wurde zur lipophilen PLA Schicht hinzugegeben, um positive Ladungen in den Film einzubringen und die Spreitung der anschließend ausgezogenen hydrophilen Alginatschicht zu verbessern. Des Weiteren konnte das negativ geladene Alginat mit den positiven Ladungen des PEI in der PLA-Schicht interagieren, was die Anhaftung zusätzlich verbesserte. Glycerin in signifikant höheren Konzentrationen als eingesetzt als Weichmacher (Kapitel 3 und 4) wurde zu der Alginatschicht gegeben, um diese klebriger zu machen und die Anhaftung an die PLA Schicht zu erhöhen. Dies erlaubte erstmals die Herstellung von zweischichtigen Filmen, in denen die beiden Lagen aneinander hafteten. Unglücklicherweise konnten die zwei Schichten aller zweischichtigen Filme nach der Trocknung leicht wieder voneinander getrennt werden. Eine zusätzliche Inkubation in Puffer zeigte, dass die Zusätze, welche die Kohäsion ermöglichten nur in den getrockneten Filmen effektiv waren und sich im Puffer sofort trennten. Daher sind die zweiseitigen Filme nur für die direkte Anwendung während einer Applikation von

Nutzen, da der mukoadhäsive Film nur für einen sehr kurzen Zeitraum an der PLA Schicht haftet.

Eine weitere Möglichkeit die Haftung der Schichten zu verbessern war die Modifikation mit Hilfe von porösen Polymernetzen. Die PLA Schicht wurde mit einem elektrogesponnenen Netz aus lipophilem PLA oder hydrophilerem PLA PEG PLA beschichtet. Obwohl Kontaktwinkelmessungen zeigten, dass die Netze einen höheren Kontaktwinkel als der Film aufwiesen, konnte die Alginatlösung, welche auf dem mit einem PLA PEG PLA Netz beschichteten PLA Film ausgezogen wurde, in das Netz eindringen und sorgte dafür, dass die beiden Schichten stärker aneinander hafteten als bei den einfachen zweischichtigen Filmen. Im Gegensatz dazu konnte die Alginatlösung jedoch nicht in das lipophile PLA Netz eindringen, was auch einen Einfluss des für das Elektrospinnen verwendeten Polymers zeigte. Anschließend durchgeführte Abschältests zeigten, dass die Trennung der zwei Schichten, welche über ein Netz miteinander verbunden waren höhere Kräfte benötigte als die Trennung von zwei Schichten ohne Netz. Demzufolge zeigten Mukoadhäsionstest, dass die hergestellten mehrschichtigen Filme stabil genug sind, um an Gewebe zu haften, ohne dass sich die Alginatschicht von der PLA Schicht trennt. Daher können mehrschichtige Filme aus unterschiedlichen Polymeren ein ideales neues Produkt darstellen, welches mukoadhäsive und antiadhäsive Eigenschaften in einem System aufweist.

9.2 Schlussfolgerung und Ausblick

Mit Hilfe unterschiedlicher chemischer und physikalischer Quervernetzungsmethoden war es möglich Polymerfilme aus den beiden hydrophilen Polymeren Alginat und Hyaluronsäure mit einstellbaren mechanischen Eigenschaften und Abbauzeiten herzustellen. Die erreichte Variabilität ist sehr nützlich, um die hergestellten Filme für die Behandlung unterschiedlicher Wunden und Verwendungszwecke anzupassen.

Mit der erfolgreichen Kombination zweier unterschiedlicher Polymerschichten konnten so zweiseitige Filme mit einer mukoadhäsiven und einer glatten Schicht hergestellt werden, was sehr nützlich ist für neuartige antiadhäsive Film mit leichterer Fixierbarkeit an der Applikationsstelle. Trotzdem müsste dies weiter untersucht und verbessert werden, indem man hydrophile Polymere benutzt, welche eine permanente kovalente Bindung mit dem verletzten Peritoneum bilden.

Die hergestellten zweischichtigen Filme könnten außerdem vielversprechend sein, um Polymere und Arzneistoffbeladungstechniken zu kombinieren und so Filme mit mehreren Freisetzungsstufen herzustellen. Dies ist interessant, um zum Beispiel eine initiale schnelle Freisetzung gefolgt von einer langsameren Freisetzung zu erreichen. So könnten initial Infektionen bekämpft werden, um anschließend die Wundheilung mit anderen Arzneistoffen zu unterstützen. Dementsprechend hergestellte ausgeklügelte Systeme müssten jedoch zunächst in-vitro und anschließend in einem angemessenen Tiermodell getestet werden.

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