# Topical delivery of $\alpha_1$-Antichymotrypsin for wound healing

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## Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 und 4 der Promotionsordnung vom 29. Januar 1998 von Herrn Prof. Dr. G. Winter betreut.

## Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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**To my parents**

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# 1 Introduction

The World Health Organisation of the United Nations prognoses the development of world-wide diabetes cases over the next decades in actual studies. Accordingly, the number of type II patients will more than double until 2030[1].
Next to the disease itself, moreover, 25% of diabetes patients frequently develop chronic wounds with about half of them requiring elaborate inpatient treatment. Therefore, the diabetic foot causes more hospitalisation than does any other complication associated with diabetes and represents approximately 2.5% of all hospital admissions[2].
Not least driven by this need wound treatment has gone through great revolution during the last decades. The paradigm shift from dry dressings based on woven fabrics towards a moist environment caused whole lots of new developments in this field.
Nevertheless, the pathological cases of chronic wounds are still difficult to handle. Even with the moist concept they require very patient and persistent treatment. Therefore, there still is strong desire for improved methods of therapy. And, due to the prognosed rise of the diabetes, this desire is likely to largely increase over the next decades.
One resort out of this situation could be revealed by biotechnology. Especially in the year 2000, the even greater revolutions in this field mostly occurring in typical scientific laboratories but praised and supported by highest authorities raised great expectations for new drug candidates. Hence for example, on international level United States President Bill Clinton announced the completion of the first survey of the entire human genome and nationally the government of the free state of Bavaria launched its high-tech-offensive endowed with investments of 1.35 billion Euros in local life science research. In this environment several small new biotech companies were founded. A considerable number of those established in Martinsried near Munich and began their research work. One of those companies is the SWITCH BIOTECH AG focusing its research on wound treatment based on peptides. One outcome of this work was the identification of $\alpha_1$-antichymotrypsin (ACT) as potential therapeutic for chronic wounds.
Hence, to take the next step in development of $\alpha_1$-antichymotrypsin (ACT) as drug candidate a collaboration between SWITCH BIOTECH AG and the Department Pharmaceutical Technology and Biopharmaceutics of the LMU Munich was

contracted in terms of a Ph. D. study at the Department under the supervision of Prof. Dr. Winter.

The aim of this study is to create formulations and drug carriers that stabilise and deliver ACT in bioactive state into the wound site. This thesis addresses introductory remarks about wound healing and protein delivery from hydrogels followed by the results of the research on the ACT formulation and concomitant carrier development.

# 1.1 Wound healing

In this section an overview over the physiology and pathophysiology of wound healing is described. This represents the medicinal context of this work and highlights the scientific rational behind the delivery of ACT in chronic wounds. Moreover, the established methods for treatment of wounds including dressings and carriers are discussed. Hereby, the methodical context of a treatment with ACT as drug product with the indication wound treatment is given.

## 1.1.1 Physiology of wound healing

The following paragraph describes the physiology of wound healing. It starts with the process of healing itself. Next, a selection of growth factors involved in the process is outlined in detail. The role of growth factors is essential for the present understanding of wound healing. Moreover, growth factors are important starting points for drug based wound treatment having led to approval of platelet-derived growth factor (PDGF), the main competitor of a maybe future product based on ACT. Consequently, proteases and their inhibitors relevant in wound healing are highlighted. As well as growth factors, proteases play important roles in wounds. And, together with their inhibitors proteases are also starting points for wound therapy. Thereby, the discussion focuses on the inhibitor ACT and its target cathepsin G because ACT and its delivery represents the main topic of this thesis.

### *1.1.1.1 Wound healing process*

The physiological wound healing process in the present understanding is usually divided into four steps – coagulation, inflammation, followed by migration and proliferation and finally the remodelling phase. These phases are not exactly distinguishable from each other, because occasionally they overlap or proceed concurrently.

In the damaged vessel wall platelets - stimulated by mediators - immediately adhere to the exposed collagen of the vessel wall. The clustered platelets partially coalesce with each other and release the platelet factors that initiate the actual clotting process. During the clotting a network of fibrin forms around the platelet plug finally filling the entire wound gap. The purpose of this fibrin network or first extracellular matrix is to retain cellular components of the blood, e.g. erythrocytes,

and thereby form a clot for the purpose of haemostasis, wound closure and provision of a matrix for the later collagen mounting[3].

*Coagulation* – this part is started by the platelet factors released by degranulating thrombocytes and by substances liberated from damaged tissue cells, for example PDGF, IGF-I, EGF and TGF-β. Following, the coagulation cascade, the well known complex chain reaction, which is initiated by injury[4] leading to the conversion of prothrombin into the enzyme thrombin is activated. Thrombin now converts fibrinogen into fibrin monomers.
Flowing blood only contains fibrinogen, the water-soluble precursor of fibrin. The conversion is solely catalyzed by thrombin located at the wound surface. Thrombin is also present in the blood as its inactive precursor prothrombin. Prothrombin and fibrinogen are coagulation or clotting factors and part of the coagulation cascade. Fibrinogen polymerises to fibrin chains, which are finally interlinked by coagulation factor XIII to form the stable fibrin network[5]. A variety of inhibitors of the coagulation factors present in the blood, for example antithrombin III, ensure that clotting is confined to the wound site. They inactivate thrombin entering the circulating blood stream. Moreover, the members of the cascade are at much lower concentration levels in the blood stream which decreases the presumption that the necessary partners meet to interact. This contributes to the confinement of the coagulation to the wound area.

*Inflammation* - once haemostasis is achieved inflammation is initiated a few hours after injury. The inflammatory phase of wound healing is characterised by recruitment and activation of granulocytes, macrophages and lymphocytes that clean the wound by phagocytosis of damaged tissue and bacteria and wound debridement by enzymatically degrading foreign matter and damaged tissue. The substances released from the cell debris resulting from tissue destruction are responsible for causing the characteristic inflammatory reactions. Vascular changes also contribute to this reaction. A fresh wound usually bleeds due to vessels rupture effecting cleansing the wound. To prevent further blood loss, the affected vessels narrow within the first minutes. This vasoconstriction is followed by vasodilation that increases the blood circulation in the wound area. Consequently, a rise in the temperature of the wound and the surrounding skin is caused. Moreover, the permeability of the capillary walls is increased by vasodilatory agents – such as histamine and serotonin – and as a result blood plasma, erythrocytes, leucocytes and platelets enter the wound. The outcome of these processes is wound oedema.

This creates the classic signs of the inflammatory process i.e. redness, swelling, functional disturbance, heat, and pain. Pain results from the wound oedema exerting increased pressure on the small nerves and nerve endings. The inflammatory reaction is induced independently of invasion by foreign organisms, and therefore inflammation can also develop in closed injuries where the skin remains intact[6].

Mediated by the chemotactic effects of cytokines and growth factors infiltrating white cells, such as neutrophils and macrophages, are attracted and activated (Fig. 1-1).

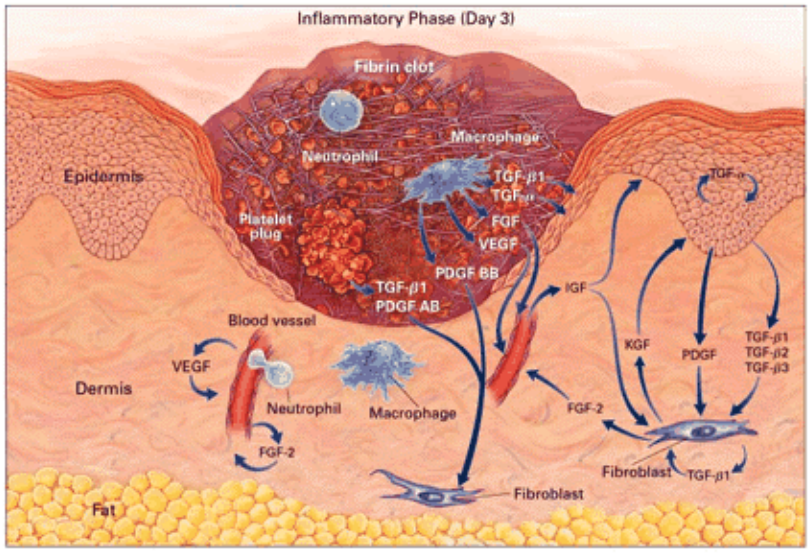


Fig. 1-1 A cutaneous wound three days after injury with growth factors thought to be necessary for cell movement into the wound – adapted from [7]

The defence cells migrate from the blood vessels that have become permeable into the wound area[7]. First to appear at the site of inflammation are neutrophils. These have the capacity to phagocytose bacteria and extracellular matrix. They also release enzymes that break down degenerating connective tissue[8]. Consequently, monocytes infiltrate attracted by chemotaxins, e.g. PDGF and TGF-β, released by aggregating platelets. Monocytes are white blood cells and precursors to macrophages. So, they are also capable of phagocytosis and once they have ingested foreign bodies they transform into macrophages[9].

Macrophages produce a large number of mediators, e.g. growth factors, of other wound healing processes and attract further phagocytic cells to the wound area[10]. Moreover, prostaglandins that sustain the inflammatory process and influence vascular dilation and IL-1 which induces fever and attracts further neutrophil granulocytes are secernated. Macrophages also release enzymes destroying tissue.

IL-1β also draws fibroblasts into the wound and up-regulates enzyme levels. Importantly, there is a balance between levels of enzymes and tissue inhibitors of these enzymes.
Inflammation physiologically last for several days. Neutrophil infiltration reaches a maximum after approximately 24 hours and declines over the next few days. These cells, once present, survive for about a further 24 hours[11].

*Migration and proliferation* - a few days after injury the migration and proliferation phase begins. Whereas catabolic processes predominate in inflammation, this phase of wound healing is characterised mainly by anabolic reactions, i.e. angiogenesis, epithelisation, and fibroplasia (Fig. 1-2). It can last for up to 24 days from the moment the wound develops.

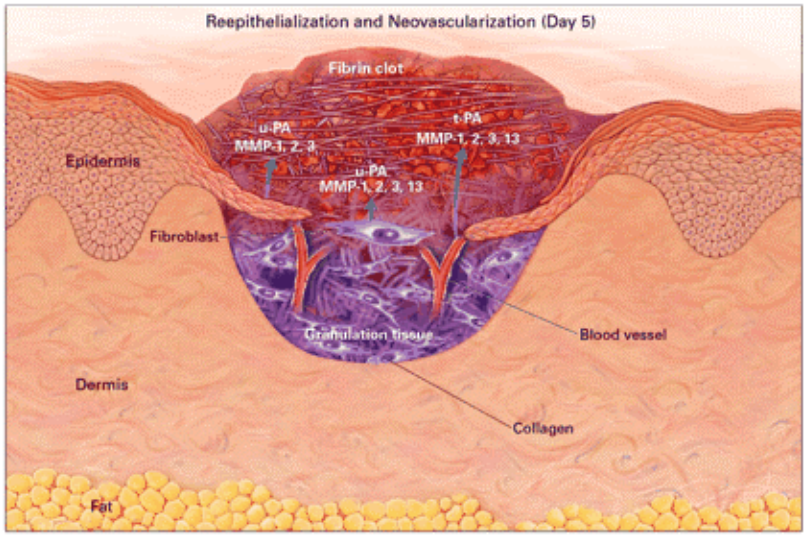


Fig. 1-2 A cutaneous wound five days after injury; blood vessels sprout into the fibrin clot as epidermal cells resurface the wound. Proteinases thought to be necessary for cell movement are shown. – adapted from [7]

The formation of blood vessels, angiogenesis, starts with an endothelial cell bud formed by existing intact vessels. Thereby, the angiogenic stimuli in the first place emanate from macrophages by secession of growth factors and cytokines. But, also keratinocytes as well as fibroblasts provide chemoattractants. So stimulated, the endothelial cells in the venules begin to produce enzymes that break down the basal membrane in the area of the stimulus[12]. Soon, endothelial cells migrate through the resulting gap in the direction of the wound following the oxygen gradient. They divide and form tubular structures that connect with other buds. As a result, during the maturation process a new basal membrane develops from the extracellular matrix components. The newly formed vascular loops then connect with intact

vessels and differentiate accordingly into capillaries, arterioles, and venules, respectively[3].

For epithelisation, proceeding in parallel to angiogenesis, keratinocytes migrate across the wound and as a result reconstitute epidermal covering from the wound margin and hair follicle remnants[13]. In addition, migration essentially requires the presence of a moist substrate, well perfused with blood, as is the case with granulation tissue. In contrast, epithelial cells are not able to migrate in a dehydrated layer. This is supposed to be a reason for the success of moist wound dressings.

Fibroplasia is determined by the chemotactically attracted migration of fibroblasts along the fibrin network into the wound site and their replicative activity there to form the new loose extracellular matrix consisting of proteoglycans as well as the water-soluble collagen fibres essential for tissue stability. Thus, especially at wound edges fibroblasts are the predominant cell type[14].

Concurrently, the provisional fibrin network, the first extracellular matrix, is broken down by fibrinolysis. This breakdown process is caused by the enzyme plasmin. Mainly, t-PA activates plasmin from its inactive precursor plasminogen[7].

Collagen is crucial to the process of wound healing as it has been identified as the most abundant connective tissue protein. Collagen is a fibrous protein synthesised in several stages. Its precursors are assembled from amino acids in the fibroblast. These protocollagen chains are twisted together in triple helical formation and get interlinked. Finally, vesicles transport the collagen to the cell membrane where they are released as soluble tropocollagen into the interstitium. Beyond, the tropocollagen molecules accumulate to form protofibrils consequently polymerising into microfibrils. Several microfibrils unite to form a collagen fibril, several of which, in turn, arrange themselves into bundles. In healthy tissue the collagen fibres are aligned in basketweave patterns. This organised structure is not achieved in wound healing as the collagen fibres at the wound site will fashion themselves in an alignment parallel to the stress lines of the wound[15]. Collagen synthesis depends on the presence of ascorbic acid as a coenzyme and further on iron and copper as cofactors. Type I and type III are the collagens most commonly found in healing wounds, although at least 19 different types of collagen have been identified and characterised[16,17]. During the process of wound healing, type III collagen and fibronectin are deposited – type III collagen later in the remodelling phase being replaced by type I.

*Remodelling* - at last, the remodelling or maturation phase finalises the wound healing process. Generally, it can take up to two years and means changes in the matrix composition over the healing time. The wound is contracted and the tensile strength of the wound cover is enhanced. The synthesis of matrix material is, as mentioned, provided by fibroblasts and regulated by growth factors, cytokines, enzymes, and prostaglandin mostly derived from macrophages and fibroblasts. The granulation tissue of the first extracellular matrix mostly consisting of keratinocytes gradually matures into scar tissue. With the formation of new fibres, the mitotic activity of the fibroblasts is concluded. They then may transform into myofibroblasts[18]. Myofibroblasts, like the muscle cells, contain contractile elements, which allow them to draw together. The collagen fibres become taut and, as far as possible, aligned to the main contours of tension in the tissue. As a result, the scar tissue shrinks and the functional cutaneous tissue at the wound margin contracts leaving only a small defect[19].

As outlined above, early collagen fibrils are laid down randomly resulting in a tensile strength of only 5 per cent of normal undamaged skin at two weeks post-injury. Over time these type III fibrils are replaced by type I collagen fibres, improving the tensile strength of the scar tissue to that of 80 per cent of normal skin. Nonetheless, scar tissue still appears different to original tissue, the former being weaker than unwounded skin. One reason for this distinction is as already mentioned that the final assembly of the collagen in granulation tissue does not resemble that of normal unwounded dermis. Moreover, since the pigment producing cells, the melanocytes, cannot be regenerated, the scar tissue does not turn brown but remains white. Besides, this tissue contains no hairs, sebaceous or sweat glands.

### *1.1.1.2 Growth factors in physiological wounds*

As described above the process of wound repair is characterised by a series of complex cellular and molecular events with a great degree of overlap and interdependence. Growth factors play fundamental roles in this process, by stimulating chemotaxis and cellular proliferation, by providing signalling among cells of the same and different type, by controlling extracellular matrix formation and angiogenesis, by regulating the process of contraction, and by re-establishing tissue integrity (Fig. 1-1). They work by binding to specific cell surface receptors and can target cells in a number of recognised modes. Release of these substances into the blood stream allows them to get to distant targets (endocrine mode). From

the cell of origin, growth factors can diffuse over short distances to affect other cells (juxtacrine mode), and to influence neighbouring cells (paracrine mode). Growth factors can also act on the cell in which they are produced (autocrine mode)[10,20].
In the following, the growth factors with greatest importance in the wound healing process are described in detail.

PDGF (platelet-derived growth factor) mainly is synthesised by macrophages, endothelial cells, fibroblasts, smooth muscle cells, and platelets. Beyond, it can be stored in platelets until cell activation for example by thrombin. The synthesis of PDGF can be induced by IL-1, IL-6, TNF-α, TGF-β and EGF. PDGF physiologically is not released into the blood stream. However, by binding to several proteins, e.g. of the extracellular matrix (ECM), local concentrations can reach increased levels. PDGF is a hydrophilic protein of 30kDa molecular weight. It is composed of two distinct polypeptide chains A and B, that form homodimers (AA or BB) or heterodimers (AB). The subunits are linked by disulfide bonds. Recently, additional C and D subunits have been described acting similarly to the A and B species [21-23]. Platelets synthesise a mixture of the three possible isoforms while fibroblasts stimulated with EGF synthesise AA homodimers. Activated macrophages produce the BB homodimer. The dimeric form of PDGF is mainly mitogenic for smooth muscle cells and vascular endothelium cells. Although, monomeric forms of PDGF are mainly chemotactic. So, PDGF is a chemoattractant for fibroblasts, monocytes and neutrophils. In addition, PDGF is a potent vasoconstrictor. However, it does not act on epithelial and endothelial cells because these cells do not express PDGF receptors[24].

B-FGF (basic fibroblast growth factor) is the prototype of the FGF family. Thereby, b-FGF shows a homology to a-FGF. Many cells, first of all endothelial cells, express b-FGF and partly store it in an inactive form. It is released after tissue injuries and during inflammatory processes. Binding of b-FGF to its receptors requires the interaction with proteoglycans of the ECM before full functional activity is obtained.
Additionally, heparin is a protectant for b-FGF, especially from the impact of proteases, acids, and heat. It also improves receptor binding and hence potentiates the biological activity of b-FGF. B-FGF stimulates the growth of fibroblasts, endothelial cells, and keratinocytes potentiated in the presence of thrombin. Additionally, b-FGF reduces the expression of the receptor for TGF-β, thus

effectively modulating the inhibitory action of TGF-β on endothelial cells. Also, FGFs control proliferation and migration of vascular endothelial cells important for angiogenesis. As well, the expression of plasminogen activator and collagenase by these cells is enhanced by b-FGF and is antagonised by TGF-β. A special member of the FGF family is FGF-7, also known as KGF, with the mentioned effects solely concentrated on keratinocytes[10,25].

EGF (epidermal growth factor) is a globular protein and is produced by cells in various organs. Following, it is present in most body fluids. It is synthesised as a larger pre-protein from which the factor itself is released by proteolytic cleavage. In addition, EGF is the prototype of a large family of EGF-like proteins (EGF-F). Particularly, TGF-α shows a strong homology to EGF. However, antibodies for EGF do not bind to TGF-α. Both factors are functionally analogous, they bind to the same receptor, and they have similar biological activities. So, EGF stimulates the proliferation of epidermal and epithelial cells, including fibroblasts and keratinocytes. This can be inhibited by the EGF inhibitor. Moreover, EGF strongly influences the synthesis of proteins of the ECM, including fibronectin, collagens, laminin, and glycosaminoglycans. Indirectly, EGF also supports angiogenesis because of its mitogenicity for endothelial cells which can be potentiated by thrombin. Besides, EGF is a chemoattractant for fibroblasts and epithelial cells[26].

TGF-α and TGF-β (transforming growth factors) are distinguished both chemically by their unique amino acid sequences and biologically by their different activities on cells. The interactions of TGF-α and TGF-β can be either synergistic or antagonistic.
TGF-α consists of a single chain peptide and is produced by keratinocytes, macrophages, and platelets. It has strong homology to EGF resulting in competition for receptor binding. The biological activities of TGF-α as well resemble those of EGF. However, some biological activities of TGF-α are stronger than those of EGF. Especially, TGF-α is mitogenic for fibroblasts and inhibits the synthesis of collagen.

TGF-β is not related to TGF-α. The biologically active form of TGF-β is a disulfide-linked homodimer. Still, TGF-β is released as the inactive complex latent-TGF with proteoglycans from the ECM. This complex should represent TGF-β molecules released by platelets after tissue injuries. This allows the factor to be stored in an inactive form. In fact, platelets contain very high amounts of TGF-β. It

is also produced for example by macrophages, lymphocytes, endothelial cells, and keratinocytes[27]. Depending upon cell type and concentration secretion of TGF-β can be induced or inhibited by a number of different stimuli. So, induction can be achieved by, for example, EGF, NGF, and IL-1. On the contrary, the synthesis can be inhibited by e.g. EGF, FGF, and calcium ions[28]. Generally, TGF-β has bifunctional effects and can either stimulate or inhibit growth of the same cells, depending on conditions. It is a potent growth inhibitor for epithelial cells, endothelial cells, fibroblasts, keratinocytes, and smooth muscle cells. It also deactivates macrophages. In many cell types TGF-β antagonises the biological activities of EGF, PDGF, and FGFs. Besides, the factor stimulates the synthesis of the major matrix proteins, including collagen, proteoglycans, glycosaminoglycans, and fibronectin. On the other hand, it inhibits their degradation mainly by inhibiting the synthesis and secretion of proteinases and by increasing their proteinase inhibitor levels. Furthermore, in monocytes TGF-β stimulates the expression of IL-1, PDGF, and FGF and inhibits the synthesis of TNF-α, TNF-β and IFN-γ. Moreover, TGF-β is a chemoattractant for neutrophils[10].

For IGF (insulin-like growth factor) two different isoforms, IGF-1 and IGF-2 of 7 kDa molecular weight, have been described. They display broad homology with insulin but can not be neutralised by antibodies directed against insulin. Both types of IGF are synthesised in many organs throughout the body finally obtained by processing of precursors. Solely IGF-1 is produced also by fibroblasts. Cell types responding to IGF-1 also include epithelial cells and fibroblasts. The factors regulating concentrations of IGF are somatotropin as well as PDGF and FGF. Thus, IGF-1 stimulates collagen and matrix synthesis. In fact, IGF-1 is considered to be one of the major anabolic factors regulating the metabolism of joint cartilage. Besides, it is also involved in angiogenesis. Like insulin, free IGF-1 causes hypoglycaemia. Therefore, binding of IGF-1 to carrier proteins prevents the establishment of a permanent hypoglycaemia in spite of high serum IGF-1 concentrations. These carrier proteins also increase plasma half life of IGF-1 and prevent the release from the blood stream into interstitial spaces. It also effects neurons and has been demonstrated to reduces neuronal loss after injury[29].

VEGF (vascular endothelial growth factor) is a homodimeric and glycosylated protein. The subunits are linked by disulphide bonds. The factor exists in several isoforms that are produced via precursors in many cells including endothelial types and macrophages. The isoforms differ in biological properties such as recognising

receptor types and interaction with proteoglycans. Consequently, shorter forms are soluble while the heavier forms are mostly bound to heparin containing proteoglycans of the ECM. VEGF is a strong angiogenic protein, especially a mitogen for vascular endothelial cells. Thereby, b-FGF and VEGF act synergistically in the induction of angiogenesis. Moreover, VEGF influences vascular permeability and is a chemoattractant for monocytes. It also induces the synthesis of clotting factors and collagenase[30,31].

### *1.1.1.3 Proteases in physiological wounds*

Proteolytic enzymes are present in all wound exudates and play an essential role in the healing of acute and chronic wounds. For wounds, proteases are mainly produced by granulocytes, keratinocytes and fibroblasts. Proteases contribute to the regulation of the balance between tissue synthesis and tissue degradation. Thus, proteolytic activity is tightly regulated with control at the transcriptional level and control by extracellular enzyme activation and inhibition. A defect in one or more of these control mechanisms would result in an increase in proteolytic activity, a trait of chronic wounds, and considered one of the primary causes of wound healing disorders. Therefore, protease control is a major goal of wound treatment. Proteases comprise endopeptidases and exopeptidases which cleave peptide bonds at points within the protein and remove amino acids sequentially from either N or C-terminus, respectively. Endopeptidases are further classified according to the structure of their active site in cysteine proteinases, aspartic proteinases, metallo proteinases and serine proteinases. In this chapter discussion focuses on serine proteases and serpins with regard to the main topic of this thesis being ACT.

*Cysteine proteases* - the cysteine proteinases family includes the lysosomal enzymes cathepsins B, K and L. Like with serine proteinases outlined below catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue.

*Aspartic proteinases* - aspartic proteinases include lysosomal cathepsin D and other proteases of the pepsin family. A second family comprises viral proteinases such as retropepsin from HIV. In contrast to serine and cysteine proteases, catalysis by aspartic proteinases do not involve a covalent intermediate though a tetrahedral intermediate exists.

*Metalloproteinases* - the known metalloproteinases (MMP) differ widely in their sequences and their structures. However, the great majority of enzymes contains conserved regions of homology and a zinc atom which is catalytically active. Zinc is in most cases bound by three amino acids, the fourth coordination site is occupied by a water molecule. The catalytic mechanism leads to the formation of a non covalent tetrahedral intermediate after the attack of the zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group. MMPs are mostly stored in leukocytes, keratinocytes and dermis cells. They are activated by removing a small peptide fragment, with the active enzyme exhibiting optimal activity around the physiologic pH. The collagenases are very specific in their function in that they split the triple helix of fibrillar collagens. The resultant denatured collagen molecule is then susceptible to attack from other proteases, in particular the gelatinases. In fact, though their substrate specifity is very different, in combination they can degrade all matrix molecules and each one deactivates inhibitors for serine proteases, mostly $\alpha_1$-PI[32].
In detail, the interstitial collagenase (MMP-1) degrades the collagen types 1, 2, 7, 8 and 10, gelatine, proteoglycans and entactin. The neutrophil collagenase (MMP-8), works very similar, it cleaves collagens 1, 2, 3, 7, and 10, gelatine, proteoglycans, bradykinin, and angiotensin I. Unlike the others, the neutrophil collagenase deactivates $\alpha_1$-antichymotrypsin. Collagenase 3 (MMP-13) degrades elastin, fibrillin, fibronectin and already denatured collagen.
Gelatinase A (MMP-2), the 72kDa enzyme, and gelatinase B (MMP-9), the 92kDa enzyme cleave collagen types 4, 5, 7, 10, and 11, gelatine, elastin, fibronectin, laminin and entactin. They also activate pro-IL-1β.
The stromelysins 1 and 2 (MMP-3 and -10) degrade collagen types 4, 5, 9, 10, and 11, fibronectin, laminin, proteoglycans and gelatine. In addition, they activate pro-MMP-1, -8 , -9 and pro-IL-1β.
Matrilysin (MMP-7) and metalloelastase (MMP-12) degrade elastin, fibronectin, laminin, entactin, proteoglycans and collagen IV[32,33].

*Serine proteases* - the serine proteinase class comprises two distinct families. The chymotrypsin family which includes enzymes such as cathepsin G, chymotrypsin, trypsin, elastase or kallikrein and the subtilisin family which include bacterial enzymes. The general structure is different in the two families but they have the same active site geometry and then catalysis proceeds via the same mechanism[34].

The serine proteinases exhibit different substrate specificities which are related to amino acid substitutions in the various enzyme subsites interacting with the substrate residues. Three residues which form the catalytic triad are essential in the catalytic process, i.e. His 57, Asp 102 and Ser 195 after chymotrypsinogen numbering (Fig. 1-3A)[34].

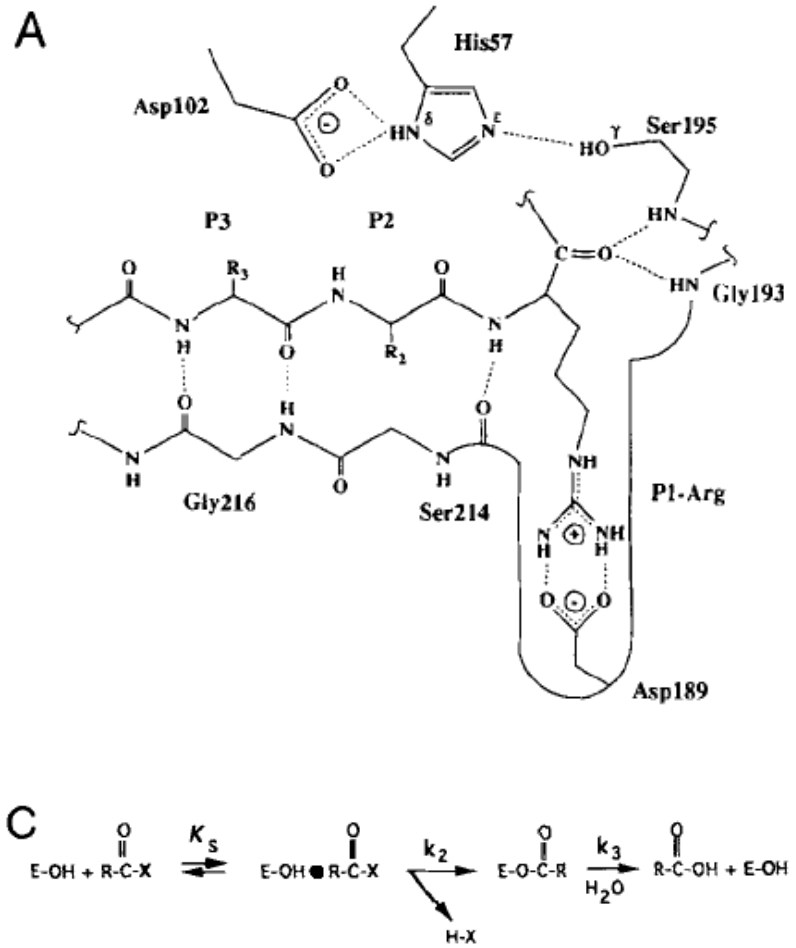


Fig. 1-3 chemical and kinetic mechanisms of catalysis for serine proteases. The catalytic groups of trypsin (A) are shown interacting with an oligopeptide substrate. C: Common kinetic mechanism of catalysis for serine proteases; adapted from[34]

The first step in the catalysis is the formation of an acyl enzyme intermediate between the substrate and the essential Serine. Formation of this covalent intermediate proceeds through a negatively charged tetrahedral transition state intermediate and then the peptide bond is cleaved. During the second step or deacylation, the acyl-enzyme intermediate is hydrolysed by a water molecule to release the peptide and to restore the Ser-hydroxyl of the enzyme. The deacylation which also involves the formation of a tetrahedral transition state intermediate, proceeds through the reverse reaction pathway of acylation. A water molecule is

the attacking nucleophile instead of the Ser residue. The His residue provides a general base and accept the OH group of the reactive Ser (Fig. 1-3C).
Cathepsin G is a cationic single chain glycoprotein of 29kDa molecular weight and is stored in active form within leukocyte granules and proinflammatory monocytes[35]. It provides a broad spectrum of biological activities, whereby around pH 7.4 its maximum activity is developed[36-38]. Cathepsin G activates other enzymes, i.e. collagenase (MMP 8) and gelatinase (MMP 9). Besides, Cathepsin G degrades macromolecules of the ECM – elastin, fibronectin, laminin, proteoglycans and collagen (type 4) – itself. Also, it appears to be necessary for proper elastase activity in the latter case. Moreover, the cytokines TNF-α and TNF-β, several lymphocyte receptors, and plasma proteins, e.g. immunoglobulins and clotting factors, are deactivated by cleavage. Platelets, on the other hand, are activated. Further, the conversion of angiotensin I into angiotensin II is catalysed by cathepsin G[39].
As the latter, elastase is a cationic single chain glycoprotein. For storage it is embedded in leukocyte granules, proinflammatory monocytes, eosinophiles, basophiles, mast cells and lymphocytes. The biological functions are similar to those of cathepsin G. Along with platelets, elastase also activates lymphocytes and the cytokines pro-IL-1b and IL-8. The secretion of signal molecules from cells is also induced by elastase, i.e. GM-CSF, IL-6, and IL-8[39,40].
Proteinase 3 is also found in monocytes and mast cells. Function and chemical class are also similar to cathepsin G and elastase.
Urokinase type plasminogen activator (uPA) is stored in monocytes, mononuclear phagocytes, T-lymphocytes and natural killer cells. Its main activity is the activation of plasmin by converting plasminogen which is present throughout body fluids. Plasmin itself degrades fibrin, laminin, fibronectin and proteoglycans activator. Besides, it activates some pro-MMPs and TGF-β[39,40].

### *1.1.1.4 Protease inhibitors in physiological wounds*

The entirety of protease inhibitors in the wound healing process is called the antiproteolytic shielding. It controls the destructive activity of proteases[41]. The plasma proteinase inhibitors, after albumin and the immunoglobulins, constitute with nearly 10% by weight of the total protein the third largest group of functional proteins in human plasma.

*Serine protease inhibitors (serpins) with ACT* - the serine proteinase inhibitors are a superfamily of proteins with a size of 350–500 amino acids. They fold into a

conserved structure and employ a unique suicide substrate-like inhibitory mechanism. Most serpins inhibit serine proteinases of the chymotrypsin family. To date around 250 serpin coding sequences are known[42]. They are divided into 16 clades and 10 highly diverged orphans.

Serpins adopt a metastable conformation that is required for their inhibitory activity. Serpins in the stable latent conformation are non-inhibitory but can be converted back to the active state by denaturation and refolding.

The conformation of serpins consists of a conserved secondary structure comprised of three β-sheets and at least seven, mostly nine, α-helices. In the metastable active form the reactive site loop containing the proteinase recognition site, is located between the first and third β-sheet. However, serpins can undergo intramolecular structural changes e.g. to convert to the more stable latent form. In that case the reactive site loop is placed into the first β-sheet while another side chain is extracted from the third sheet[43].

The most stable state for inhibitory serpins is a form, in which the loop has fully inserted into the first β-sheet, as in the latent conformation, but without the extraction of the side chain from the third β-sheet. The *Tm* for unfolding of such conformation is about 120 °C, compared to about 60 °C for the native state[44].

Protein proteinase inhibitors act competitively by allowing their target enzymes to bind directly to a substrate-like region contained within the amino acid sequence of the inhibitor. This reaction between enzyme and inhibitor is essentially second order, and the resultant complex generally is equimolar[45].

Serpins inhibit serine proteinases by an irreversible suicide substrate mechanism. The proteinase initially is bound in a non-covalent complex with serpin. Secondly, the active site serine of the protease forms a covalent ester with a carbonyl of the inhibitor. Following, the peptide bond is cleaved and the reactive site loop is inserted into the first β-sheet and transports the covalently bound proteinase with it. Upon complete loop insertion the active site catalytic triad of the proteinase is distorted and therefore deactivated.

This conformational rearrangement is driven by the greater stability of the cleaved loop-inserted conformation compared with the native-like conformation. Thus, the acyl-intermediate is kinetically trapped due to slowing of the deacylation steps of the normal substrate reaction. In fact, serpin-proteinase complexes would be cleared long before complex decay could occur.

Though, in the case that the described reactions are somehow impeded, the enzyme may successfully complete the deacylation step and escape before entrapment. This yields an active proteinase and a cleaved, inactive serpin. The ratio of complex and

cleaved serpin products is determined by the competition between the rate of ester hydrolysis and that of loop insertion and proteinase distortion. So, chymotrypsin and cathepsin G produce a modified inactive, no longer inhibitory form of ACT. A negative effect of the need for a metastable conformation in the active state is that inappropriate loop insertion can occur mediated by several factors including formulation excipients and processes. More precisely, by reaction of the loop of one molecule and the beta-sheet of another, aggregation to dimers and higher order oligomers can result[45,46]. Therefore, one major physical instability of ACT during formulation studies is estimated to be the dimer formation and perhaps the formation of higher order aggregates.

A1-antichymotrypsin (ACT) is a plasma glycoprotein first isolated and characterised in 1962[47] (Fig.1-4). The inhibitor is a major acute phase protein, whose concentration increases rapidly and dramatically after a variety of events, ACT shows the most immediate response as an acute phase protein, doubling in concentration from 250μg/ml normal concentration in plasma within eight hours of insult.

Fig. 1-4 Crystal structure of ACT with partial loop insertion; adapted from[48]

Three laboratories isolated ACT using a variety of conditions[47,49,50]. Significantly, ACT can be isolated from serum through its ability to bind to DNA [51]. In all cases the product obtained stoichiometrically inhibited chymotrypsin to produce an equimolar complex that was denaturation resistant to dissociation. The molecular weight of the native protein was between 58,000 and 68,000; the differences were

attributable to the methodology and the high carbohydrate content (about 26%) of this glycoprotein. Thus, ACT is a specific inhibitor of chymotrypsin-like proteinases, forming stable complexes with chymotrypsin[49]and neutrophil cathepsin G[46]. No inhibition of either human trypsin or neutrophil elastase has been found[52]. ACT rapidly forms complexes with the mentioned chymotrypsin-like serine proteinases; the rate is by far the fastest with cathepsin G ($k = 5{,}1 \times 10^7\ M^{-1}\ s^{-1}$), and much slower with chymotrypsin[53].

*Inhibitors of matrix metallo proteases* - recently, a number of inhibitors for MMPs have been described [54]. The best known substances are TIMP-1 and -2 (tissue inhibitor of MMPs) and $\alpha_2$-macroglobuline. These inhibitors selectively bind to MMPs and deactivate them.

## 1.1.2 Pathophysiology of chronic wounds

This paragraph outlines the pathophysiology of chronic wounds both on cellular and clinical levels.

A chronic wound is defined as one in which the normal process of healing is disrupted at one or more points in the phases of haemostasis, inflammation, proliferation and remodelling[55]. Chronic wounds, unlike acute wounds, do not undergo the ordered molecular and cellular processes of physiological tissue repair previously discussed. However, the healing process of chronic wounds is thought to be stuck in inflammation.

Chronic wounds can also be considered to be an imbalance between tissue deposition stimulated by growth factors, and tissue destruction mediated by proteases[56]. Hereby, the imbalance favours the destructive process. Thus, the molecular and cellular processes are disrupted leading to significant differences in the microenvironment of the wound, both in terms of the constituents of the exudates and the cellular components of the wound area. In addition, oxidative damage by free radicals, condition specific factors of underlying diseases, and accumulated necrotic tissue as well contributes to the chronic state. The further healing of those wounds results in skin defects of excessive fibrous appearance, for instance keloids and scar contractures, or alternatively in insufficient tissue replacement, i.e. a non-healing wound.

### *1.1.2.1 Cellular and biochemical imbalance in chronic wounds*

Moreover, the persisting inflammatory phase leads to wound exudate showing - in comparison to acute wounds – increased protease concentration and reduced levels of growth factor activity.

During the inflammatory phase chemotactically attracted and activated macrophages secrete inflammatory cytokines, which increase protease production and reduce the synthesis of inhibitors.

In a physiologically healing wound there is also a balance of pro-inflammatory cytokines and their natural inhibitors. In chronic wounds on the contrary, the levels of these cytokines are increased. Mainly, the persistent inflammatory stimulus is caused by repetitive trauma, local tissue ischaemia, necrotic tissue, heavy bacterial burden, or tissue breakdown[57].

As well, in acute wounds proteases and their inhibitors are in equilibrium, but protease concentrations are elevated in chronic wounds. So, levels of collagenase, gelatinase A, and gelatinase B (MMP-1, -2, -9) have been shown to be elevated in fluid derived from pressure ulcers and venous leg ulcers[58,59]. Other proteases, such as neutrophil elastase, have also been observed to be higher in chronic wounds[60]. Hence, elevated levels of serine proteases cause degradation of extracellular matrix, resulting in impaired cell migration and connective tissue deposition. Furthermore, they degrade growth factors and their target cell receptors[59]. Growth factors applied externally to the wound are degraded the same way. To sum up, the wound healing balance is shifted in favour of destructive processes.

Emerging from cell membrane lipids break down caused by exaggerated cell necrosis associated with impaired wound healing effects higher numbers of cell death. Certainly, increased amounts of active oxygen species, for example hydroxyl radicals, peroxide anions, hydroperoxyl radicals or nitric oxide (NO) are known to be key negative factors in a number of inflammatory conditions[61,62].

Furthermore, in chronic wounds the specific cell populations and processes that are essential for wound repair are disrupted. So, epithelial cells fail to migrate across the wound tissue. Hence, hyperproliferation of cells occurs at the wound edges and interferes with normal cellular migration. As a result, the proliferation rate of fibroblasts is reduced and their apoptosis is inhibited[63-65]. And generally, the response of cells to growth factors is reduced because the failure to re-epithelialise, the most obvious clinical feature of chronic wounds, is due to a failure in migration rather than proliferation of the keratinocytes[66].

In fact, these processes effectively hold the wound in the inflammatory phase and therefore prevent a wound from entering the proliferative phase and a physiological ongoing of the healing process.

### *1.1.2.2 Clinics of chronic wounds*

According to their causale chronic wounds may be categorised as diabetic foot ulcers, venous or arterial leg ulcers, pressure ulcers, tumours, burns or even post-surgical wounds. But, the most common are the venous leg ulcer, the pressure ulcer and the diabetic foot ulcer. These types appear different externally, but all share common characteristic features. In detail, recurrent trauma, ischaemia, and prolonged inflammation are apparent.

Ulcus cruris disorders are divided into venous and arterial ulcerations. Venous ulcerations are the most common type of ulcer affecting the lower extremities. Here, a chronic venous reflux disorder occurs because of inherited or postthrombotic varicosis. With vein valves becoming incompetent the resulting backflow of blood causes venous congestion. The lymphatic system compensates the oedema in the first part, but soon it as well suffers damage from the overload. This results in oedema and a decreased oxygen supply in the surrounding skin. In arterial ulcers a complete or partial arterial blockage mostly resulting from arteriosclerosis lead to similar inadequate supply of surrounding tissue. Consequently, in both cases tissue necrosis and ulceration will develop.

The pressure ulcer is a compressive-ischaemic skin lesion predominantly occurring above bony prominences such as the sacrum, heel, or ankle. Ulceration is again caused by inadequate supply due to ischaemia resulting from abnormal pressure on the tissue on a cellular level. The pressure to tissue is usually applied between a bony prominence and a hard surface, for example ankles in inappropriate foot wear. Beyond, an ischaemic lesion of the skin develops which after a short latent period leads to ischaemic skin necrosis.

Diabetic foot ulcers are a common complication of diabetes mellitus because diabetes as underlying disease can cause damage to the nerve and vascular supply in the feet and legs. So, diabetics are prone to foot ulcerations due to both neuropathic and ischaemic complications. Next to the consequences of vascular damage outlined above neuropathy contributes to ulceration. In detail, neural damage also leads to lacking supply, and due to the loss of sensation the risk of trauma is severely increased[67].

#### *1.1.2.3 Infection of wounds*

Bacterial bioburden can cause a delayed or impaired healing. In detail, endotoxins and proteases stimulate an inflammatory wound environment, further the clotting mechanisms, leukocyte function, angiogenesis, and formation of granulation and scar tissue are disordered. Defined by extent and necessary treatment bacterial burden present in the wound is divided into several degrees[68]. Contamination is defined as the presence of non-replicating bacteria. This is a normal condition in chronic wounds and does not contribute to impaired healing. Colonisation is defined as the presence of replicating bacteria without a host reaction. Replicating bacteria colonise and contaminate all chronic wounds not meaning that these wounds are infected. Bacterial colonisation does not contribute to impaired healing. Critical colonisation is defined as the presence of replicating microorganisms, which are beginning to cause local tissue damage. There may be subtle local indications that a change in the equilibrium, or increasing bioburden, could be contributing to delayed healing[69]. Infection occurs when healing is impaired because bacteria have invaded tissue, are multiplying, and are causing a host reaction.

Although bacteria are present in all chronic wounds, generally, only critical colonisation and infection indicate an antimicrobial treatment. But, additional other factors are to be maintained for every case individually, i.e. the balance between host resistance and the quantity and virulence of bacteria, second concomitant medications including immunosuppression, and any underlying diseases such as diabetes.

Biofilms are an element of wound infection that has recently become apparent. Bacteria proliferating in wounds form microcolonies attaching to the wound and secreting a biofilm that protects the organisms. So, biofilms are protected areas of infection and bacterial resistance within the wound, protecting bacteria from the effects of antimicrobial agents such as antibiotics and antiseptics[70].

### 1.1.3 Treatment of chronic wounds

In this section the methods of treatment of chronic wounds are outlined. Although, many older but obsolete methods are still in use in clinical practice solely the modern state-of-the-art methods are described. As a first treatment the wound is debrided. After that, under a moist dressing depending on the wound type the healing process is allowed to proceed in moist environment. Where necessary, an infection controlling treatment is conducted. For further support of the healing

process skin substitutes are available as well as vacuum treatment devices. And, for the correction of the above described imbalance phenomena products for the control of growth factors and protease levels in the wound can be applied. An ACT delivering device would also belong to this last group of products and thus, in that section also the scientific rational behind the delivery of ACT into wounds is discussed.

### *1.1.3.1 Debridement*

Debridement – the removal of devitalised tissue - is facilitated by natural mechanisms in every wound, but accelerating this process makes healing more efficient. It may be necessary because devitalised tissue in the wound bed supports bacterial growth and is a physical barrier to healing. Devitalised tissue may also cause excessive amounts of proteases to be released. The methods of debridement in today's clinical practice are surgical, enzymatic, autolytic, mechanical and biologic.

Sharp surgical debridement is a very fast and efficient way to remove necrotic tissue from the wound bed. It is performed where there is an extensive amount of necrotic tissue or there is a widespread infection requiring infected material to be removed.

Enzymatic debridement means the use of manufactured proteolytic enzymes, i.e. collagenases. These support naturally occurring enzymes to degrade necrotic tissue.

Autolytic debridement is a process performed by phagocytic cells and proteolytic enzymes in the wound site liquefying and separating necrotic tissue from healthy tissue. Wound dressings, which maintain a moist wound bed, can provide an optimal environment for debridement, as they allow migration of the phagocytic cells. Unsurprisingly, the process of autolytic debridement can result in increased wound fluid requiring appropriate dressing.

Mechanical debridement is a method that physically removes debris from the wound. Examples of mechanical debridement include conventional dressings causing mechanical separation of necrotic tissue from the wound bed once the dressing is removed and wound irrigation using a pressurised stream of water to remove necrotic tissue.

Biologic larval therapy is an alternative method using sterile maggots that break down, liquefy and remove dead tissue secreting powerful proteolytic enzymes followed by eating of the digested tissue[71].

### *1.1.3.2 Moist wound treatment*

#### *1.1.3.2.1 History of moist wound treatment*

Prior to late 20th century, wounds were felt to heal better if exposed under a scab. This process produced surface desiccation and eschar formation now known to deepen the wound, but was felt to protect the wound from outside influence. The thinking is understandable given the fact that the most severe effect of wounding at that time was infection and no antibacterial agents were available for treatment. This concept remained popular until the mid-20th century.

Between 1948 and 1958 several articles were published describing accelerated healing of acute wounds under occlusion. But, the primary death from wounds, especially burns, was still infection and therefore standard care returned to exposure, especially with the advent of a topical antibiotic silver sulfadiazine cream to be applied twice daily. This and following topical antibiotics could control infection in exposure but also retarded healing, especially epithelialisation, which nevertheless was considered as secondary.

A landmark study in 1962 by George Winter, from Smith &Nephew Inc. and considered the father of moist wound healing, demonstrated that wounds epithelialised more rapidly under occlusive dressings with the reason being that occlusive dressings maintained a moist wound surface. This study was conducted on pigs showing that the moist environment accelerated the epithelialisation process about 30% compared to air dried wounds[72]. Numerous studies followed which demonstrated that wound occlusion and moisture increased all phases of healing. Wound bacterial colonisation which was demonstrated to be higher in a case study in a moist healing environment did not appear to retard healing or cause sepsis[73]. However, the risk of severe burden and infection decreasing the healing process was proven to be lower in occluded wounds[74]. In 1994, U.S. authorities published a guideline for treatment of pressure ulcers comprising occlusion for the purpose of autolytic debridement and provision of a moist environment. Following until present, wet treatment has been established in clinical practice as standard care.

#### *1.1.3.2.2 Effects of moist wound treatment*

In general, during a moist treatment the likelihood of scarring is reduced because there is no scab formation. In addition, moisture is essentially required for the already described activity of growth factors and proteolytic enzymes (section

1.1.1.2, 1.1.1.3 ). It is as well necessary for surface oxygen delivery and an efficient nutrient delivery. As a result, moisture improves the processes of the migration and proliferation phase by providing the ability of cells to migrate across the wound surface. So, an increased rate of epithelisation and angiogenesis is reached and further, fibroblast proliferation and thus collagen synthesis is improved.
Next to an improvement of the healing by biochemical means there is also an improvement for direct patients' concern. Pain is a major complication for wound treatment because in open wounds, the nerve endings are exposed and the wound can feel painful. With a moist environment the nerve endings are cushioned and protected which gives relief from pain.
On the contrary, any surface desiccation decreases all phases of healing. In fact, surface drying was shown to lead to an increase in wound depth and a higher risk of infection[75].

#### *1.1.3.2.3 Products for moist wound treatment*

The topical wound management product chosen will depend on wound characteristics, including amount of exudate, wound size, the presence of infection, and the characteristics of the surrounding skin. Especially the amount of exudate is important because low levels of moisture may lead to the discussed disadvantages but exaggerated levels of moisture lead to unwanted counterproductive maceration of tissue including intact skin. Hence, moisture-retentive dressings like okklusive films, hydrogels, and hydrocolloids are preferably selected for wounds with light to moderate drainage. Absorbent dressings like foams and alginates tend to be selected for wounds with moderate to heavy exudate.
*Film dressings* – occlusive films are semi-permeable polyurethane dressings that are coated with an adhesive. They are used for minor exudating wounds. Their purpose is to prevent bacterial infection by shielding, to absorb low amounts of exudate and to maintain a moist wound environment for fresh epithelial tissue. The dressings insure a gaseous exchange for vaporising superfluous liquid.
*Hydrogels* - hydrogels and hydrogel dressings are used to treat wounds with low exudate levels. With these products only low amounts of exudate are necessary to provide a moist milieu, since they contain high amounts of water themselves. Most products contain sodium carboxymethyl cellulose or polyacrylates swollen to an amorphous gel in a propylenglycol water mixture. Hydrogel dressings are used to hydrate necrotic tissue, facilitating autolytic debridement, while being able to absorb exudate. They can also be used to provide a moist wound environment

during the later stages of wound closure. In clinical practice, hydrogels are often additionally covered by a film dressing.

*Hydrocolloids* - for moderate exudation hydrocolloid dressings can be used. They contain a layer of hydrocolloid. This is defined as liquid absorbing particles in an elastic, self-adhesive mass. The particles mostly consist of sodium carboxymethyl cellulose, calcium alginate, pectine, and gelatine, respectively. The elastic mass contains different synthetic polymers. The wound exudate binds to the absorbing particles of the hydrocolloid matrix to form a cohesive gel maintaining a moist wound environment. Most products as well are covered on the upper side by a semi-permeable polyurethane film.

*Foams* - foam or hydrocellular dressings are double-layer dressings consisting of a polyurethane film carrier and a polyurethane foam layer on the wound side. They are used for moderate to heavily exuding wounds. The foam may be combined with polyacrylate particles supporting the liquid absorption. The foam core binds high amounts of debris and exudate. The film again provides gaseous exchange, but provides shielding against bacteria. So, a balance of absorbed and vaporising liquid establishes a moist milieu. Speciality absorbent dressings can be used as secondary dressings.

*Alginates* - alginate dressings are used to cover heavily exuding wounds. They mostly contain a combination of calcium and sodium alginate fibres. Alginate dressings are highly absorbent and can incorporate high amounts of exudate by forming a hydrogel. Thereby, the calcium alginate polymers are soaked with exudate. After that, due to the high amounts of sodium in the exudate there is a diffusional exchange of calcium and sodium enabling the resulting sodium alginate to swell and form a hydrogel. Moreover, alginates support healing by binding bacteria and debris inside the gel structure and by providing a moist environment[75].

### *1.1.3.3 Infection control in wounds*

The most frequently used topical antimicrobials in modern wound care practice include octenidine, iodine, and silver containing products. Chlorhexidine, hydrogen peroxide, and honey as well are in discussion but seem to be used more rarely. In the past acetic acid, sodium hypochlorite, potassium permanganate and proflavine have been used.

*Iodine* – iodine as element was used in treating wounds mainly in the 19$^{th}$ century. Due to its heavy adverse effects it is obsolete today. Therefore, the safer formulations povidone iodine and cadexomer iodine have been developed.

Povidone iodine is a polyvinylpyrrolidone - iodine complex; cadexomer iodine is composed of beads of dextrin and epichlorhydrin that carry iodine. Whereas its efficacy as a skin disinfectant is undisputed, numerous publications describe the use of iodine in cleansing wounds, and as a topical agent to prevent or treat localised wound infections, but controversy surrounds its safety and efficacy[76].

*Silver* - silver also has a long history as an antimicrobial agent, especially since the late 19th century[77]. Metallic silver is not active, but in aqueous environments silver ions are released and antimicrobial activity depends on the intracellular accumulation of low concentrations of silver ions. These bind to negatively charged components in proteins and nucleic acids, thereby effecting structural changes in bacterial cell walls, membranes and nucleic acids that affect viability[78]. The complex issues concerning the toxicity of silver to mammalian systems, and its effects on the healing process are not completely discussed. Skin discolouration and irritation associated with the use of silver nitrate is well documented; absorption of silver, systemic distribution and excretion in urine has also been reported[79]. In wound care silver has been utilised in several formulations. Silver nitrate application is rare, but silver sulphadiazine, colloidal and nanocrystalline elemental silver dressings have recently been developed and are widely used. These function by the sustained release of low concentrations of silver ions over time, and generally appear to stimulate healing, as well as inhibiting micro-organisms[80].

### *1.1.3.4 Skin substitutes for wound healing*

Tissue engineering has added several skin substitutes to the variety of dressings available for wound treatment. These products for example consist of fibroblasts and keratinocytes grown on collagen matrices. In clinical evaluation the application of Apligraf® has been shown to accelerate wound closure compared to control[81].

### *1.1.3.5 Growth factors control in chronic wounds*

For the described inductive effects of growth factors on cell migration the potency of these substances has been evaluated in numerous experiments. Convincing results of these efforts have been published in an unmanageable amount of publications. Following the results of research, many clinical trials with growth factors externally applied on wounds have been conducted. But, various degrees of success have been reported. For example, Richard et al conducted a trial with b-FGF on diabetic foot ulcers with no seen advantage of verum over the placebo control[82]. Also, EGF was exogenously applied to patients with diabetic foot

ulcers[83]. There, a significant enhancement of healing and a reduction of healing time was reported. But, Falanga et al[84] treated patients with venous ulcers in a study with EGF. Although they showed EGF was safe and significantly reduced the size of the ulcers, it failed to enhance epithelialisation. Robson et al applied KGF-2 or repifermin on chronic venous ulcers during clinical trial. Thereby, a significant acceleration of wound closure was achieved[85].

For PDGF-BB (platelet-derived growth factor consisting of BB-homodimer) or becaplermin several clinical trials finally leading to the approval of Regranex® in 1999 for the treatment of diabetic foot ulcers have also been published. Efficacy and safety in diabetic foot ulcers have been proofed[86-88]. So, the application of Regranex® achieved a 43% increase of incidents of wound closure and a 32% decrease of time until wound closure. Similar trials, e.g. concerning pressure ulcers, acute, and open surgical wounds have also been conducted with promising results but not yet leading to an approval[89-91]. In Regranex® PDGF is formulated in an aqueous carboxymethyl cellulose hydrogel. Further, the formulation contains an acetate buffer, lysine hydrochloride, and sodium chloride.

Another new technology for augmenting levels of growth factors in wounds is by gene transfer. Andree et al used particle-mediated and microseeding gene transfer to deliver human EGF to porcine wounds[92,93]. A high expression of EGF, as well as a significant acceleration of healing, was shown in the transfected wounds. For PDGF a clinical trial using a viral vector is planned[94].

In summary, there are several growth factors being evaluated in clinical trials, but given by the very diverse results, the type of the individual wound is an essential criteria for the choices of growth factors. Therefore, the approval of Regranex® only for diabetic foot ulcers is feasible.

To overcome this problem and to make allowance to the thought of growth factors acting in concert, methods of autologous growth factor application have been developed. Thereby, a sample of the patient's blood is taken and separated by centrifugation. The necessary fraction, e.g. platelets, is isolated and applied to the wound area as appropriate. However, effects are not well proven and questionable[95,96].

### *1.1.3.6 Protease control in chronic wounds*

Next to the delivery of growth factors the protease levels in chronic wounds have been identified as efficient starting point for treatment. Generally, as described in section 1.1.2.1 protease levels in chronic wounds are increased. Therefore, a

decrease of these levels is a goal of treatment. For that purpose, so-called active dressings are available that modulate these protease levels unspecifically. Moreover, the delivery of a protease inhibitor represents a potent possibility to balance the exaggerated lytic activity in chronic wounds in a very distinctive way. Despite many small chemical entities being protease inhibitors have been patented none of those has led to approval[97]. But, a physiological inhibitor could be an alternative to these substances due to a bandwidth of effects outlined below. Therefore, the topic of this thesis is the delivery of ACT, a physiological protease inhibitor, into wound sites.

#### *1.1.3.6.1 Active dressings for chronic wounds*

Some polymers, i.e. collagen and oxidative regenerated cellulose, proved to modulate the wound environment at the biochemical level. In detail, the levels of proteolytic enzymes in wound fluid are reduced by physically entrapping and mechanically inhibiting their activity. This is thought to originate the described decrease of tissue destruction and prevention of growth factor degradation, leading to an overall increase in granulation tissue formation and faster wound repair. Therefore, wound dressings consisting of collagen or oxidative regenerated cellulose were developed[98,99].

#### *1.1.3.6.2 Delivery of **ACT** in chronic wounds*

It has been shown by works of SWITCH BIOTECH AG that chronic, diabetic ulcera in humans have strongly reduced capability for up-regulation of the level of expression of ACT. In healthy humans, the expression of ACT increases drastically in the wound tissue following wounding. Further, it has been established that besides the reduced levels of ACT transcripts, the activity of the ACT polypeptides is also selectively decreased in poorly-healing diabetic wounds compared to the oberserved increase in activity in normally healing wounds as well as in venous ulcers. Thus, it is the increase of both expression and function, particularly the activity, which leads to a strengthening of the antiprotease shield, and which in turn allows an increased neosynthesis of collagen and consequently supports rapid wound healing in normally healing wounds. Furthermore, the results show that this disturbance in the ACT protease inhibitor equilibrium is specific for the poorly healing diabetic wounds[97,100].

As a consequence, application of ACT into chronic wounds should support the wound healing process in a variety of ways. It can protect the extracellular matrix

via inhibition of mast cell chymase and cathepsin G. Cathepsin G itself is capable of activation of MMP 8 and MMP 9. So, delivery of ACT can at least partly decrease the enzymatic activity of this group of proteases. In similar way, elastase activity was shown to be dependent on the presence of cathepsin G. Furthermore, ACT has been reported to inhibit the neutrophil chemotaxis and superoxide generation. Both factors contribute to the inflammation at the wound site. For this multitude of effects the protein protease inhibitor ACT is likely to be superior over small chemical entities designed to inhibit one enzyme[39].

Therefore, the delivery of ACT into the wound area promises improvement of the disturbed healing of particularly diabetic wounds.

## 1.2 Protein delivery from hydrogel formulations

"Hydrogels are three-dimensional, hydrophilic, polymeric networks capable of imbibing large amounts of water or biological fluids. The networks are composed of homopolymers or copolymers, and are insoluble due to the presence of chemical crosslinks (tie-points, junctions), or physical crosslinks, such as entanglements or crystallites. The latter provide the network structure and physical integrity. These hydrogels exhibit a thermodynamic compatibility with water which allows them to swell in aqueous media"[101].

Hydrogels have been in use in the pharmaceutical, medicinal, and cosmetic field for many years. Mainly, they have been applicated topically with or without a drug substance for local treatment. An exception of course are the matrix based transdermal therapeutic systems for e.g. systemic delivery of hormones. But, with the availability of large molecular weight protein drugs and the grown demands for a controlled release of drugs in modern medicine, hydrogels have earned further increasing attention as drug delivery systems for the systemic delivery of both peptide and small chemical compounds[101].

For the desired controlled delivery of drugs by gel matrices the release mechanism is a decisive parameter. Generally, for the release from polymeric drug delivery devices three main mechanisms are described and reviewed[102].

*Diffusion control* - in a diffusion controlled system the drug is distributed homogeneously in the gel matrix. For release, the protein drug permeates through the continuum of the carrier to the release site. Hence, diffusion of the protein through the matrix is the rate-limiting step.

*Chemical control* - in the case of chemical control, the polymer is degraded resulting in an erosion of the matrix and release of the drug.

Figure 1-5 displays the different types of polymer degradation mechanisms. In (1) a biodegradable bond is incorporated into the polymer backbone. Chemical or enzymatic cleavage of the bond converts a water-insoluble polymer into water-soluble, low molecular weight polymer fragments. In (2) the gel exists as a covalently or ionically cross-linked matrix. Therefore, cleavage of unstable linkages in the crosslinks leads to a breakdown of the network structure. Covalently crosslinked hydrogels and ionically cross-linked polymers degrade by this mechanism. For these two mechanisms a further differentiation can be made concerning physical terms. Hydrolysis can occur at an even rate throughout the whole polymer matrix indicating a bulk erosion phenomenon. Contrarily, in surface erosion the delivery system degrades only at its surface because the degradation is

blocked inside the matrix e.g. by excipients changing the pH to value unfavourable for hydrolysis. Besides, in some systems the drug can be attached to the polymer by a covalent bond that is degraded chemically triggering the release of the drug.
Example (3) of Fig. 1-5 describes polymer solubilisation as degradation mechanism which is not actually related to a chemical reaction. Rather, the system dissolves and liquefies as water diffuses into the network leading to swelling and simple dilution of the polymers (Fig. 1-5).

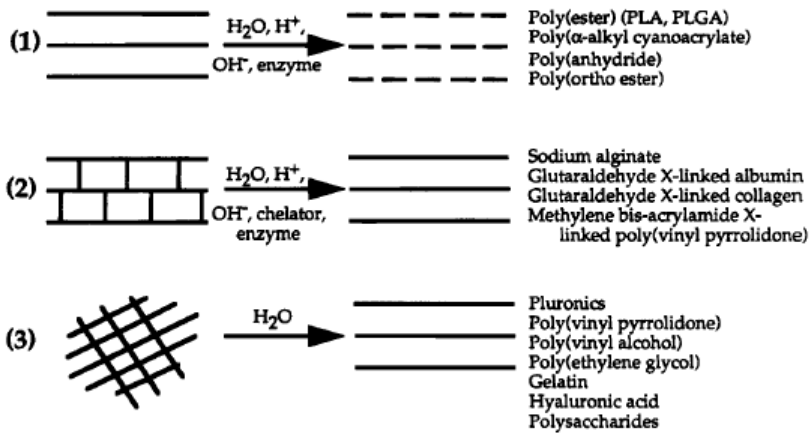


Fig. 1-5 Polymer degradation mechanisms in hydrogels and related devices; (1) hydrolysis of the backbone; (2) hydrolysis of the crosslinked polymer network; (3) hydration and solubilisation of a polymer matrix;[102]

*Solvent activation* - a third mechanism is solvent activation. The drug can be released either by swelling of the polymer in which the drug was previously locked into place within the polymer matrix in a glassy state or by an osmotic effect, which can be accomplished by external water entering the drug delivery system because of an osmotic driving force and subsequently driving the drug out of the system.

## 1.2.1 Suitability of hydrogels for protein delivery

Protein drugs place stringent demands on their delivery systems due to their structure, physicochemical properties, stability, pharmacodynamics, and pharmacokinetics. More specifically, peptides and proteins must retain their structural integrity until they reach their delivery site and must not be degraded upon enzymatic interactions. In addition, the physiological barriers, e.g. skin and cell membranes, are obstacles for the successful penetration of such drugs to their site of action.

Thus, next to protein integrity, a delivery device is required to deal satisfactorily with technological factors such as protein loading efficiency and desired release characteristics.

Due to their high water content, hydrogels are generally considered as biocompatible materials. So, they provide minimal mechanical irritation upon in-vivo implantation mainly due to their soft and rubbery nature which is similar to natural tissue[101].
Generally, the mostly aqueous environment of hydrogels appears very similar to the physiological environment for proteins and peptides in the body and therefore is supposed to be favourable for stability and activity of these substances. Moreover, due to the low interfacial tension between water and hydrogels, tendencies to protein adsorption and cell adhesion are very low. Furthermore, hydrogels offer a broad acceptability for individual drugs with different hydrophilicities and molecular sizes. Also, maximum drug load is expected to be uncritical for proteins in most cases because the high water content provides a high solubility for hydrophilic protein substances[101]. Therefore, hydrogel carriers are favourable for the delivery of peptide and protein drugs.

## 1.2.2 Protein delivery from hydrogels

In the following an overview over protein delivering hydrogel formulations published during the last decades is outlined in dependence of the application site. Thereby, the application of hydrogels in wounds, on skin for transdermal delivery, in the gastrointestinal tract via oral administration, and for injection is highlighted.

### *1.2.2.1 Application in wounds*

For the application of protein drug loaded hydrogels in wounds, a multitude of reports has been published. These mainly deal with the delivery of growth factors aiming towards an acceleration and improvement in wound closure. Also, a whole variety of gelling agents has been used for the various approaches. A series of examples is given that report improving or accelerating impact of growth factors on wound healing.
Hydroxyethyl cellulose was used to incorporate a-FGF in a gel formulation[103]. A preparation of 1mg/ml a-FGF stabilised by heparin was evaluated in vitro and in vivo. In release studies in a special apparatus in vitro indicated that 90% of total a-

FGF content were liberated and recovered in the acceptor consisting of PBS/heparin. Moreover, in a diabetic mouse model an improvement of wound healing was found.
B-FGF further was delivered from crosslinked chitosan gels[104]. Thereby, in vitro release from a 1mg/ml b-FGF containing gel into PBS solely under non-degrading conditions was studied in a well plate without a membrane. Thereby, a maximum of 20% was released within 24 hours in PBS as acceptor. It was concluded that the remaining substance was locked in the matrix, while in vivo degradation was expected to liberate the whole dosis. Besides, in a diabetic mouse model an improvement of wound healing was found.
Further, collagen gels have been reported to effectively deliver EGF and TGF-β[105]. As well, collagen was used as carrier for delivery of PDGF, TGF-β, and FGF[106]. Furthermore, gelatine was tested on the delivery of FGF[107]. Besides, a gelatine gel crosslinked with dextran dialdehydes was used on EGF[108]. KGF was administered in a fibrin gel carrier[109]. Moreover, TGF-β was administered in a Poloxamer® gel as well as a polyethylene glycol gel[110]. Furthermore, a PEG-PLGA-PEG blockcopolymer was used on a TGF-β plasmid[111]. And, the thrombin receptor agonist peptide was embedded in a polyvinyl caprolactam - calcium alginate film[112]. Also, methylcellulose gels delivered TGF-β[113]. EGF further was delivered from chitosan gels[114].
The research on the delivery of PDGF in a carboxymethyl cellulose hydrogel has finally led to approval of Regranex® (see section 1.1.3.5).
The variety of combinations of proteins and gellant types researched for wound treatment indicates a broad compatibility of proteins and hydrogels. Nevertheless, data given in these publications mostly are written from the medical point of view and therefore focus on in vivo effects. Stability and release concerns of the proteins, however, are not always investigated thoroughly.

### *1.2.2.2 Transdermal delivery*

Recently, research on transdermal delivery of proteins is focusing on electrically-assisted mechanisms, using iontophoresis and electroporation[115]. Polyacrylamide hydrogel formulations are being investigated as vehicles for transdermal iontophoresis to obtain the enhanced permeation of luteinising hormone releasing hormone into hairless rat skin[116].
Moreover, a methyl cellulose hydrogel was used as a viscous ultrasonic coupling medium for transdermal sonophoresis. It was concluded that an enhanced

permeation of insulin and vasopressin out of a gel vehicle across human skin driven by sonophoresis is possible[117].

### *1.2.2.3 Oral delivery*

Orally administered peptides and proteins are usually prone to hydrolysis and digestion in the harsh, acidic, and enzymatically active environment of the stomach. To overcome this obstacle various approaches have been made to protect the drug from degradation by pH sensitive polymers in form of matrices or coatings.

For example, a protection mechanism for oral delivery of insulin was investigated using a pH-responsive complexation hydrogel matrix[118]. The hydrogels used to protect insulin were crosslinked matrices consisting of a copolymer of polymethacrylic acid and polyethylene glycol. Complexation of the polymeric carrier occurs via the hydrogen bonding between the carboxyl group of the methacrylic acid and the oxygen of the PEG chains, which protects the drug in the low pH environment of the stomach. As the environmentally sensitive polymeric carrier passes into the small intestine, the pH is shifted to neutral, causing deprotonation of the carboxyl group on the acrylic acid and thus creating repulsion between the polymer chains. The increased mesh size, due to the ionic repulsion and the uptake of water in the decomplexed state, allows for release of the drug at the targeted site of absorption.

In vivo experiments with microparticles in oral administration studies using both healthy and diabetic rats indicated that insulin was released in the neutral and basic regions of the intestine. Moreover, a strong dose-dependent hypoglycaemic effect was demonstrated.

Besides the intestine, the colon is considered as promising absorption site for oral administration of proteins and peptide drugs as well. In contrast to the upper gastrointestinal tract, the colon as delivery site offers very low proteolytic enzyme activities. Therefore, the route of oral administration of proteins to target the release of the drug to colon is under research. Early approaches used polymer coatings with azo aromatic crosslinks that are degraded by specific microflora, e.g. Bifidobacteria, in the colon. With this system the peptide hormones vasopressin and insulin could be delivered to the colon of rats after oral administration[119].

Later approaches aim towards embedding of the proteins in biodegradable matrices and hydrogels[120]. Thereby, polymers are preferred that are degraded by colon specific microflora, such as inulin. Till date, in vitro studies on the release of bovine serum albumin from methacrylated inulin hydrogels have been published[121]. To

further improve the shielding against degradation in the upper gastrointestinal tract these gel systems may be combined with coatings of pH-sensitive, biodegradable, or bioadhesive polymers.

### *1.2.2.4 Ophthalmic delivery*

Maleic anhydride-alkyl vinyl ether copolymers have been used to fabricate polymeric films containing α-interferon[122]. The interferon was coincorporated with human serum albumin as a suspension into gels. Albumin was used both as a diluent for the interferon and as an intermolecular binder for the polymer matrix. The gels were processed as ophthalmic implants. It was reported that the erosion of the polymer matrix next to diffusion of interferon contributes to the release profile.

### *1.2.2.5 Delivery by injection and general approaches*

The development of protein drug loaded hydrogels applicated by injection aims towards a parenteral depot form for these substances desirably with a controlled release behaviour. Therefore, most reports deal with the liberation characteristics of the peptides from hydrogels preferably with a prolonged release period with little or no burst effects.
In general, for subcutaneous or intraperitoneal delivery of proteins in gel matrices, the basic semi-solid hydrogel formulation seems inappropriate. Therefore, rather than semi-solid gels, other hydrogel-related application forms are in the focus of delivery research. These are e.g. stimuli-sensitive hydrogels that are injectable at room temperature but form a gel when heated to body temperature. Another approach is the in situ photopolymerisation of the polymers leading to gel formation. Examples are given below followed by reports about more general approaches on protein delivery from hydrogels.

A very well investigated thermoresponsive hydrogel is formed by Poloxamer® 407. In general, Poloxamers® are block copolymers of polyethylene oxide and polypropylene oxide. Aqueous solutions at concentrations of 20% Poloxamer® 407 are injectable liquids that rapidly gel when exposed to body temperature after subcutaneous or intraperitoneal injection. There, they are not metabolised by the body, but the gels dissolve over time getting cleared finally. A formulation of interleukin-2 in Poloxamer® 407 gels has shown good effects and biocompatibility in vivo. Hereby, interleukin-2 lost 15% of activity over a 3 days storage at 37°C, but

could be stabilised over that period by human serum albumin. However, in vitro studies indicated a release of greater 80% within 8 hours[123].
Moreover, the release of human growth hormone from Poloxamer® 407 gels was investigated in vitro and in vivo. A release of close to zero order kinetics was found over three days. After subcutaneous administration in rats the formulation was reported to liberate the protein over one week[124].

Another group of polymers exhibiting reverse thermal gelation properties are low molecular weight PLGA-PEG-PLGA copolymers. This system, known as ReGel®, was evaluated as a pharmaceutical vehicle by Zentner et al.[125]. In vivo biodegradation studies in rats revealed that the formulation was completely resorbed from the injection site within 4–6 weeks. The ReGel® system has also been tested for the parenteral delivery of peptides. Thereby, formulations consisted of the peptide in an appropriate buffer system with the gellant but no further stabilisers. Zn-insulin release from ReGel® occurred over approximately one week and even longer depending on the basic triblock copolymer composition. Moreover, the in vitro studies on porcine growth hormone (pGH) and Zn-pGH indicated a continuous liberation over two weeks with no initial burst and mass balance recovery of drug. In vivo results support these results. Furthermore, G-CSF was released from ReGel® to 85% over 12 days. Thereby, no degradation of G-CSF was detected during the release period. Again, this was supported by in vivo data.

Moreover, Hubbell et al. introduced block copolymers of lactic acid and polyethylene glycol as protein delivery vehicles[126]. The polymers are terminally diacrylated and are photopolymerised in an aqueous precursor solution forming a hydrogel. From that preparation insulin, lysozyme, lactate dehydrogenase, ovalbumin, and bovine serum albumin were liberated in vitro. But, release of all substances was finished within 2 to 5 days depending on their molecular weight.
A later report by Hubbell et al. focuses on formation of a bilayer hydrogel depot on polyethylene glycol basis. In detail, it consists of a higher permeability intimally-adherent layer, containing the drug, and a lower permeability luminal layer. Both layers are formed by photopolymerisation of polyethylene glycol diacrylate precursors of different molecular weight. In this system, horseradish peroxidase was used as a model protein, and delivery to the arterial media was measured in rat carotid arteries ex vivo. Thereby, the lower permeability luminal layer served to

enhance delivery of the model protein into the arterial media for delivery periods of three days.

As a more general approach, Hennink and coworkers reported on degradable dextran hydrogels[127]. There, the release of interleukin-2 from non-biodegradable methacrylated dextran and biodegradable lactate-hydroxyethyl methacrylated dextran hydrogels was investigated in vitro. The latter are crosslinked via hydrolysable ester bonds. Interestingly, from non-degradable hydrogels with an initial water content above 70%, the protein release followed Fickian diffusion, whereas from gels with an initial water content of 70% or lower the protein was fully entrapped in the hydrogel meshes. The release from the biodegradable system lasted over 5 to 15 days depending on the crosslinking density. Thereby, the liberation is determined by degradation and diffusion resulting in a nearly zero order profile. Further, interleukin-2 was recovered as monomer in all cases.
Later the group around Hennink reported on degradable dextran hydrogels based on stereocomplex formation between enantiomeric oligomeric lactic acid chains. Release of lysozyme and immunoglobulin G in vitro were finished after six days. Thereby, the proteins were quantitatively released from the gels and with full preservation of lysozyme activity[128].

### 1.2.3 Summary

To conclude, there is a broad variety of publications dealing with the delivery of proteins out of hydrogel matrices. But, research on that topic seems mainly motivated by the goal to create a controlled and sustained releasing drug delivery system. An exception might be the application on wounds. Because, these are widely based on in vivo experiments leading to results that a clinical effect is seen after administration of the preparation or not.
However, the release from hydrogels is mainly diffusion controlled in most cases. According to Fick's law the release rate in this mechanism solely depends on the diffusion coefficient which is closely related to the molecular weight of the diffusing substance. Hence, the liberation rate of these systems is not very sustained but hard to be influenced[129]. Though there are certain possible manipulations reported, e.g. concerning the crosslinking density of the network and swelling behaviour of the gel, to prolong the release, most of the aqueous gel preparations outlined offer a maximum releasing period from days to weeks for protein drugs (see references above).

Thus, to provide a further sustained release profile, for example to reach a continuous release over three months, other delivery devices that release the drug load by different mechanisms, e.g. non-aqueous systems being prone to chemical degradation, have to be evaluated[130,131].

As mentioned the major part of publications concentrate on the release characteristics of the protein drug - hydrogel system. However, if at all, the stabilisation of proteins in the particular hydrogels is addressed only concerning the release period. Longer term stability of proteins embedded in the hydrogels like it is necessary for the further development of a commercial formulation seems to be investigated only to a minor degree.

## 1.3 Aim of the thesis

The major aim of this work was to formulate ACT in suitable carriers that provide proper stability and delivery of this protein drugs into wound sites. The studies are to be performed in vitro.

The application form for the delivery of ACT should be based on hydrogels. Because as outlined in the first introductory parts, hydrogels are the state of the art application form in wound healing. Besides, as presented in the later part of the introduction chapter, they are also known to be suitable for protein drug delivery.

In addition, several key data have been specified by the collaboration partners based on the company medicinal research prior to this work. So, the concentration level of ACT is set to 60µg/ml in the hydrated state. The shelf life of the protein delivering product should be 18 months or greater to ensure proper marketing and logistics for a future commercialised product. Thereby, a water containing product can be stored in a refrigerated state, but a dry product should enable a storage at room temperature to limit costs of product shipping and distribution. Moreover, the preparations should release ACT in considerable amount within one to five days in concordance with common change of dressing intervals in clinical practice. For the selection of ingredients and manufacturing methods after the concerns of protein stability and suitability for wound treatment also the regulatory status should be considered to not unnecessarily hamper a later approval process.
The research work is finished with the presentation and recommendation of formulations for ACT meeting the requirements given above. Subsequently to this collaboration, the formulations are to be introduced in animal experiments with prospect to clinical trials by the collaboration partner.

This thesis is divided into three main chapters. The theoretical introductory remarks about wound healing and protein delivery from hydrogels are outlined before. This is followed by chapter 2 describing materials and methods used in this work. Consequently, chapter 3 with results and discussion is presented. There, the structure follows the milestones of development, i.e. stabilisation of ACT in solution, development of semisolid hydrogel carriers and stabilisation of ACT therein, development of dried gel forms – xerogels and films – and stabilisation of ACT therein, and finally the release of ACT from the reconstituted dry systems.

Chapter 4 summarises the experimental outcome, aims for appropriate conclusions and puts the results into perspective. Chapter 5 is addressed to listen special and continuative literature referred to in the thesis.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 $\alpha_1$-Antichymotrypsin (ACT)

$\alpha_1$-antichymotrypsin (ACT) is a human protein proteinase inhibitor. It is isolated from human plasma and is purchased from Calbiochem, Darmstadt, Germany as lyophilisate (from 188µl solution consisting of 1mg ACT, 150mM NaCl, 20mM Tris, pH 7.4) to be stored at –20°C.

```
Met Glu Arg Met Leu Pro Leu Leu Thr Leu Gly Leu Leu Ala Ala Gly
1               5                  10                  15
Phe Cys Pro Ala Val Leu Cys His Pro Asn Ser Pro Leu Asp Glu Glu
            20                  25                  30
Asn Leu Thr Gln Glu Asn Gln Asp Arg Gly Thr His Val Asp Leu Gly
        35                  40                  45
Leu Ala Ser Ala Asn Val Asp Phe Ala Phe Ser Leu Tyr Lys Gln Leu
    50                  55                  60
Val Leu Lys Ala Pro Asp Lys Asn Val Ile Phe Ser Pro Leu Ser Ile
65                  70                  75                  80
Ser Thr Ala Leu Ala Phe Leu Ser Leu Gly Ala His Asn Thr Thr Leu
                85                  90                  95
Thr Glu Ile Leu Lys Gly Leu Lys Phe Asn Leu Thr Glu Thr Ser Glu
            100                 105                 110
Ala Glu Ile His Gln Ser Phe Gln His Leu Leu Arg Thr Leu Asn Gln
        115                 120                 125
Ser Ser Asp Glu Leu Gln Leu Ser Met Gly Asn Ala Met Phe Val Lys
    130                 135                 140
Glu Gln Leu Ser Leu Leu Asp Arg Phe Thr Glu Asp Ala Lys Arg Leu
145                 150                 155                 160
Tyr Gly Ser Glu Ala Phe Ala Thr Asp Phe Gln Asp Ser Ala Ala Ala
                165                 170                 175
Lys Lys Leu Ile Asn Asp Tyr Val Lys Asn Gly Thr Arg Gly Lys Ile
            180                 185                 190
Thr Asp Leu Ile Lys Asp Leu Asp Ser Gln Thr Met Met Val Leu Val
        195                 200                 205
Asn Tyr Ile Phe Phe Lys Ala Lys Trp Glu Met Pro Phe Asp Pro Gln
    210                 215                 220
Asp Thr His Gln Ser Arg Phe Tyr Leu Ser Lys Lys Lys Trp Val Met
225                 230                 235                 240
Val Pro Met Met Ser Leu His His Leu Thr Ile Pro Tyr Phe Arg Asp
                245                 250                 255
Glu Glu Leu Ser Cys Thr Val Val Glu Leu Lys Tyr Thr Gly Asn Ala
            260                 265                 270
Ser Ala Leu Phe Ile Leu Pro Asp Gln Asp Lys Met Glu Glu Val Glu
        275                 280                 285
Ala Met Leu Leu Pro Glu Thr Leu Lys Arg Trp Arg Asp Ser Leu Glu
    290                 295                 300
Phe Arg Glu Ile Gly Glu Leu Tyr Leu Pro Lys Phe Ser Ile Ser Arg
305                 310                 315                 320
Asp Tyr Asn Leu Asn Asp Ile Leu Leu Gln Leu Gly Ile Glu Glu Ala
                325                 330                 335
Phe Thr Ser Lys Ala Asp Leu Ser Gly Ile Thr Gly Ala Arg Asn Leu
            340                 345                 350
Ala Val Ser Gln Val Val His Lys Ala Val Leu Asp Val Phe Glu Glu
        355                 360                 365
Gly Thr Glu Ala Ser Ala Ala Thr Ala Val Lys Ile Thr Leu Leu Ser
    370                 375                 380
Ala Leu Val Glu Thr Arg Thr Ile Val Arg Phe Asn Arg Pro Phe Leu
385                 390                 395                 400
Met Ile Ile Val Pro Thr Asp Thr Gln Asn Ile Phe Phe Met Ser Lys
                405                 410                 415
Val Thr Asn Pro Lys Gln Ala
            420
```

Fig. 2-1 Amino acid sequence of human ACT with numbering;

ACT has a molecular weight of 68kDa and consists of 423 amino acids (Fig. 2-1). Moreover, it is heavily glycosylated with 40 neutral sugar residues, 35

acetylglucosamine residues, and 14 acetylneuraminic acid residues per molecule adding up to about 25% sugar content of the total molecular weight.

## 2.1.2 Excipients and chemicals

| Substance | Quality | Supplier |
|---|---|---|
| Sodium chloride, Potassium chloride, Magnesium chloride, Ethylenediamine tetra acetate, Sodium sulphate, Ammonium chloride, Tetramethylammonium chloride, Potassium bromide, Phosphoric acid, Potassium hydroxide | p.a. | Merck KGaA, Darmstadt, Germany |
| Polysorbate (Tween®) 20 | Ph. Eur. 2004 | Serva, Heidelberg, Germany |
| Poloxamer® 188 | Ph. Eur. 2004 | BASF, Ludwigshafen, Germany |
| Solutol® HS15 | Purest | BASF, Ludwigshafen, Germany |
| Sucrose | Ph. Eur. 2004 | Merck KGaA, Darmstadt, Germany |
| Trehalose | Purest | British sugar, Peterborough, UK |
| Glycerol | Ph. Eur. 2004 | Merck KGaA, Darmstadt, Germany |
| Propylenglycol | Ph. Eur. 2004 | Merck KGaA, Darmstadt, Germany |
| Mannitol | Ph. Eur. 2004 | Merck KGaA, Darmstadt, Germany |
| Sorbitol | Purest | Merck KGaA, Darmstadt, Germany |
| α-cyclodextrin, β-cyclodextrin, Hydroxypropyl-β-cyclodextrin; γ-cyclodextrin | Purest | Wacker, Burghausen, Germany |
| Urea | Ph. Eur. 2004 | Merck KGaA, Darmstadt, Germany |
| Glyine, Alanine, Phenylalanine, Arginine, Methionine, Lysine, Cysteine | Ph. Eur. 2004 | Merck KGaA, Darmstadt, Germany |
| Benzylalcohol | Ph. Eur. 2004 | Sigma, Taufkirchen, Germany |
| Chlorokresol | Ph. Eur. 2004 | Sigma, Taufkirchen, Germany |
| Parabene | Purest | Sigma, Taufkirchen, Germany |
| Phenol | Ph. Eur. 2004 | Sigma, Taufkirchen, Germany |

## 2.1.3 Polymers

All polymers were purchased as Ph. Eur. 2004 grade where available. Else, pharma grade was ordered.

### *2.1.3.1 Cellulose ethers*

| Polymer | Brand name | Supplier |
|---|---|---|
| Hydroxyethyl cellulose 100.000 | Natrosol® 250 HHX | Aqualon, Düsseldorf, Germany |
| Hydroxyethyl cellulose 30.000 | Tylose® H 30.000 | Clariant, Wiesbaden, Germany |
| Hydroxyethyl cellulose 10.000 | Tylose® H 10.000 | Clariant, Wiesbaden, Germany |
| Hydroxyethyl cellulose 4.000 | Tylose® H 4.000 | Clariant, Wiesbaden, Germany |
| Hydroxyethyl cellulose 1.000 | Tylose® H 1.000 | Clariant, Wiesbaden, Germany |
| Hydroxyethyl cellulose 300 | Tylose® H 300 | Clariant, Wiesbaden, Germany |
| Carboxymethyl cellulose Na 30.000 | Tylopur® C 30.000 | Clariant, Wiesbaden, Germany |
| Carboxymethyl cellulose Na 10.000 | Blanose® 7HF | Aqualon, Düsseldorf, Germany |
| Carboxymethyl cellulose Na 1.000 | Tylopur® C 1.000 | Clariant, Wiesbaden, Germany |
| Carboxymethyl cellulose Na crosslinked | AcDiSol® | FMC, Newark, USA |
| Methyl cellulose 1.000 | Tylose® MH 1.000 | Clariant, Wiesbaden, Germany |
| Hydroxypropyl cellulose 100.000 | Klucel® HF | Aqualon, Düsseldorf, Germany |
| Hydroxypropylmethyl cellulose 100.000 | Methocel® K100M | Colorcon, Kent, UK |
| Hydroxypropylmethyl cellulose 15.000 | Methocel® K15M | Colorcon, Kent, UK |

### *2.1.3.2 Gellan gum*

Gellan gum is produced by Pseudomonas elodea. It is an anionic polysaccharide consisting of a repeating linear tetrasaccharide unit. The latter is a sugar sequence of β-D-glucose, β-D-glucuronic acid, β-D-glucose, and α-L-rhamnose. Native gellan gum - Kelcogel® LT100 – is partly acetylated at the $C_6$-atom of the first glucose unit. Moreover, there is a glyceryl at the same glucose unit (Fig. 2-2). The acetylated polymers form soft and very elastic gels because the acetyl groups disturb and therefore reduce the intermolecular forces. The deacetylated polysaccharides – Kelcogel® F – form harder but more brittle gels (Fig. 2-3). Both gellan types form thermoreversible gels. Therefore, gel manufacture is described as cooling of a warm gellan solution. In our case this step is replaced by steam sterilisation.

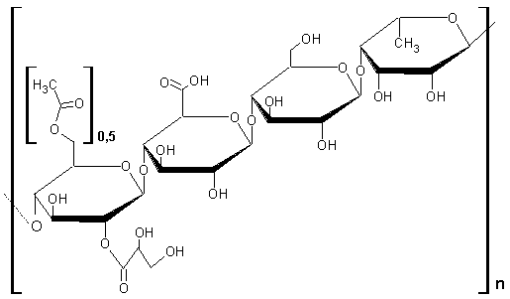

Fig. 2-2 native gellan gum;

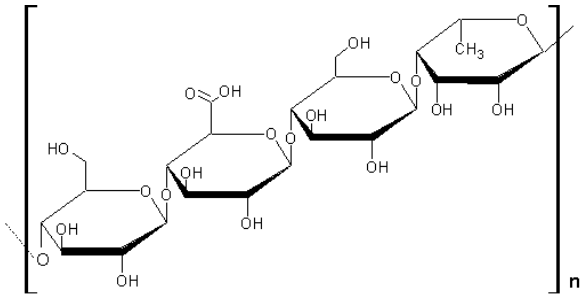

Fig. 2-3 deacetylated gellan gum;

Gellan gum molecules are parallel double helices. For gelation, moreover, cations like sodium, potassium or calcium are used for their support of interlinking gellan gum polymers. In detail, the helices are linked via electrostatic interactions between monovalent cations, water molecules and a carboxyl group[132]:

double helix - $K^+$ - water - $K^+$ - double helix

Bivalent cations form direct complexes with two carboxyl groups.

### *2.1.3.3 Other polymers*

| **Polymer** | **Brand name** | **Supplier** |
|---|---|---|
| Polyethylene glycol (PEG) 400, 2000, 6000, 20.000 | | Clariant, Wiesbaden, Germany |
| Alginate Na | Protanal® HF120 RBS | FMC, Newark, USA |
| Xanthan gum | Xanthural® 11k | CP Kelco, Lille, Denmark |
| Gellan gum F | Kelcogel® F | CP Kelco, Lille, Denmark |
| Gellan gum LT 100 | Kelcogel® LT100 | CP Kelco, Lille, Denmark |
| Gelatine A | | Sigma, Taufkirchen, Germany |
| Polyacrylate | Carbopol® 940 | BF Goodrich, Cleveland, USA |
| Eudragit® | Eudragit® L100 | Röhm, Darmstadt, Germany |
| Polyvinyl pyrrolidone 17 | Kollidon® 17PF | BASF, Ludwigshafen, Germany |
| Polyvinyl pyrrolidone 90 | Kollidon® 90 | BASF, Ludwigshafen, Germany |
| Polyvinyl alcohol 100.000 | | Sigma, Taufkirchen, Germany |
| Lutrol® F127 | | BASF, Ludwigshafen, Germany |

# 2.2 Methods

## 2.2.1 Characterisation of ACT

### *2.2.1.1 ACT activity assay*

In order to determine the ACT activity, an activity assay based on Cathepsin G binding is performed. During the reaction a complex is formed:
Cathepsin G/ACT/Antibody 1/Antibody 2
The antibody 2 is linked to horseradish peroxidase catalysing the detectable colour reaction.
96-well plates are coated with BSA and subsequently with Cathepsin G (Calbiochem, Darmstadt, Germany). After washing, ACT samples are added and incubated for 30min at 37°C. After three times washing with PBS-T (phosphate buffered saline with 0.05% Tween®) buffer , a rabbit anti human ACT antibody (DAKO, Glostrup, Denmark) is added to the wells and is incubated for 30min at 37°C. After washing three times with PBS-T buffer, a goat anti rabbit IgG antibody conjugated with horseradish peroxidase (DAKO, Glostrup, Denmark) is added and is again incubated for 30 min at 37°C. The wells are again washed three times with PBS-T buffer. Subsequently, OPD (1,2-Diaminobenzene, Sigma, Taufkirchen, Germany) substrate solution is prepared according to the manufacturer's protocol with hydrogen peroxide (Sigma, Taufkirchen, Germany), added to the wells and incubated at room temperature in the dark. After 10 minutes the reaction is stopped by adding 100µl 0.5M sulphuric acid per well. Immediately after stopping the reaction the absorption at 490 nm is determined.

### *2.2.1.2 ACT ELISA*

In order to determine the ACT content, an ELISA is performed. During the reaction a complex is formed: Antibody1/ACT/Antibody2
The antibody2 is linked to horseradish peroxidase catalysing the detectable colour reaction.
96-well plates are coated with rabbit anti human ACT antibody (DAKO, Glostrup, Denmark) over night at 6°C. After washing and blocking with PBS-T/BSA, ACT samples are added and incubated for 120min at 37°C. After three times washing with PBS-T/BSA, a anti human ACT antibody conjugated with horseradish peroxidase (Biotrend, Cologne, Germany) is added to the wells and is incubated for

120min at 25°C. Subsequently, OPD (1,2-Diaminobenzene) substrate solution is prepared according to the manufacturer's protocol (Sigma, Taufkirchen, Germany), added to the wells and incubated at 25°C in the dark. After 15 minutes the reaction is stopped by adding 100µl 0,5M $H_2SO_4$ per well. Immediately after stopping the reaction the absorption at 490 nm is determined.

#### *2.2.1.3 Gel electrophoresis*

Protein integrity was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions using an XCell II Mini cell system (Novex, San Diego, USA). Samples were diluted in a pH 6.8 Tris-buffer, containing 2% SDS and 2% glycerin for 30 min at 90°C and subsequently loaded into gel wells (NuPAGER Novex 10% Bis-Tris Pre-cast Gel 1.0 mm from Invitrogen, Groningen, Netherlands). Electrophoresis was performed in a constant current mode of 60 mA in a Tris-glycine/SDS running buffer. After staining with coomassie blue staining kit (Novex Colloidal blue stain kit), the gels were dried using a DryEaseR Gel Drying System (Invitrogen).

### 2.2.2 Manufacture of matrices

#### *2.2.2.1 Wet film manufacture with the scraper*

For the casting of wet films a scraper is used (Erichsen, Hemer, Germany, Model 360, 03216). It is 6cm wide and provided with defined four gaps of 500µm, 1000µm , 1500µm, and 2000µm height, respectively. After loading the scraper with approx. 10 ml of gel the apparatus is drawn over a glass surface (retail window glass prepared by float-technique) with a slow constant speed.

#### *2.2.2.2 Freeze-drying*

Lyophilisation is operated in a two chamber freeze-dryer ε12G (Christ, Osterode, Germany). Primary packaging material for production of placebo xerogels are petri dishes, whereas ACT loaded matrices are produced in 2R glass vials, glass type I with Teflon® coated chlorobutyl rubber stoppers.

#### *2.2.2.3 Warm air drying*

For manufacture of polymer films the warm air dryer reported earlier[165] is used (Barkey GmbH & Co. KG, Leopoldshöhe, Germany) (Fig. 2-4).

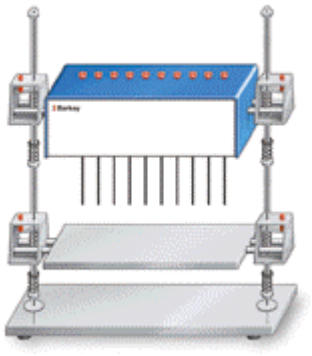

Fig. 2-4 Warm air drier for vials and eppendorf caps;

Drying is performed with dry and tempered nitrogen gas injected through nozzles into heated vials. All temperatures are set to 25°C. Nitrogen flow rate is 1.0 l/min at a pressure of 0.5 bar.

### 2.2.3 Characterisation of matrices

#### *2.2.3.1 Viscometry*

Viscometric measurements on hydrogels with pseudoplastic behaviour are conducted on a rotating cylinder viscometer DV-II+ (Brookfield, Middleboro, USA). Complex rheology is measured with a MCR 100 cone plate viscometer (PaarPhysika, Ostfildern, Germany). Therefore, a cone (50mm in diameter, 1° angle; CP 50-1) is used at an oscillation frequency of 10 1/s.

#### *2.2.3.2 Mechanical tests*

The mechanical properties of the matrices are evaluated using a Texture Analyser model TA.Xtplus (Stable Microsystems, Godalming, UK).
*Compression test method* - For compression tests on xerogels a cylindrical probe of 0.5" in diameter is used. The compression strain is set to 50% deformation, crosshead speed is 1mm/min.

*Tensile test method* - The test apparatus for measuring the tensile strength of films consists of two mechanic grips and the test procedure is based on the ASTM D822-75 method. The extension speed is 1mm/min. Film specimens which break directly at the grips are discarded.
*Puncture test method* – Films are punctured by a driving ball probe of 0.5" in diameter. Fixation of the specimen is provided by a film holder. The dry rectangular film specimens are positioned between two mounting plates and are fixed with four screws. The plates contain a hole of 22mm in the centre. The ball probe is driven downwards through the mounted film at a crosshead speed of 1mm/min. The calculation of the nominal puncture strength and the elongation at rupture is comparable to the tensile test method. However, the elongation is based on the displacement of the radius instead of the length.

### *2.2.3.3 In vitro Release tests*

Release tests are performed in vitro by using a modified Loth chamber[134]. The chamber body consists of Plexiglas®. Associated material is an IPC 12 channel hose pump (Ismatec, Wertheim, Germany), tubing material (Tygon R3603), cellulose acetate membrane filters 0.45µm (Sartorius, Göttingen, Germany), and 2ml syringes (HSW, Tuttlingen, Germany).

### *2.2.3.4 Karl Fischer Titration*

Residual moisture is determined via coulorimetric Karl Fischer titration with generating the iodine electrolytically to keep the reaction ongoing. Therefore, a coulorimetric Karl Fischer titrator with a head space oven is used (Analytik Jena AG, Jena, Germany). Sealed samples are heated to 80°C in the oven chamber. For measurement the vaporised water is transported into the coulorimetric cell filled with Hydranal® Coulomat AG (Riedel-de Haen) via a needle system.

### *2.2.3.5 Differential scanning calorimetry (DSC)*

Measurements (DSC 204, Netzsch, Selb, Germany) are performed using 5mg to 10mg of sample. Heating and cooling were conducted at a scan rate of 5 K/min from 25°C to 100°C.

### *2.2.3.6 X-ray diffraction*

Wide-angle X-ray scattering (WAXS) was performed by an X-ray Diffractometer XRD 3000TT, (Seifert, Ahrensburg, Germany) equipped with a copper anode (40kV, 30mA, wavelength 0.154178 nm). Experiments were conducted at a scan rate of 2 theta = 0.05° in a 5° to 40° range.

# 3 Results and Discussion

The development of ACT for topical delivery into wound sites is mainly diverted into stabilisation studies on ACT and the development of the (placebo) carrier systems. Thereby, the application forms of solution, hydrogel, xerogel, and polymer film are run through. During all these stages on the one hand the aspects of the delivery vehicle, namely its suitability for protein stabilisation, as its suitability for wound treatment, are investigated. And, on the other hand the aspects of ACT stabilisation and later the release of ACT are researched.

In detail, after a discussion of analytical tools the stabilisation of ACT in solutions is described in a first section. Secondly, the development of particular hydrogels for wound treatment and the stabilisation of ACT within these hydrogels is discussed. A third section outlines the xerogel formation by lyophilisation of the named hydrogels. Moreover, again the suitability of xerogels for application on wounds and their ability to stabilise ACT is researched. The fourth section discusses polymer films as alternative to xerogels with similar aspects. Further, the release of ACT from the preparations is summarised in a fifth section.

# 3.1 Analytical tools for the characterisation of ACT during formulation development

This section describes evaluations for the characterisation of ACT during formulation development.

Due to its unique mode of action described in chapter 1 and the fact that its native and active state represents only a state with a relative minimum of free energy, ACT is likely to be prone to deactivation by refolding to the non-active energy minimum state and dimerisation or formation of higher order aggregates by entanglement of the loops of two ACT molecules during loop movement.

Analytical tools therefore have to be chosen to mainly detect inhibitory activity and aggregate formation of ACT. Furthermore, it is desirable to characterise the tertiary structure especially the folding behaviour of ACT during experiments. Unfortunately, the low concentration of ACT in measurable samples (1µg/ml to 60µg/ml) and low sample amounts resulting from high drug prices lead to unsuitability of many standard methods for these tasks due to inappropriate detection limits and minimal sample volumes[135].

These methods include separation methods with UV-detection, i.e. reversed phase liquid chromatography, size exclusion chromatography, capillary electrophoresis, and asymmetric flow field-flow fractionation. Further, several analytical methods describing protein structure, such as fourier transform infrared spectroscopy, circular dichroism, and differential scanning calorimetry are not able to generate sensible signals for ACT in the required concentration. So is the case with turbidity and particulate matter detection.

Thus, the immunochemical methods outlined in chapter 2 are used for routine detection because of their high specificity and lowest detection limits. However, these methods hold high error and standard deviation compared to e.g. established chromatographic methods. The activity assay based on a cathepsin G/ACT/antibody sandwich and a linked colour reaction is used to determine in vitro inhibitory activity of ACT. Further, the ELISA is used for determination of total ACT content during release experiments. Moreover, a SDS-PAGE method with coomassie blue detection is used for quantitative detection of aggregation and fragmentation in selected samples. Hereby, it has to be kept in mind that SDS-PAGE is suitable for aggregates formed by covalent binding but non-covalently bound aggregates may be undiscovered.

## 3.2 Stabilisation of ACT in solution

As a first step in formulation development, short-term stability studies on ACT in aqueous solution are reported. Therefore, the aim of that series of experiments is to identify buffer and pH conditions, stabilising additives, and other suitable technical ingredients. Hereby, both thermal stress and freeze/thaw (F/T) stress conditions are investigated. The outcome is used as an approach to further formulation development, but if appropriate the formulations at this stage also can be used as early formulation for e.g. animal experiments or toxicology testing itself.
Samples are prepared in aliquots of 0.1ml with 60µg/ml ACT content in eppendorf caps and are stressed either at 40°C in an air bath or by freeze/thawing in liquid nitrogen and room temperature, respectively. Readout is done by activity assay and SDS-PAGE for some samples.

### 3.2.1 Effects of pH, buffers, and electrolytes on ACT solution stability

The pH is a very powerful tool to control both the physical stability[136] and the chemical stability of protein drugs by its well known general influence on chemical reactions.
Electrolytes including buffer salts can affect a protein solution by indirect mechanisms like changing the solvent properties or a non-specific electrostatic shielding effect. Besides, very specific ion binding phenomena between salts and protein are known[137]. These effects can also stabilise, behave inert and destabilise the protein in solution, respectively, depending on the definite situation in the formulation. Moreover, the content of salts can effect protein stability directly by hydrophilic or hydrophobic interactions depending on their position in the Hoffmeister lyotropic series[138,139]. Exceeding the above mentioned, bi- or polyvalent ions can bind to charged positions within the protein molecule, but may also catalyse oxidative reactions or bind catalysers, e.g. by chelation, which can result in both stabilisation and destabilisation, respectively.

#### *3.2.1.1 Effect of pH on ACT solution stability*

For the investigation of the effect of pH on its stability ACT is formulated in a buffer solution containing 50mM potassium phosphate. The pH is adjusted with

potassium hydroxide to prevent the known pH shift phenomena of sodium phosphate buffered systems during freezing in later experiments[140,141]. For the temperature studies each sample is kept on an elevated stress level of 40°C for 5 days. As described, analysis is carried out by activity assay and SDS-PAGE.
The results of recovered activity after stressing show a strong dependency of ACT activity on the pH of the solution. In the acidic region up to a pH value of 5.5 almost no activity can be measured. But there is activity between 50 to 60 % recovered around the neutral point peaking between pH 7.0 and 7.6 (Fig. 3-1).

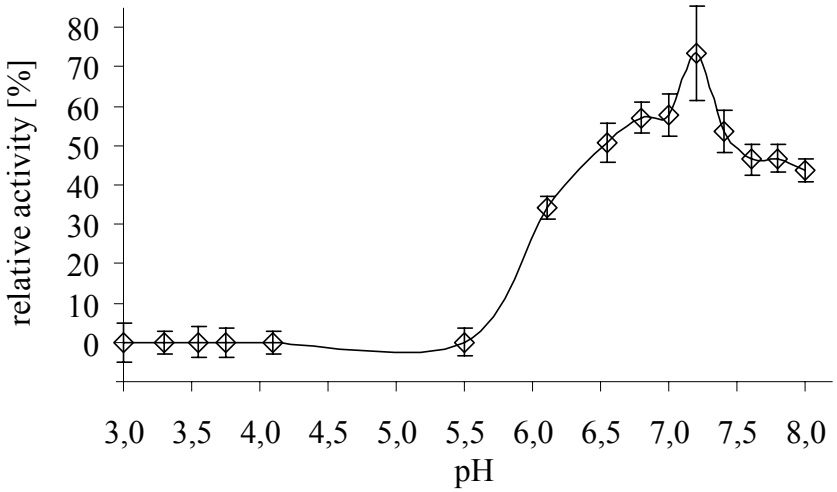


Fig. 3-1 pH stability profile of ACT in 50mM potassium phosphate buffer after a 5 days storage at 40°C measured by activity assay;

Gel electrophoresis also detects pH dependent degradation. In acidic buffer samples the fragment bands are striking. Lane 2 of Fig. 3-2 show the main fraction of 85% at the monomer band at around 60kDa, an aggregate band at about 100kDa to 120kDa representing 9.7% of total content, and two fragment bands with 4.7% of total content each at approx. 40kDa and 20kDa molecular weight. Similar fragmentation is seen in lane 1. In neutral and basic pHs, lane 3 to 6 of Fig. 3-2, solely the aggregate band can be seen.

Taking results together it can be concluded that in acidic pHs fragmentation as well as aggregation leads to inactivation of ACT. In the neutral and basic regions mainly aggregation is responsible for loss of activity.

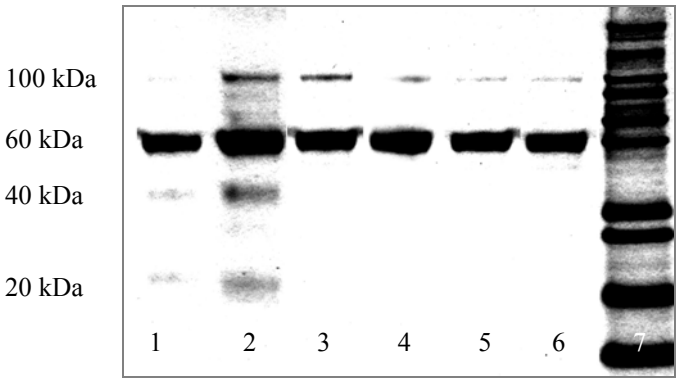


Fig. 3-2 pH dependent stability of ACT in 50mM potassium phosphate buffer after a 5 days storage at 40°C measured by SDS-PAGE:
lane 1: pH 2.2 with 0.5% 100kDa aggregate, 3.2% 40kDa, 0.7% 20kDa fragments; lane 2: pH 4.5 with 9.7% 100kDa aggregate, 3.8% 40kDa, 0.9% 20kDa fragments; lane 3: pH 6.5 with 8.5% 100kDa aggregate; lane 4: pH 7.4 with 4.7% 100kDa aggregate; lane 5: pH 8.0 with 2.5% 100kDa aggregate; lane 6: pH 11.0 with 1.7% 100kDa aggregate; lane 7: marker;
control without degradation is visualised in Fig. 3-4;

But, one may recognise that although practically no activity is determined in the acidic, the main fraction of ACT is still detected as monomer in gel electrophoresis. This might be due to the fact that SDS-PAGE is solely sensitive to aggregates with covalently bound monomers because non-covalent aggregates are dissociated by the preparation with SDS. The latter thus are also detected as monomers. Granted that ACT forms inactive, non-covalent aggregates in the first part the given results could be explained. And, this is very likely due to the mode of action of ACT described in chapter 1. Another explanation instead could be the formation of monomeric inactive forms of ACT during stressing by refolding to the inactive state of minimum energy which unfortunately can not be further detected due to the described analytical problems (section 3.1). This interpretation is also feasible for the neutral and basic pHs because the detected amount of aggregates of around 10% is by far lower than the inactive ACT amount calculated from activity assay being about 40 to 50%.

For exact determination of the pH-optimum between pH 7.0 and 7.6 a kinetics study is done over a period of twelve days in a narrower pH spectrum. Activity there decreases according to first order kinetics. Thus, a logarithmic curve can be fitted to the results with its slope indicating the activity loss rate. So, the rate is almost constant between pH 7.0 and 7.4 indicated by similar values for the slope of the fitted function. But, at pH 7.6 a higher rate is detected by a larger negative slope (Tab. 3-1). As a result, the optimum pH for temperature studies is ascertained to pH 7.2 representing the centre of a plateau of constant low decrease rate.

| pH | slope of logarithmic curve | $R^2$ |
|---|---|---|
| 7.0 | - 38.7 | 0.979 |
| 7.2 | - 42.6 | 0.994 |
| 7.4 | - 44.9 | 0.996 |
| 7.6 | - 55.5 | 0.993 |

Tab. 3-1 Slope and correlation coefficient of the fitted logarithmic first order kinetics equation on pH stability kinetics of ACT in 50mM potassium phosphate buffer after a 12 days storage at 40 °C measured by activity assay;

A similar setting as for the temperature stress study is performed for the F/T tests consisting of 5 cycles of freezing in liquid nitrogen and thawing at room temperature. The resulting curve of recovered activity like above shows values of around 60% between pH 7.2 and 7.6 and a total loss in the acidic region (Fig. 3-3). Again, gel electrophoresis indicates dimer formation during the F/T stressing (Fig. 3-4).

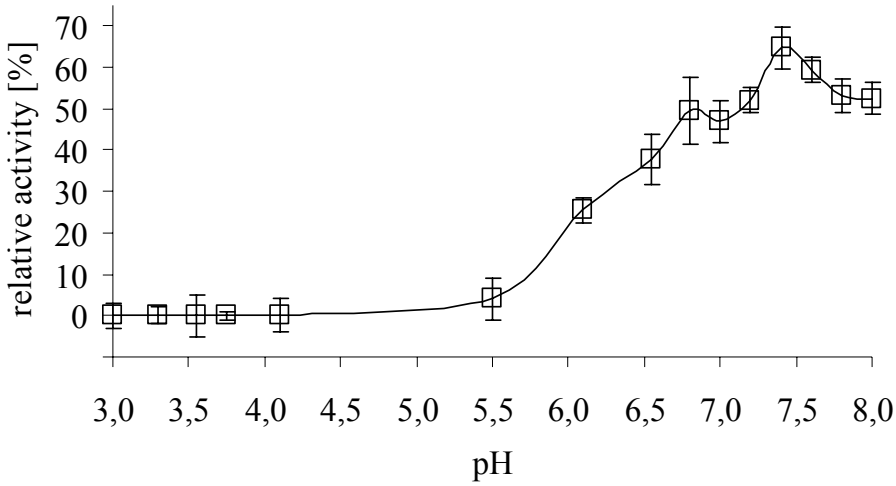


Fig. 3-3 pH stability profile of ACT in 50mM potassium phosphate buffer after 5 F/T cycles in liquid nitrogen and 25°C, resp. measured by activity assay;

As a matter of fact, the working pH for further studies is determined at pH 7.2. It is located in the centre of an area of low decrease rate for both stress types. That is why not only the stabilising effect of the solution is maximised but due to the plateau also the best possible robustness against small accidental changes of pH within the system is created.

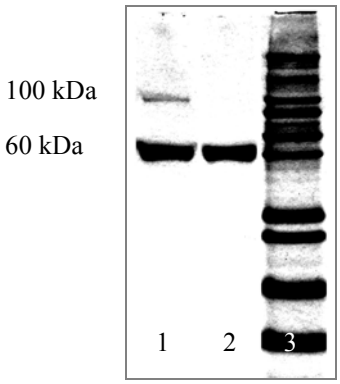


Fig. 3-4 SDS-PAGE gel for evaluation of freeze/thaw stress dependent stability of ACT in 50mM potassium phosphate buffer pH 7.2 after 5 F/T cycles:
lane 1 F/T stressed sample with 9.8% 100kDa aggregate; lane 2 unstressed control sample without fragment and aggregate formation; lane 3 marker;

### *3.2.1.2 Effect of buffer species on ACT solution stability*

As a consequence of the foregoing section, a choice of buffers with a maximum capacity around the neutral point is tested at the pH level of 7.2. The content of buffer for the F/T-stress is lowered to 10mM instead of 50mM in the temperature treated samples.
For the temperature stress as well as for the F/T-stress next to the phosphate buffer, also TrisHCl and a phosphate/citrate mixture show comparable recovered relative activities and therefore comparable stabilisation characteristics (Tab. 3-2). As a result, these systems act as alternatives to the standard phosphate buffer for future development.

| buffer pH 7.2 | stress condition | rel. activity [%] ±sd |
|---|---|---|
| 50mM phosphate | 40°C/9 days | 78.2±0.5 |
| 50mM TrisHCl | 40°C/9 days | 68.5±5.1 |
| 25/25mM phosphate/citrate | 40°C/9 days | 80.2±6.8 |
| 10mM phosphate | F/T/5 cycles | 42.5±7.2 |
| 10mM TrisHCl | F/T/5 cycles | 41.0±7.1 |
| 10/10mM phosphate/citrate | F/T/5 cycles | 46.1±4.5 |

Tab. 3-2 Recovered relative activities of ACT after temperature and freeze/thaw stress in dependence of the buffer system measured by activity assay;

The phosphate/citrate mixture is further investigated concerning the relation of concentration of the components. Hence, for temperature stress to a 25mM phosphate buffer increasing amounts of citrate resulting in concentrations from 10mM to 50mM are added. For F/T treatment the contents are lowered to 5mM phosphate and 2mM to 20mM citrate.

Results after temperature stressing at that point do not show remarkable differences. However, for the freeze/thaw stress an optimum arises for low, 5 to 10mM, citrate contents (Fig. 3-5). So, an equimolar buffer appears the best choice for the phosphate/citrate mixture.
To summarise, to date the phosphate buffer pH 7.2 is chosen as standard buffer, but the TrisHCl system and the equimolar phosphate/citrate mixture can also be considered if advantage or necessity arises during further development. All buffer systems are approved by FDA for injection up to a content of 1% at least which represents solutions of 70mM to 80mM.

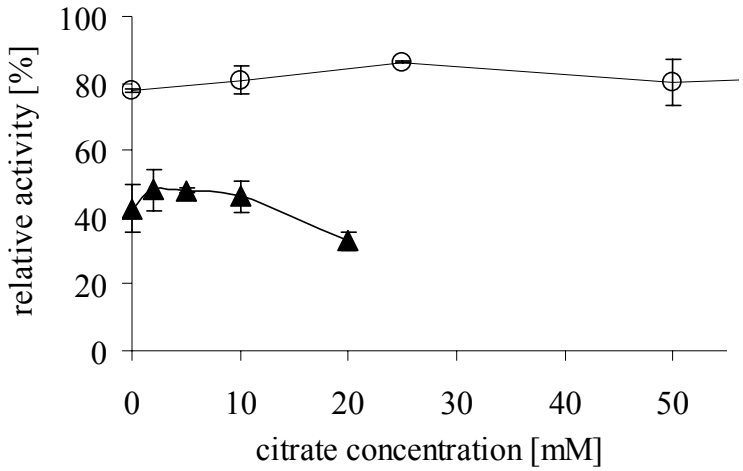


Fig. 3-5 Recovered relative activities of ACT after 9 days at 40°C storage in 25mM phosphate buffer and varying citrate contents (○) and after 5 F/T cycles in 5mM phosphate buffer and varying citrate contents (▲) measured by activity assay;

### *3.2.1.3 Effect of salts on ACT solution stability*

For the use as osmotic agent during formulation and for general use, e.g. during drug substance manufacturing, a variety of salts is tested in temperature stress tests. Samples are buffered in 50mM potassium phosphate buffer pH 7.2 and salts are added resulting in concentrations of 100mM, except 10mM for magnesium chloride and EDTA sodium. Other than before, stressing at 40°C lasts for 6 days for technical reasons.
As a result, none of the samples shows a major change of activity after stressing compared to the untreated material and therefore all of them can be used with ACT. Besides, generally, all tested salts are compatible with the assay, but the differences between some controls could be related to assay interaction or in the

case of sodium containing samples to pH changes during freezing for transportation (Fig. 3-6).

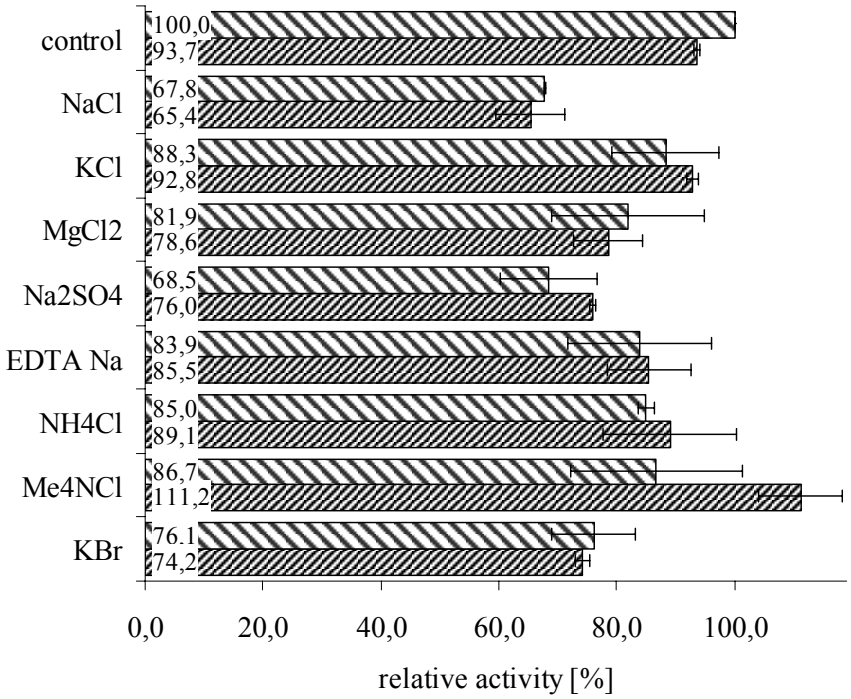


Fig. 3-6 Recovered relative activity of ACT in 50mM potassium phosphate buffer pH 7.2 with different salt additives before (above) and after (below) a 6 days storage at 40°C measured by activity assay;

### *3.2.1.4 Effect of buffer content and osmolarity on ACT solution stability*

Next, the optimal phosphate buffer content and total osmolarity for an ACT stabilising solution is to be determined.

In order to investigate the influence of these two closely related factors of the solution on ACT activity under temperature stress a factorial experimental design is created. The first relevant factor is the concentration of the potassium phosphate buffer system. It is varied from a minimum of 5mM to a maximum of 100mM. The other factor is the concentration of potassium chloride as osmotic agent, hereby being 0mM the low value and 100mM the high value. With these two factors a simplex-lattice design of 3$^{rd}$ order degree, i.e. on four levels, is calculated by the Statistica 6.1 software. For interpretation a 2$^{nd}$ degree multiple regression is carried out resulting in a corresponding response surface function (Fig. 3-7).

For the pure buffer samples (Fig. 3-7 edge behind on left) an increase of activity is measured from the minimum to about 25mM of phosphate concentration. To higher pure buffer contents more or less a plateau on high level can be seen.

Osmolarity there is determined between 150mosm/kg and 200mosm/kg. The addition of KCl as osmotic agent works counterproductive in most cases. Only with very low buffer contents of 10mM to 20mM an improvement of stability of ACT can be obtained by an optimum concentration of KCl which is around 50mM. For these samples osmolarity is measured as 154mosm/kg. In conclusion, the favourable range of osmolarity is supposed to be between 150mosm/kg to 200mosm/kg. Unless, except for the case of low buffer contents the addition of an osmotic agent is not necessary and recommended.
Recovered activity values in this chart for technical reasons exceed the values from former experiments. This arises from the reference to a different control sample with a lower measured activity.

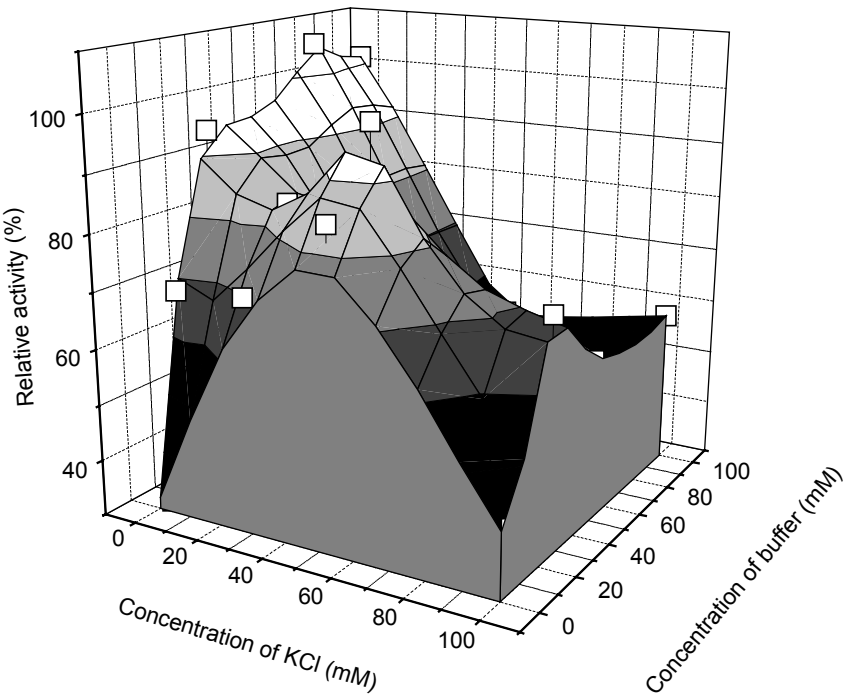


Fig. 3-7 Response surface calculated from recovered relative activities of ACT in samples with potassium phosphate buffer pH 7.2 and salt (KCl) contents according to a simplex-lattice design after temperature treatment – 40°C for 9 days;

For F/T-studies on the buffer and electrolyte content solely the buffer concentration is altered without further addition of electrolytes. 5 cycles of the usual F/T-stressing are performed. From the recovered activity curve against

buffer concentration a clear maximum at 10mM can be determined (Fig. 3-8). Therefore, in contrast to temperature studies a lower buffer content is to be used.

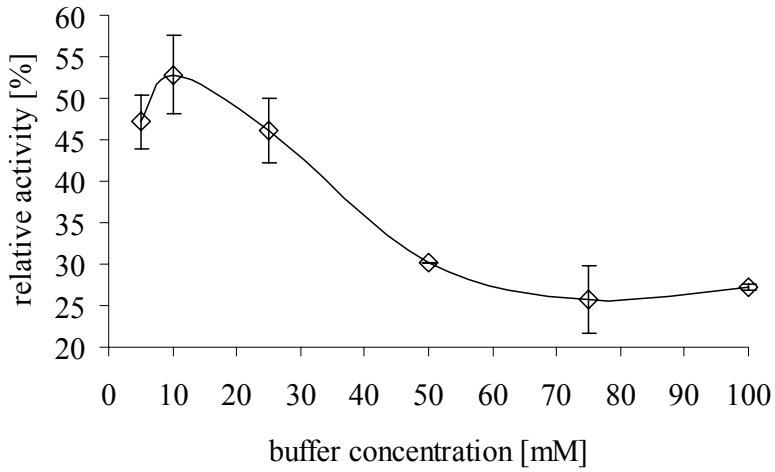


Fig. 3-8 Recovered relative activities of ACT after 5 F/T cycles in varying potassium phosphate buffer contents pH 7.2 measured by activity assay;

### *3.2.1.5 Summary of the effects of pH, buffer, and electrolytes on ACT solution stability*

During the experiments of this section buffer systems and electrolyte contents of an ACT stabilising solution are investigated. In detail, optimal pHs, buffer species, buffer contents, compatible salts and suitable buffer/salt combinations are evaluated.

In fact, for temperature stress a potassium phosphate buffer concentration of 50mM at a pH of 7.2 and for F/T stress a 10mM phosphate buffer at same pH is to be recommended. For temperature stress, alternatively, a solution containing 20mM phosphate buffer and 50mM potassium chloride is suitable. Despite, for further development the pure buffer alternatives are determined as standard buffer systems.

## 3.2.2 Effects of stabilisers and excipients on ACT solution stability

In this section protein stabilisers are screened in the same manner as the buffer/electrolyte systems. Besides, substances able to stabilise during drying processes and substances for other technical purposes are tested in this setting.
The basis for the samples are 0.1ml solution of 60µg/ml ACT in a 50mM potassium phosphate buffer pH 7.2 for temperature stressing and a reduced buffer content of 10mM for the freeze/thaw experiments.
In literature, various stabilisers different in their chemical structure and mode of action are described[137]. In the course of this work, theses substances have to be applicable on open wounds next to their ability to stabilise. So additionally, for a first consideration the regulatory status of the substances is taken into account.
The following is dealing with the testing of surfactants, sugars, polyols, cyclodextrins, amino acids, and preservatives. Polymeric adjuvants are described in a later section (3.3.2.1).

### *3.2.2.1 Surfactants for the stabilisation of ACT in solution*

Besides their ability to reduce surface tension in liquids surfactants are known to bind at hydrophobic surfaces[142], gas-liquid interfaces[143] and at the protein molecule itself mostly at hydrophobic areas[144]. Therefore, mechanisms of protein protection by surfactants based on a competitive situation for adsorption on denaturing interfaces between protein and surfactant are discussed[145]. Further, a mechanism relating to a direct binding of the surfactant to the protein, marked by a higher necessary concentration of surfactant that depends on the protein content in the solution has been reported[146]. But surfactants are also able to prevent chemical degradation in some cases [147]. Ionic surfactants are usually avoided because of their ability to bind to polar as well as to unpolar groups and therefore denature proteins [148]. For that reason only a choice of non-ionic surfactants is investigated in this study.
Tween® 80, Poloxamer® 188, and Solutol® HS15 are added to the standard phosphate buffer to a maximum content of 0.2%.

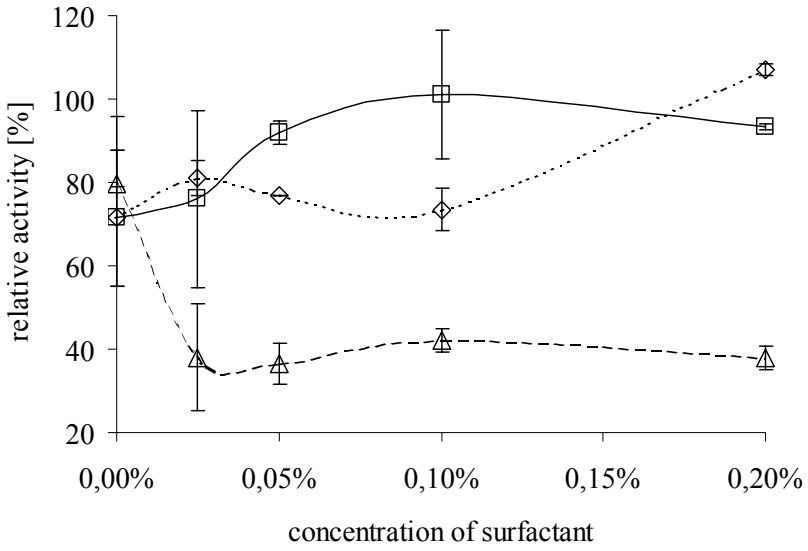


Fig. 3-9 Recovered relative activity of ACT in dependence of the concentration of surfactants in ACT samples in 50mM potassium phosphate buffer after a 9 days storage at 40°C measured by activity assay; □: Poloxamer® 188, ◇: Tween® 80, △: Solutol® HS15;

In the temperature stress Solutol® HS15 causes a severe decay in ACT activity in all tested concentrations. For that reason, Solutol® HS15 appears to be completely incompatible with ACT and the investigation on that surfactant is stopped here. Poloxamer® 188 and Tween® 80 are compatible with the protein and moreover can effectively improve its stability particularly when surfactant content exceeds 0.1% (Fig. 3-9).

In the corresponding F/T-stress experiments the effect is even more convincing. One may notice that the control is close to zero when the stress level hereby is raised to 15 F/T cycles. Indeed, Poloxamer® 188 and Tween® 80 completely prevent the ACT activity loss even at 15 F/T cycles. Hereby, Poloxamer® 188 is effective from 0.05% and Tween® 80 from 0.1% (Fig. 3-10).

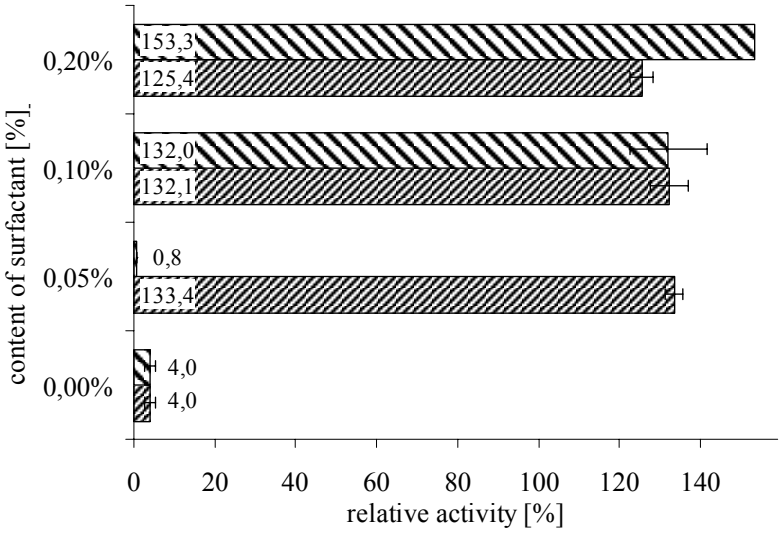


Fig. 3-10 Recovered relative activity of ACT in dependence of the concentration of surfactants in ACT samples in 10mM phosphate buffer pH 7.2 after 15 F/T cycles measured by activity assay; above: Tween® 80; below: Poloxamer® 188;

To sum up, the tested surfactants Poloxamer® 188 and Tween® 80 prove high efficiency in stabilising ACT during temperature and even more in F/T stressing. Hereby, a concentration of 0.1% is recommended representing a compromise between stabilising efficiency and physiological tolerance. The regulatory status of these two surfactants is satisfactory. Poloxamer® 188 is approved by FDA to a maximum potency of 0.6% for intravenous injection and 0.3% for subcutaneous injection[149], whereas Tween® 80 is approved also by FDA to a maximum potency of 0.2% for intralesional injection and 8% for intravenous injection[149]. Solutol® HS15 destabilises ACT and is therefore unsuitable for further studies.

### *3.2.2.2 Sugars and polyols for the stabilisation of ACT in solution*

Sugars and the related groups of polyols are very often reported as non-specific protein stabilisers. Applied in a concentration of at least 5% their stabilisation mechanism is interpreted as a preferential exclusion phenomenon[150,151]. Though, in some cases sugars and polyols can as well inhibit chemical degradation by complexation effects in a similar way as polyvalent ions. However, because of the well known Maillard reaction between amino groups and reducing sugars only non-reducing sugars are considered[152]. For the use as stabilisers in solution and in prospect of the use in later manufacturing steps, e.g. as bulking agents in freeze-drying, a variety of sugars and sugar alcohols is tested in this section.

Samples based on 0.1 ml 60µg/ml ACT solutions in standard phosphate buffers pH 7.2 are loaded with excipient to a resulting concentration of 5% sugar and polyol, respectively. Stressing is done at 40°C for 9 days.

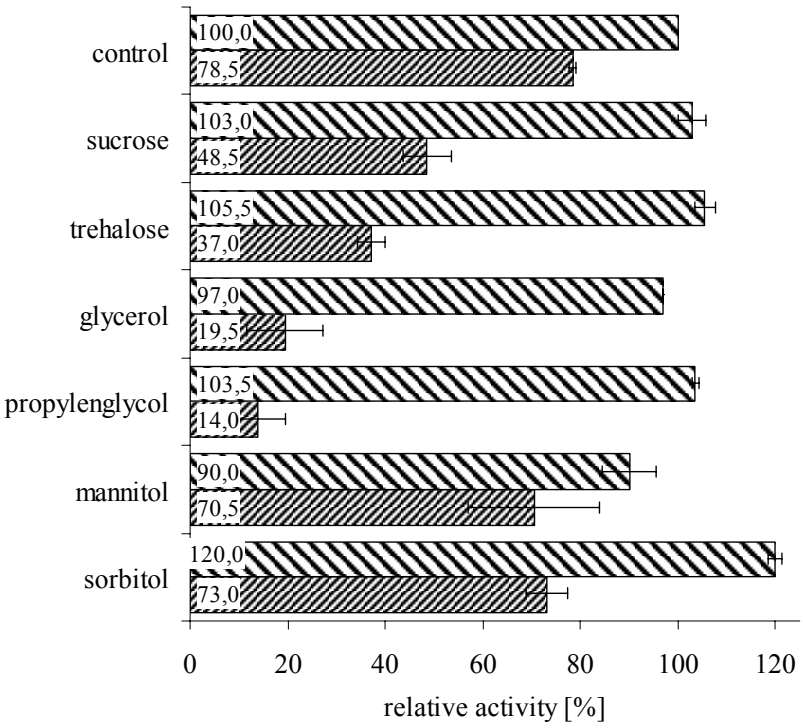


Fig. 3-11 Recovered relative activity of ACT in 50mM potassium phosphate buffer pH 7.2 with sugar/polyol additives before (above) and after (below) a 9 days storage at 40°C measured by activity assay;

Despite the well known stabilising effects of sugars, the preservation of ACT activity by these additives is quite disappointing. All samples without stressing are in comparable range with the control without excipient. Thus, the tested substances are compatible with the assay. Therefore, the huge losses of activity during stressing at 40°C are proven to be due to a destabilising effect on ACT. Only the sugar alcohol samples containing mannitol and sorbitol are in comparable range with the control sample in heat stressing and thus can be considered as inert.
In the corresponding F/T experiments (data not shown) none of the candidates shows stabilising effects on ACT.
Consequently, only mannitol and sorbitol though having no stabilising potential can be considered to be added to an ACT formulation for technical reasons. Also, the regulatory status of these substances is satisfactory. Mannitol is approved by FDA to a maximum potency of 13% for intravenous injection and 10.66% for

intralesional injection[149]. Sorbitol is approved also by FDA to a maximum potency of 45% for intralesional injection and 30% for intravenous injection[149].

### *3.2.2.3 Cyclodextrins for the stabilisation of ACT in solution*

Cyclodextrins are cyclic oligosaccharides consisting of six, seven, and eight glucose monomers, respectively. Nomenclature corresponds to the molecule size rising from α-CD to β-CD to γ-CD. In the ring the polar hydroxyl groups are located on the outside, whereas the etherlike oxygen atoms in the inside form a nonpolar cavity. The exterior allows cyclodextrins to dissolve in water while the cavity forms inclusion complexes with hydrophobic molecules, e.g. the hydrophobic residues in proteins. Depending on the number of glucose units the cavity grows from α- to β- to γ-CD. So, the size relation of cavity and including molecule also influences complex characteristics. This way of complexation can affect the protein in two ways. On the one hand, it can stabilise the unfolded state of proteins by intercalating the hydrophobic residues in proteins. On the other hand, by intercalating these hydrophobic residues it can prevent proteins from aggregation[153,154].

In the stability study, α-, β-, Hydroxypropyl-β-, and γ-cyclodextrin are tested. As above, samples based on 0.1ml 60µg/ml ACT solutions in standard phosphate buffers pH 7.2 are loaded with cyclodextrin to a resulting concentration of 2% which means a major excess of cyclodextrin based on molecular numbers. Stressing is done at 40°C for 9 days.

Again, all unstressed samples are in comparable range (Fig. 3-12 above) indicating all additives being compatible with the assay. The stressed samples of β-, HP-β-, and γ-CD are comparable with the stressed control sample (Fig. 3-12 below). Solely in the α-cyclodextrin samples a remarkably reduced recovered activity is measured. Therefore, α-CD proves to destabilise ACT, whereas the other derivatives are more or less indifferent against ACT activity after temperature stressing.

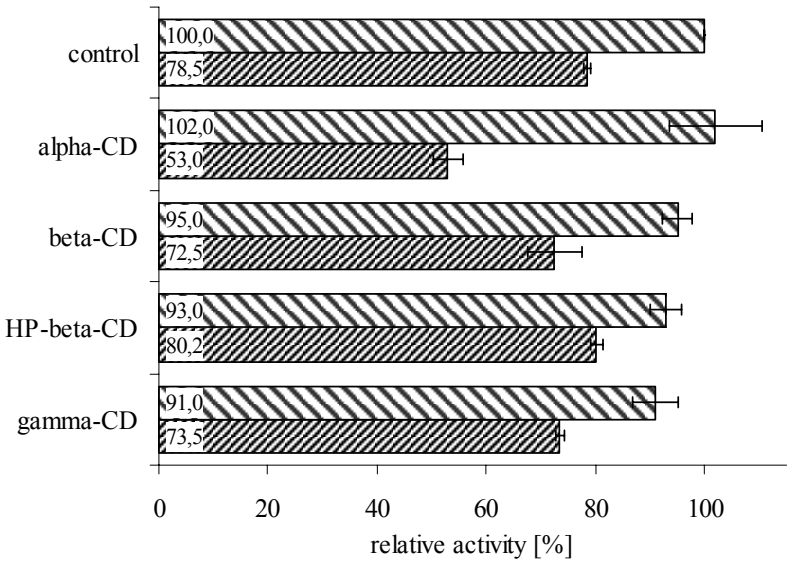


Fig. 3-12 Recovered relative activity of ACT in 50mM potassium phosphate buffer pH 7.2 with cyclodextrin additives (2%) before (above) and after (below) a 9 days storage at 40°C measured by activity assay;

The non-destabilising cyclodextrins are further tested in F/T studies. The testing is conducted in 0.1ml ACT solution 60µg/ml in 10mM phosphate buffer at a stress level of 15 F/T cycles with 2% cyclodextrin. In this test, β-cyclodextrin and, most notably, HP-β-cyclodextrin achieved remarkable stabilising effects. On the contrary, γ-CD is not very effective in this stress situation (Fig. 3-13).

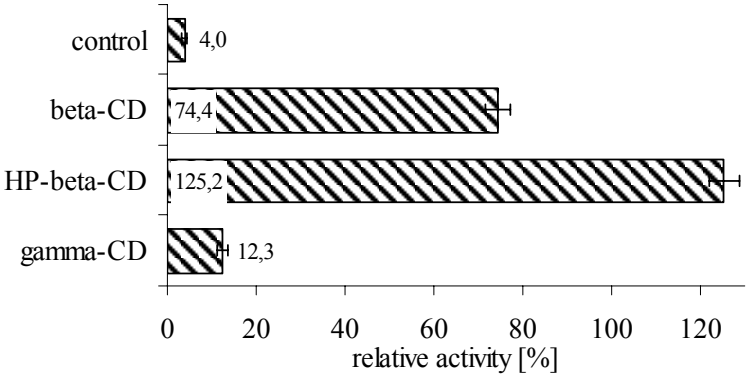


Fig. 3-13 Recovered relative activity of ACT in dependence of cyclodextrin addition (2%) in ACT samples in 10mM potassium phosphate buffer pH 7.2 after 15 F/T cycles measured by activity assay;

For HP-β-CD an additional experiment investigating the concentration dependence is conducted. Thus, a concentration series from an equimolar ratio to a 100-fold excess in molarity of cyclodextrin (0,015%) over ACT is tested in the same F/T setting. This shows an increasing protection up to a nearly complete recovery for

HP-β-CD detected for the maximum concentration (Fig. 3-14). Therefore, a stabilising interaction of HP-β-CD with ACT can be postulated, but an estimation of coordination numbers is not possible. Rather, an equilibrium between complex and dissociated molecules is to be supposed, whereby the necessary excess of HP-β-CD pushes the equilibrium towards the stabilising complex.

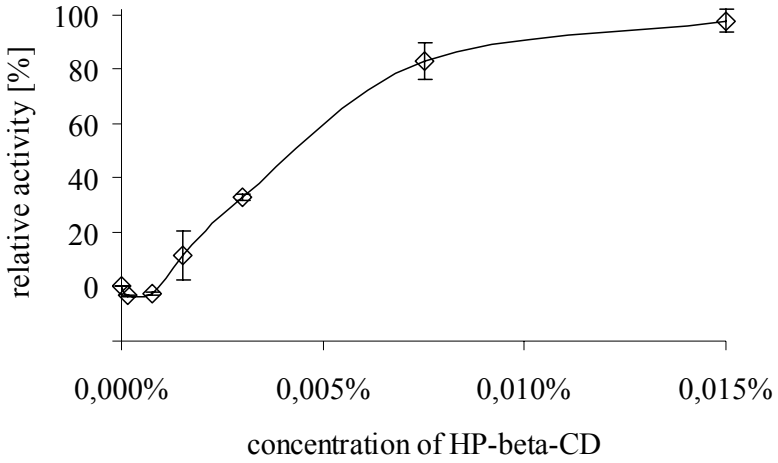


Fig. 3-14 Recovered relative activity of ACT in dependence of HP-β-cyclodextrin concentration in ACT samples in 10mM potassium phosphate buffer pH 7.2 after 15 F/T cycles measured by activity assay; data points correspond to molecular ratios of ACT : HP-β-CD being 1:0, 1:1, 1:5, 1:10, 1:20, 1:50, 1:100;

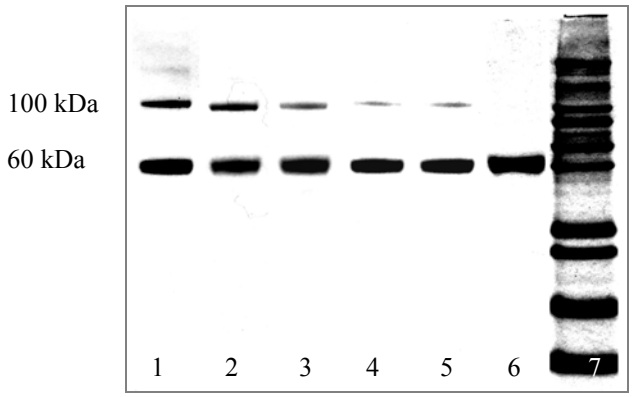


Fig. 3-15 HP-β-CD concentration dependent stability of ACT in 10mM potassium phosphate buffer pH 7.2 after 15 F/T cycles measured by SDS-PAGE:
Concentration is given in molecular ratio ACT : HP-β-CD; lane 1: 1:1, 11% aggregate; lane 2: 1:5, 8.5% aggregate; lane 3: 1:10, 5.8% aggregate; lane 4: 1:50, 3.2% aggregate; lane 5: 1:100, 1.5% aggregate; lane 6: untreated control; lane 7: marker;

From this experimental setup, additionally, readout by SDS-PAGE is operated. It indicates a decrease in aggregate detection with rising HP-β-CD content (Fig. 3-15 lanes 1 to 5) by thinning 100kDa bands. Most remarkable, like in the other read

outs, the amount of lost activity in the activity assay and the detected aggregates by SDS-PAGE are not in the same magnitude.

Therefore, HP-β-CD can be used as stabiliser mainly for F/T stabilisation. Concentration is tested suitable between 0.015% and 2%. But, HP-β-CD is approved by FDA for intravenous injection to a maximum potency of 0.4%[149].

### *3.2.2.4 Amino acids for the stabilisation of ACT in solution*

As sugars and sugaralcohols amino acids are supposed to be able to stabilise proteins by preferential exclusion. Moreover, in special cases some amino acids inhibit chemical degradation, e.g. methionine may work as antioxidant and so reduce oxidative degradation of the protein. For these purposes, a selection of amino acids already used in protein stabilisation are investigated[137].
The 50mM phosphate buffers are produced with a 2% addition of the particular amino acid. The pH of 7.2 then is adjusted with potassium hydroxide. Stressing is done on 0.1ml 60µg/ml ACT solutions at 40°C for 9 days.

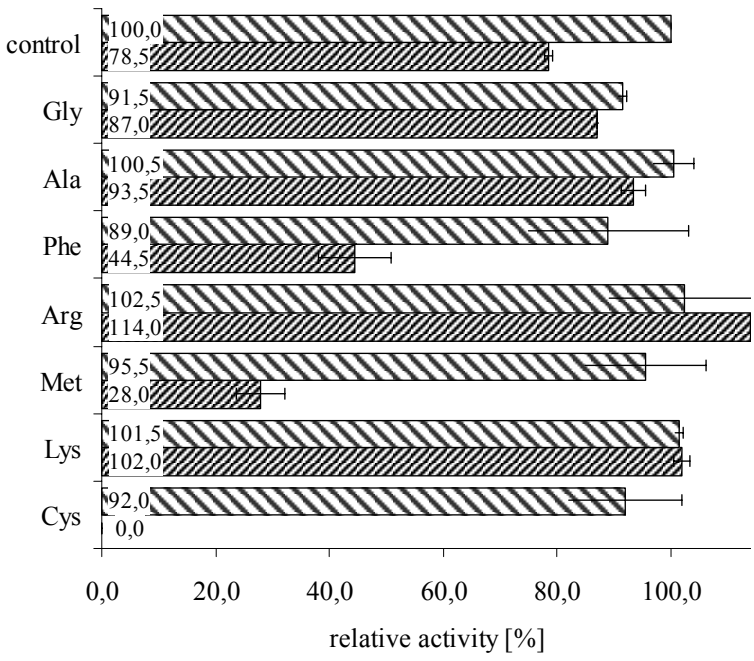


Fig. 3-16 Recovered relative activity of ACT in 50mM potassium phosphate buffer pH 7.2 with different amino acids before (above) and after (below) a 9 days storage at 40°C measured by activity assay;

All tested substances are interoperable with the assay indicated by the untreated samples being in the same range as the buffer control (Fig. 3-16 above). Among the

stressed samples glycine, alanine, arginine, and lysine allow hardly any damage to ACT activity during temperature stressing. On the contrary, the sulphur containing amino acids methionine and cysteine drop out as well as phenylalanine (Fig. 3-16 below).

From this experiment readout by SDS-PAGE is conducted. In the presence of methionine, phenylalanine, and lysine fragments in amounts smaller 10% are observed. With methionine, phenylalanine, cysteine, glycine, and alanine dimer amounts of 1.4 to 21.6% are detected (Fig. 3-17).

Although SDS-PAGE again only roughly correlates with activity assay results, it can be concluded that arginine, glycine, and alanine perform best in both analytical techniques.

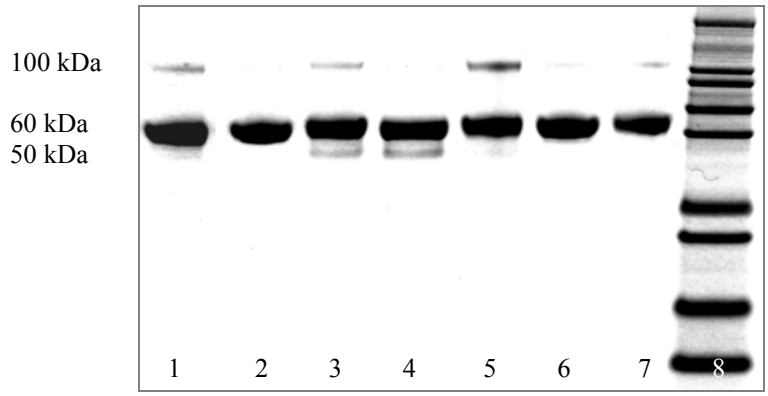


Fig. 3-17 SDS-PAGE gel on influence of amino acids on stability of ACT in 50mM potassium phosphate buffer pH 7.2 after thermal treatment (9 days, 40°C): lane 1: methionine 9.6% dimer, 1.2% fragment; lane 2: arginine only monomer; lane 3: phenylalanine 4.5% dimer, 3.6% fragment; lane 4: lysine 6.7% fragment; lane 5: cysteine 21.6% dimer; lane 6: glycine 1.4% dimer; lane 7: alanine 3.6% dimer; lane 8: marker;

Unfortunately, during F/T experiments the tested amino acids did not show any stabilising effect (data not shown).

For its proven stabilisation on ACT and its known stabilisation qualities during protein drying arginine was chosen to be examined in further concentration studies[155]. This series was studied in the same setting as described above. The results curve shows a clear optimum at 10mM (Fig. 3-18), because the measured ACT activity after thermal treatment is at its maximum at that point.

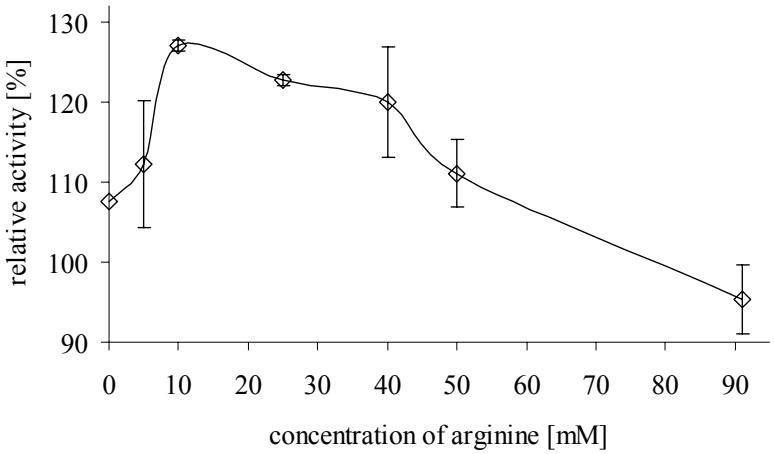


Fig. 3-18 Recovered relative activity of ACT in 50mM phosphate buffer pH 7.2 with different arginine contents after a 9 days storage at 40°C measured by activity assay;

Indeed, arginine can be used for stabilisation of ACT at an optimal concentration of 10mM, correspondent to 0.17%. FDA approval is given for up to 88% for intravenous injection[149].

### *3.2.2.5 Preservatives for ACT containing solutions*

Finally, a choice of parenterally usable preservatives is tested as excipients in this series. In the standard phosphate buffer the following substances are tested: 1% benzyl alcohol, 0.1% 4-chloro-m-cresol, and a paraben mixture, i.e. p-hydroxybenzoic acid methyl ester/ p-hydroxybenzoic acid propyl ester 0.1%/0.04%.

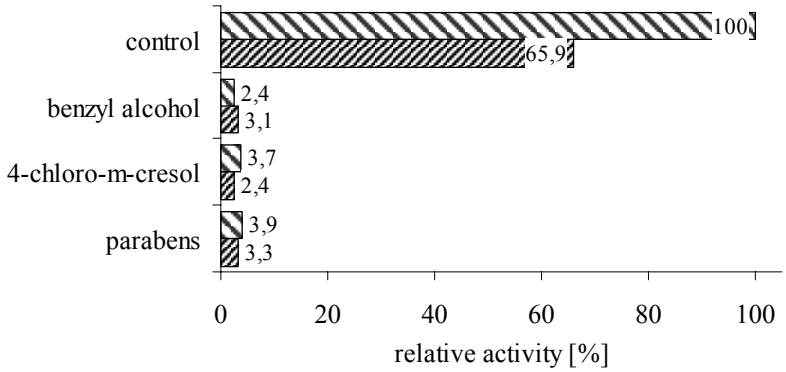


Fig. 3-19 Recovered relative activity of ACT in 50mM potassium phosphate buffer pH 7.2 with preservatives before and after a 9 days storage at 40 °C measured by activity assay;

Unfortunately, these substances are not interoperable with the assay because neither the unstressed samples nor the stressed samples show remarkable activity

(Fig. 3-19). So, no information about their behaviour against ACT can de obtained. Due to this interference with analysis, these preservatives cannot be used in a formulation for ACT.

## 3.2.3 Summary of ACT solution stability studies

In the course of this chapter the stabilisation of ACT in solution is evaluated. The main degradation pathway for ACT seems to be dimer formation and presumably later higher order aggregation. Only in acidic pHs and with some amino acids a fragmentation can be seen.
A pH of 7.2 turned out to be the best choice for a buffered solution. However, the optimal range of pH values is very narrow. First of all, low pH levels can harmfully damage, fragment and inactivate ACT. So, for application on a wound site which can provide an acidic pH a buffer system is recommended in the delivery device to guarantee suitable pH for non-liberated protein. Therefore, phosphate, Tris, and phosphate/citrate buffers can be used. For phosphate the optimal content and relating osmolarity have been examined. Moreover, from a variety of substances surfactants, cyclodextrins, and amino acids have proven stabilising potential on ACT.

| stabiliser | liquid solution | frozen solution |
|---|---|---|
| buffer | 50mM potassium phosphate pH 7.2<br>alternative: 20mM potassium phosphate / 50mM potassium chloride pH 7.2<br>alternative: Tris HCl pH 7.2<br>alternative: potassium phosphate/citrate pH 7.2 | 10mM potassium phosphate pH 7.2 |
| surfactant | 0.1% Poloxamer® 188<br>alternative: 0.1% Tween® 80 | 0.1% Poloxamer® 188<br>alternative: 0.1% Tween® 80 |
| cyclodextrin | possibly: HP-β-CD | 0.015-2% HP-β-CD |
| amino acid | 10mM arginine | possibly: arginine |

Tab. 3-3 Recipes of stabilising aqueous solutions for 60µg/ml ACT in liquid solution (from temperature stress study) and in frozen solution (from F/T stress study);

Indeed, depending on the stress type two recipes for stabilising solutions can be formulated relating to solutions to be kept in liquid state and frozen solutions, respectively (Tab. 3-3). All ingredients are already approved for parenteral use by FDA.

# 3.3 Hydrogels as delivery system for ACT into wounds

In this chapter the manufacture of hydrogels as ACT loaded delivery devices for wound healing is evaluated. Therefore, the gels are optimised concerning their sterilisation and rheologics to meet the requirements of wound dressings. And, in a second step stabilisation studies of ACT in dependence of the polymer and in the total semi-solid formulation are conducted.

## 3.3.1 Development as delivery system for wound healing

From the great variety of pharmaceutically relevant polymers[156] a selection is assembled according to these criteria:

- The polymer is already in use in medical products for wound treatment.
- The regulatory status of the polymer promises uncomplicated approval by regulatory authorities for application on open wounds.
- The polymer is readily available to affordable pricings and does not require excipients with toxic or protein destabilising potential for gelling.
- The polymer must not release monomers or oligomers during (bio-) degradation with disturbing activity on protein stability[157].

The resulting choice is listed in Table 3-5.
Following, in the course of the development of the hydrogel as wound dressing material the parameters concerning sterility and mechanical properties have to be evaluated.
Sterility in general can be produced by several well known techniques, but steam sterilisation is the most effective and safe method. Therefore, gelling agents and the hydrogels made thereof that enable autoclavation without remarkable change in properties are in favour for the choice as formulation ingredient.
The mechanical properties in the first place are described by the rheological behaviour of the gel. In detail, the viscosity of the gel has to be adjusted to provide proper manufacture and application, and while on the wound site staying in place

and providing intimate contact to the wound ground essential for effective drug release.

### *3.3.1.1 Sterilisation of hydrogels*

A variety of gels (Tab. 3-5) is evaluated concerning possible sterilisation methods. As outlined above, the first to choose technique for sterilisation of the hydrogels is steam sterilisation. Therefore, standard conditions like they are described in the pharmacopoeia are used, i.e. a temperature of 121°C at 2 bar steam pressure is applied for 15 min.

From the cellulose derivatives the hydroxyethyl- and the carboxymethyl cellulose sodium species are suitable for autoclavation. Resulting gels are homogeneous, free of air bubbles, and completely swollen. Moreover, changes of viscosities are negligible. Further, xanthan gum and gellan gum can be autoclaved successfully. In addition, autoclavation can replace the heat treatment of these polymers obligatory for gel formation.

Besides, alginate gels are liquefied during autoclavation, while methyl-, hydroxypropyl- and hydroxymethylpropyl cellulose precipitate due to their well known paradox temperature solubility.

### *3.3.1.2 Viscosity of hydrogels*

Preparations with low viscosity tend to evaporate fast and cannot provide satisfactory contact with the wound surface because their tendency to adhere to the wound site is too low. A very high viscosity, in contrast, complicates the manufacturing processes and a convenient application. So, a reasonable viscosity range of the gels has to be determined which is done by comparison with commercially available semisolid products indicated for wound treatment. Thus, in a first series of experiments a choice of market products is characterised by viscosity measurement. As all the measured gels are pseudoplastic, experiments are conducted in a Brookfield rotating cylinder viscometer.

For interpretation of the measurements the rheograms are considered. In these diagrams the shear rate $\chi$ is drawn against the shear stress $\sigma$ which results in the typical curves. For these gels the power-law (Ostwald-de Waele) for pseudoplastic materials is used (Equ. 3-1). The equation is linearised by the logarithm and is written as Equ. 3-2.

$$\sigma = k \cdot \chi^{n} \qquad \ln \sigma = \ln k + n \cdot \ln \chi$$

Equ. 3-1 Equ. 3-2

Equ. 3-1&3-2 Power or Ostwald-de Waele law 3-1, in linearised, logarithmic form 3-2 with shear stress σ [dyn/cm$^2$ = 10Pa], shear rate χ [1/s], consistency coefficient k [0,1Pa s$^n$], and flow behavior index or Power-law exponent n [-]; n<1 meaning pseudoplastic and n>1 shear thickening behaviour[158];

For graphic interpretation, the measured curves are charted into double logarithm plots. The linearised equation (Equ. 3-2) now can be fitted to the results disclosing the slope n and the y-intercept ln k. The correlation coefficient R indicates the quality of fitting (Fig. 3-20, Tab. 3-4).

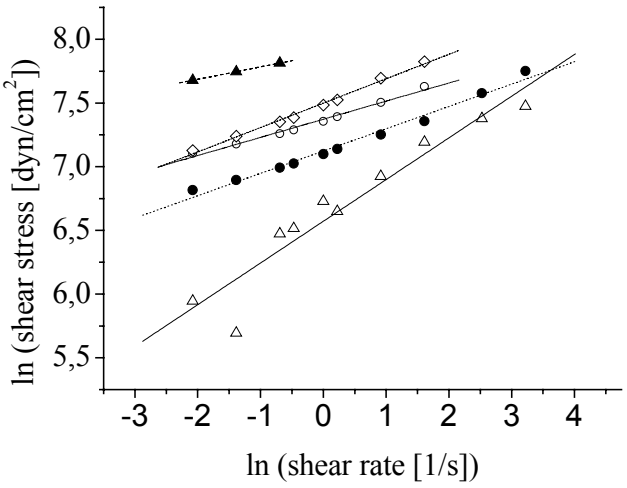


Fig. 3-20 Rheograms of market products listed in table 3-4 in double-logarithm plot with fitted evens; legend and rheological data from fitted function parameters ln k, n, and $R^2$ from power law (Equ. 3-2) are listed in table 3-4

| | | ln k | n | $R^2$ |
|---|---|---|---|---|
| Traumon Gel® | ▲ | 7.37 | 0.14 | 0.994 |
| Traumasept® | ◇ | 7.49 | 0.19 | 0.996 |
| Tyrosur® | ○ | 7.12 | 0.17 | 0.992 |
| Decoderm Basiscreme® | ● | 6.57 | 0.32 | 0.954 |
| Multilind Heilpaste® | △ | 7.88 | 0.09 | 0.999 |
| Suprasorb G® | | 8.27 | 0.39 | 0.995 |
| Askina Braun® | | 8.34 | 0.32 | 0.999 |

Tab. 3-4 Legend and rheological data from fitted function parameters ln k (consistency coefficient, flow behaviour index n, and correlation coefficient $R^2$ from power law (Equ. 3-2);

The resulting even of the rheograms is parallel to a major extend indicating a similar flow behavior index n and therefore a similar pseudoplastic flow behaviour.

Therefore, the logarithm of the consistency coefficient k (ln k) can be used as characteristic parameter for describing viscosity in this context. Ln k of most products developed for application on wounds is between 7.0 and 7.5. The products with higher ln k values are gels of high viscosity that are difficult to handle for further preparations (Tab. 3-4).

Thus, the viscosity of the hydrogels to be developed are adjusted to the lower ln k values of 7.0 to 7.5. For that purpose, a set of gels from several gellants is prepared always including a concentration series for every gelling agent. Where possible, steam sterilisation was carried out before measurement. The corresponding rheograms for every gel are determined as above. It has been found that ln k is directly proportional to the concentration of most tested gelling agents (Fig. 3-21). From that proportion an equation combining ln k and the gellant concentration (Equ. 3-3) is gained from linear regression.

$$\ln k = A + B \cdot concentration\left(gelling\ agent\right)$$

Equ. 3-3 Relation of consistency coefficient k and concentration of gellant in tested gels;

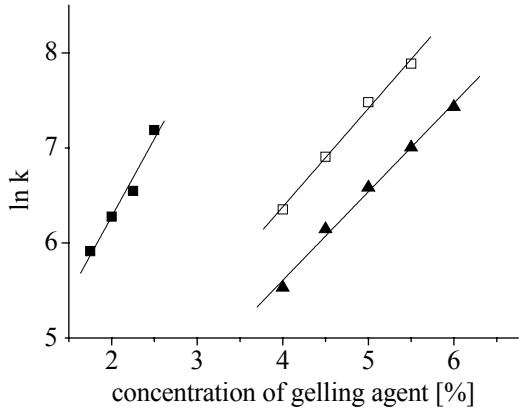


Fig. 3-21 Ln k values of carboxymethyl cellulose sodium 10.000 (□), hydroxyethyl cellulose 100.000 (■), and hydroxyethyl cellulose 10.000 (▲) gels in dependence of the gelling agent content measured after steam sterilisation;

Following, in a simple calculation using Equ. 3-3 the concentration resulting from the required ln k between 7.0 and 7.5 described above is determined for every gelling agent. That is the concentration of the gelling agents to be used in the manufacture of the hydrogel as wound dressing and drug carrier in following studies (Tab. 3-5).

| Polymer | Quality | Intercept A | Slope B | Correlation coefficient $R^2$ | Concentration (polymer) [%] |
|---|---|---|---|---|---|
| Alginate Sodium | SF250 | 4.53 | 0.58 | 0.986 | 4.5 |
| Carboxymethyl cellulose sodium | 1.000 | 1.62 | 0.98 | 0.993 | 6.0 |
| Carboxymethyl cellulose sodium | 10.000 | 2.24 | 1.04 | 0.986 | 5.0 |
| Carboxymethyl cellulose sodium | 30.000 | 0.56 | 1.41 | 0.985 | 10.5 |
| Carboxymethyl cellulose sodium | cross-linked | 0.37 | 0.60 | 0.973 | 11.5 |
| Hydroxyethyl cellulose | 300 | 1.74 | 0.72 | 0.982 | 8.0 |
| Hydroxyethyl cellulose | 4.000 | 2.16 | 0.89 | 0.975 | 6.0 |
| Hydroxyethyl cellulose | 10.000 | 1.88 | 0.93 | 0.978 | 5.5 |
| Hydroxyethyl cellulose | 100.000 | 3.00 | 1.64 | 0.988 | 2.5 |
| Hydroxypropyl cellulose | 100.000 | 4.19 | 1.07 | 0.999 | 2.5 |
| Hydroxypropylmethyl cellulose | 15.000 | 4.14 | 0.96 | 0.989 | 3.5 |
| Hydroxypropylmethyl cellulose | 100.000 | 4.3 | 1.28 | 0.998 | 2.5 |
| Methyl cellulose | 1.000 | 1.18 | 0.82 | 0.999 | 7.0 |
| Carboxymethyl starch sodium | | 5.82 | 0.20 | 0.981 | 8.5 |
| Gellan gum | LT100 | 5.31 | 1.22 | 0.992 | 1.5 |
| Gellan gum | F | 5.26 | 1.18 | 0.985 | 1.5 |
| Xanthan gum | | 0.26 | 5.35 | 0.978 | 7.0 |
| Polyacrylate Sodium | Carbopol 940 | 6.21 | 1.04 | 0.975 | 1.0 |
| Polyvinyl pyrrolidone | Kollidon 90F | -1.04 | 0.24 | 0.998 | 35 |
| Polyethylen glycol | 20000 | -6.2 | 0.19 | 0.998 | 72 |

Tab. 3-5 Gelling agents, regression parameters, and determined concentration to be used in wound healing gels;

### *3.3.1.3 Viscosity of gellan gum/hydroxyethyl cellulose hydrogels*

Since gels are mobile they offer the advantage of intimate contact with the surface of a wound, but this advantage is, however, tempered by the conflicting needs of making the gel sufficiently mobile for application but viscous enough to prevent fast flow out of the wound under the influence of gravity. The latter disadvantage of free-flowing gels could be overcome by crosslinking of polymers, but this implies major challenges for manufacturing and application.

Hence, the rheology of gellan gum could be a back door out of this dilemma because it is sensitive to monovalent cations, i.e. gellan gum forms non free-flowing but very brittle hydrogels in the presence of e.g. sodium and potassium salts.

This is described by the flow curve of such preparations in comparison to a hydroxyethyl cellulose gel (Fig. 3-22).

For this purpose the rotating cylinder method in the Brookfield viscometer used till date is not sufficient. Rather an oscillating cone plate method is used for these evaluations. In detail, an amplitude sweep test with constant oscillation frequency ω and controlled shear deformation is performed. That means that the angle of deflection φ of the cone and with that the shear rate γ is defined by the method. The variable parameters are the corresponding shear stress τ and loss angle δ that are obtained from measurement.
The change of shear rate over time given by the apparatus in this experiment follows a sinus curve, whereas the resulting change of shear stress over time follows a shifted sinus curve. This phase shift between the two sinus curves is described by the loss angle δ. From these values the storage modulus G´ and the loss modulus G´´ can be calculated (Equ. 3-4, Equ. 3-5, Equ. 3-6).

$$G' = \frac{\tau_A}{\gamma_A} \cdot \cos\delta \qquad G'' = \frac{\tau_A}{\gamma_A} \cdot \sin\delta \qquad \tan\delta = \frac{G``}{G`}$$

Equ. 3-4 Equ. 3-5 Equ. 3-6

Equ. 3-4 & Equ. 3-5 & Equ. 3-6 Storage modulus G`, loss modulus G``, and loss factor tan δ; with loss angle δ, amplitude shear stress $\tau_A$, and shear rate amplitude $\gamma_A$;

Thereby, the storage modulus G´ describes the elastic properties of the sample that are responsible for a reversible deformation storing the deformation energy within the system. The loss modulus G´´ is a measure for the plastic or viscous behaviour of the specimen being responsible for irreversible deformation and a loss of deformation energy for the matrix. Furthermore, the loss factor tan δ describes the relation of viscous and elastic character in the test sample.
Thus, these three values are used to describe the rheological behaviour of the gel system. The relative positions of moduli in the double logarithm plot against shear stress and the value of the loss factor being < 1 indicate that the elastic properties prevail over plastic behaviour.
This domination of the elastic character is seen in both the hydroxyethyl cellulose gel and the gellan gum swollen in sodium chloride solution (Fig. 3-22). But for the gellan curves with sodium chloride addition this issue is much more pronounced due to the higher difference between the curve levels. In fact, the lower loss factor marks a rheology tending to be like the behaviour of solids.
Moreover, the gellan moduli curves cross each other after very abrupt change in runs of the curves, describing a break down of the gel structure at a threshold of

shear stress. There, a yield value $\tau_{Y\ gellan}$ can be calculated. Taken together, this characterises a very hard but brittle gel with a very distinctive yield point. Compared to gellan gum, hydroxyethyl cellulose is characterised by both very low pronounced elasticity and yield point describing a soft almost free-flowing gel (Fig. 3-22).

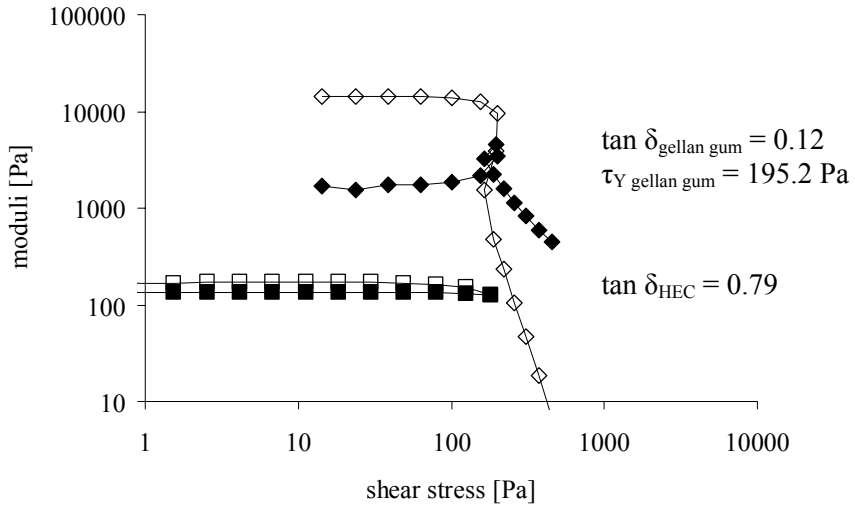


Fig. 3-22 Comparison of gellan gum gel and hydroxyethyl cellulose gel flow curves; measurement in amplitude sweep with controlled shear deformation and a constant frequency ω=10 1/s of 1.0% gellan gum in 0.9% sodium chloride with storage modulus G' (◇), loss modulus G'' (◆), loss factor tan $\delta_{gellan}$, yield point $\tau_{Y\ gellan}$; 2.5% hydroxyethyl cellulose 100.000 with storage modulus G' (□), loss modulus G'' (■), loss factor tan $\delta_{HEC}$

However, the brittleness of gellan gum gels is inappropriate for a wound dressing and so further modifications are necessary. It has been found that a combination of the two very different gellants gellan gum and hydroxyethyl cellulose forms suitable gels for wound treatment.

As already mentioned, a higher loss factor expresses a less brittle and softer behaviour of the gel. And, the low loss factor of gellan gum alone can be increased by partly substitution by hydroxyethyl cellulose in isotonic sodium chloride containing gels (Tab. 3-6).

| hydroxyethyl cellulose | gellan gum | loss factor tan δ |
|---|---|---|
| 0 % | 1.5 % | 0.12 |
| 2.0 % | 1.0 % | 0.18 |
| 2.25 % | 0.75 % | 0.40 |
| 2.5 % | 0 % | 0.79 |

Tab. 3-6 Loss factor tan δ in dependence of hydroxyethyl cellulose and gellan gum content in hydrogels containing 0.9% sodium chloride;

Although an effective increase of the loss factor values is monitored with contents lower 1.0% gellan gum, the 2.0% hydroxyethyl cellulose : 1.0% gellan gum mixture is chosen for further development due to its convenient sensory properties, i.e. how it is felt on skin. Further, it is observed that the gel system containing the two gellants still provides the sensitivity against monovalent cations. Hence, it is castable like a liquid without salt content and forms non-free flowing but soft gels with salt content.

Moreover, the surrogating of the sodium chloride solution against the potassium phosphate buffer used in ACT stabilising formulations is investigated. Therefore, the rheological behaviour of the 2.0% hydroxyethyl cellulose : 1.0% gellan gum system is examined in water and in 50mM potassium phosphate buffer pH 7.2, respectively (Fig. 3-23). Experimental setting of the rheometer method is as above.

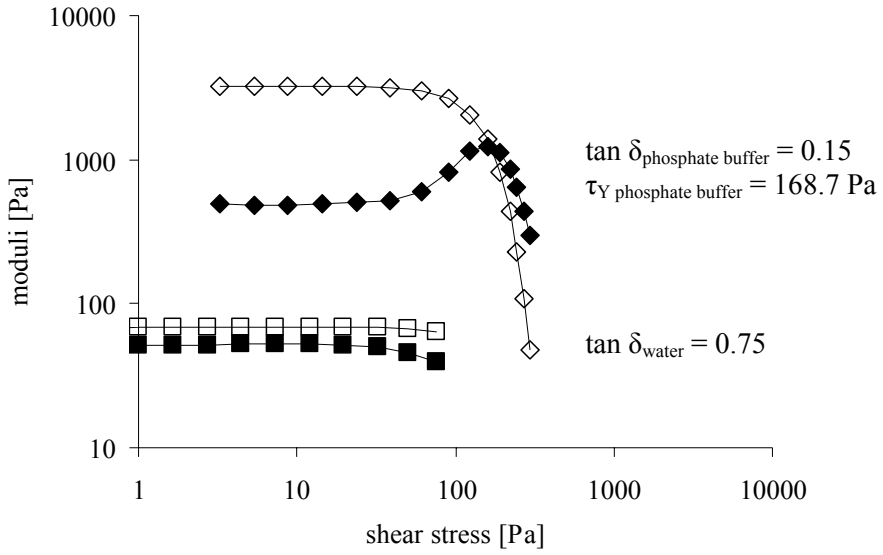


Fig. 3-23 Flow curves in amplitude sweep of 2.0% hydroxyethyl cellulose / 1.0% gellan gum; in 50mM potassium phosphate buffer pH 7.2 with storage modulus G' (◇), loss modulus G'' (◆), loss factor tan $\delta_{phosphate\ buffer}$, yield point $\tau_{Y\ phosphate\ buffer}$; in water with storage modulus G' (□), loss modulus G'' (■), loss factor tan $\delta_{water}$ ;

For interpretation, again the relative position of moduli is considered. For influencing the gel behaviour isotonic sodium chloride solution (tan $\delta_{0.9\%\ NaCl}$ = 0.18) is nearly on a par with 50mM potassium phosphate buffer pH 7.2 (tan $\delta_{phosphate\ buffer}$ = 0.15) like it is developed for some ACT formulations. The gels also provide a yield point $\tau_Y$ which is slightly reduced by the hydroxyethyl cellulose surrogate ($\tau_{Y\ 0.9\%\ NaCl}$ = 169.0Pa, $\tau_{Yphosphate\ buffer}$ = 168.7Pa) compared to gellan gum ($\tau_{Y\ gellan\ gum}$ = 195.2Pa) alone (Fig. 3-23).

Therefore, the desired plastic behaviour of gellan gum can also be activated in the mixture by addition of the usual phosphate buffer. On the contrary, without salt content the hydroxyethyl cellulose fraction dominates the viscosity of the gel forming a free flowing mass.

To conclude, the 2.0% hydroxyethyl cellulose : 1.0% gellan gum mixture conveniently combines the properties of a soft free-flowing gel necessary for manufacture, drug loading, and provision of wound contact with the mechanic advantages of cross-linked matrices. Thus, this system provides a very attractive alternative to pseudoplastic hydrogels made from a single polymer. Unfortunately, gellan gum till date is only approved for ophthalmic solutions up to 0.6% by FDA[149].

## 3.3.2 Stability of ACT in hydrogel formulations

Based on the results of the stabilisation studies on ACT in aqueous solution a group of polymers is tested in similar manner for short term temperature stress and F/T stress. Consequently, these short term studies are followed by mid term stability tests in semi-solid hydrogels according to ICH guideline. Prior to these tests the manufacture of sterile ACT loaded gels is highlighted. The outcome of the stability study is assessed for the purpose of evaluating the possibility of hydrogels as application form and as storage form for ACT.

### *3.3.2.1 Effects of polymers on ACT stability in hydrated formulations*

Polymers are a chemically heterogeneous group of substances. Therefore, their effects on proteins can not be outlined in a straight way. Generally, polymer interaction with proteins is of great similarity to other discussed substance classes depending on their underlying chemical structure. So, for example, surface activity, preferential exclusion, steric hindrance, and viscosity limiting structural movement are important stabilising interactions with proteins[137].

Polymers are tested not only because of their own protein stabilising potency but also because of their necessity for gel forming. In fact, the polymers are tested for a non-destabilising effect in the formulation instead of an active stabilising effect. That is why the experimental setting is changed concerning sample composition. Test samples, thus, basically already consist of an improved phosphate buffered

(pH 7.2) and surfactant, 0.1% Poloxamer® 188, containing 60µg/ml ACT solution wherein the polymer is added. The polymer content is reduced to 0.5% in these experiments to keep the samples in a liquid state. This is done to provide the possibility of unchanged liquid handling during sample preparation and analytics without further stress factors influencing ACT activity.

As before, unstressed material is measured first to detect incompatibilities with the assay (data not shown). In this set of experiments the polyacrylate polymers, Carbopol® and Eudragit®, failed due to complete deactivation of ACT even without stressing. Methyl cellulose and Lutrol® F127 were stopped here because of their middle-rate stabilising qualities and their unfavourable mechanical characteristics in xerogel and film formation (see sections there). The other tested polymers showed acceptable compatibility and are tested further on (listed in Fig. 3-24).

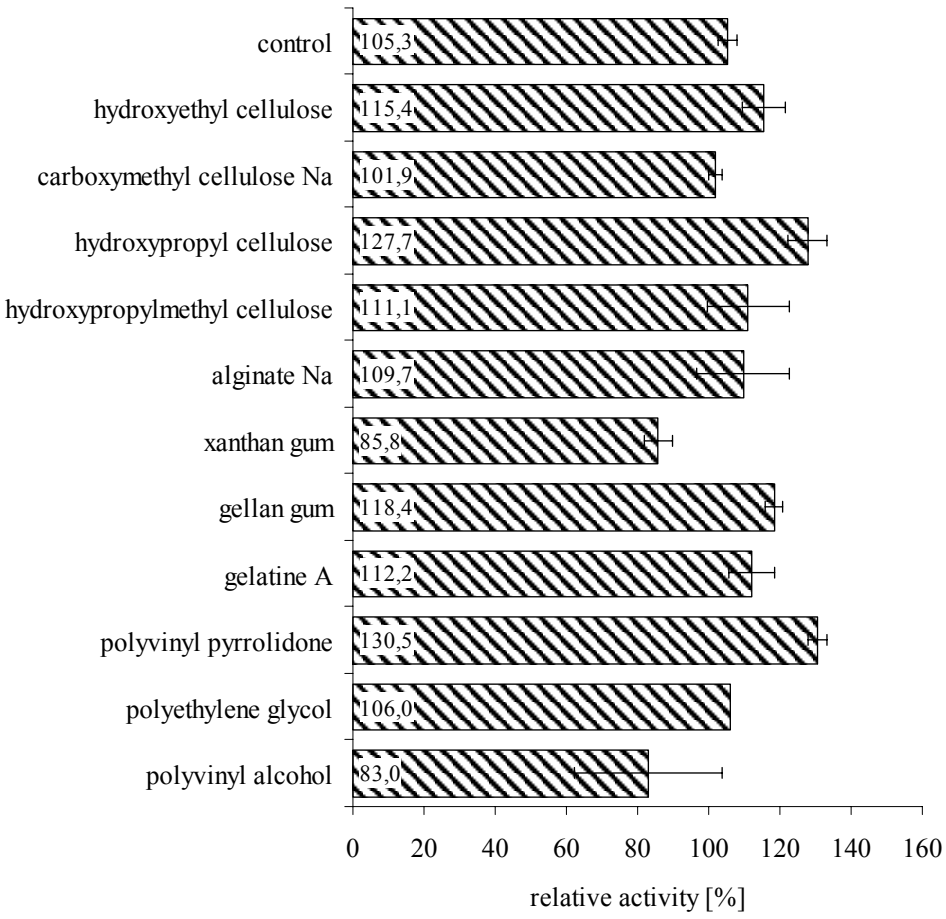


Fig. 3-24 Recovered relative activity of ACT in 50mM potassium phosphate buffer pH 7.2 and 0.1% Poloxamer® 188 with various polymers at 0.5% content after a 9 days storage at 40°C measured by activity assay; polymers are HEC 10.000, CMC Na 10.000, HPC 100.000, HPMC 15.000, gellan gum LT100, PVP 17, PEG 2000, PVA 100.000;

With the latter the stressing experiments are carried out. Samples for these experiments are prepared in the same improved formulation as above and are treated at 40°C for 9 days and 15 F/T cycles, respectively. The control sample contains no polymer.

Most of the samples are within the range of the control, where no loss of activity can be detected. For xanthan gum and polyvinyl alcohol samples lower values of relative activity are obtained (Fig. 3-24).

For the F/T studies a solution is used containing 10mM potassium phosphate buffer pH 7.2 and 0.1% Poloxamer® 188 as surfactant. Here, none of the chosen polymers caused a destabilisation of ACT activity during 15 F/T cycles (Fig. 3-25).

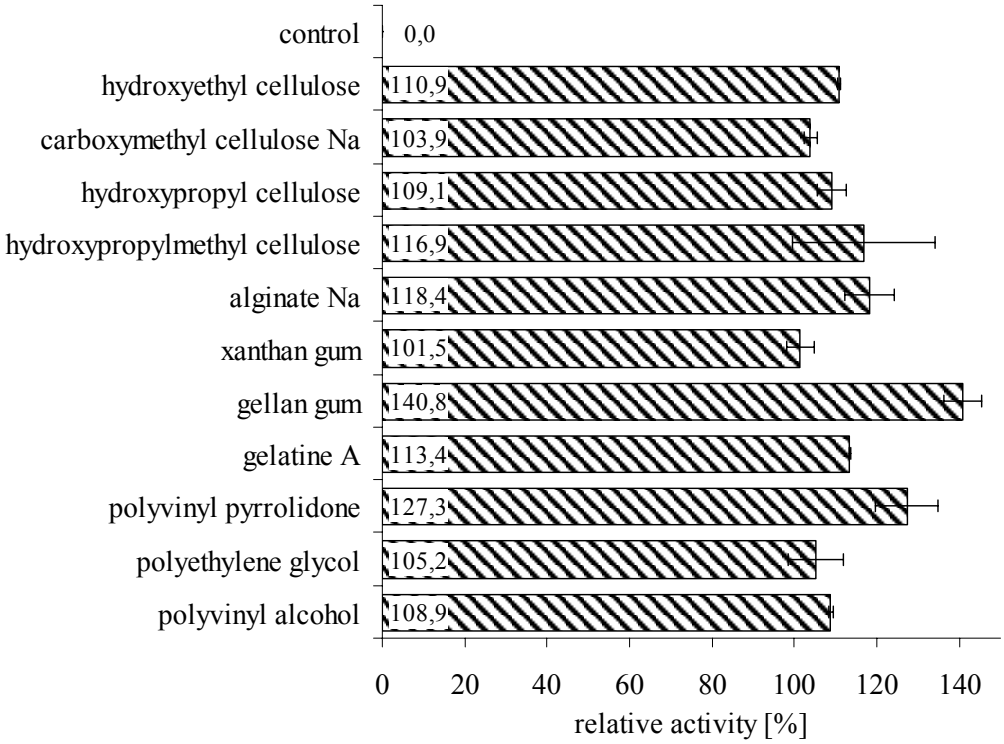


Fig. 3-25 Recovered relative activity of ACT in 10mM potassium phosphate buffer pH 7.2 and 0.1% Poloxamer® 188 with various polymers at 0.5% content after 15 freeze/thaw cycles in liquid nitrogen measured by activity assay; control is formulated in buffer; polymers are HEC 10.000, CMC Na 10.000, HPC 100.000, HPMC 15.000, gellan gum LT100, PVP 17, PEG 2000, PVA 100.000;

In conclusion, hydroxyethyl cellulose, carboxymethyl cellulose Na, hydroxypropyl cellulose, hydroxypropylmethyl cellulose, alginate Na, gellan gum, gelatine A, polyvinyl pyrrolidone, and polyethylene glycol are compatible with the analytical tool and are not depleting ACT activity in temperature- and F/T stress.

The regulatory status of the polymers by the FDA for drug applications is quite complex. Gelatine, polyethylene glycol, and carboxymethyl cellulose sodium are approved for intramuscular injection up to a content of 16%, 3 to 65% depending on PEG species, and 0.9% respectively. Polyvinyl pyrrolidone and hydroxyethyl cellulose are approved for use in ophthalmic solutions and transdermal delivery systems. Hydroxypropyl cellulose is approved for topical, hydroxypropylmethyl cellulose for ophthalmic administration. Alginate is solely approved for oral delivery[149].
Although, especially alginate and hydroxyethyl cellulose are used in medical devices for wound healing. So, an approval as drug containing material for parenteral use with blood contact by authorities should be manageable in spite.

### *3.3.2.2 Aseptic manufacture of ACT loaded hydrogels*

Obviously, final sterilisation of the gel product is not possible with thermal treatment due to protein instability. And, hydrogels are not suitable for sterile filtration because of their viscosity. Thus, a separate sterilisation of protein solution and hydrogel carrier is needed followed by aseptic manufacturing.
Where possible, steam sterilisation is conducted on the intermediate drug-free hydrogel products. The other polymers are dispersed in 70% ethanol for 15 minutes. After this disinfection time ethanol evaporates and leaves a dry aseptic polymer that gets hydrated with sterile solution to form the hydrogel under aseptic conditions.

Polymer screening till date has been performed with reduced polymer contents to maintain the liquid state. There, a simple mixing of drug solution with polymer solution is appropriate for sample preparation. However, for the manufacture of semisolid ACT loaded hydrogels another drug loading procedure has to be developed.
Thus, as first method the homogenisation of a sterile, previously made hydrogel with sterile filtered ACT solution using a static mixer is evaluated[159](Fig. 3-26).
The sterile placebo gel is filled into a first syringe. The mixer unit is connected to the syringe and the system is filled with gel to remove air. A second syringe containing the concentrated ACT solution is connected without trapping air within the system. The mass is pumped back and forth 10 times for complete homogenisation.

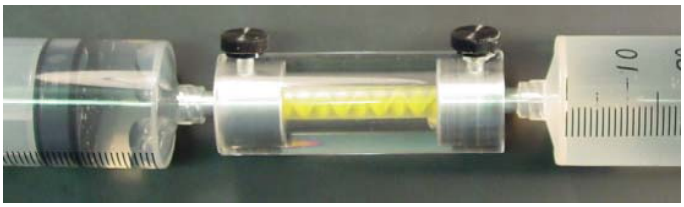

Fig. 3-26 Static mixer with mounting device between two syringes; gel mass is pumped back and forth 10 times for homogenisation;

For validation of this procedure the number of homogenisation steps is evaluated with fluorescein sodium. The gel was split into 0.5ml portions which were analysed by fluorimetric detection. It turned out that 10 homogenisation steps are appropriate for effective drug loading indicated by a relative standard deviation of 2.7% for the concentration of fluorescein in the measured samples. Moreover, in the used setting a minimum of 3ml total amount is shown to be necessary whereby 1ml is lost in the apparatus.
Unfortunately, the following experiments for loading of ACT into gels show that activity of ACT decreases to a huge extent of over 85% during that loading procedure. It can be concluded that not only temperature and freeze/thaw stress but also the shear stress in the static mixer device can damage ACT molecules and so deactivate them. That is why the method of loading ACT in a gel base with the use of the static mixer device is abandoned at that point.

So, another method of loading ACT into a sterile hydrogel preparation with only mild stress burden has to be developed. For the reason that a direct drug loading by mixing is not possible due to deactivation of ACT by shearing an indirect method via a sterile, dry gel intermediate is evaluated. Thereby, the polymer swells into the drug solution until homogeneity avoiding shearing in favour of a longer duration of the loading process.
Practically, a steam-sterilised gel is poured into petri dishes or cast out using a scraper to a defined thickness of 2mm. Under aseptic conditions, this wet film then is dried to a solid film under flowing air. Consequently, the film is cut in shape and hydrated in appropriate vases, e.g. petri dishes, glass vials, or well plates, with sterile filtered ACT solution. Hereby, one layer film is used for 2mm solution filling height and two layers are used for 4mm filling height each resulting in correspondent gel thickness. The swelling to homogeneity is finalised after 48 hours for all polymers at 6°C. Activity measurements indicate that activity of ACT is not reduced during this procedure (data not shown). Therefore, for the following experiments the described indirect method is used for manufacture of ACT gels.

### *3.3.2.3 Analysis of ACT loaded hydrogels*

Analysis of these gels holds similar difficulties as the manufacture. For reliable and reproducible ACT activity determination by the used assay a liquid sample with the analyte in solution is necessary. Moreover, only mild shearing should be applied as results above indicate. So, to liquefy the gel formulation an enzymatic method and a method by simple dilution are evaluated.
The idea behind the enzymatic method is to degrade the cellulose backbone of polymers into shorter fragments being unable to maintain the semi-solid gel structure. The tested enzyme, cellulase, is tested in a preliminary series concerning its influence on ACT activity in liquid solution. Unfortunately it again has turned out that this convenient method is not suitable for ACT because no ACT activity could be recovered form test samples incubated over 8 hours at 6°C. The molecular impact of cellulase on ACT was not further investigated, but it can be speculated that the heavy glycosylation of ACT is a point of attack for the enzyme leading to this unwanted complete loss of activity.
For the reason that a chemical degradation of the polymer is unsuitable a physical degradation by dilution was investigated. It was found that a dilution of 1:8 with 50mM potassium phosphate buffer with 0.1 % Poloxamer® 188 and a repeated mild shaking is sufficient to form a liquid with no detectable loss on ACT activity. Unless, the dilution of 1:8 which results in concentrations of a maximum of 7.5 µg/ml can be coped with by the activity assay protocols. As before, most other analytical techniques especially with direct UV detection fail because of a higher detection limits. Indeed, the described dilution method was used for sample preparation prior to activity analysis for all described experiments.

### *3.3.2.4 Mid term stability of ACT in hydrogel formulations*

Based on the results of the stabilisation in liquids a three months stability test in semi-solid gels is launched with a set of formulations. All alternatives for the stabilisers are considered. But, for capacity reasons solely for the polymers a selection was done, this in prospect of later discussed results for dry forms and suitability for steam sterilisation. In fact, only hydroxyethyl cellulose 100.000 and mixtures of hydroxyethyl cellulose with acylated and deacylated derivatives of gellan gum - quality LT100, and F - are tested. Additionally, soluble polymers are tested together with the formulations. These polymers - PVP and PEG - fulfil technical purposes outlined in the sections describing the development of xerogels and films.

Samples of ACT loaded gels are prepared under aseptic conditions as outlined above. Packaging containers are glass vials that are sealed with a Teflon®-coated rubber stopper. The concentration of ACT was 60µg/ml, sample volume was 0.4 ml. So prepared samples are stored at three temperature levels, namely 6°C, 25°C, and 40°C. After 1, 4, 8, and 12 weeks storage time samples are drawn and stored at –80°C until analysis. But, before analysis by activity assay the samples are prediluted 1:8 to form a liquid gel dispensable like a liquid for handling reasons according to the evaluated method.

Calculations described in detail below are conducted to estimate reaction kinetics and shelf lives under storage conditions; 6°C simulates storage in a refrigerator, 25°C simulates storage at room temperature, and 32°C simulates temperature condition during application on the wound (adapted from[160]). The data on 32°C level is gained from Arrhenius calculations below.

The assessment of samples is done in pursuance with three benchmarks. First is the suitability of the hydrogel formulation as future storage form for stocking at room temperature. Therefore the 25°C estimation has to exceed 18 months above the lower specification limit. Second is the same question for stocking in the refrigerator. And, third is the suitability for application of the hydrogel formulation into the wound site. For this purpose, the estimated shelf life of the hydrogel at 32°C should exceed one week.

#### *3.3.2.4.1 Principles of data interpretation*

Generally, results are gained as a set of curves of residual activity over time for every formulation and temperature level. An example is shown in Fig. 3-27. From a starting value of about 100% the activity declines over the 3 months of storage time.

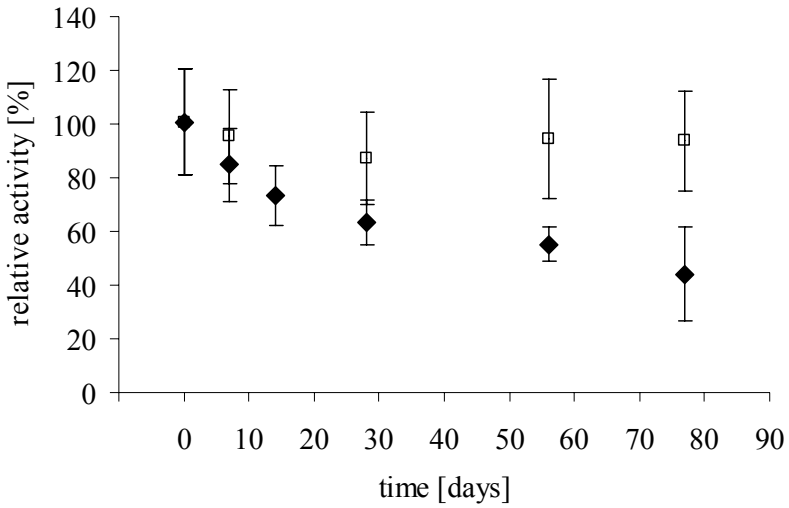


Fig. 3-27 Recovered relative activities of a hydrogel formulation consisting of 60µg/ml ACT, 10mM arginine, 0.1% Tween® 80, 0.05% PVP 17, 2.5% hydroxyethyl cellulose in a 50mM potassium phosphate buffer pH 7.2 at 6 °C (□) and 40°C (◆) in linear scale measured by activity assay;

For an estimation of the reaction kinetics of the loss of ACT activity over time the relative activity results are drawn against sample time. Additionally, for higher reaction orders mathematical modifications are implemented on the relative activity values for linearisation according to the well known time laws:

Equ. 3-7 0. order $$c(t) = c(0) - k_0 \cdot t$$

Equ. 3-8 1st order $$\ln c(t) = \ln c(0) - k_1 \cdot t$$

Equ. 3-9 2nd order $$\frac{1}{c(t)} = \frac{1}{c(0)} + k_2 \cdot t$$

Equ. 3-7 & 3-8 & 3-9 Time laws for reaction kinetics for 1st, 2nd, and 3rd order; c(t) is relative activity at time t, c(0) starting relative activity, $k_{1/2/3}$ are velocity constants, and t is time in days;

For these three cases functions are fitted over the modified curves and next to the equation parameters the correlation coefficient is determined and used for assessing the feasibility of the fitted reaction order. Fig. 3-28 shows an example for the fitting of the first order time law.

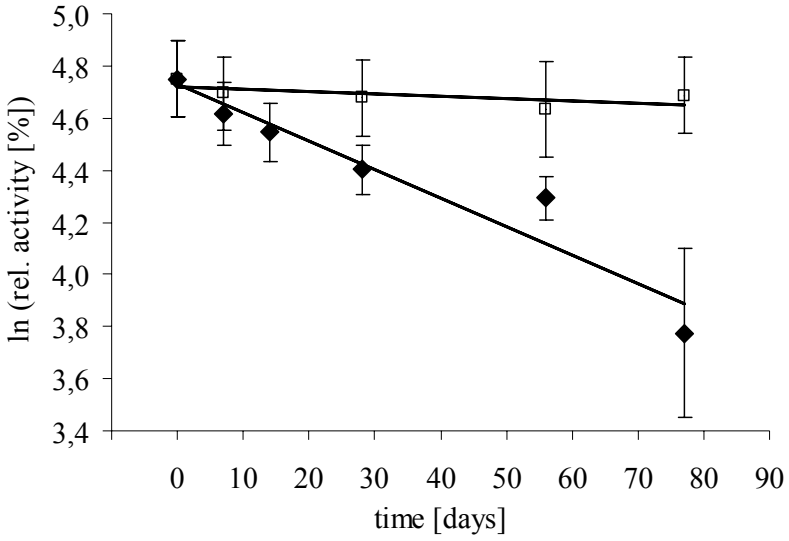


Fig. 3-28 Recovered relative activities of a hydrogel formulation consisting of 60µg/ml ACT, 10mM arginine, 0.1% Tween® 80, 0.05% polyvinyl pyrrolidone 17, 2.5% hydroxyethyl cellulose in a 50mM potassium phosphate buffer at 6°C (□) and 40°C (◆) in logarithmic concentration scale with fitted logarithmic functions resulting in evens in the one-sided logarithmic scale as predicted by first-order time law; measured by activity assay;

As shown in this example the best fitting results given by correlation coefficients are gained for the first order equation (Equ. 3-8), i.e. a logarithmic scale for relative activity results.

According to the guideline ICH-QA1 the datasets are tested on change-over-time, indicated by the p-value of the slope of the fitted line exceeding 0.25 for no change-over-time. And, in the case of no change-over-time variability is tested to confirm statistical conformity of stability data. Further, for an estimation of the shelf life the 95%-confidence intervals are calculated for the curves. The intercept of the lower confidence limit with the lower specification limit – in our case 10 % loss of relative activity - indicates the end of the shelf life. An example is seen in Fig. 3-29.

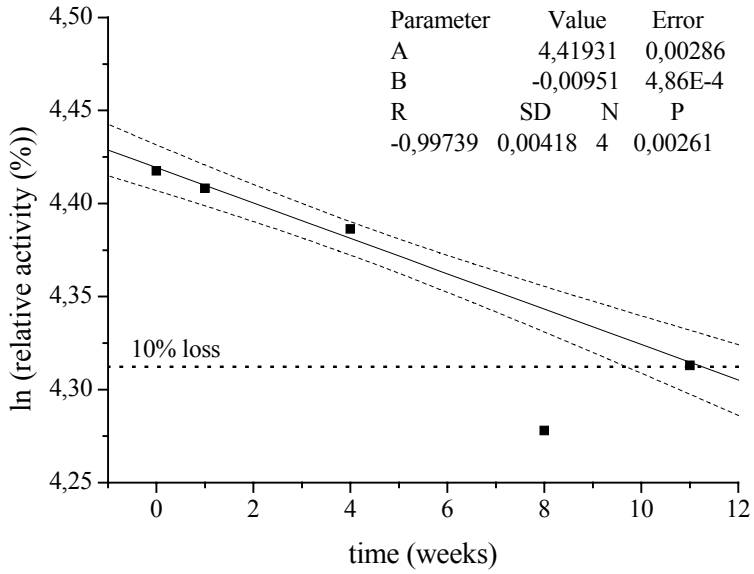


Fig. 3-29 Recovered relative activities of a hydrogel formulation consisting of 60µg/ml ACT, 10mM arginine, 0.1% Poloxamer® 188, 1.5% polyvinyl pyrrolidone 17, 1.0% PEG 400, 1.0% gellan gum, 2.0% hydroxyethyl cellulose in a 50mM potassium phosphate buffer at 6°C measured by activity assay; relative activity is in logarithmic scale; fitted logarithmic function and 95% confidence intervals are shown; 10% loss line marks the lower specification limit;

Moreover, via the Arrhenius-equation (Equ. 3-10) the reaction constants are linked to temperature.

$$\ln k_1 = -\frac{E_A}{R \cdot T} + \ln A$$

Equ. 3-10 Arrhenius equation; $k_1$ is the first order velocity constant, $E_A$ is the activation energy [J/mol], R is the gas constant [J/(K*mol)], T is the absolute temperature [K], and A is the collision factor

By inserting the known values for k and T for the three temperature levels, an even can be calculated from where the missing values of $E_A$ and A can be determined. In detail, the slope is equal to $-E_A/R$ and the intercept is equal to ln A (Fig. 3-28). This equation can be used to calculate the velocity constant of any temperature lying between the data limits. In fact, the calculations are conducted for 6°C, 25°C and 32°C. Based on that, estimating calculations of shelf lives on these temperature levels are conducted.

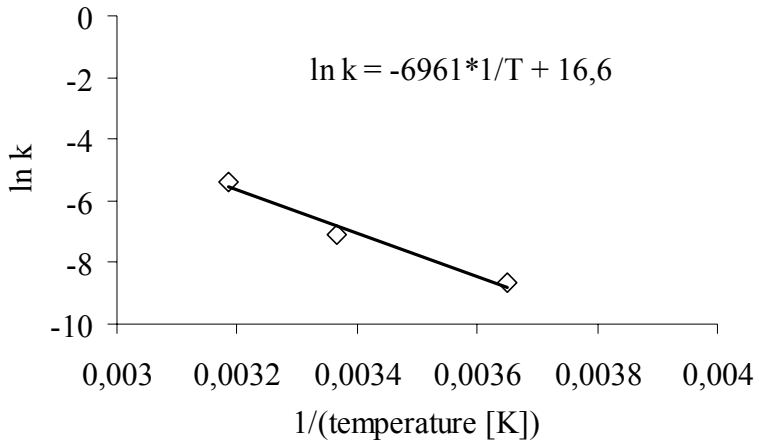


Fig. 3-30 Velocity constants k in logarithmic plot against temperature in reciprocal plot with fitted function; slope of the function is equal to $-E_A/R$, intercept is equal to ln A from the Arrhenius equation (Equ. 3-10)

### *3.3.2.4.2 Experimental results and discussion*

The kinetics of the curves follow most likely the first order time law (Fig. 3-26) because correlation coefficients are best for the first order kinetics in most cases (data not shown).

But, all datasets show change-over-time and additionally many are variable according to ICH-QA1 guideline (calculations not shown). Thus, confidence intervals of most datasets are too large to allow a sensible, statistically profound estimation of shelf life as described above.
Instead, an estimation is made by simple extrapolation of the fitted curve to the intercept with the 10% loss level as lower specification limit. For all formulations, consequently, first order reaction constants for every temperature level are calculated and corresponding shelf lives are estimated (Tab. 3-7).

(1)

| | | | |
|---|---|---|---|
| 60µg/ml | $\alpha_1$-antichymotrpsin | reaction constant k (1°C) | $2.99 \cdot 10^{-4}$ 1/day |
| 50mM | phosphate buffer | reaction constant k (24°C) | $1.41 \cdot 10^{-3}$ 1/day |
| 10mM | arginine | reaction constant k (41°C) | $1.05 \cdot 10^{-2}$ 1/day |
| 0.1% | Tween® 8o | shelf life (6°C) | 253.4 days |
| 0.05% | PVP 17 | shelf life (25°C) | 50.1 days |
| 2.5% | HEC 100.000 | shelf life (32°C) | 22.1 days |

(2)

| | | | |
|---|---|---|---|
| 60µg/ml | $\alpha_1$-antichymotrpsin | reaction constant k (1°C) | $7.19 \cdot 10^{-4}$ 1/day |
| 50mM | phosphate buffer | reaction constant k (24°C) | $1.05 \cdot 10^{-3}$ 1/day |
| 10mM | arginine | reaction constant k (41°C) | $3.63 \cdot 10^{-3}$ 1/day |
| 0.1% | Poloxamer® 188 | shelf life 6°C | 138.0 days |
| 0.05% | PVP 17 | shelf life 25°C | 67.4 days |
| 2.5% | HEC 100.000 | shelf life 32°C | 47.0 days |

(3)

| | | | |
|---|---|---|---|
| 60µg/ml | $\alpha_1$-antichymotrpsin | reaction constant k (1°C) | $1.58 \cdot 10^{-3}$ 1/day |
| 50/50mM | phosphate/citrate buffer | reaction constant k (24°C) | $2.15 \cdot 10^{-3}$ 1/day |
| 10mM | arginine | reaction constant k (41°C) | $5.29 \cdot 10^{-3}$ 1/day |
| 0.1% | Poloxamer® 188 | shelf life 6°C | 63.3 days |
| 0.05% | PVP 17 | shelf life 25°C | 37.0 days |
| 2.5% | HEC 100.000 | shelf life 32°C | 28.3 days |

(4)

| | | | |
|---|---|---|---|
| 60µg/ml | $\alpha_1$-antichymotrpsin | reaction constant k (1°C) | $1.70 \cdot 10^{-4}$ 1/day |
| 50mM | phosphate buffer | reaction constant k (24°C) | $8.16 \cdot 10^{-4}$ 1/day |
| 10mM | arginine | reaction constant k (41°C) | $4.61 \cdot 10^{-3}$ 1/day |
| 0.1% | Poloxamer® 188 | shelf life 6°C | 439.3 days |
| 0.05% | PEG 2000 | shelf life 25°C | 96.8 days |
| 2.5% | HEC 100.000 | shelf life 32°C | 45.1 days |

(5)

| | | | |
|---|---|---|---|
| 60µg/ml | $\alpha_1$-antichymotrpsin | reaction constant k (1°C) | $9.31 \cdot 10^{-4}$ 1/day |
| 50mM | phosphate buffer | reaction constant k (24°C) | $1.44 \cdot 10^{-3}$ 1/day |
| 10mM | arginine | reaction constant k (41°C) | $6.25 \cdot 10^{-3}$ 1/day |
| 0.1% | Poloxamer® 188 | shelf life 6°C | 105.7 days |
| 0.05% | PVP 17 | shelf life 25°C | 45.6 days |
| 2.0% | HEC 100.000 | shelf life 32°C | 29.8 days |
| 1.0% | gellan gum LT100 | | |

(6)

| | | | |
|---|---|---|---|
| 60µg/ml | $\alpha_1$-antichymotrpsin | reaction constant k (1°C) | $6.48 \cdot 10^{-3}$ 1/day |
| 50mM | phosphate buffer | reaction constant k (24°C) | $6.62 \cdot 10^{-3}$ 1/day |
| 10mM | arginine | reaction constant k (41°C) | $8.41 \cdot 10^{-3}$ 1/day |
| 0.1% | Tween® 8o | shelf life 6°C | 16.3 days |
| 0.05% | PVP 17 | shelf life 25°C | 14.5 days |
| 2.0% | HEC 100.000 | shelf life 32°C | 13.7 days |
| 1.0% | gellan gum LT100 | | |

(7)

| | | | |
|---|---|---|---|
| 60µg/ml | $\alpha_1$-antichymotrpsin | reaction constant k (1°C) | $1.36 \cdot 10^{-3}$ 1/day |
| 50mM | phosphate buffer | reaction constant k (24°C) | $3.50 \cdot 10^{-3}$ 1/day |
| 10mM | arginine | reaction constant k (41°C) | $1.05 \cdot 10^{-2}$ 1/day |
| 0.1% | Poloxamer® 188 | shelf life 6°C | 63.1 days |
| 1.5% | PVP 17 | shelf life 25°C | 24.8 days |
| 1.0% | PEG 400 | shelf life 32°C | 15.4 days |
| 2.0% | HEC 100.000 | | |
| 1.0% | gellan gum LT100 | | |

(8)

| | | | |
|---|---|---|---|
| 60µg/ml | $\alpha_1$-antichymotrpsin | reaction constant k (6°C) | $9.95 \cdot 10^{-4}$ 1/day |
| 50mM | phosphate buffer | reaction constant k (24°C) | $2.84 \cdot 10^{-3}$ 1/day |
| 10mM | arginine | reaction constant k (41°C) | - |
| 0.1% | Poloxamer® 188 | shelf life 6°C | 105.9 days |
| 0.05% | PVP 17 | shelf life 25°C | 37.1 days |
| 2.0% | HEC 100.000 | shelf life 32°C | 24.2 days |
| 1.0% | gellan gum F | | |

(9)

| | | | |
|---|---|---|---|
| 60µg/ml | $\alpha_1$-antichymotrpsin | reaction constant k (1°C) | $2.28 \cdot 10^{-3}$ 1/day |
| 50mM | Tris buffer | reaction constant k (24°C) | $5.93 \cdot 10^{-3}$ 1/day |
| 10mM | arginine | reaction constant k (41°C) | $2.50 \cdot 10^{-2}$ 1/day |
| 0.1% | Poloxamer® 188 | shelf life 6°C | 46.3 days |
| 0.05% | PVP 17 | shelf life 25°C | 17.8 days |
| 2.5% | HEC 100.000 | shelf life 32°C | 7.7 days |

(10)

| | | | |
|---|---|---|---|
| 60µg/ml | $\alpha_1$-antichymotrpsin | reaction constant k (1°C) | $2.53 \cdot 10^{-3}$ 1/day |
| 50mM | Tris/NaCl buffer | reaction constant k (24°C) | $9.80 \cdot 10^{-3}$ 1/day |
| 10mM | arginine | reaction constant k (41°C) | $2.08 \cdot 10^{-2}$ 1/day |
| 0.1% | Poloxamer® 188 | shelf life 6°C | 41.6 days |
| 0.05% | PVP 17 | shelf life 25°C | 10.8 days |
| 2.0% | HEC 100.000 | shelf life 32°C | 8.3 days |
| 1.0% | gellan gum F | | |

Tab. 3-7 Formulations of the mid term stability testing with ingredients (left column), reaction constants k at the experimental stress levels, and estimated shelf lives at the required temperature levels (right column);

Unless data show variation according to ICH-QA1 several conclusions can be made. For wet state hydrogels stored in glass vials the phosphate buffer has greater stabilising potential than the Tris and citrate/phosphate systems. This is manifested in shelf lives of formulation (2) in comparison with (3) and (9), as well as formulation (8) compared to (10). The surfactant Poloxamer® 188 works better than the Tween® 80 alternative. See comparison of formulation (1) with (2), and (6) with (7). For the soluble polymers polyethylene glycol is favourable over polyvinyl pyrrolidone as shelf lives of formulation (2) and (4) indicate. Moreover, the hydroxyethyl cellulose gelling agent is favourable over each tested alternatives. This results from comparison of formulation (2) with (5) and (8). However, no formulation offers enough stabilising potential to enable a hydrogel as storage form at room temperature or in the refrigerator. Because, neither at 6°C nor at 25°C storage temperature the estimated shelf life of any formulation reaches the required limit of 18 months (550 days). But, for all tested formulations stability is above the lower specification limit for over 7 days at 32°C. All formulations, hence, fulfil the stability requirements for topical, local application in wounds. Thus, the gained data on advantage and disadvantage of excipients do not display knock-out criteria for choice of ingredients. Indeed, the spectrum of excipient candidates for further development is not narrowed.

#### *3.3.2.4.3 Summary*

Results indicate that reaction kinetics of activity loss of ACT is highly likely to follow a first order equation. Moreover, a commercial formulation of ACT in a wet hydrogel is not possible due to insufficient shelf life. In contrast, all formulations can be applicated into the wound site as far as stability of ACT is concerned. As well, several formulations are applicative as early refrigerated formulation for the purpose of animal experiment or early clinical trial where only limited shelf life is required.

### *3.3.2.5 Freeze/thaw stability of ACT in hydrogel formulations*

For verification of the results of F/T studies on ACT in solution and with polymer ingredients similar hydrogel formulations as in the prior section are tested in F/T studies. Sample preparation and analysis are performed as described above. Stressing is done by repeated freezing in liquid nitrogen and thawing at room temperature.
Samples contain 60µg/ml ACT in 2.5% hydroxyethyl cellulose gels. Other ingredients are listed in Tab. 3-8.
Results indicate a total recovery of ACT after 15 F/T cycles for all excipient combinations. In the buffer control without stabilisers activity is completely lost after 15 cycles (Fig. 3-31). Therefore, it can be concluded that all hydrogel formulations are cryoprotective on ACT.
This is an important pre-requisite for a successful lyophilisation and enables frozen hydrogels for the use as early formulations[161 162].

| buffer control □ | formulation 1 ■ | formulation 2 ◇ | formulation 3 ▷ | formulation 4 ○ |
|---|---|---|---|---|
| 10 mM phosphate | 10 mM phosphate<br>10 mM arginine<br>0.1% Poloxamer® 188<br>0.05%PVP 17 | 10 mM phosphate<br><br>0.1% Poloxamer® 188<br>0.05%PVP 17 | 10 mM phosphate<br>10 mM arginine<br>0.1% Tween® 80<br><br>0.05%PVP 17 | 10 mM phosphate<br>10 mM arginine<br>0.1% Poloxamer® 188<br>0.05% PEG 2000 |

Tab. 3-8 Recipes for F/T studies containing 60µg/ml ACT and 2.5% hydroxyethyl cellulose;

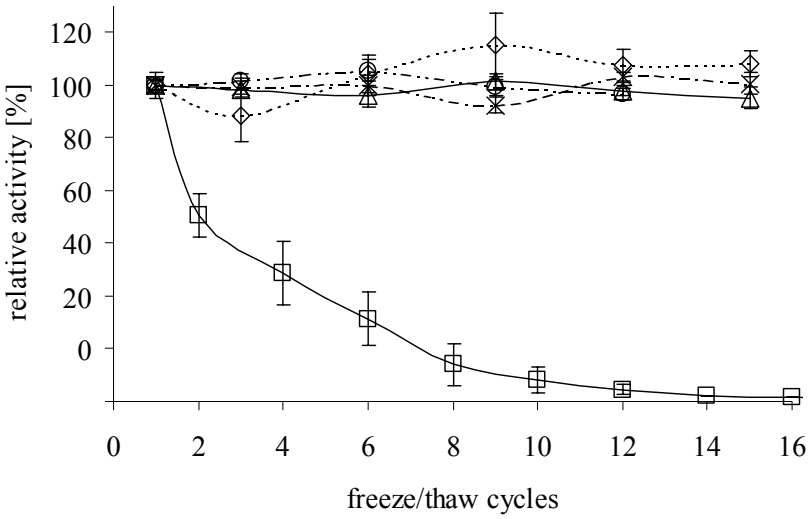


Fig. 3-31 F/T stability of ACT in hydrogel formulations; kinetics of decay of relative activity in formulations listed in Tab. 3-8 during F/T treatment in liquid nitrogen; formulation 1 (■), formulation 2 (◇), formulation 3 (▷), formulation 4 (○), buffer control (□);

### 3.3.3 Summary of hydrogels as ACT delivery systems for wound healing

During these studies hydrogels are developed for both the concerns of a wound dressing material and the needs of the drug stability of ACT.

In a first approach the placebo hydrogel carriers are evaluated with regard to ability for sterilisation and rheological properties. Secondly, short term stabilisation studies on ACT with suitable polymers are performed. Consequently, ACT stabilising hydrogel formulations are created containing excipients being suggested by short term stability studies. Following, drug loading procedures into hydrogels are evaluated. After that, the named formulations are introduced in mid term stability studies and F/T studies. Results indicate that hydrogels are suitable application forms for ACT. They also can represent early formulations contingently in frozen state. But, hydrogels can not be used as storage form for a future drug product based on ACT due to the limited shelf lives of such products.

## 3.4 Dry delivery systems

In the last section it has been shown that aqueous carriers are suitable for application of ACT on wounds and for early formulations of ACT. In contrast, it has been shown that aqueous carriers are not suitable for long term storage. Thus, a dry form is to be developed that stabilises ACT during long term storage. Moreover, when hydrated just before application a hydrogel is to be formed suitable as wound dressing and releasing ACT in suitable period of time. For this task, development starts from the gel bases discussed in the hydrogel section.

In Chapter 1 wound dressings that are stored as dry matrices and that form gels when put in contact with aqueous solution have been outlined. For the commercial production of many of these dressings, e.g. alginate or carboxymethyl cellulose dressings, jetting or extrusion of gels or polymers followed by spinning or felting of resulting fibres is used[163]. This forms very fast hydrating fibre matrices due to the thin fibres and wide meshed structure. Other products, for example collagen sponges, are produced by lyophilisation of hydrogels[164].

Although lyophilisation is much more expensive and time consuming, the extrusion or jetting techniques are inappropriate for protein drug load due to the extended shearing of the material which is very likely to cause damage to the protein molecules during fabrication.
Therefore, the first technique used for drying of ACT loaded hydrogels during this formulation study is lyophilisation. The resulting matrices are xerogels. In addition, a second technique – the warm air drying - is evaluated. Warm air drying has been successfully used for drying of protein solutions[165]. From this technique swellable, self-supportive polymer films are formed.
In the first part of this chapter the development of xerogels as placebo dressings and drug loaded carriers is described. In a second part the development of films also in placebo state and as drug loaded matrices is outlined.

## 3.4.1 Xerogels as drug delivery systems for wound healing

Today, there are several lyophilised and swellable polymer containing products used as drug free wound dressings in clinical practice. For example, Suprasorb G® consists of collagen, and Promogran® contains a mixture of oxidised regenerated cellulose and collagen.
Besides, many patent applications have been submitted on drugs in xerogel or sponge dressing materials. Some of these matrices also contain protein drugs[166-176]. But, despite the large number of patent applications about mainly growth factors in lyophilised polymer matrices back in the 1990s none of those has led to approval by authorities let alone to market launch, yet.
In this section, the production of placebo xerogel carriers and investigations of their mechanical properties are described. Thereby, influence of the lyophilisation process, of the polymer composition, and of further formulation ingredients are highlighted.
The goals of these studies are to ensure the suitability of the xerogel as wound dressing and to investigate the compatibility with protein formulation ingredients. Xerogels are meant to be reconstituted before or in the moment of application. So, the wound will not be confronted with the dry xerogel pad. It rather will get in contact with the yet hydrated gel state. Therefore, the swelling behaviour is the most important technical feature for xerogels. In fact, a fast and complete swelling is essential for such a product. Moreover, a homogeneous appearance, a convenient and soft consistency is desirable for compliance reasons. Furthermore, a residual moisture of less than 2% is essential for protein drug stability.

### *3.4.1.1 Lyophilisation process*

For the development of a suitable lyophilisation process for the manufacture of xerogels the cryostabilising hydrogel formulations described in the prior section are taken as starting points.
For preliminary studies differential scanning calorimetric experiments are conducted on placebo gels from where temperature parameters of the freeze drying process are gained. Following, placebo xerogels are examined optically and by x-ray analysis.

### *3.4.1.1.1 DSC studies*

For examination of placebo gels by DSC specimen are prepared according to the results of the prior ACT stabilisation studies.
The thermograms solely show a freezing and melting peak (Fig. 3-32). Therefore, a standard freeze drying program with a freezing phase leading to temperatures below the crystallisation temperature of around –15°C is adequate.

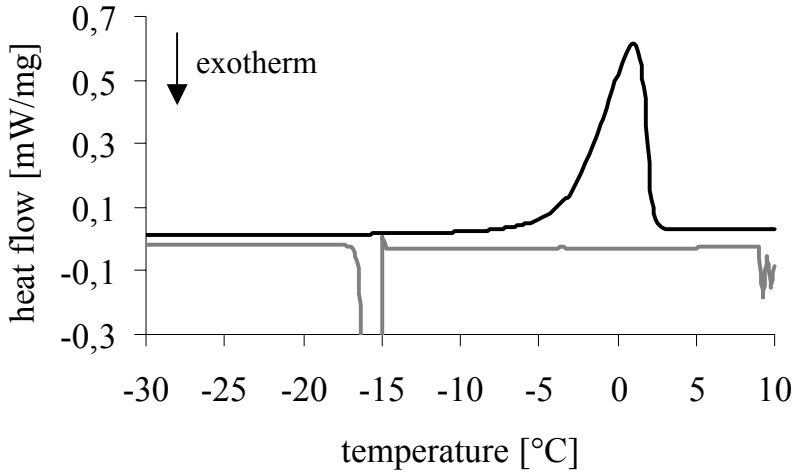


Fig. 3-32 DSC thermogram of 2.5% hydroxyethyl cellulose 100.000 gel swollen in 10mM phosphate buffer pH7.2, 10mM arginine, 0.1% Poloxamer® 188; freezing curve (below) indicates a crystallisation at –14.8°C, melting curve (above) shows a melting peak at –0.9 °C

### *3.4.1.1.2 Lyophilisation program*

| time [h] | plate temperature [°C] | cabin pressure [mbar] |
|---|---|---|
| 0 | 20 | 1013 |
| 1 | -45 | 1013 |
| 1.5 | -45 | 1013 |
| 0.1 | -45 | 0.1 |
| 1 | -30 | 0.1 |
| 33 | -30 | 0.1 |
| 5 | 20 | 0.1 |
| 0.1 | 20 | 0.011 |
| 8 | 20 | 0.011 |

Tab. 3-9 Program of the conventional freeze drying process for xerogels

The first experiments on lyophilisation of hydrogels were conducted with a standard freeze drying program according to the preliminary DSC tests. So, the samples are filled in petri dishes to a filling height of 4 mm. These are placed in the

drying chamber at room temperature and following freezing is done at very fast rates of 1.1 K/min. After a retaining phase a conservative main drying step is proceeding at –30°C. The subsequent secondary drying is also conservatively conducted at 20°C (Tab. 3-9).

Unfortunately, the resulting xerogel pads appear very rough in visible structure which may lead to disturbed compliance. The reason for this appearance was found in the random crystallisation of the gels during freezing. Indeed, the crystal forming in the liquid status starts anywhere in the gel and grows through the gel until completeness. And, the way of growth seems to be preserved in the pad (Fig. 3-33).

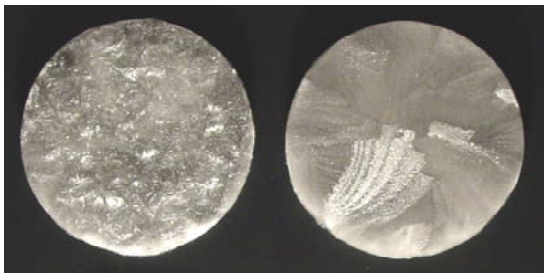

Fig. 3-33 Xerogel pads consisting of hydroxyethyl cellulose 100.000 made from 2.5% hydrogels by conventional freeze drying process without annealing; left: top, right: bottom

Several attempts aiming to the controlled induction of seed crystals like placing a cooled needle into the gel, ultrasonic vibration, and induction by rime formation on the surface by opening the dryer chamber door for a short time[177], respectively, did not succeed.
But, the introduction of an annealing step into the freezing phase of the lyophilisation process led to a more desirable, homogeneous appearance (Fig. 3-34)[178].

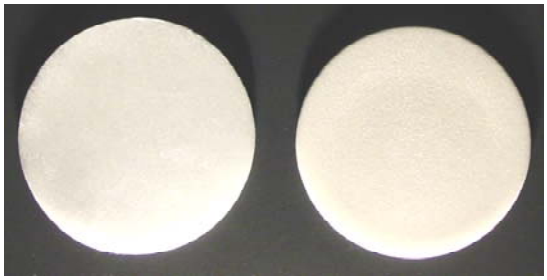

Fig. 3-34 Xerogel pads consisting of hydroxyethyl cellulose 100.000 made from 2.5% hydrogels by the freeze drying process with annealing step during freezing; left: top, right: bottom

This annealing step is carried out as follows: The gels are frozen under the crystallisation point until the described random crystal forming process is completed. After that, the gels are heated up again close below their freezing point (-3°C) to remain there for 90 minutes. Consequently, the gels are cooled again at a very slow rate of 7 K per hour to –45°C initialising the primary drying after three hours of balancing time. The process is visualised in Tbl. 3-10 and Fig. 3-35. In fact, the annealing step at that point is not introduced for the purpose of protein stabilisation but only for matrix considerations.

| time [h] | plate temperature [°C] | cabin pressure [mbar] |
|---|---|---|
| 0 | 20 | 1013 |
| 0.5 | -17 | 1013 |
| 1 | -17 | 1013 |
| 1 | -3 | 1013 |
| 1.5 | -3 | 1013 |
| 0.75 | -25 | 1013 |
| 0.3 | -45 | 1013 |
| 1.5 | -45 | 1013 |
| 0.1 | -45 | 0.1 |
| 1 | -30 | 0.1 |
| 33 | -30 | 0.1 |
| 5 | 20 | 0.1 |
| 0.1 | 20 | 0.011 |
| 8 | 20 | 0.011 |

Tab. 3-10 Program of the freeze drying process with annealing step for xerogels

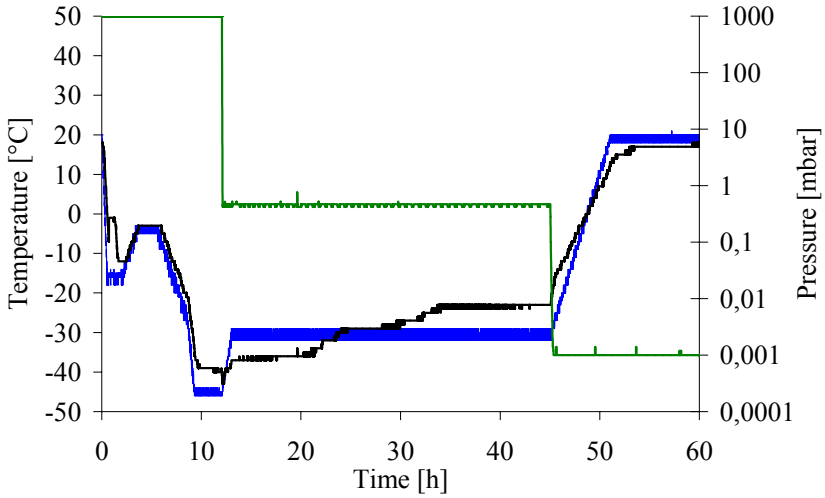


Fig. 3-35 Temperature/pressure – time diagram of freeze drying process with annealing step during freezing; --- plate temperature, --- product temperature, --- cabin pressure

Although there is clearly visible change in optical appearance in the xerogels made from lyophilisation with conventional program and program with annealing step, respectively, no difference is detected in x-ray analysis. In detail, no remarkable

peak formation at all is seen in the x-ray patterns (Fig. 3-36). It can be concluded that due to the lack of regular assembly of polymer molecules no reflections of x-rays can be detected. This indicates an amorphous instead of a crystalline state of the molecules in the matrix.

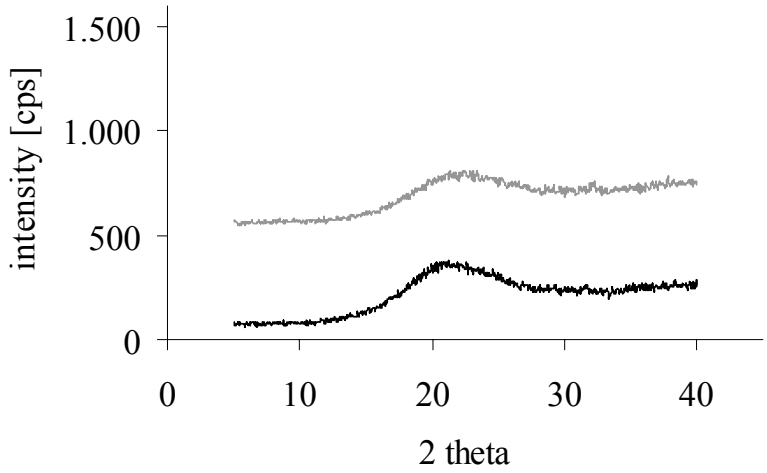


Fig. 3-36 X-ray patterns of xerogels consisting of hydroxyethyl cellulose 100.000 made from 2.5% hydrogels without (above) and with (below) annealing step during lyophilisation; curves are shifted on the y-axis for better demonstration;

### *3.4.1.2 Gel composition for xerogel formation*

For lyophilisation and xerogel formation a variety of polymers is screened for eligibility. The concentration of the gellant in the hydrogel was chosen according to the results of the rheological studies during hydrogel development. After freeze drying with the developed process, the xerogels are examined concerning optical appearance, texture analysis, swelling behaviour, and residual moisture.

As an example for the studies on different gelling agents the studies on hydroxyethyl cellulose are described briefly. After an evaluation of parameters related to the polymer, excipients for protein stabilisation and for adjustment of mechanical properties are incorporated into the matrices and their influence investigated. Similarities and differences of the example hydroxyethyl cellulose to other gellants are outlined. Moreover, the formation of a xerogel matrix made from two gellants – hydroxyethyl cellulose and gellan gum - is described.

#### *3.4.1.2.1 Hydroxyethyl cellulose qualities for xerogel formation*

The nomenclature of the different molecular weights of hydroxyethyl cellulose qualities is taken over from the Tylose® brand nomenclature where the characteristic number expresses the viscosity of a 2% solution measured in [mPa·s]. Differences in molecular weight in cellulose derivatives result in different polymer contents in the gel needed for attaining the same desired viscosity. After freeze-drying, the total content of polymer obviously is closely related to the total dry mass, the density, and the pore structure of the lyophilisate. It is easy to imagine that those factors strongly interact with the mechanical properties of the dry pad and the reconstitution behaviour when the pad is hydrated. Both is essential for the development of the xerogels as wound dressings.
In this series xerogels are prepared from gels with the standard viscosity highlighted in the hydrogel section but with hydroxyethyl cellulose qualities differing in their chain length. In detail, a series of gels from 2.5% of HEC 100.000 to 8.0% of HEC 300 was manufactured. As before, lyophilisation was carried out with the special program with annealing step (see section 3.4.1.1) and analysis was performed by swelling studies, texture analysis and residual moisture detection.
Swelling behaviour is examined by weighing the mass difference between the dry xerogel pad and the swollen pad. Therefore, the xerogel pad is soaked in water or the particular reconstitution medium. After three minutes swelling time the pad is removed from the medium. Consequently, excessive solution adhering to the gel is removed by a paper towel and the swollen gel is weighed. Finally, the swelling value q is calculated (Equ. 3-11).

$$Swelling\ q = \left( \frac{m(swollen\ gel) - m(Xerogel)}{m(Xerogel)} \right)$$

Equ. 3-11 Swelling value q calculated from dry weight of the pad (m(Xerogel)) and the weight after 3 minutes exposure to water (m(swollen gel);

In other words, the swelling value describes how many times the xerogel can take up its own dry weight of water within three minutes.
All samples are acceptable concerning optical appearance measured by sensory valuation. The swelling of the tested xerogels instead turned out to be strongly dependent on the chain length of the used polymer. So, xerogels with higher chain length polymers combined with a lower total polymer content swell faster in the given period than xerogels made from polymers with shorter molecules (Fig. 3-37).

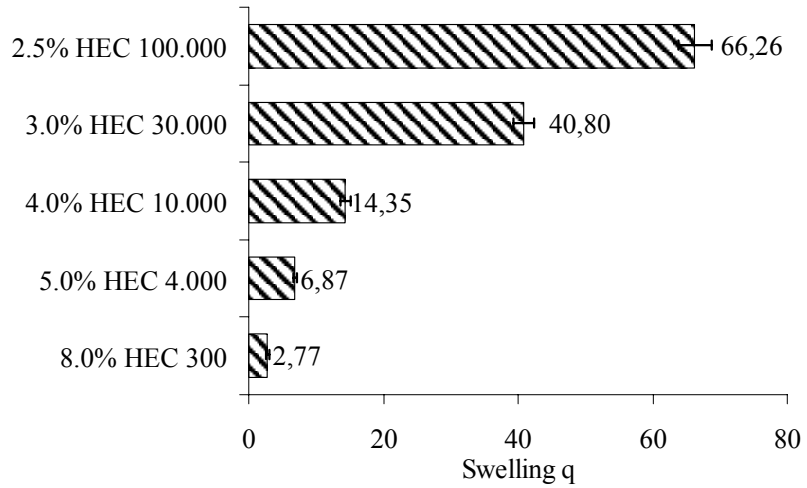


Fig. 3-37 Swelling of xerogels after 3 minutes exposure to water; matrices are made from different hydroxyethyl cellulose qualities in different concentrations but comparable viscosity in the hydrated state;

In the same way mechanics are effected. Measurements are conducted by texture analysis as compression tests of the matrices with a cylinder probe. This method delivers the deformation energy during compression by the probe, restoring energy during release of the probe and the elastic modulus calculated as quotient of compression stress and strain. The modulus can also be expressed as average slope of the force diagram (Fig. 3-38). In fact, matrices from short chain polymers show higher elastic moduli and slightly higher restoring energies which delivers a more stiff impression (Tab. 3-12).

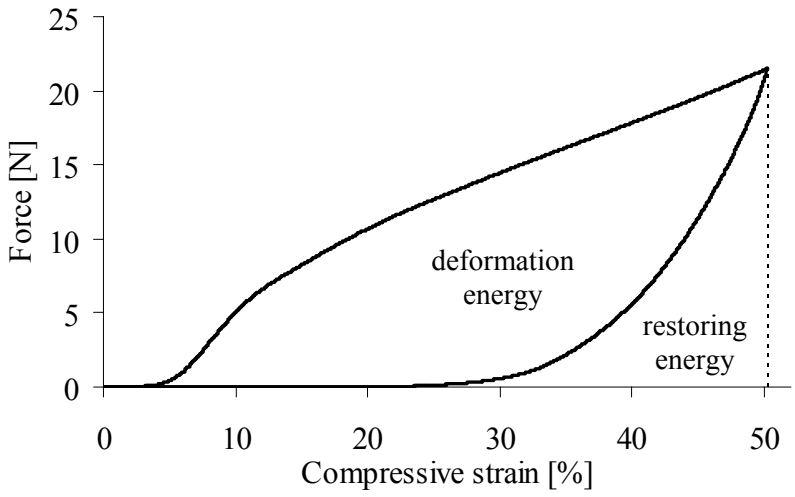


Fig. 3-38 Force diagram of texture analysis of xerogels made form 5% hydroxyethyl cellulose 4000; xerogel is compressed to 50% of height followed by release to beginning; integrated areas are deformation and restoring energy;

| Polymer | Quality | Concentration in the hydrogel [%] | Elastic modulus [mN/mm$^2$] | Restoring energy [%] |
|---|---|---|---|---|
| Hydroxyethyl cellulose | 100.000 | 2.5 | 22.0 | 28.2 |
| Hydroxyethyl cellulose | 30.000 | 3.0 | 28.3 | 29.8 |
| Hydroxyethyl cellulose | 10.000 | 4.0 | 45.6 | 29.6 |
| Hydroxyethyl cellulose | 4.000 | 5.0 | 49.2 | 30.8 |
| Hydroxyethyl cellulose | 300 | 8.0 | 59.3 | 38.2 |

Tab. 3-12 Mechanical properties of xerogels made of different hydroxyethyl cellulose qualities and different concentrations with comparable viscosity gained from texture analysis;

As already pointed out, higher chain length polymers effectuate lower densities of the porous freeze-dried material. This results in higher pore sizes which is supposed to be the reason for the faster swelling. Indeed, water can penetrate faster into the core structure through the wider pores and hydrate the as well lower amount of polymer in these xerogels for gelling. Mechanical properties are effected for the same reason because a more dense matrix due to a higher dry weight of polymer effectuates higher resistibility to compression forces and so higher elastic moduli, i.e. increased stiffness. Restoring energy seems to be affected to a minor extent.
Furthermore, residual moisture detection which is a crucial factor for protein stabilisation is carried out by Karl-Fischer-Titration. The samples for moisture detection were ventilated and stored under in nitrogen atmosphere until analysis. Fortunately, moisture content in all samples was measured between 0.55% and 0.67% which is promising for feasibility of a dry protein formulation.

To summarise, hydroxyethyl cellulose polymers of higher chain length produce xerogels with as well improved hydration properties as mechanical properties. The low residual moisture values are constant in all polymer qualities. Therefore, the quality 100.000 appears as best choice for further development.

#### *3.4.1.2.2 Excipients in hydroxyethyl cellulose xerogels*

After the polymers as main ingredients for xerogels have been characterised the effects of excipients in xerogel matrices are evaluated. Xerogels are prepared as above but the underlying hydrogels contain the additives to be tested. The

excipients are substances supporting the stability of the protein drug to be loaded on the one hand, i.e. electrolytes including buffer salts and various groups of protein stabilisers. On the other hand, substances useful for technical and mechanical reasons, especially improvement of swelling capacities are tested. Among these are soluble polymers as hydrophilisers and tablet disintegrants for improvement of swelling. So, the first group is tested for compatibility with the matrix and extent of disturbance of the xerogel formation and mechanical features. The second group is evaluated for the purpose of improvement of matrix formation and mechanics. Despite the slightly different objective, the influence of the particular excipients on the produced xerogels is evaluated in the same experimental setting, i.e. with regard to compatibility with the matrix, influence on hydration, influence on mechanical properties and residual moisture. The test series is conducted with the polymer HEC 100.000.

*Screening* - for hydroxyethyl cellulose nearly all of the tested substances are compatible with the xerogel matrix (Tab. 3-13).

From the group of stabilisers only sodium sulphate and glycine could not be incorporated. In detail, in these cases crystals are visible on the surface of the xerogel pads. Nevertheless, electrolytes and sugar-like structures tend to harden the matrix and decrease swelling. Surfactants and soluble polymers reduce the brittleness of the xerogels to some degree. The swellable polymers, crosslinked carboxymethyl cellulose, carboxymethyl starch, xanthan gum, and gellan gum, also harden the matrix. The hoped for increasing effect on swelling could not be verified. Unless, the addition of gellan gum showed the change in flow behaviour of the hydrated gel already discussed in the hydrogel section. Residual moistures are mainly between 0.5% and 1.5% and therefore suitable for protein stabilisation.

After the screening, buffers, surfactants, and soluble polymers are further tested for their influence on swelling in dependence of their concentration. Also a mixture of components describing a formulation suitable for stabilisation of ACT is tested.

| Excipient | Concentration in hydrogel | Elastic modulus [mN/mm$^2$] | Restoring energy [%] | Swelling q | Residual moisture [%] |
|---|---|---|---|---|---|
| Control | | 22.0 | 28.2 | 66.26 | 0.5 |
| Potassium phosphate buffer pH 7.2 | 50 mM | 39.2 | 12.8 | 50.8 | 0.6 |
| Potassium citrate buffer pH 7.2 | 50 mM | 41.1 | 15.0 | 33.5 | 0.6 |
| Tris HCl buffer pH 7.2 | 50 mM | 20.3 | 42.3 | 34.4 | 0.5 |
| Sodium chloride | 0.9% | 35.3 | 15.6 | 32.4 | 0.5 |
| Potassium chloride | 0.9% | 34.9 | 13.2 | 33.8 | 0.5 |
| Calcium chloride | 0.5% | 45.3 | 15.9 | 24.3 | 0.8 |
| Magnesium chloride | 0.5% | 40.8 | 22.3 | 28.7 | 0.7 |
| EDTA Na | 0.5% | 35.7 | 12.2 | 32.6 | 0.7 |
| Arginine | 2% | 29.9 | 13.5 | 45.8 | 1.0 |
| Tween® 80 | 0.2% | 24.9 | 28.3 | 69.7 | 0.9 |
| Poloxamer® 188 | 0.2% | 25.2 | 25.1 | 67.0 | 0.8 |
| Sucrose | 2% | 48.7 | 40.8 | 22.3 | 1.8 |
| Mannitol | 2% | 46.3 | 43.2 | 25.8 | 1.5 |
| β-Cyclodextrin | 1% | 42.6 | 35.7 | 32.4 | 1.4 |
| Polyvinyl pyrrolidone 17PF | 2% | 19.6 | 45.3 | 52.8 | 0.9 |
| Polyvinyl pyrrolidone 90F | 2% | 18.6 | 48.7 | 38.6 | 0.8 |
| Polyethylenglycol 2.000 | 2% | 17.8 | 52.8 | 36.1 | 0.8 |
| Polyethylenglycol 20.000 | 2% | 19.2 | 59.3 | 55.2 | 0.9 |
| Polyvinyl alcohol 100.000 | 0.05% | 22.3 | 31.2 | 34.1 | 0.5 |
| Crosslinked carboxymethyl cellulose sodium | 5% | 42.1 | 25.3 | 36.2 | 1.2 |
| Carboxymethyl starch | 4% | 25.3 | 30.2 | 31.1 | 1.4 |
| Xanthan gum | 0.5% | 23.2 | 32.1 | 36.1 | 1.1 |
| Gellan gum | 0.5% | 29.2 | 48.3 | 50.6 | 0.9 |

Tab. 3-13 Excipients in hydroxyethyl cellulose matrices; xerogels are formed from 2.5% HEC 100.000 hydrogels with named excipients and concentrations; given is the influence on dry mechanics by elastic modulus and restoring energy, the influence on swelling behaviour and on residual moisture after lyophilisation (n=2);

*Buffers* – Both tested buffer types – potassium phosphate and Tris hydrochloride – decrease swelling in all tested concentrations. Except for very low contents there seems to be only minor dependence on the concentration of the buffer. The disturbance of swelling is far more pronounced with Tris hydrochloride than with the phosphate system (Fig. 3-39).

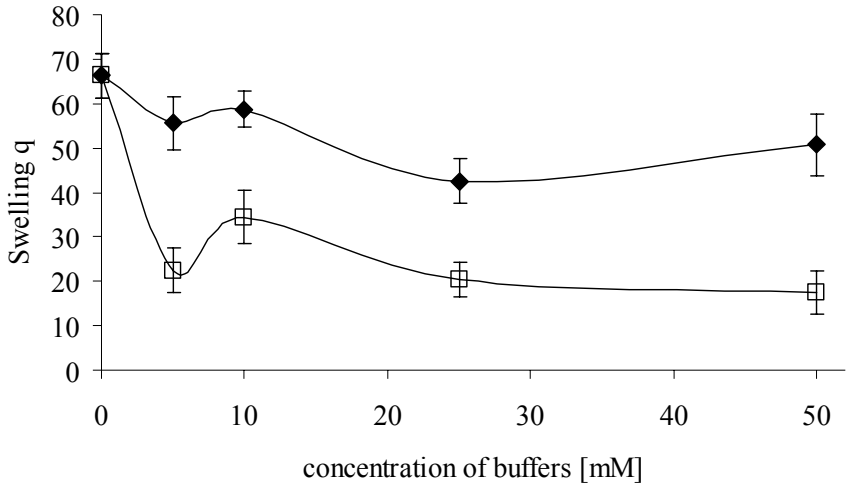


Fig. 3-39 Swelling behaviour of xerogels made from hydrogels containing 2.5% hydroxyethyl cellulose 100.000 and various phosphate buffer (◆) and TrisHCl buffer (□) contents;

*Surfactants* – Both candidates increase swelling at all concentration levels. But lower concentrations seem to be more effective. Also, with Tween® 80 this effect is more pronounced than with Poloxamer® 188 (Fig. 3-40). Further, not only the swelling value but also the speed of soaking with liquid and swelling is increased (data not shown).

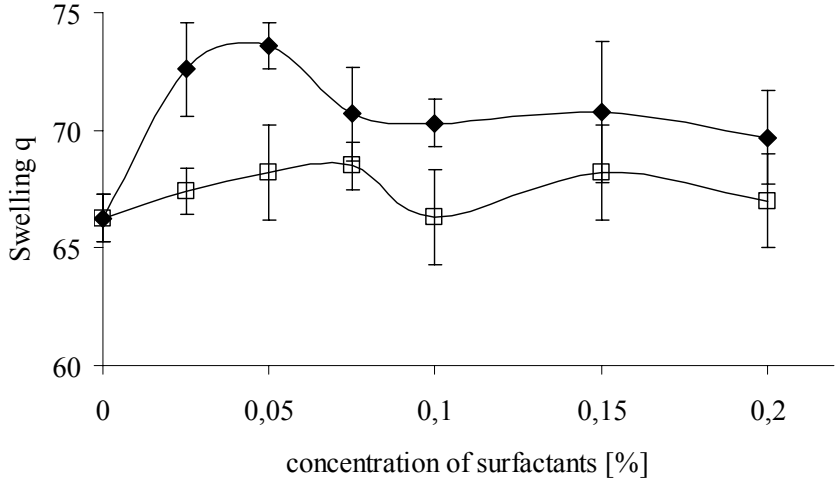


Fig. 3-40 Swelling behaviour of xerogels made from hydrogels containing 2.5% hydroxyethyl cellulose 100.000 and various Tween® 80 (◆) and Poloxamer® 188 (□) contents;

*Soluble Polymers* – Depending on concentration these substances can do both an increase and decrease of xerogel swelling. Very low contents of less than 0.1% effect a high increase in swelling, whereas concentrations exceeding 0.1% lead to a decrease (Fig. 3-41).

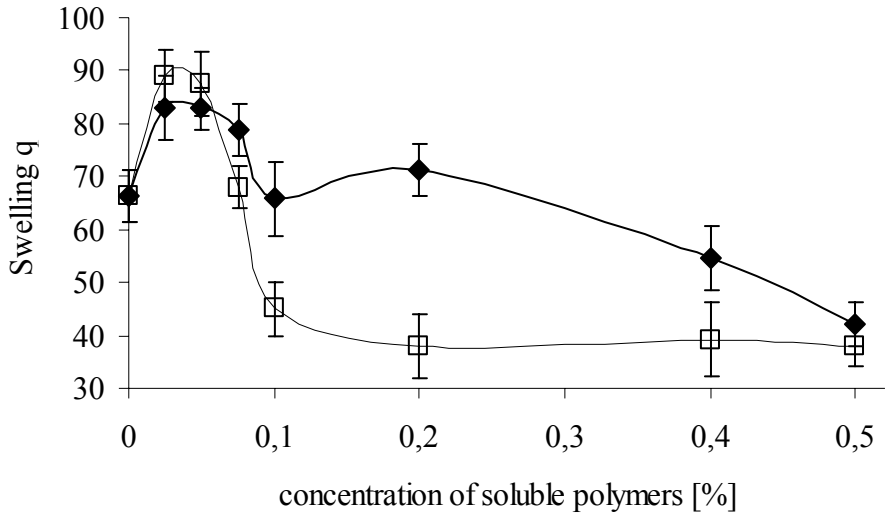


Fig. 3-41 Swelling behaviour of xerogels made from hydrogels containing 2.5% hydroxyethyl cellulose 100.000 and various polyvinyl pyrrolidone 17 (◆) and polyethylene glycol 2.000 (□) contents

*Formulation Mixture* – Finally, formulations suitable for stabilisation of ACT are evaluated. The formulations are made from hydrogels consisting of 10mM buffer, 10mM amino acid, 0.1% surfactant, 0.05% soluble polymer, and 2.5% hydroxyethyl cellulose 100.000. According to this pattern the substances potassium phosphate, Tris hydrochloride, potassium phosphate/citrate, arginine, Tween® 80, Poloxamer® 188, polyvinyl pyrrolidone 17PF, and polyethylene glycol 2000 are combined in all possible variations.

All prepared xerogel pads appear acceptable. Swelling is satisfactory in all cases with values gained exceeding 65.0. Residual moistures have been detected lower than 1%.

The observed hardening of the xerogels with some excipients should be explained by a support of the porous polymer structure by substances with stronger intermolecular bindings, e.g. ionic bindings in the case of electrolytes and hydrogen bonds in the case of sugars. On the contrary, intermolecular bonds between gellant molecules should be plastisised by surfactants and other soluble polymers leading to a softening of the pad.

For visualisation of the reconstitution of placebo xerogel formulations one example is displayed in Fig. 3-42.

The xerogel pad is soft and has a favourable as well as homogeneous appearance. When put in contact with aqueous solution the pad soaks within seconds until completeness without air bubbles being entrapped. For the next two to three

minutes the pad keeps its shape before the gel forming process is finished and the gel starts flowing like it is typical for non-crosslinked gels (Fig. 3-42). So, from a practical point of view this provides enough time after external reconstitution for application into the wound site.

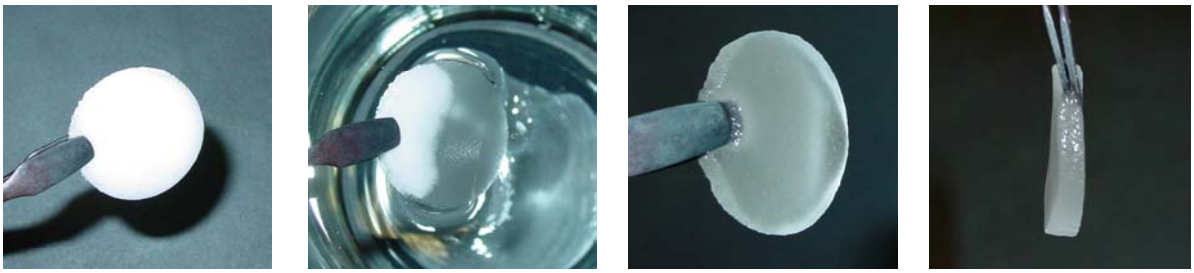

Fig. 3-42 Swelling behaviour of xerogels made from hydrogels containing 2.5% hydroxyethyl cellulose 100.000, 10 mM potassium phosphate buffer pH 7.2, 10 mM arginine, 0.1% Tween® 80, 0.05% polyvinyl pyrrolidone 17; left: dry pad, 2nd from left: pad is soaking when put in contact with water, 3rd from left and right: soaked pad stays in shape for 2-3 minutes before flowing starts

To conclude, the development of hydroxyethyl cellulose xerogels with formulation ingredients suitable for delivery of ACT into open wounds has grown to an acceptable state. These patterns are used as starting points for the development of ACT loaded xerogels.

#### *3.4.1.2.3 Hydroxyethyl cellulose / gellan gum mixtures for xerogel formation*

For its convenient mechanics in the hydrogel state the mixture of hydroxyethyl cellulose 100.000 with gellan gum F is also investigated for xerogel formation. According to the rheological examinations the relation of gellant components in the mixture is tested. The concentration range thereby is between 1.5% and 3.0% for hydroxyethyl cellulose and between 0.7% and 1.5% for gellan gum. Due to the sensitivity of the rheology of gellan gum to ionic additives this examination is done both without any further excipients and with the addition of mixtures of formulation excipients, respectively. Moreover, swelling studies are conducted with water and isotonic sodium chloride solution, respectively, as reconstitution media.

All the tested xerogels show an acceptable optical appearance. Moreover, they do not differ much in terms of mechanical properties, swelling, and residual moisture. So, instead of detailed datasets on all ingredient variations more generalised values are given.

The pads are harder than the pure hydroxyethyl cellulose pads. So, the elastic moduli are measured between 27.5 and 32.1, and restoring energies are found around 45%. Residual moistures are detected around 1%.
Swelling is also hardly affected by gellant composition in the given range, i.e. q values of about 50 are found for the swelling without excipients. With formulation additives swelling decreases slightly to a q value of 35 to 45 but is still excellent. The reconstitution with water and isotonic sodium chloride solution, respectively, solely differs in the rheology of the resulting hydrogel. The resulting gel pads provide the same differences in rheological properties as the underlying hydrogels (Fig. 3-43). The reconstitution process itself seems unaffected.

Fig. 3-43 Hydroxyethyl cellulose / gellan gum xerogel pad produced from a hydrogel containing a 2% / 1% mixture of hydroxyethyl cellulose 100.000 and gellan gum F swollen in water; left: dry pad before reconstitution; center: flowing gel pad 5 min after reconstitution with water; right: non flowing gel pad 5 min after reconstitution with isotonic sodium chloride solution;

Therefore, it can be concluded that the mixture of hydroxyethyl cellulose and gellan gum is suitable for xerogel formation.
Finally, for further studies the relation of the two gellants in the mixture is taken over from the hydrogel section. This is done because all tested relations are acceptable for xerogel formation but in the hydrated state an optimum has been found for a 2.0% / 1.0% mixture. This mixture, therefore, is acceptable for both the dry and hydrated state. In fact, further studies are conducted with a 2.0% / 1.0% hydroxyethyl cellulose 100.000 / gellan gum mixture.

#### *3.4.1.2.4 Other polymers for xerogel formation*

*Screening* - next to hydroxyethyl cellulose further series of experiments were conducted on a variety of polymers. Xerogels were prepared from hydrogels consisting of the polymer swollen in water. Autoclavation was carried out where possible as outlined in the hydrogel section. The mechanical testing concerning

swelling and texture analysis as well as moisture measurement were performed as above (Tab. 3-14).

| Polymer | Quality | Concentration in hydrogel [%]l | Elastic modulus [mN/mm$^2$] | Restoring energy [%] | Swelling q | Residual moisture [%] |
|---|---|---|---|---|---|---|
| Alginate Sodium | SF250 | 4.5 | 57.4 | 56.0 | 12.6 | 1.4 |
| Carboxymethyl cellulose sodium | 1.000 | 6.0 | 58.7 | 32.8 | 11.2 | 0.9 |
| Carboxymethyl cellulose sodium | 10.000 | 5.0 | 55.3 | 30.2 | 15.8 | 0.8 |
| Carboxymethyl cellulose sodium | 30.000 | 10.5 | 51.0 | 31.8 | 36.9 | 0.9 |
| Carboxymethyl cellulose sodium | crosslinked | 11.5 | 12.1 | 9.5 | 67.4 | 0.4 |
| Hydroxypropyl cellulose | 100.000 | 2.5 | 21.0 | 26.2 | 56.1 | 0.6 |
| Hydroxypropylmethyl cellulose | 15.000 | 3.5 | 29.0 | 28.2 | 6.8 | 0.7 |
| Hydroxypropylmethyl cellulose | 100.000 | 2.5 | 22.3 | 30.2 | 58.6 | 0.9 |
| Methyl cellulose | 1.000 | 7.0 | 29.1 | 39.9 | 4.5 | 1.1 |
| Carboxymethyl starch sodium | | 8.5 | 35.0 | 41.7 | 27.3 | 1.3 |
| Gellan gum | LT100 | 1.5 | 35.2 | 44.2 | 45.8 | 1.1 |
| Gellan gum | F | 1.5 | 34.4 | 41.6 | 56.1 | 0.9 |
| Xanthan gum | | 7.0 | 29.3 | 22.4 | 16.0 | 1.0 |
| Dextran | 60 | 40 | - | - | - | - |
| Gelatine | A | 10 | 53.5 | 45.8 | 2.97 | 2.0 |
| Polyacrylate Sodium | Carbopol 940® | 1.0 | 19.0 | 10.2 | 88.5 | 1.5 |
| Eudragit® | L100 | 1.0 | - | - | - | - |
| Lutrol® | F127 | 15 | 25.0 | 36.1 | 6.2 | 2.5 |
| Polyvinyl pyrrolidone | Kollidon® 90F | 35 | - | - | - | - |
| Polyvinyl pyrrolidone | Kollidon® 17PF | 45 | - | - | - | - |
| Polyethylen glycol | 20000 | 75 | - | - | - | - |
| Polyethylen glycol | 2000 | 75 | - | - | - | - |

Tab. 3-14 Screening of gelling agents for lyophilisation; xerogels are formed from named polymers in listed concentrations; given is the influence on dry mechanics by elastic modulus and restoring energy, the influence on swelling behaviour and on residual moisture after lyophilisation (n=2);

Concerning optical appearance and residual moisture with most of the listed materials satisfactory xerogel formation is possible. However, with polyvinyl pyrrolidone, polyethylene glycol, and Eudragit® no acceptable xerogel formation was achieved. Therefore, these polymers were not further investgated.

Within the optically acceptable samples, though, swelling performance during hydration and texture properties are very diverted. The best performing polymers in this setting are the group of non-ionic cellulose ethers, i.e. hydroxypropyl cellulose, hydroxypropylmethyl cellulose, and methyl cellulose with properties similar to hydroxyethyl cellulose outlined above. Hereby, again the higher molecular weight turned out to be advantageous. More lipophilic derivatives provide reduced swelling properties. This is supposed to be related to reduced interactions with hydrophilic reconstitution media.
Non-crosslinked carboxymethyl cellulose sodium, alginate sodium, and xanthan gum form harder and more slowly swelling matrices due to the strong ionic binding character of these polymers. The crosslinked carboxymethyl cellulose sodium and carboxymethyl starch that are commonly used as tablet disintegrants provide an amazing swelling behaviour. But, the crosslinked carboxymethyl cellulose matrix is not coherent and disintegrates into powdery snatches under slight mechanical stress. Carboxymethyl starch suffers from the hardness of the xerogel structure. A huge swelling capacity is also seen with pure gellan gum, but it as well suffers from hardness of the matrix. Polyacrylate sodium also swells to huge extend but on the contrary forms an only very weak structure due to the low content. Moreover, gelatine forms very hard and hardly swelling matrices.

*Excipients* - excipient compatibility studies according to those with hydroxyethyl cellulose are conducted on carboxymethyl cellulose 10.000, hydroxypropylmethyl cellulose 15.000, hydroxypropylmethyl cellulose 100.000, and hydroxypropyl cellulose 100.000. Although experiments are performed as previously described results are given out in a short summary for reasons of lucidity.
Concerning compatibility for all tested types of xerogels the influence of additives is similar as on hydroxyethyl cellulose xerogels. However, in all samples hydroxypropylmethyl cellulose and hydroxypropyl cellulose matrices still show a reduced swelling compared to those of hydroxyethyl cellulose. Experimental values are gained between 17 and 45 for both polymers. Furthermore, as carboxymethyl cellulose matrices generally are harder in texture due to higher density and the ionic structure the effects of additives are reduced. Also, an improvement of swelling behaviour by excipients is provoked only to a very limited extent.

## 3.4.2 Stability of ACT in xerogel formulations

According to the results of the mechanical studies on xerogels most of the relevant protein stabilisers are compatible with the xerogels from that point of view and so can be used for further development of ACT stabilising formulations. Therefore, excipients are introduced in lyophilisation studies on ACT formulations that were tested positively in the prior stability studies on ACT concerning temperature and freeze/thaw stressing on the one hand, as well as in mechanical studies on xerogels on the other hand. The investigation of excipients in lyophilisation tests additionally to F/T stress – described in the hydrogel section - is necessary because denaturation can be induced by both freezing and subsequent drying[179]. First, the lyophilisation process itself as stress factor is investigated. Second, a three months stability study on xerogels is conducted.

### *3.4.2.1 Stability of ACT during the lyophilisation process*

Due to the fact that most sugarlike substances demonstrated a destabilising effect on ACT in solution the polymers are used as bulking agents. Thereby, we have to accept that the known protective effect of disaccharides on proteins may not be mimicked by the polymers[180].
Sample gels are prepared in eppendorf caps to 0.1ml volume with 60µg/ml ACT and the excipients to be tested. In the further descriptions all concentrations of substances refer to the hydrated state of the particular gel. After swollen homogeneously the gels are freeze-dried using the procedure with annealing step described above whereby the xerogels are formed. These are reconstituted with 40mM potassium phosphate buffer pH 7.2 immediately after lyophilisation adding up to 50mM buffer defined as standard in the solution stability testing. Due to the high resulting viscosities samples are diluted for analysis as before with 50mM potassium phosphate buffer pH 7.2 containing 0.1% Poloxamer® 188.
*Buffers* - the first group of ingredients to be tested are the buffer components. Thus, 10mM potassium phosphate, 5/5mM potassium citrate/phosphate mixture, 10mM Tris hydrochloride, 10mM arginine phosphate, and 5/5mM arginine citrate/phosphate mixture are tested in 2.5% hydroxyethyl cellulose matrices (Fig. 3-44).
In all buffer systems losses between 30% to 45% are detected. This indicates the necessity of further stabilisers. Although arginine seems to effectuate further reduction of recovered ACT activity compared to arginine free samples differences

are too marginal to lead to a decision over a favourite buffer system. This is to be evaluated during mid term stability tests.

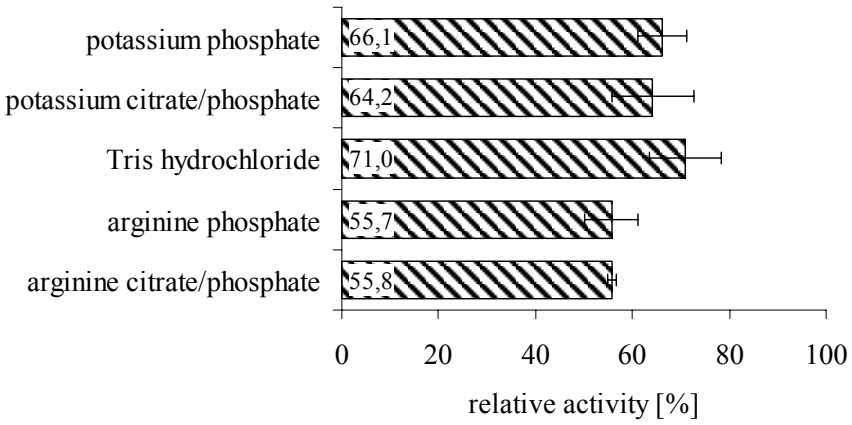


Fig. 3-44 Relative activity of ACT in 2.5% hydroxyethyl cellulose 100.000 xerogels with 10mM buffers after reconstitution with 40mM potassium phosphate buffer pH 7.2 measured by activity assay;

*Surfactants and soluble polymers* - in the next series the influence of surfactants and soluble polymers is studied. Though, generally surfactants are not known to inhibit protein unfolding during dehydration[181], they are tested for lyoprotection because they have been proven to be beneficial during freezing in the solution stability section. The soluble polymers are also known as lyoprotectants of proteins[182,183].
Samples with a basic matrix consisting of 2.5% hydroxypropylmethyl cellulose 100.000 and 10mM potassium phosphate buffer are spiked with 0.1% Poloxamer® 188, 0.1% Tween® 80, 0.5% PEG 2000, and 0.5% PVP 17, respectively. The control sample next to ACT solely contains gellant and buffer. Freeze drying, dilution and analysis is carried out as above.
Surfactants clearly improve the stabilisation of ACT during lyophilisation, whereby Tween® 80 appears more effective than Poloxamer® 188. In accordance with literature, this may be explained by the protective activity of surfactants during freezing. PEG 2000 as well leads to higher recovered ACT activity. In contrast, the PVP 17 sample is comparable to the control, so PVP can be considered as inert (Fig. 3-45). It has been reported that soluble polymers like PEG[184], PVP[185 186], and PVA[187] interact with proteins during freezing and drying e.g. by preferential steric exclusion. These interactions may lead to either stabilisation, destabilisation, or no change in protein stability depending on the particular protein and other factors, e.g. concentration.

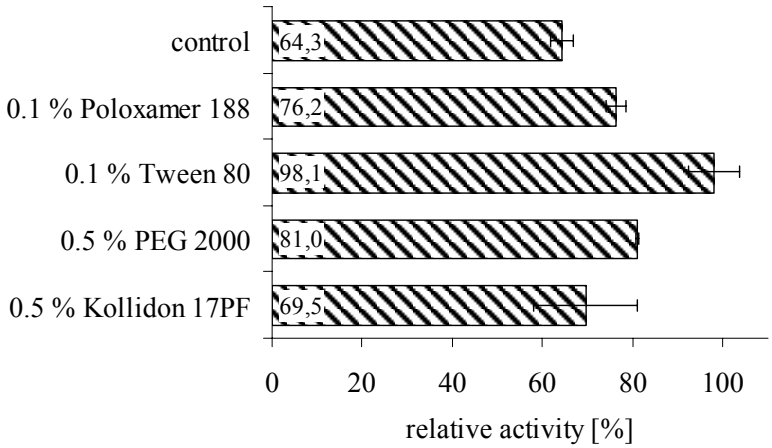


Fig. 3-45 Relative activity of ACT in 2.5% hydroxypropylmethyl cellulose xerogels with 10mM potassium phosphate buffer pH 7.2 after reconstitution with 40mM potassium phosphate buffer pH 7.2 measured by activity assay; control without additional spiking, samples with surfactant and soluble polymer, respectively;

*Gelling agents and hydroxypropyl-β-cyclodextrin* - the different gelling agents are tested in already improved formulations. Indeed, they contain 10mM potassium phosphate buffer pH 7.2, 0.1% Tween® 80, 0.05% PEG 2000, and variing polymers as gelling agents. These are hydroxypropyl cellulose, hydroxypropylmethyl cellulose, and hydroxyethyl cellulose. Moreover, a gellan gum/hydroxyethyl cellulose mixture, carboxymethyl cellulose sodium, and alginate sodium are tested. A variation with hydroxypropyl-β-cyclodextrin added to each gellant was conducted in parallel. Without cyclodextrin the non-ionic polymers perform better than the ionic and sodium containing gelling agents. The gellan gum/HEC mixture surprisingly shows highest recovered ACT activities. It has been reported that this class of substances can provide stabilising capacity on proteins[188]. The ionic, sodium containing polymers may interact with the phosphate buffer system and lead to the well known pH shift during freezing. This may lead to the detected loss of activity. The series with cyclodextrin addition features generally lower activity values than without cyclodextrin. Most notably, the samples with the more lipophilic agents suffer from the cyclodextrin influence (Fig. 3-46).

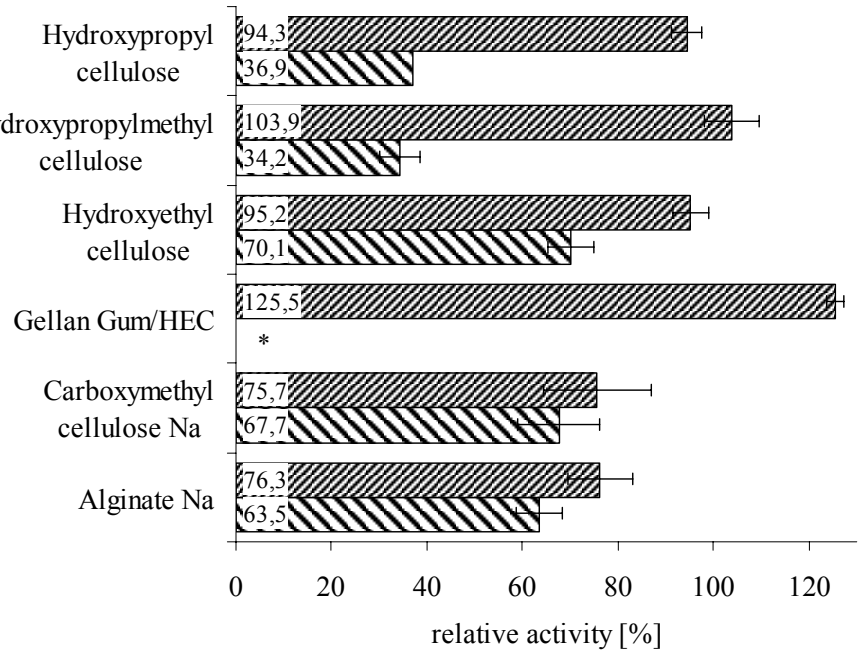


Fig. 3-46 Relative activity of ACT in xerogels with 10mM potassium phosphate buffer, 0.1% Tween® 80, 0.05% PEG 2000 and gelling agent (above) and additional 0.0075% hydroxypropyl-β-cyclodextrin spike (below) after reconstitution with 40mM potassium phosphate buffer measured by activity assay; gellants are 2.5% hydroxypropylmethyl-, hydroxypropyl-, hydroxyethyl cellulose 100.000, gellan gum/hydroxyethyl cellulose 100.000 1%/2% mixture, 3.0% carboxymethyl cellulose sodium 10.000, 5.0% alginate sodium; * sample not measured;

To summarise, in this section the influence of the lyophilisation process for xerogel formation as stress factor on ACT stability is evaluated. It is found that xerogel formation with embedded active ACT is possible. Excipients can be chosen in concordance with results from studies on solution, hydrogel, and placebo xerogel. Buffer variations can be used as tested although arginine seems to be awkward. Surfactants and soluble polymers can be used without constraints. For the gelling agents non-ionic species are preferable. The gellan gum containing samples surprisingly turn out to provide the most effective stabilisation on ACT.

### *3.4.2.2 Mid term stability of ACT in xerogel formulations*

Based on the previous results of the stabilisation in hydrogels and xerogels a three months stability test in xerogels was launched with a set of formulations. Variations are made in excipient composition. For the surfactant Tween® 80 and Poloxamer® 188 are evaluated, for buffer systems phosphate, phosphate/citrate, and Tris hydrochloride are tested. As in the hydrogel tests, for gellants only hydroxyethyl cellulose and mixtures of hydroxyethyl cellulose with acylated (LT100) and

deacylated (F) derivatives of gellan gum are investigated. Despite other non-ionic cellulose ethers could also be considered according to the given results hydroxyethyl cellulose is chosen for its suitability for autoclavation. Additionally, the soluble polymers are varied between polyvinyl pyrrolidone and polyethylene glycol.
Samples of ACT loaded gels were prepared under aseptic conditions as before. Packaging containers are glass vials that are sealed with a Teflon®-coated lyophilisation rubber stopper. The concentration of ACT in hydrated state was 60 µg/ml, sample volume was 0.4 ml. After swollen homogeneously the gels are freeze-dried using the procedure with annealing step described above whereby the xerogels are formed.
Xerogels are then stored at two temperature levels, 25°C and 40°C. After 4, 8, and 12 weeks storage time samples are drawn and stored at –80°C until analysis. Xerogel samples then are reconstituted and prediluted as described above. Readout is again done by activity assay. For the samples stored for three months SDS-PAGE is performed in addition.
Calculations described in detail in the hydrogel section are conducted to estimate reaction kinetics and shelf lives under storage conditions. The 25°C level simulates real conditions for storage at room temperature, 40°C simulates temperature stress conditions. Other than in the hydrogel study the 6°C temperature level was omitted in this setting. This was done because the changes of measured values were estimated to be too small in comparison to the spreading of measured values delivered by the activity assay. In that case reasonable conclusions would be impossible. Moreover, a refrigerated storage of a lyophilised product at this temperature level would be hardly acceptable due to marketing concerns.
The assessment of samples is done in pursuance with the suitability of the xerogel formulation as future storage form for stocking at room temperature. Therefore, the 25°C estimation has to exceed 18 months above the lower specification limit.

#### *3.4.2.2.1 Principles of data interpretation*

Generally, results are gained as a set of activity loss curves over time for every formulation and temperature level. An example is shown in Fig. 3-47. From a starting value of about 100% the activity declines over the 3 months of storage time.

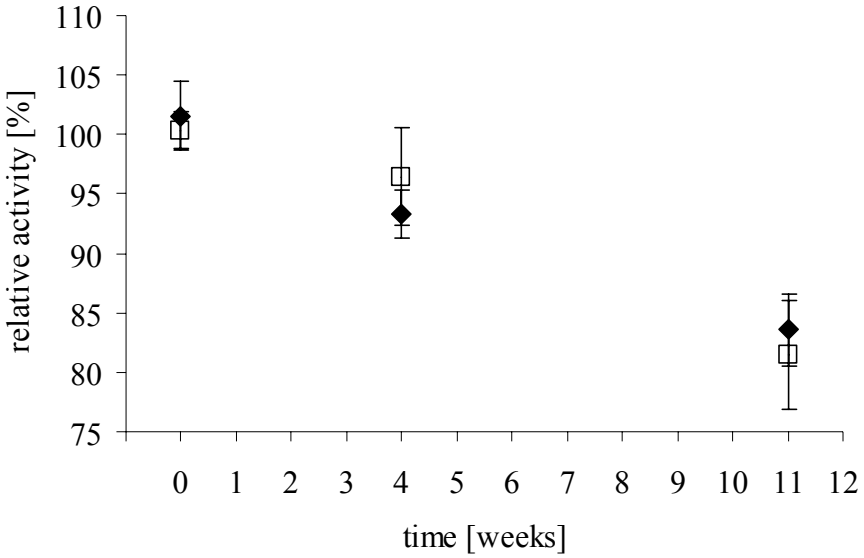


Fig. 3-47 Recovered relative activities of a xerogel formulation consisting of 60µg/ml ACT, 10mM arginine, 0.1% Poloxamer® 188, 0.05% PEG 2000, 2.5% hydroxyethyl cellulose 100.000 in a 10mM potassium phosphate buffer pH 7.2 at 25°C (◆) and 40°C (□) in linear scale measured by activity assay;

As in the mid-term stability study on hydrogels reaction kinetic functions are fitted to the modified curves and next to the equation parameters the correlation coefficient is determined and used for assessing the feasibility of the fitted reaction order. Fig. 3-48 shows an example for the fitting of the first order time law.

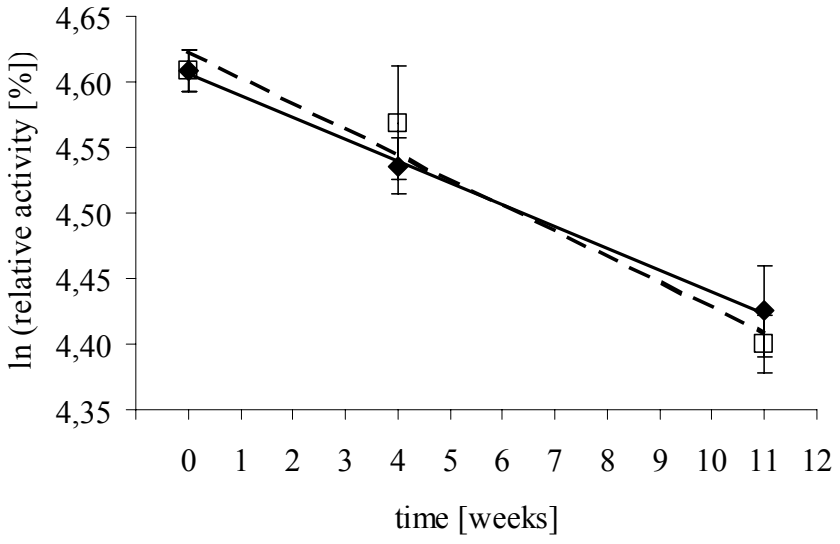


Fig. 3-48 Recovered relative activities of a xerogel formulation consisting of 60µg/ml ACT, 10mM arginine, 0.1% Poloxamer® 188, 0.05% PEG 2000, 2.5% hydroxyethyl cellulose 100.000 in a 10mM potassium phosphate buffer at 25°C (◆) and 40°C (□) in logarithmic concentration scale with fitted logarithmic functions resulting in evens in the one-sided logarithmic scale as predicted by first-order time law; measured by activity assay;

Results indicated that the best fittings given by correlation coefficients are gained for the first order equation, i.e. a logarithmic scale for relative activity results. In fact, the coefficients for 1st order are between 0.985 and 0.999, for zero order coefficients are between 0.950 and 0.992, and coefficients for 2nd order are below 0.96.

Like in the hydrogel section datasets are tested according to the guideline ICH-QA1. Further, for an estimation of the shelf life the 95%-confidence intervals are calculated for the curves where appropriate. The intercept of the lower confidence limit with the lower specification limit – in our case 10 % loss of relative activity - indicates the end of the shelf life. Alternatively, the intercept of the extrapolated decay even with the lower specification limit is equated with the end of shelf life.

#### *3.4.2.2.2 Experimental results and discussion*

The kinetics of the decay curves follow most likely the first order time law (Fig. 3-48) because correlation coefficients are best for the first order kinetics in most cases (data not shown).

But, all datasets show change-over-time and additionally many are variable according to ICH-QA1 guideline (calculations not shown). Thus, confidence intervals of most datasets are too large to allow a sensible, statistically profound estimation of shelf life as described above.

Instead, the described alternative is applied. The estimation is made by simple extrapolation of the fitted curve to the intercept with the 10% loss level as lower specification limit. For all formulations, consequently, first order reaction constants for every temperature level are calculated and corresponding shelf lives are estimated (Tab. 3-15).

(1)

| | | | |
|---|---|---|---|
| 60µg/ml | α1-antichymotrypsin | reaction constant k (24°C) | $8.71 \cdot 10^{-4}$ 1/day |
| 10mM | phosphate buffer | reaction constant k (40°C) | $3.08 \cdot 10^{-3}$ 1/day |
| 10mM | arginine | shelf life 25°C | 121.0 days |
| 0.1% | Poloxamer® 188 | shelf life 40°C | 34.3 days |
| 0.05% | PVP 17 | | |
| 2.5% | HEC 100.000 | | |

(2)

| | | | |
|---|---|---|---|
| 60µg/ml | α1-antichymotrypsin | reaction constant k (24°C) | $1.48 \cdot 10^{-4}$ 1/day |
| 10mM | phosphate buffer | reaction constant k (40°C) | $1.21 \cdot 10^{-3}$ 1/day |
| 10mM | arginine | shelf life 25°C | 711.3 days |
| 0.1% | Tween® 80 | shelf life 40°C | 87.3 days |
| 0.05% | PVP 17 | | |
| 2.5% | HEC 100.000 | | |

(3)

| | | | |
|---|---|---|---|
| 60µg/ml | α1-antichymotrypsin | reaction constant k (24°C) | $1.17 \cdot 10^{-3}$ 1/day |
| 10mM | phosphate buffer | reaction constant k (40°C) | $3.53 \cdot 10^{-3}$ 1/day |
| 10mM | arginine | shelf life 25°C | 89.9 days |
| 0.1% | Poloxamer® 188 | shelf life 40°C | 29.9 days |
| 0.05% | PEG 2000 | | |
| 2.5% | HEC 100.000 | | |

(4)

| | | | |
|---|---|---|---|
| 60 µg/ml | α1-antichymotrypsin | reaction constant k (24°C) | $8.68 \cdot 10^{-4}$ 1/day |
| 10mM | phosphate buffer | reaction constant k (40°C) | $3.47 \cdot 10^{-3}$ 1/day |
| 10mM | arginine | shelf life 25°C | 121.4 days |
| 0.1% | Poloxamer® 188 | shelf life 40°C | 30.4 days |
| 0.05% | PVP 17 | | |
| 2.0% | HEC 100.000 | | |
| 1.0% | gellan gum LT100 | | |

(5)

| | | | |
|---|---|---|---|
| 60µg/ml | α1-antichymotrypsin | reaction constant k (24°C) | $1.92 \cdot 10^{-4}$ 1/day |
| 10mM | phosphate buffer | reaction constant k (40°C) | $1.40 \cdot 10^{-3}$ 1/day |
| 10mM | Arginine | shelf life 25°C | 547.7 days |
| 0.1% | Poloxamer® 188 | shelf life 40°C | 75.5 days |
| 0.05% | PVP 17 | | |
| 2.0% | HEC 100.000 | | |
| 1.0% | gellan gum F | | |

(6)

| | | | |
|---|---|---|---|
| 60µg/ml | α1-antichymotrypsin | reaction constant k (24°C) | $4.06 \cdot 10^{-4}$ 1/day |
| 10mM | Tris HCl buffer | reaction constant k (40°C) | $1.19 \cdot 10^{-3}$ 1/day |
| 10mM | Arginine | shelf life 25°C | 259.3 days |
| 0.1% | Poloxamer® 188 | shelf life 40°C | 88.4 days |
| 0.05% | PVP 17 | | |
| 2.0% | HEC 100.000 | | |

Tab. 3-15 Formulations of the mid term stability testing with ingredients (left column), first order reaction constants k at the experimental stress levels, and estimated shelf lives at the temperature levels (right column);

Unless data show variation according to the ICH-QA1 guideline several conclusions can be made. For xerogels stored in glass vials the Tris hydrochloride system has greater stabilising potential than the phosphate buffer. This is manifested in shelf lives of formulation (1) in comparison with (6). The surfactant

Tween® 80 performs better than the Poloxamer® 188 alternative. See comparison of formulation (1) with (2). For the soluble polymers PVP is favourable over PEG as shelf lives of formulation (1) and (3) indicate. Moreover, the HEC / gellan gum F gellant system is favourable over pure hydroxyethyl cellulose and the HEC/gellan gum LT100 alternatives. This results from comparison of formulation (1) with (4) and (5).
So, there are the two formulations – (2) and (5) - with shelf lives of about or greater 550 days (18 months). Thus, these meet the stability requirements for a storage form for stocking at room temperature defined at the beginning of development.

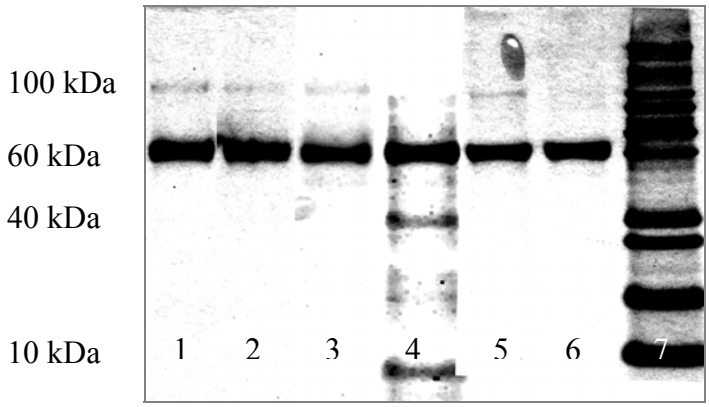


Fig. 3-49 Stability of ACT in formulations after a 3 months storage at 25 °C measured by SDS-PAGE:
lane 1: formulation (1) with 9.4% 100kDa aggregate; lane 2: formulation (2) with 4.7% 100kDa aggregate; lane 3: formulation (3) with 3.5% 100kDa aggregate; lane 4: formulation (4) with 1.7% 100kDa aggregate, 7.2% 40kDa, 10.7% 10kDa, and other fragments; lane 5: formulation (5) with 3.5% 100kDa aggregate; lane 6: formulation (6) with 0.7% 100kDa aggregate; lane 7: marker;
control without degradation is visualised in Fig. 3-4

The readout by SDS-PAGE for samples stored over three months at 25°C shows the dimer formation again as main instability. Again the addition of Tween® 80, Tris hydrochloride and the HEC/gellan gum F are favourable over particular alternatives. With gellan gum LT100 an extensive fragmentation is detected. Hence, the formulations (2) and (5) can be recommended for further development.
However, the greatest stabilising potential of a xerogel formulation is to be estimated for a combination of Tween® 80 and HEC/gellan gum F maybe in a Tris hydrochloride buffer system. Although, this combination is not yet tested and therefore cannot be recommended for immediate further development. But, in future studies, e.g. adjustment of the formulation for market launch, this can be taken into account.

#### *3.4.2.2.3 Summary*

Results indicate that reaction kinetics of activity decay of ACT in xerogels is highly likely to follow a first order equation. Main instability again is the loss of activity and dimer formation. Moreover, a commercial formulation of ACT in a xerogel is possible due to sufficient shelf life. Furthermore, the two suitable formulations after reconstitution can be applicated into the wound site as far as stability of ACT is concerned. As well, these formulations are applicative for the purpose of animal experiment or clinical trial. Results also indicate that a not yet tested excipient combination may be the most effective stabilising formulation. This can be evaluated within later studies, e.g. adjustment of the product for market launch.

## 3.4.3 Polymer films as drug delivery systems for wound healing

To date, there are several non-swellable polymer film products used as drug free wound dressings in clinical practice. For example, OpSite® by Smith&Nephew consists of a polyurethane film with a polyacrylate adhesive.
Besides, few patents have been applicated on swellable film dressing materials[189]. Some of these films also contain protein drugs[190]. But, like with xerogel matrices none of those developments has led to approval by authorities let alone to market launch, yet.
In this section, the development of a production process for placebo swellable film carriers and investigations of their mechanical properties are described. Thereby, influence of the drying process, of the polymer composition, and of further formulation ingredients are highlighted.
These studies are aiming at ensuring the suitability of the films as wound dressing and at investigating the compatibility with protein formulation ingredients. The development strategy, the used methods, and experimental design is similar to the xerogel development outlined in the previous chapter. For that reason, to avoid redundancy the description of methods and discussions is shortened with reference to the particular chapter of the xerogel studies. Moreover, the differences between xerogel and film development are stressed.
So, one major difference is the swelling behaviour of films compared to that of xerogels. Films do not provide an open pore structure. Rather, they swell from outside to inside which is a much slower process than the soaking and swelling of xerogels. Thus, the swelling is not supposed to be completed when the film is applicated into the wound site. The wound will be confronted with a semi-reconstituted state of the film. Therefore, the texture is the most important mechanical feature for polymer films. In fact, a certain robustness and a soft consistency is highly desirable for application and compliance reasons.

### *3.4.3.1 Production process*

There are several methods in use for production of polymer films. In the field of oral dosage forms polymer films serve as coating material. There, the film is formed by spraying a polymer dispersion onto the tablet core followed by evaporation of the dispersion media. For self supportive films the most common method of film manufacture is blown film extrusion. The process involves extrusion of a plastic

melt through a circular die to form a thin walled tube, followed by bubble-like expansion by air being introduced via a hole in the centre of the die. The outcome of this process is a film tubing that is cooled and flattened to create a lay-flat tube of film. The regulation of film width and thickness is done by control of the volume of air in the bubble and the output of the extruder. Polyethylene is the most common polymer in use for blown film. Next to that, films can be formed by precipitation out of particular baths. But, the method mainly used for water swellable polymers is the dry-cast method. Thereby, a melt or a solution of the polymer is extruded through a die onto a roll. There, the mass cools down to form a film robust enough to be transferred onto a mesh for drying. The drying may be completed in a drying tunnel. This procedure is appropriate for many pharmaceutically relevant polymers such as gelatine and the cellulose ethers. A special application of this method is the production of transdermal therapeutic systems. Thereby, the polymer matrix is not directly cast onto the roll but onto a backing layer being placed between the roll and the matrix. Maybe, the mass is cast on a web before the backing is added. Further transportation is also supported by the backing layer.

Although these procedures are widely used, they are not suitable for the minimal amounts of lab scale protein drug delivery. For that reason, two scale-down methods depending on the gel amount to be dried are investigated.

For higher gel amounts a method involving a scraper is used. Thereby, the gel is filled in the scraper apparatus which consequently is drawn over a glass plate. By mild extrusion through the defined gap resulting from the movement over the plate a wet film of constant thickness is formed (Fig. 3-50). This film can be dried under laminar flow at room temperature or in a cabinet drier at any desired temperature. When dry, the self supportive film is removed from the plate (Fig. 3-50). When later preparing ACT loaded films in very low scale the appropriate amount of gel is placed on the bottom of an eppendorf cap or a vial. Consequently, the mass is dried under a flow of nitrogen in a special device controlling both gas and product temperature[191]. The film forms on the bottom and side walls of the container (Fig. 3-50).

Fig. 3-50 Films made from 2.5% hydroxyethyl cellulose 100.000 hydrogels; left: 2R glass vial with dried film on the bottom an lower side walls; center: wet film being cast out with scraper; right: self supportive film after removal from plate;

### *3.4.3.2 Gel composition for polymer film formation*

A variety of polymers is screened for eligibility for warm air drying and film formation. The concentration of the gellant in the hydrogel is chosen according to the results of the rheological studies during hydrogel development. After drying the films are examined concerning optical appearance, texture analysis, swelling behaviour, and residual moisture. Thereby, as during xerogel development optimised film compositions are to be developed concerning texture properties and embedding of protein stabilisers.
Hence, films consisting of pure gelling agent are screened. In addition, texture properties of film compositions containing hydroxyethyl cellulose as gelling agent are optimised. Moreover, the formation of a film matrix made from two gellants – hydroxyethyl cellulose and gellan gum - is described. The compatibility of excipients with the particular film matrices is investigated subsequently.

#### *3.4.3.2.1 Gelling agents for film formation*

The screening of gelling agents is conducted in similar manner as during xerogel development described above.
From the variety listed in Tab. 3-12 only the cellulose derivatives, alginate, xanthan gum, and gellan gum form acceptable films concerning their optical appearance after drying. However, the ionic polymers carboxymethyl cellulose sodium and alginate sodium form very hard and stiff films. Especially alginate films tend to break before drying is completed. These characteristics again should be related to the strong ionic intermolecular forces in these polymers. Besides, xanthan gum and

gellan gum form softer but very brittle films. This implicates as well a tendency of breaking of the film in the later stages of drying. Finally, the non-ionic cellulose derivatives turn out as the most promising candidates. They form the softest and most elastic films in these experiments.
The hardness and stiffness of films can be counteracted by a plasticiser. In the film structure solely consisting of gelling agent, the individual molecules of the polymer lack mobility because of their mutual interference which is much higher when ionic binding is involved. The use of a plasticiser implicates the introduction of a lower molecular weight substance into the structure that acts as a molecular lubricant, physically separating the chains and allowing them some mobility, thus giving flexibility. Obviously, the larger the volume of plasticiser is, the greater is the flexibility and softness. Common plasticisers in pharmaceutical products are esters of organic acids, e.g. citric acid and phthalic acid, polyalcohols and esters thereof, as well as polyethylene glycol derivatives. Although even very hard films should get softened by plasticiser addition, the film specimen already being softer without plasticiser, however, are taken as starting point for further development.
Furthermore, generally, it is seen that the higher the concentration of the gelling agent in the hydrogel the more stiff appear the resulting films. That is obviously related to higher thickness of the film also resulting in a delayed swelling behaviour of these matrices. Unfortunately, this is hardly measurable in the swelling experiments but is noticeable by the degree and speed of softening of the films during hydration. Because a fast softening during hydration is desirable for a wound dressing, the focus of development concerning gelling agents is on high molecular weight polymer qualities.
Although the film samples produced in this series from pure gelling agent are promising, none of those can be used as a wound dressing without further modifications. Because, the samples providing enough flexibility lack of a certain robustness required for proper handling. On the contrary, the more robust films fail to be flexible enough for a convenient application. Therefore, the use of plasticisers and strengtheners as additives on pure gelling agent with special focus on HEC and HEC/gellan gum is evaluated.

#### *3.4.3.2.2 Polymers as additives to hydroxyethyl cellulose films*

It has been pointed out that films solely consisting of a pure cellulose derivative cannot be recommended for use as a wound dressing. Therefore, the addition of

further excipients, namely polymers, is investigated to cope with the major weak points of the cellulose ethers in the formation of self supportive films.

3.4.3.2.2.1 *Swelling*

The films examined until this point of development offer a constantly low swelling rate at least compared to the xerogels. Therefore, the addition of tablet disintegrants – carboxymethyl starch and crosslinked carboxymethyl cellulose - to the film formulation is investigated to increase swelling of these matrices.

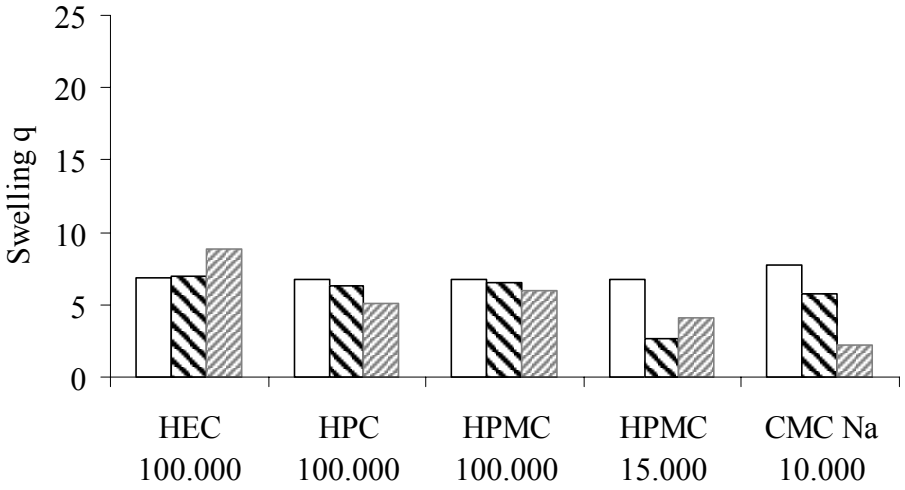


Fig. 3-51 Swelling of film matrices made from hydrogels containing 2.5% hydroxyethyl cellulose 100.000, 2.5% hydroxypropyl cellulose 100.000, 2.5% hydroxypropylmethyl cellulose 100.000, 3.5% hydroxypropylmethyl cellulose 15.000, and 5.0% carboxymethyl cellulose sodium 10.000, respectively (left bar); swelling with addition of 0.5% carboxymethyl starch to the basic hydrogels (center bar); swelling with addition of 0.5% crosslinked carboxymethyl cellulose to the basic hydrogels (right bar);

Unfortunately, the hoped for effect of improvement of the swelling behaviour of the film matrices by adding disintegrants can not be verified. All swelling values are taken after three minutes exposure to water. Most of the values range between 5 and 7 and therefore are 2 to 10-fold lower than the corresponding xerogel values (Fig. 3-51).

Next to these experiments neither with surfactants nor with soluble polymers a major improvement of the swelling characteristics can be achieved.

As a result, the application of films is supposed to be different from the application of xerogels. With the xerogels it is found that reconstitution is completed before or in the moment of application. In contrast, the films can not swell in that short period of time. Following, the film is to be wetted before placing onto the wound.

At that point reconstitution is started and will proceed in the wound with wound fluid or solution provided externally beneath and on top of the film. As a matter of fact, the wound will be confronted with a semi-reconstituted film not yet completely transformed into a hydrogel. For that reason, the texture properties of films play a very important role for the applicability of these matrices. Consequently, these are optimised by texture analysis outlined below.

*3.4.3.2.2.2 Texture*

For the optimisation of the mechanical characteristics a system consisting of three excipients is evaluated – the gelling agent, a strengthener, and a plasticiser. These studies are conducted on hydroxyethyl cellulose 100.000 as gellant, polyvinyl pyrrolidone 17 as strengthener, and polyethylene glycol 400 as plasticiser.
However, due to extensive interaction the particular influences of these three substances on mechanics cannot be treated separately. Hence, all tested samples contain the three ingredients in varying concentrations. For evaluation of this complex system a simplex-lattice factorial design with the three factors on five levels is created. In detail, the concentration levels of HEC 100.000 are 1.13%, 2.25%, 3.0%, 4.0%, and 5.0%. For PVP 17 and PEG 400, the levels are 0%, 1.0%, 2.25%, 3.0%, and 4.5%. Response surfaces are calculated from average values of two measurements.
Characterisation is done by texture analysis. Thereby, the tensile strength at rupture is gained from a tensile test method where the film is placed between two grips and is extended until rupture (Fig. 3-52).

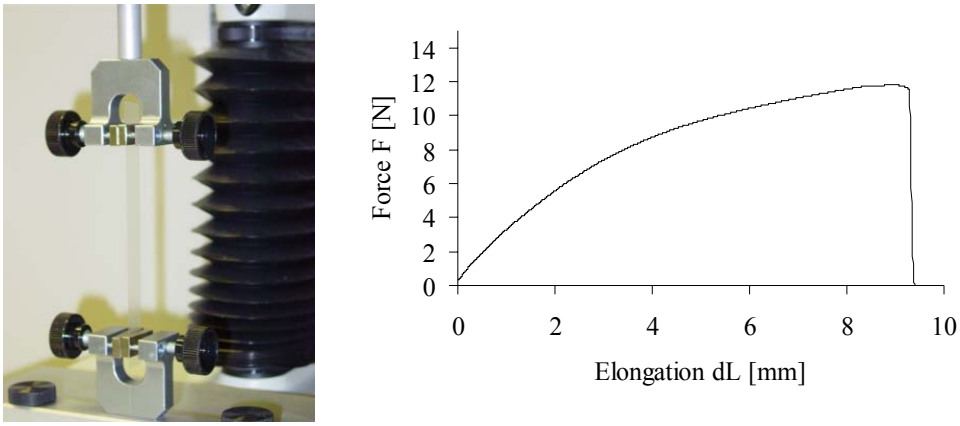


Fig. 3-52 Measurement of tensile strength with texture analyser (left); force-distance diagram of tension experiments with rupture at 11.8 N (right);

The tensile strength value is calculated from applied force at rupture and the cross section area of the film specimen[192] (Equ. 3-12).

$$TS = \frac{F}{A}$$

Equ. 3-12 Tensile strength TS [N/mm$^2$]; F is applied force at rupture [N], A is cross-sectional area [mm$^2$];

Moreover, the elastic modulus is tested in a puncture test where the sample is expanded and penetrated by a ball probe[192] (Fig. 3-53).

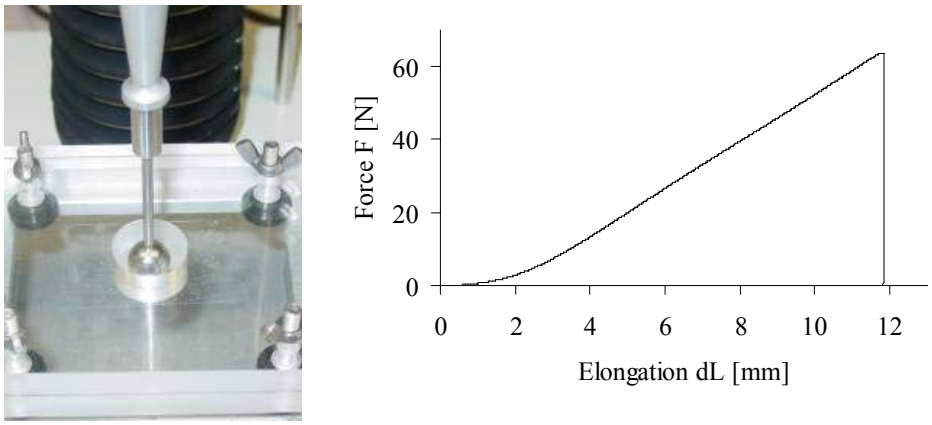


Fig. 3-53 Measurement of elastic modulus with texture analyser (left); force-distance diagram of penetration experiments with rupture at 61.5 N (right);

From the resulting load-displacement diagrams and the physical dimensions of the construction elastic moduli are calculated (Equ. 3-13).

$$E = \frac{stress}{strain} = \frac{F/A}{dL/L}$$

Equ. 3-13 Young's modulus of elasticity E [N/mm$^2$]; F is applied force [N], A is cross-sectional area [mm$^2$], dL is change in length [mm], L is unstressed length [mm];

For visualisation of the different aspects of mechanical characterisation the response surfaces calculated by a 3$^{rd}$ order model with the Origin® Software are given describing the interaction of two particular factors. The third factor thereby is kept on a constant level. Like before, concentrations given in this section refer to

the hydrogel state before drying. Film specimen are gained by drying of a wet film of 2 mm height consisting of the particular hydrogel.

*Robustness* - Surely, a certain robustness of the films is necessary to ensure a proper handling of the product by patients and medical personnel in practice. Thus, as a measure for robustness the tensile strength of the film specimen are investigated by texture analysis.

Yet, the tensile strength of the system depends on all components of the ternary mixture. First of all, a set of gels is examined with a constant PEG content and varying HEC and PVP contents. As expected, the tensile strength of the film increases with rising HEC contents. But, tensile strength values of about 4.5 $N/mm^2$ gained for the practical concentrations of HEC between 2% to 3% are not satisfactory. But, with the addition of PVP an increase in tensile strength can be detected. Although values exceeding 10 $N/mm^2$ can be achieved a reasonable content should be between 2.0% to 3.5% leading to strength values of 8.0 to 9.0 $N/mm^2$ (Fig. 3-54).

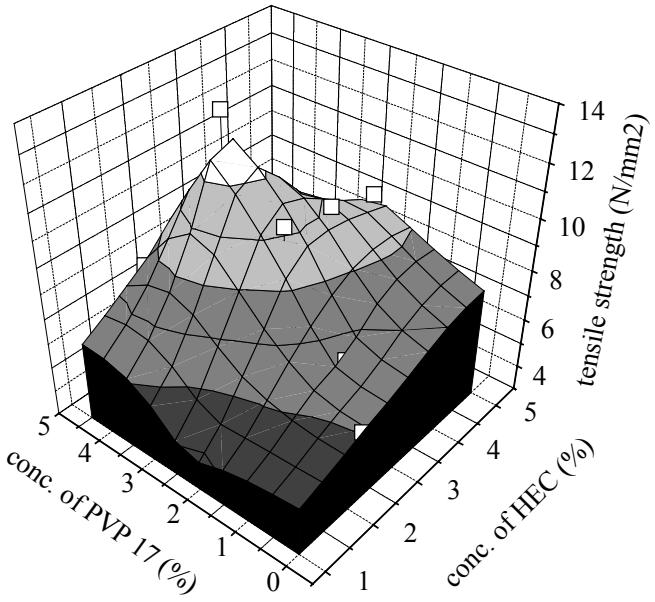


Fig. 3-54 Response surface calculated from tensile strength values of samples with 2.25% polyethylene glycol 400; hydroxyethyl cellulose 100.000 and polyvinyl pyrrolidone 17 contents vary according to a simplex-lattice design; concentrations refer to the hydrated state before drying;

Further, the effect of the plasticiser is clearly visible in Fig. 3-55. The content of PVP in this setting is kept at constant level - 2.25% in the hydrogel state. Variables are the HEC and PEG content. Here, a clear optimum for the PEG content is detected, i. e. from 0% until 2% PEG content an increase of strength values can be

seen. However, from 2% to higher concentrations of PEG a decrease of tensile strength is following. In fact, there is a clear maximum of tensile strength gained at 1.5% to 2.25% polyethylene glycol content for all tested formulations.

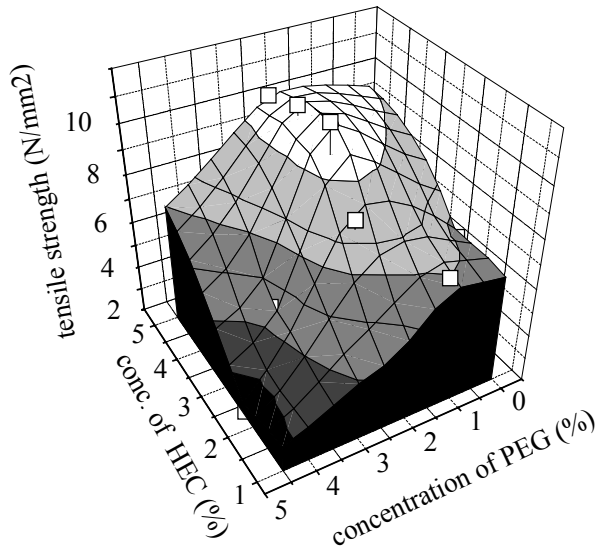


Fig. 3-55 Response surface calculated from tensile strength values of samples with 2.25% polyvinyl pyrrolidone 17; polyethylene glycol 400 and hydroxyethyl cellulose 100.000 contents vary according to a simplex-lattice design; concentrations refer to the hydrated state before drying;

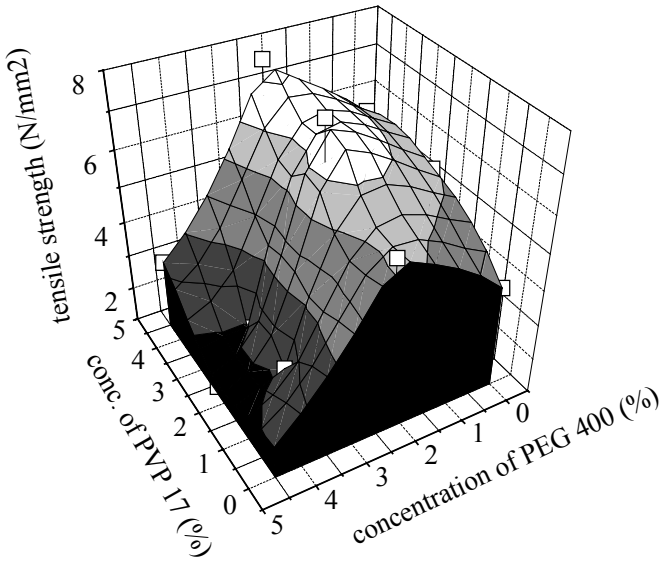


Fig. 3-56 Response surface calculated from tensile strength values of samples with 2.25% hydroxyethyl cellulose 100.000; polyethylene glycol 400 and polyvinyl pyrrolidone 17 contents vary according to a simplex-lattice design; concentrations refer to the hydrated state before drying;

The described maximum of tensile strength at an optimal PEG concentration is also visible in the third group of interacting partners. Because, for varying PVP contents and a constant HEC concentration the same maximum around 2% PEG content is obtained (Fig. 3-56).

*Flexibility* - For a convenient and free of pain application as well as for provision of efficient contact to the wound ground a high flexibility of the film matrix is desirable. Thus, the elastic modulus E which is a measure for stiffness and a reciprocal measure for flexibility is calculated from texture analysis as given in Equ. 3-13.

Other than the tensile strength the elastic modulus is mainly dependent on the PEG content. Only at very low concentrations of PEG influences by the other components are noticeable in the tested concentration range. Indeed, at the PEG level detected as optimal for tensile strength – around 2% concentration in hydrogel state - the elastic moduli of the systems are at a constant low level nearly independent of the gellant (Fig. 3-57) and strengthener content (Fig. 3-58).

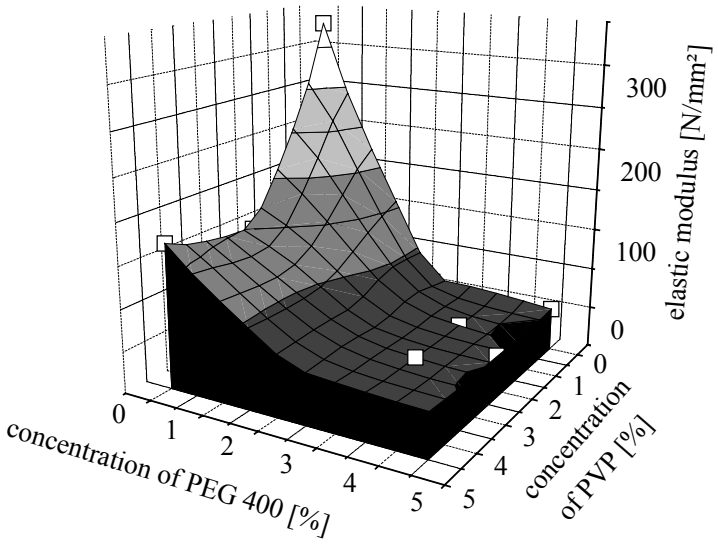


Fig. 3-57 Response surface calculated from elastic moduli of samples with 2.25% hydroxyethyl cellulose 100.000; polyethylene glycol 400 and polyvinyl pyrrolidone 17 contents vary according to a simplex-lattice design; concentrations refer to the hydrated state before drying;

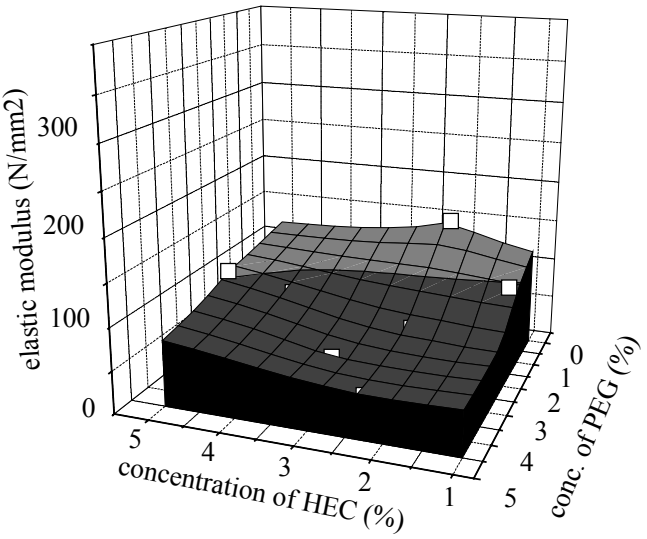


Fig. 3-58 Response surface calculated from elastic moduli of samples with 2.25% polyvinyl pyrrolidone 17; polyethylene glycol 400 and hydroxyethyl cellulose 100.000 contents vary according to a simplex-lattice design; concentrations refer to the hydrated state before drying;

To sum up, by the addition of polymers the mechanics of swellable, self-supportive polymer films made from cellulose ethers as main ingredient can be influenced and optimised to a major extent. It has been shown that a film with favourable handling and applicative properties can be manufactured from formulations of the following pattern:

| | |
|---|---|
| 2.0% – 2.5% | Hydroxyethyl cellulose 100.000 |
| 2.0% - 3.5% | Polyvinyl pyrrolidone 17 |
| 1.5% - 2.5% | Polyethylene glycol 400 |

Concentrations given refer to the hydrogel state before drying. However, the low swelling rate of polymer films could not be improved by addition of polymers to hydroxyethyl cellulose films.

#### *3.4.3.2.3 Hydroxyethyl cellulose / gellan gum mixtures for film formation*

For its convenient rheology in the hydrogel state the mixture of hydroxyethyl cellulose 100.000 with gellan gum is also investigated for film formation. Thereby, the relation of gellant components in the mixture is tested with the concentration range being between 1.5% and 5.0% for hydroxyethyl cellulose and between 0%

and 1% for gellan gum. Each film additionally contains 2.25% PEG 400 and 2.25% PVP 17 according to the results with pure hydroxyethyl cellulose films outlined above. These films are tested by texture analysis and swelling studies that are conducted with water and isotonic sodium chloride solution, respectively, as reconstitution media.

All the tested films show an acceptable optical appearance. Residual moistures of small scale samples dried under flowing nitrogen in glass vials are determined as between 1.5% and 2.0%. Moreover, swelling is hardly affected by gellant composition in the given range, i.e. values of about 8 to 10 are obtained. Like with the corresponding xerogels the reconstitution with water and isotonic sodium chloride solution, respectively, leads to differences in the rheology of the resulting hydrogels.

Mechanical properties of the produced films are similar to those of the pure hydroxyethyl cellulose films discussed above. With higher hydroxyethyl cellulose contents tensile strength increases (Fig. 3-59). However, with the addition of gellan gum the tensile strength stays on a high level up to a gellan gum concentration of about 0.3%. For a gellan gum content exceeding 0.3% a clear decrease of strength values is found (Fig. 3-59). Macroscopically, this is manifested in a more brittle appearance of these film specimen. Though, these samples still appear acceptable for wound treatment.

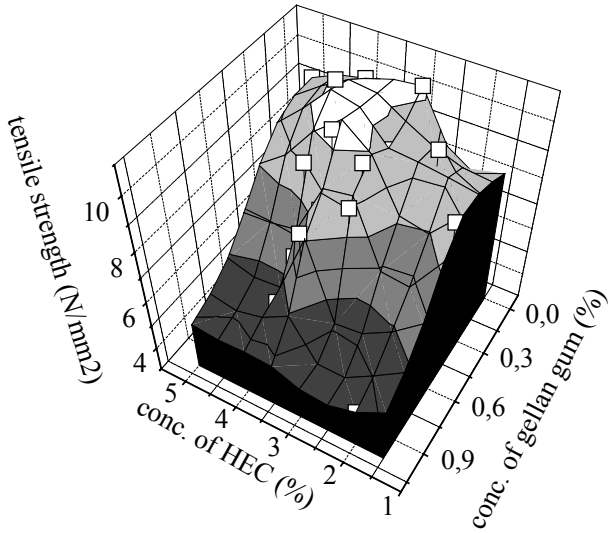


Fig. 3-59 Response surface calculated from tensile strength values of film samples with variable hydroxyethyl cellulose 100.000 and gellan gum F contents according to a simplex-lattice design; films also contain 2.25% polyethylene glycol 400 and 2.25% polyvinyl pyrrolidone 17; concentrations refer to the hydrated state before drying;

The elastic modulus of the system is also slightly increased with higher gellan gum contents. Despite, all measured values are in acceptable low range (Fig. 3-60). Besides, as found in the section above the main dependence should be on the plasticiser contents rather than on gellants which was not further tested. Therefore, as far as flexibility is concerned all tested formulations can be used.

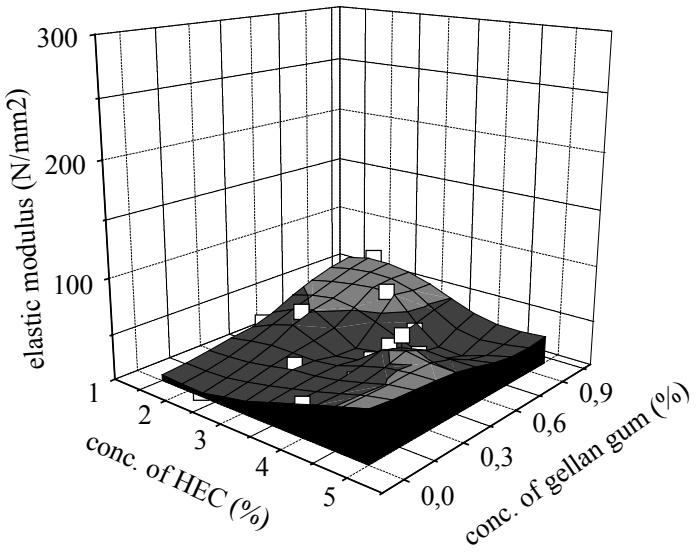


Fig. 3-60 Response surface calculated from elastic moduli of film samples with variable hydroxyethyl cellulose 100.000 and gellan gum F contents according to a simplex-lattice design; films also contain 2.25% polyethylene glycol (PEG) 400 and 2.25% polyvinyl pyrrolidone (PVP) 17; concentrations refer to the hydrated state before drying;

Therefore, it can be concluded that the mixture of hydroxyethyl cellulose and gellan gum is suitable for film formation. The relation of gellants found during the hydrogel studies can be taken as film composition as well. But, for a higher robustness a bisection or even further reduction of the gellan gum content maybe favourable. Although, further studies are conducted with a mixture of 2.0% hydroxyethyl cellulose 100.000 / 1.0% gellan gum mixture.

### *3.4.3.2.4 Protein stabilisers in polymer films*

The effects of protein stabilizers added to films were found to solely regard optical appearance and homogeneity of the matrices. The swelling behaviour is hardly affected as well as mechanical properties.
The effects again are described by means of hydroxyethyl cellulose matrices. These are made from hydrogels - 2.5% hydroxyethyl cellulose 100.000 with excipient to

be tested in water – that are cast out as wet films of 2 mm height. Contents given refer to the hydrogel state.
Phosphate buffer is suitable up to a concentration of 5mM. However, higher contents lead to crystallisation on the film surface. Crystals on the film surface are also seen with the following substances given with their tested concentration: sodium chloride (1%), potassium chloride (1%), sodium sulphate (1%), mannitol (2%), and glycine (2%). Moreover, surfactants just combined with gellant provoke shrinking of the film during drying at room temperature in every tested concentration up to 0.2%. Therefore this class can be used only in combination with other excipients, e.g. the shrinking can be partly impeded by the use of Tris as buffer component. Shrinking is also seen with sucrose (2%), high molecular weight polyethylene glycol (2%), and crosslinked carboxymethyl cellulose (5%). Furthermore, Tris hydrochloride (50mM), arginine (2%), calcium and magnesium chloride (0.5%), EDTA sodium (0.5%), and β-cyclodextrin (1%) can be incorporated without noticeable disturbance.
The tests described by means of hydroxyethyl cellulose as example are also conducted on other gallants. With carboxymethyl cellulose sodium, hydroxypropylmethyl cellulose, hydroxypropyl cellulose, and a hydroxyethyl cellulose/gellan gum mixture these effects basically are seen as well. Yet, the described negative effects of the tested stabilizers are more distinct with the more lipophilic matrices. This should be related to the hydrophilic character of most protein stabilizers and their reduced interaction with the lipophilic polymers.

### 3.4.4 Stability of ACT in film formulations

According to the results of the mechanical studies on polymer films most of the relevant protein stabilizers are compatible with the films from that point of view and so can be used for further development of ACT stabilising formulations. Therefore, excipients are introduced in film forming studies on ACT formulations that are tested positively in the prior stability studies on ACT concerning temperature stressing on the one hand, as well as in mechanical studies on polymer films on the other hand. First, the warm air drying process itself as stress factor is investigated. Second, a three months stability study on film formulations is conducted.

### *3.4.4.1 Stability of ACT during the film manufacturing process*

For the drying of the prepared hydrogel samples the warm air drying apparatus[165] is used. Thereby, drying is carried out with flowing nitrogen for 12 hours, the gas stream and the gel containing eppendorf caps being tempered to 25°C. Reconstitution and dilution for analysis are conducted as described in the xerogel section.

In this experimental set up the investigations on stabilising potency are conducted on formulation level. The formulations contain the substances according to Table. 3-16 with ACT in a hydroxyethyl cellulose matrix. Hereby, the preferable excipients are detected by comparison of the recovered ACT activity in the different formulations.

| formulation | | | | relative activity [%]±sd |
|---|---|---|---|---|
| (1) | phosphate | Poloxamer® 188 | | 94.5±3.49 |
| (2) | phosphate | Poloxamer® 188 | PVP 17 | 104.5±10.17 |
| (3) | phosphate | Poloxamer® 188 | PEG 400 | 98.1±1.49 |
| (4) | arginine phosphate | Poloxamer® 188 | | 111.0±11.03 |
| (5) | arginine phosphate | Poloxamer® 188 | PVP 17 | 119.1±21.07 |
| (6) | arginine phosphate | Poloxamer® 188 | PEG 400 | 118.7±0.16 |
| (7) | phosphate | Tween® 80 | | 106.3±1.21 |
| (8) | phosphate | Tween® 80 | PVP 17 | 100.2±10.17 |
| (9) | phosphate | Tween® 80 | PEG 400 | 102.4±5.3 |
| (10) | arginine phosphate | Tween® 80 | | 120.5±0.75 |
| (11) | arginine phosphate | Tween® 80 | PVP 17 | 125.2±0.02 |
| (12) | arginine phosphate | Tween® 80 | PEG 400 | 112.2±10.26 |

Tab. 3-16 Relative activity of ACT in films after reconstitution with 45mM potassium phosphate buffer with standard deviation measured by activity assay; underlying hydrogels contain 60µg/ml ACT, 5mM potassium phosphate buffer, 10mM arginine, 0.1% surfactant, 1.5% soluble polymer, and 2.5% hydroxyethyl cellulose 100.000;

*Arginine* – results indicate that arginine has a stabilising effect on ACT during film manufacture. This is gained by comparison of formulations (1)-(3) against (4)-(6) and (7)-(9) against (10)-(12), respectively. The stabilising potential of arginine on proteins during vacuum drying has been reported previously[155] (Tab. 3-16).

*Surfactants* – Tween® 80 is observed to be more effective than Poloxamer® 188 for ACT stabilisation during drying. This conclusion can be drawn from the comparison of formulation (1)-(6) with (7)-(12). There, most of the Tween® 80 formulations show higher residual activity than the particular Poloxamer® 188 (Tab. 3-16). This was also seen in hydrogel and xerogel formulations.

*Soluble polymers* – polyvinyl pyrrolidone that is used as strengthener in the film matrix in most samples as well shows a stabilising effect. Comparing formulation

(1) with (2), (4) with (5), (7) with (8), and (10) with (11) indicates mostly higher recovered ACT activities. Polyethylene glycol is used as plasticiser in the film. In the Poloxamer® 188 containing samples an additional stabilisation on ACT is shown. This results from the comparison of formulation (1) with (3) and (4) with (8). On the contrary, comparing formulation (7) with (9) and (10) with (12) indicates that in the Tween® containing samples there is no additional stabilisation proven by polyethylene glycol (Tab. 3-16).

*Gelling agent* – for evaluation of the gelling agents a standard formulation was chosen – 60 µg/ml ACT, 5mM potassium phosphate pH 7.2, 0.1% Tween® 80, 1,5% PEG 400 - with variable polymer types as gellants. These are hydroxypropyl cellulose, hydroxypropylmethyl cellulose, and hydroxyethyl cellulose. Moreover, a gellan gum/hydroxyethyl cellulose mixture, carboxymethyl cellulose sodium, and alginate sodium are tested. Unlike with xerogels, the more hydrophilic and ionic polymers perform better than the more lipophilic gelling agents - HPC and HPMC. The gellan gum/HEC mixture again shows highest recovered ACT activities (Fig. 3-61).

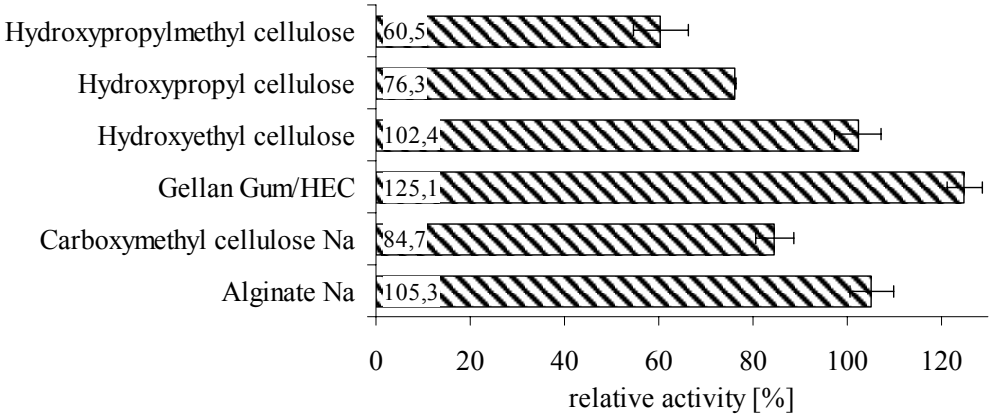


Fig. 3-61 Relative activity of ACT in films with 5mM potassium phosphate buffer, 0.1% Tween® 80, 1.5% PEG 400 and gelling agent after reconstitution with 45mM potassium phosphate buffer measured by activity assay; gellants are 2.5% hydroxypropylmethyl-, hydroxypropyl-, hydroxyethyl cellulose 100.000, gellan gum/hydroxyethyl cellulose 100.000 1%/2% mixture, 3.0% carboxymethyl cellulose sodium 10.000, 5.0% alginate sodium;

To summarize, in this section the influence of the drying process for polymer film formation as stress factor on ACT stability has been evaluated. It has been found that film formation with embedded active ACT is possible. Excipients can be chosen in concordance with results from studies on solution, hydrogel, and placebo films. Phosphate buffer can be used as tested. As well, arginine, surfactants and soluble polymers can be used without constraints. For the gelling agents

hydroxyethyl cellulose and the ionic species are preferable. The gellan gum containing samples again turned out to provide the most effective stabilisation on ACT.

### *3.4.4.2 Mid term stability*

Based on the previous results of the stabilisation in hydrogels and films a three months stability test in polymer films is launched with a set of formulations. Excipient composition is varied by similar means as in the xerogel mid term stability setting. Additionally, the samples contain polyvinyl pyrrolidone and polyethylene glycol as evaluated in the texture studies. Gel samples are dried using the small scale drying apparatus as described above whereby the films are formed. Sample plans, reconstitution of samples, and analysis including preparations are again described in the xerogel section (see chapter 3.4.2.1).
As expected, the data patterns gained from activity measurements by the activity assay correspond to those gained in the xerogel studies. Again, activity decrease over time is supposed to follow first order kinetics. Therefore, a linearisation is achieved in a one-sided logarithm plot. Moreover, a linear function can be fitted to the decrease (Fig. 3-62). The slope of the fitted curve delivers the negative reaction constant k.

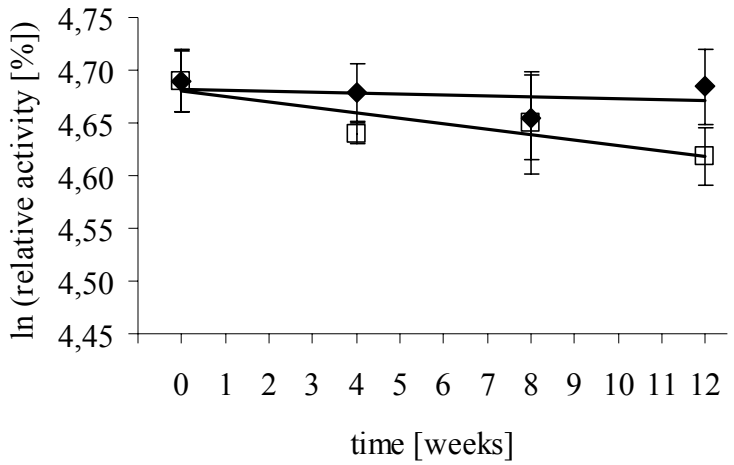


Fig. 3-62 Recovered relative activities of ACT measured by activity assay of a film formulation consisting of 60µg/ml ACT, 10mM arginine, 0.1% Tween® 80, 2.0% PVP 17, 2.0% PEG 400, 2.5% HEC 100.000 in a 5mM potassium phosphate buffer at 25°C(◆, $k=1.30\cdot10^{-4}$ 1/day) and 40°C(□, $k=7.43\cdot10^{-4}$ 1/day) in logarithmic concentration scale with fitted logarithmic functions resulting in evens in the one-sided logarithmic scale as predicted by first-order time law;

Further sample compositions and corresponding results after data processing like above are displayed in Tab. 3-17.

| | | | | |
|---|---|---|---|---|
| (1) | 60 µg/ml | α1-antichymotrypsin | reaction constant k (24°C) | $7.90 \cdot 10^{-4}$ 1/day |
| | 5 mM | phosphate buffer | reaction constant k (40°C) | $8.76 \cdot 10^{-4}$ 1/day |
| | 10 mM | arginine | shelf life 25°C | 133.4 days |
| | 0.1 % | Poloxamer® 188 | shelf life 40°C | 120.3 days |
| | 2.0 % | PVP 17 | residual moisture at start | 1.5% |
| | 2.0 % | PEG 400 | residual moisture after 3 | |
| | 2.5 % | HEC 100.000 | months | 2.0% |
| (2) | 60 µg/ml | α1-antichymotrypsin | reaction constant k (24°C) | $1.22 \cdot 10^{-3}$ 1/day |
| | 10 mM | Tris HCl buffer | reaction constant k (40°C) | not evaluable |
| | 10 mM | arginine | shelf life 25°C | 86.3 days |
| | 0.1 % | Poloxamer® 188 | shelf life 40°C | not evaluable |
| | 2.0 % | PVP 17 | residual moisture at start | 2.0.% |
| | 2.0 % | PEG 400 | residual moisture after 3 | |
| | 2.5 % | HEC 100.000 | months | 3.5% |
| (3) | 60 µg/ml | α1-antichymotrypsin | reaction constant k (24°C) | $1.30 \cdot 10^{-4}$ 1/day |
| | 5 mM | phosphate buffer | reaction constant k (40°C) | $7.43 \cdot 10^{-4}$ 1/day |
| | 10 mM | arginine | shelf life 25°C | 810.5 days |
| | 0.1 % | Tween® 80 | shelf life 40°C | 141.8 days |
| | 2.0 % | PVP 17 | residual moisture at start | 1.9% |
| | 2.0 % | PEG 400 | residual moisture after 3 | |
| | 2.5 % | HEC 100.000 | months | 2.8% |
| (4) | 60 µg/ml | α1-antichymotrypsin | reaction constant k (24°C) | not evaluable |
| | 5 mM | phosphate buffer | reaction constant k (40°C) | $2.52 \cdot 10^{-3}$ 1/day |
| | 10 mM | arginine | shelf life 25°C | not evaluable |
| | 0.1 % | Poloxamer® 188 | shelf life 40°C | 41.9 days |
| | 2.0 % | PVP 17 | residual moisture at start | 2.1% |
| | 2.0 % | PEG 400 | residual moisture after 3 | |
| | 2.0 % | HEC 100.000 | months | 3.5% |
| | 1.0 % | gellan gum LT100 | | |
| (5) | 60 µg/ml | α1-antichymotrypsin | reaction constant k (24°C) | $1.04 \cdot 10^{-4}$ 1/day |
| | 5 mM | phosphate buffer | reaction constant k (40°C) | $4.20 \cdot 10^{-4}$ 1/day |
| | 10 mM | arginine | shelf life 25°C | 1010.3 days |
| | 0.1 % | Poloxamer® 188 | shelf life 40°C | 250.9 days |
| | 2.0 % | PVP 17 | residual moisture at start | 1.2% |
| | 2.0 % | PEG 400 | residual moisture after 3 | |
| | 2.0 % | HEC 100.000 | months | 2.1% |
| | 1.0 % | gellan gum F | | |

Tab. 3-17 Formulations of the mid term stability testing with ingredients (left column), reaction constants k at the experimental stress levels, estimated shelf lives at the required temperature levels, and residual moisture at start as well as after 3 months storage (right column);

Generally, the stabilising potential of excipients in films is very similar to the results gained from xerogel mid-term stability studies. Nevertheless, for films stored in

glass vials the phosphate buffer has greater stabilising potential than Tris hydrochloride. This is manifested in shelf lives of formulation (1) in comparison with (2).
As in xerogels, the surfactant Tween® 80 performs better than the Poloxamer® 188 alternative. See comparison of formulation (1) with (3). Moreover, the HEC/gellan gum F gellant system is favourable over pure hydroxyethyl cellulose and the HEC/gellan gum LT100 alternatives. This results from comparison of formulation (1) with (4) and (5) (Tab. 3-17).
So, there are the two formulations – (3) and (5) - with shelf lives of greater 550 days (18 months). Thus, these meet the stability requirements for a storage form for stocking at room temperature defined at the beginning of development.
Moreover, studies on residual moisture by Karl-Fischer-titration produce values of 1.5% to 2.1% for all formulations at the beginning of storage. After three months values of 2.0% to 3.5% residual moisture are detected on both temperature levels (Tab. 3-17). Thereby, gellan gum LT100 and Tris hydrochloride containing samples tend to show higher values. However, these values are good starting points, but further optimisation of e.g. the drying process to gain values less than 2.0% residual moisture may be appropriate.

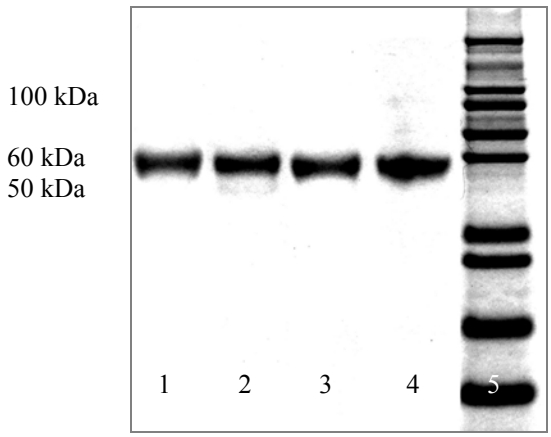


Fig. 3-63 Stability of ACT in formulations after a 3 months storage at 25 °C measured by SDS-PAGE:
lane 1 formulation (1) with no aggregate detected; lane 2 formulation (2) with 0.7% 50kDa fragment; lane 3 formulation (3) with no aggregate detected; lane 4 formulation (5) with 0.9% 100kDa aggregate; lane 5 marker;
control without degradation is visualized in Fig. 3-4

The readout by SDS-PAGE for samples stored over three months at 25°C surprisingly shows hardly any dimer formation (Fig. 3-63). Only formulation (4) is not evaluable. This specimen can not be filled properly in the electrophoresis gel

due to its elastic rheology even when heated. However, this is a promising result for the formulations. Concerning choice of excipients no conclusions can be drawn out of these experiments, unfortunately.
Hence, the formulations (3) and (5) can be recommended for further development. However, like with xerogels the greatest stabilising potential of a film formulation is to be estimated for a combination of Tween® 80 and HEC/gellan gum F. Although, this combination is as well not yet tested for the polymer films and therefore cannot be directly recommended supported by data for immediate further development. But, in future studies, e.g. adjustment of the formulation for market launch, this can be taken into account.

#### *3.4.4.3 Summary*

Results indicate that reaction kinetics of activity decay of ACT in films is highly likely to follow a first order equation. Main instability again is the loss of activity. In contrast to xerogel studies dimer formation is not found during film studies. Moreover, a commercial formulation of ACT in a swellable polymer film is possible due to sufficient shelf life. Furthermore, the two suitable formulations after reconstitution can be applicated into the wound site as far as stability of ACT is concerned. As well, these formulations are applicative for the purpose of animal experiment or clinical trial. Results also indicate that a not yet tested excipient combination may be the most effective stabilising formulation. This can be evaluated within later studies, e.g. adjustment of the product for market launch.

### 3.4.5 Summary for dry matrices as ACT delivery systems for wound healing

During the ACT stability studies in solution and hydrogels it has turned out that the shelf life of such formulations is very limited. Therefore, formulations with higher stabilising potential had to be developed that also have to fit the requirements of modern wound care. So, a dry form appears the best choice for improvement of drug stability, and hydrogels still are the state-of-the-art application form on wounds.
As a result, dry forms had to be developed that form hydrogels after reconstitution. Based on the hydrogels reported in the prior chapter two alternatives were investigated to meet these aims - the xerogels and the polymer films.

For both forms carrier development was carried out by establishing production procedures, optimising ingredient composition for both mechanical properties of the products and stabilising ACT in the matrices. Characterisation thereby is done by examination of texture properties, swelling behaviour, residual moisture, ACT stability during drying, and during storage.

Two xerogel formulations are identified that meet all the requirements. They have a homogeneous and soft appearance, they swell spontaneously when hydrated to a hydrogel, and they deliver shelf lives for ACT exceeding 18 months.

For the films as well two formulations turn out to fulfil the aims. These matrices offer very convenient texture properties, as well form suitable hydrogels after hydration, and also provide shelf lives greater 18 months for ACT.

Thus, development of stabilising formulations for ACT suitable for application into wound sites is finished successfully. In the following, the release of ACT from the preparations is studied in vitro.

# 3.5 Release of ACT from dry delivery systems

In this chapter the release behaviour of ACT from the matrices reported in the prior sections is investigated. For that purpose an in vitro method for release studies is established.
For the release period a time frame of one to five days is desirable from the medical point of view because a fast release and absorption of the drug is favourable. The common change of dressing intervals in clinical practice of wound care are between three to five days. Therefore, for highest effectiveness, the maximum release period preferably should not exceed five days.
Moreover, the in vivo release site to be simulated is an open wound. Although a topical delivery is described, wounds are not supposed to provide a major diffusion barrier for a drug substance. Thus, there is no physiological diffusion barrier like skin to be mimicked in vitro. Rather, a system providing a very low resistance to diffusion of the drug out of the matrix is preferable. In addition, the release temperature is set to 32°C representing the USP specification for dermal release[195].
After the experimental setup the first studies on model substances are described. Following, release studies of ACT from xerogels and polymer films are reported.

## 3.5.3 Experimental setup

For the setup of this series of in vitro experiments the type of chamber model, a proper acceptor medium and a suitable membrane is discussed. The membrane is placed between the chambers to separate donor and acceptor and so is penetrated by the diffusing substance during release.

### *3.5.3.1 Membrane*

To avoid dilution, erosion, and dissolution of the swellable donor gel with the liquid acceptor during the release experiment a separation of donor and acceptor chamber is obligatory. Therefore, a membrane is necessary that is not used for simulation of a physiological barrier, e.g. skin, rather it should mechanically separate the media. Thus, in preliminary tests a membrane is evaluated that allows an unimpeded diffusion of model substances but keeps the major part of the gelling agent on the donor side. In preliminary studies, it turns out that a cellulose

acetate filter membrane of 0.45µm pore size meets the named requirements at best[196] (data not shown).
It holds the gelling agent back in the donor chamber and provides one of the fastest release rates for FITC-Dextran 70kDa in a membrane testing series. Membranes with smaller pore sizes, e.g. 0.1µm and 0.22µm, show slower release and, therefore, seem to impede diffusion out of the gel. But, membranes with larger pores, e.g. 0.8µm and 1.2µm, do not provide further increased release rates compared to 0.45µm. Thus, diffusion of the drug is not affected by these membranes. However, the gelling agent can pass through the pores of 0.8µm and 1.2µm diameter. This is detected by an increase in viscosity of the acceptor solution. Moreover, gel is found on the acceptor side of the membrane having penetrated through the pores.
Cellulose acetate is non-lipophilic and water permeable. And, according to manufacturers` instructions this material has also a low protein adsorption tendency.

### *3.5.3.2 Acceptor medium*

To simulate the conditions in a chronic wound site a solution similar to wound exudate would have been a reasonable choice. Unfortunately, the composition of wound exudate varies in very broad ranges[197]. Hence, an exact copy of a standard wound exudate is not possible. And more important, priorities are set on the second factor influencing the evaluation of the acceptor solution which is the stabilising potency on ACT. Release samples to be analysed are taken from the acceptor solution following a sample plan and are kept in frozen state until analysis and for later storage. Therefore, a degradation of the drug substance in the acceptor medium during the experiment and a degradation by freezing stress would adulterate the releasing profiles. This confines the composition of the medium, e.g. sodium is not suitable because of the pH shifting interaction with phosphate buffer during freezing. Further, a surfactant is desirable to minimize material loss due to adsorption to the membrane, the chamber and tubing walls. Thus, an ACT stabilising solution consisting of 10mM potassium phosphate buffer pH 7.2 and 0.1% Poloxamer® 188 is chosen as acceptor. This solution is proven to be suitable for stabilisation in solution state during the release experiment as well as for stabilisation during storage of samples as frozen solution (section 3.2.2).
Furthermore, the release medium has to provide sink conditions for the diffusion of the drug substance. That means a sufficient amount of acceptor medium has to

be offered in relation to the amounts of drug substance and donor medium. In detail, according to common definitions, sink conditions for a substance are provided if its concentration at the end of the experiment does not exceed 10% of its saturation solubility in the medium. Thus, for the low amounts of model drugs and ACT, sink is provided even with rather low acceptor volumes. Therefore, the minimum of acceptor medium is determined by the sample volume to be taken out of the reservoir until the experiment is finished.

### *3.5.3.3 Chamber model*

For evaluation of methods it turned out to be important that the donor gel is mechanically fixed in the chamber. Because, by using a water permeable membrane slight differences in pressure between donor and acceptor might lead to an unwanted transport of water from one chamber into the other. This is to be avoided because both a dilution or shrinking of the gel and a change of acceptor volume would produce non-evaluable results. Therefore, the release tests are performed in a modified Loth model[134].

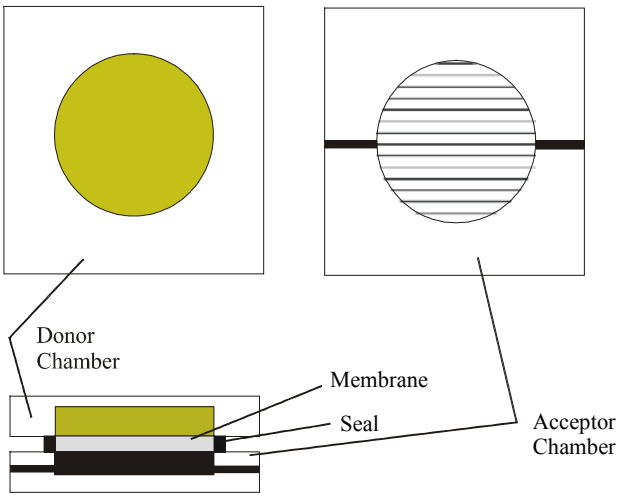


Fig. 3-64 Modified Loth chamber made of acrylic glass for release studies; the donor chamber (left and above) keeps 1.25ml of donor medium at a layer thickness of 4mm; the acceptor chamber (right and top) provided with a ripple plate supporting the membrane is filled with acceptor medium and is connected to the medium reservoir (not shown); chambers are separated by a cellulose acetate filter membrane with 0.45µm pore size; the system is sealed by a polyurethane rubber o-ring;

There, the gel containing chamber is mechanically fixed. Due to the higher osmolarity of the donor medium a slight permanent pressure results in the donor chamber that is absorbed mechanically by the bending membrane and the chamber

body. Moreover, the model is modified in size that the donor sample cavity is circular in shape with 2.0cm in diameter and has a height of 0.4cm, resulting donor volume is only 1.25ml. The acceptor, moreover, provides a ripple plate supporting the membrane and providing contact between acceptor solution and the membrane. Particular drill holes in the body enables the acceptor medium to be pumped through the chamber (Fig. 3-64). The whole apparatus is tempered to 32°C for measurement in a cabinet heater.

For the provision of the acceptor medium to the chamber two alternative systems are evaluated. In the first system – the dynamic model - the acceptor medium is pumped in closed circuit by a hose pump with a flow rate of 10ml/min. The total volume is 20ml. Samples are taken from the reservoir without replacement according to a sample plan. In the alternative system – the static model – two syringes are connected to the acceptor chamber (Fig. 3-65). A 2ml portion of acceptor medium is filled in the syringes and provided to the diffusion site. Following a sample plan, sample collection is done by complete exchange of the portion against fresh medium.

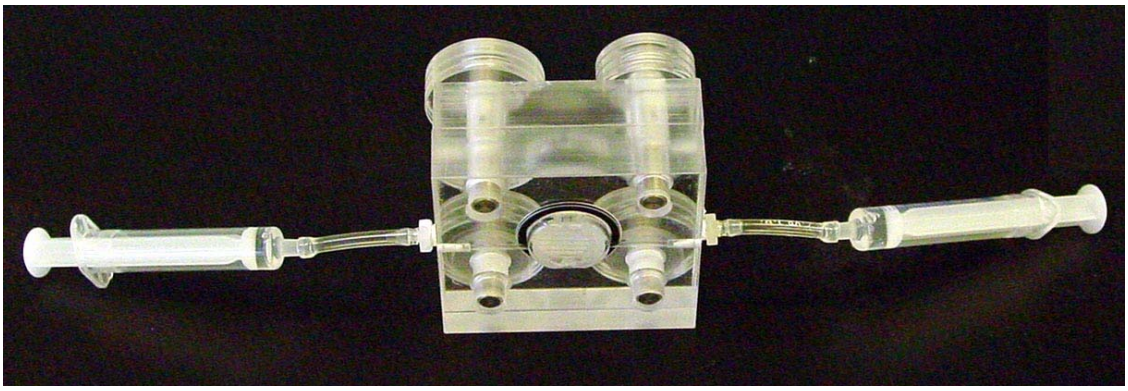

Fig. 3-65 Modified Loth chamber for release studies equipped as static model with two syringes providing the acceptor solution;

## 3.5.4 Theoretical background and data interpretation

For the interpretation of release kinetics the theoretical background given by Higuchi is used. These equations are solutions to Fick's second law. So, they describe the diffusion process of a dissolved substance from a semi-solid base into a sink. Thereby, the diffusion within the donor to the releasing interface is the rate-limiting step in the overall process. Equation 3-14 describes the whole process. But,

due to its complexity a simplified form (Equ. 3-15) of this relation is used for calculations[199].

$$c_A = h \cdot c_0 \cdot \left[ 1 - \frac{8}{\pi^2} \cdot \sum_{m=0}^{\infty} \left( \frac{e^{-\left( \frac{D \cdot (2m+1)^2 \cdot \pi^2 \cdot t}{4h^2} \right)}}{(2m+1)^2} \right) \right] \qquad c_A = 2 \cdot c_0 \cdot \sqrt{\frac{D \cdot t}{\pi}}$$

Equ. 3-14 Equ. 3-15

Equ. 3-14&3-15 Model functions after Higuchi describing the diffusion of a substance totally in solution out of a semi-solid matrix into a sink; equ. 3-14 describes the correlation more exactly; equ. 3-15 is a simplification strictly valid in the first third of the process; hereby, is $c_A$ = concentration in acceptor at time t [mol/l]; $c_0$ = starting concentration in donor at time t=0 [mol/l]; t = time t [min]; D = diffusion coefficient [m/min]; h = thickness of diffusion layer [m];

For data interpretation the released amounts of substance are drawn against releasing time. The resulting curves describe a square root function. Therefore, this chart can be Linearised when drawn against the square root of time. Consequently, a linear function is fitted to the resulting even delivering the slope as a measure for the release rate. Further, the amounts released at a certain time point are read from this chart.

## 3.5.5 Release of model substances from gel based matrices

In a first series of experiments the release of model drugs of different molecular weight from standard hydrogel matrices are examined using the dynamic model. Thereby, hydrogels consisting of 2.5% hydroxyethyl cellulose 100.000 in water are used as standard. The hydrogels, moreover, contain Fluorescein sodium, FITC-Dextran 19kDa, and FITC-Dextran 70kDa, respectively, as model drugs. Quantification is carried out spectrofluorimetrically in microwell plates at $\lambda_{ex}$ = 493 nm and $\lambda_{in}$ = 515 nm. The released amounts of the particular model drugs - given in percentage of total content - are drawn against the diffusion time (Fig. 3-66).

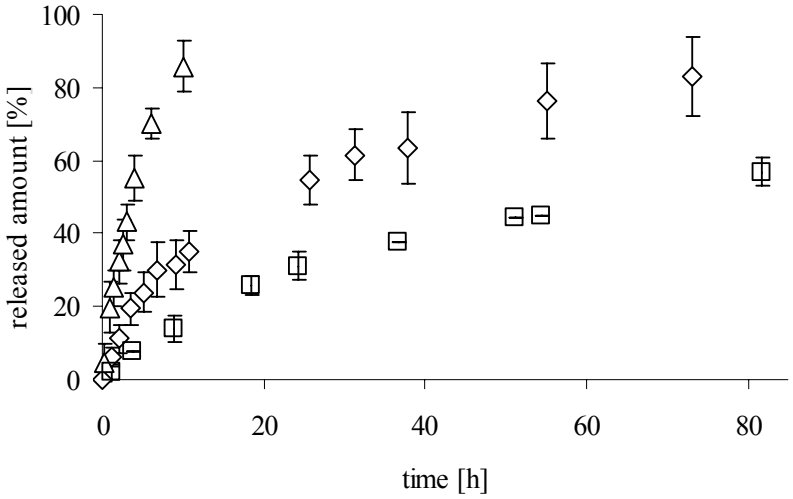


Fig. 3-66 Release diagram of Fluorescein Na (△), FITC-Dextran 19kDa (◇), FITC-Dextran 70kDa (□), respectively, from hydrogels containing 2.5% hydroxyethyl cellulose 100.000 in water; release medium is a 10mM potassium phosphate buffer pH 7.2, 0.1% Poloxamer® 188;

With all tested substances the model tests follow the theoretical predictions of a square root of time function and so can be Linearised in a square root of time chart (Fig. 3-67). It is seen that the release rate of the substance is clearly dependent on its molecular weight.

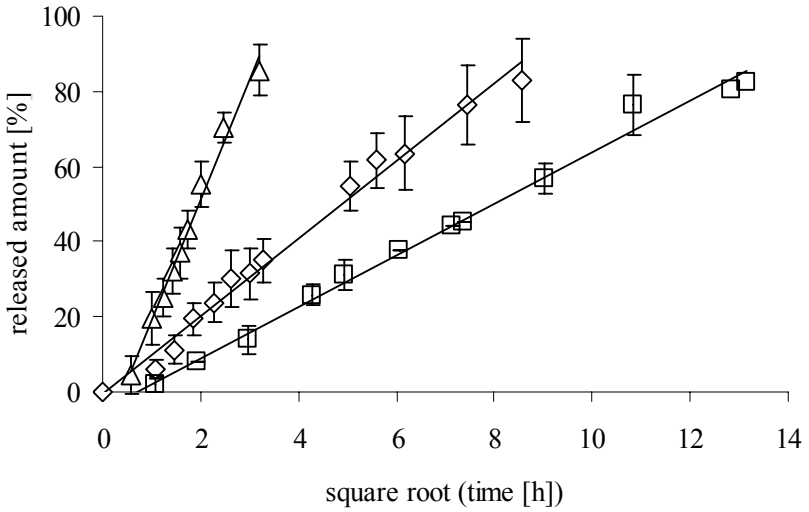


Fig. 3-67 Release diagram of Fluorescein Na (△, slope=34.4, $R^2$=0.98), FITC-Dextran 19kDa (◇, slope=12.3, $R^2$=0.99), FITC-Dextran 70kDa (□, slope=7.2, $R^2$=0.99), respectively, from hydrogels containing 2.5% hydroxyethyl cellulose 100.000 in water drawn against the square root of time; release medium is a 10mM potassium phosphate buffer pH 7.2, 0.1% Poloxamer® 188;

Bearing in mind that the diffusion coefficient of a substance is also related to its molecular weight, the relation of the release rate to the diffusion coefficient becomes apparent. This also is predicted by theory (Equ. 3-15). Thus, the model can be used for release studies of molecules of the size of proteins, especially ACT with 68kDa molecular weight.

One may doubt that the different release rates detected in Fig. 3-67 are determined by the different diffusional behaviour of the molecules in the gel matrix rather than by a restricted passage through the membrane due to the pore structure. To support our view about that topic, an estimation of molecule sizes is given in Fig. 3-68. The molecule diameters of FITC-Dextran derivatives are given as Stokes diameters[200]. The diameter of ACT is calculated by an approximation according to Equ. 3-16[201].

$$V_{protein\,molecule} = 1.21 \cdot M_{protein\,molecule}$$

Equ. 3-16 Approximation of the volume of a protein molecule $V_{protein\ molecule}$ [$10^{-3}nm^3$] by its molecular weight $M_{protein\ molecule}$ [Da]; the correction factor is related to the average partial specific volume of proteins being 0.73 $cm^3/g$[201];

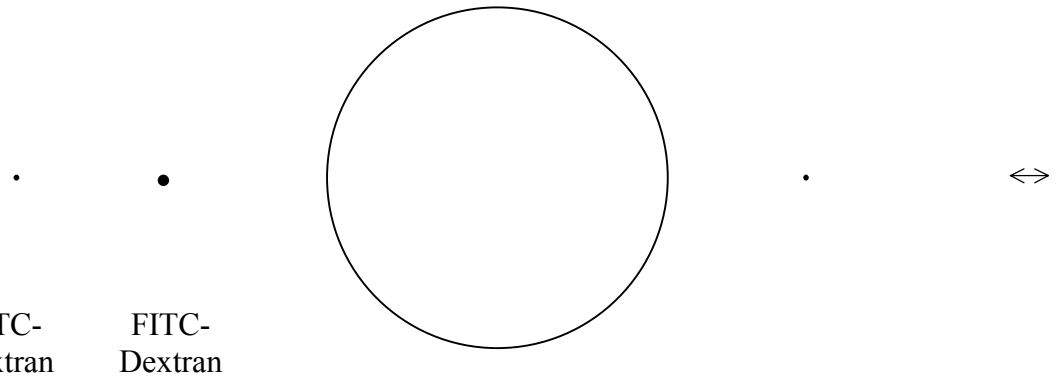


Fig. 3-68 Visualisation of molecule sizes in comparison to membrane pore size; FITC-Dextran molecule sizes are stokes diameters - 6.6nm for 19kDa derivative, 12.0nm for 70kDa derivative[200]; membrane pore size is given as 450nm, and ACT diameter is estimated as 5.4nm;

From the relation of diameters it can be stated that it is highly unlikely that differences in the diffusional behaviour of substances of different molecular weight displayed in Fig. 3-67 are related to different degrees of restriction for the diffusion of the given substances through the membrane.

Nevertheless, a certain restriction for the diffusion is provided by the membrane because the pore structure effects a reduction of the diffusional area in the system

compared to e.g. an in vivo situation where the gel has direct and intimate contact with the wound ground. Therefore, the release in vivo is supposed to be faster to some degree compared to in vitro studies due to the lack of the membrane.

In the following, the different application forms under development during this work are tested with a standard load of FITC-Dextran 70kDa. Xerogel and film samples are prepared as described for the ACT loaded specimen (section 3.3.2.2.). Samples are reconstituted with water for one minute before the release test in the dynamic model is started. The release curves are again linearised and displayed in Fig. 3-69.

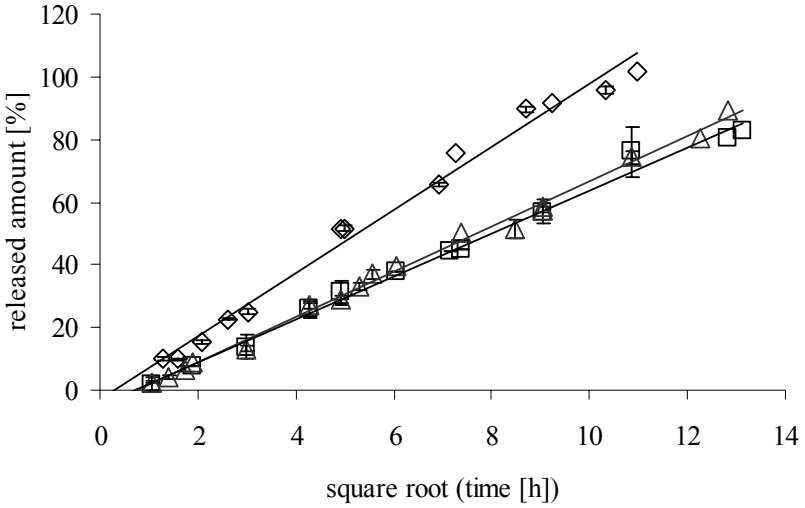


Fig. 3-69 Release diagram of FITC-Dextran 70kDa from hydrogel (△, slope=7.2, $R^2$=0.99), film (◇, slope=10.0, $R^2$=0.98), xerogel (□, slope=6.9, $R^2$=0.99), respectively, from compositions containing 2.5% hydroxyethyl cellulose 100.000 against the square root of time; release medium is a 10mM phosphate buffer pH 7.2, 0.1% Poloxamer® 188;

The release rates from xerogels and hydrogels are very similar. As a result, no differences are seen between xerogels after reconstitution and the underlying hydrogels as far as release characteristics are concerned.
From films, the release rate slightly exceeds the rate from the corresponding wet hydrogel. This should be rooted in a higher concentration gradient of the drug in the film experiment. Unlike gels, films start the diffusion with a very low volume. Though it of course increases during the hydration period, at least in the first part the concentration in the film is higher than in the gel. That results in a higher driving force for the diffusion and with that in a higher release rate from films.
Nevertheless, the release from the dry matrices is still in comparable range with that from the underlying hydrogels. Further, it can be estimated that a protein like

ACT with around 68kDa molecular weight will be released to 90% within approximately five days. The slope of the Linearised release curve of ACT is expected to be in the range of 6.5 to 10.5. However, the differences in chemistry and molecular weight of the molecules might influence the kinetics to a major degree.
To sum up, the model studies indicate that the present system is suitable for in vitro release studies of drugs with the required molecular weight from wet or reconstituted gel preparations.

## 3.5.6 Release of ACT from formulations

In this section the release of ACT from the developed dry matrices is investigated. The formulations are taken over from the mid term stability studies reported in chapter 3.4. After the model evaluation the experiments on xerogels and films are reported.

### *3.5.6.1 Dynamic model*

Based on the convincing results of the liberation of the model compound the first release studies on ACT are also performed in the dynamic system. Unfortunately, the read out methods of ELISA and activity assay deliver different results on the release curves. According to ELISA results, ACT is liberated from the preparations, and the release curves follow the expected kinetics very closely. Thereby, a maximium of 90% total content released is gained after 55 hours which means a slightly faster release rate than the rate gained with FITC-Dextran. Indeed, the active amount of ACT liberated increases in the first few hours as well. But in contrast to total content, after reaching a maximum at around 50 hours of releasing time the measured ACT activity declines rapidly to zero level.
However, in the release phase before the activity decline starts slopes can be read that are higher than those gained in control experiments with FITC-Dextran 70kDa (Fig. 3-70).
The reason for the decline of activity in the acceptor medium is found in the system of the dynamic release model. The medium is pumped through plastic tubing during the entire testing time by a hose pump. Moreover, the medium in the supply is homogenised by a magnetic stirrer.

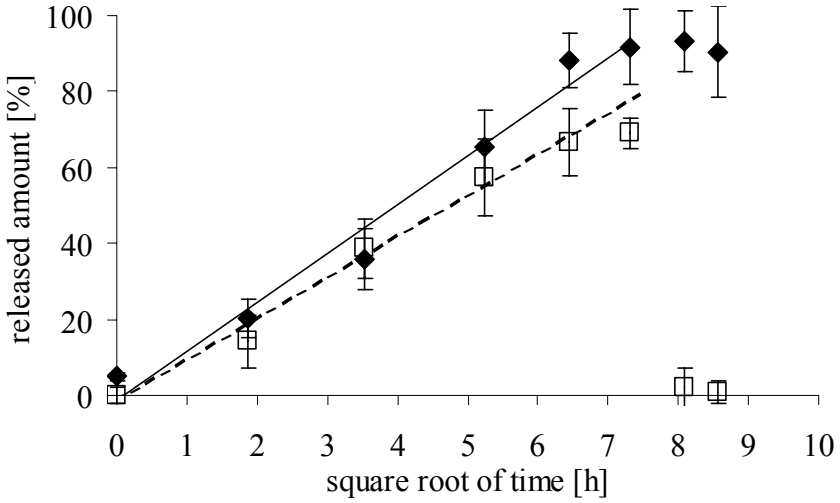


Fig. 3-70 Linearised release diagram of ACT from a xerogel formulation in the dynamic model; release profiles are given as active ACT gained by activity assay (□, slope=10.8, $R^2$=0.98) and total ACT content measured by ELISA (◆,slope=12.8, $R^2$=0.98); underlying hydrogel contains 60µg/ml ACT, 10mM potassium phosphate buffer pH 7.2, 10mM arginine, 0.1% Poloxamer® 188, and 2.5% hydroxyethyl cellulose 100.000 in water; release medium is a 10mM phosphate buffer pH 7.2 with 0.1% Poloxamer® 188;

In control experiments an ACT solution similar to the acceptor medium is stressed by stirring and stirring in combination with pumping, respectively. Both factors put shear stress on the ACT molecule that leads to deactivation. Thereby, the pumping is more harmful than the stirring alone (Fig. 3-71).

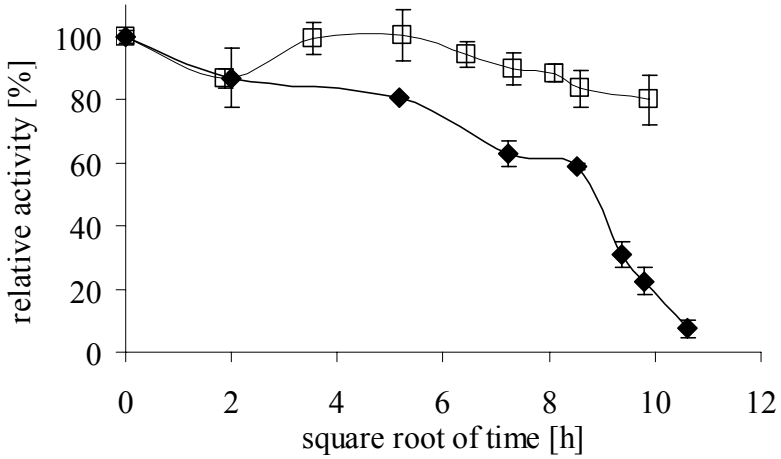


Fig. 3-71 Decrease of relative activity of a 60µg/ml ACT solution (10mM potassium phosphate buffer pH 7.2, 0.1% Poloxamer® 188) during stirring in the acceptor surplus (□) and during pumping through the tubes and stirring in the surplus (◆);

Therefore, it can be concluded that the dynamic system is unfavourable for the release studies on ACT. The sensitivity of ACT against shear stress also detected during experiments with static mixers (section 3.3.2.2) impedes the use of hose pumps. In the following, the static model is used for further investigations.

## *3.5.6.2 Static model*

### *3.5.6.2.1 Evaluation of the model*

Before the actual release tests in the static model, control experiments are performed to evaluate the loss of activity and total content during the actual release tests.

Therefore, a solution of 60µg/ml ACT formulated in a 50mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188 is exposed to the tubing material, the chamber material, and the membrane, respectively. A control is kept in a glass vial. For analysis by activity assay and ELISA, samples are drawn after 18 hours and 75 hours.

The loss of activity is minimal in the glass vials. But, in the samples exposed to tubing, chamber, and membrane loss of activity is observed between 10% to 25% after 75 hours (Fig. 3-72).

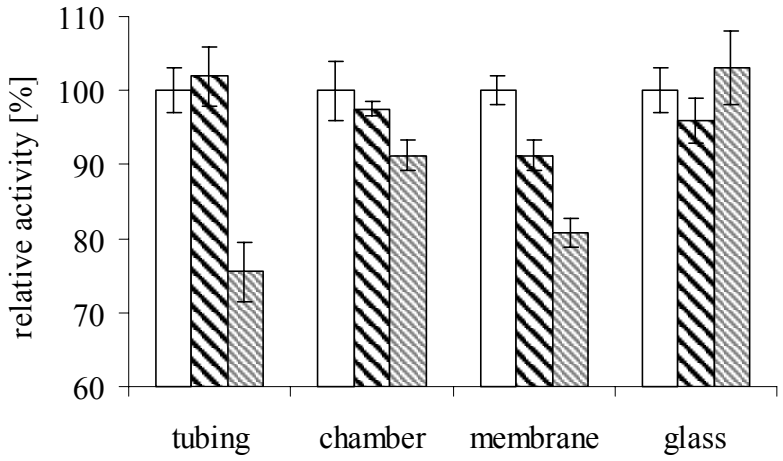


Fig. 3-72 Residual activity of a 60µg/ml ACT solution formulated in a 50mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188 during exposure to tubing material, chamber material, membrane material, and glass, respectively, measured by activity assay; left: control before exposure; centre: exposure for 18 hours, right: exposure for 75 hours;

The total content measured by ELISA shows a decrease over time as well, but to much lower extent. In detail, glass again shows almost no loss, but in the other samples only 90% to 95% of total content are recovered (Fig. 3-73).

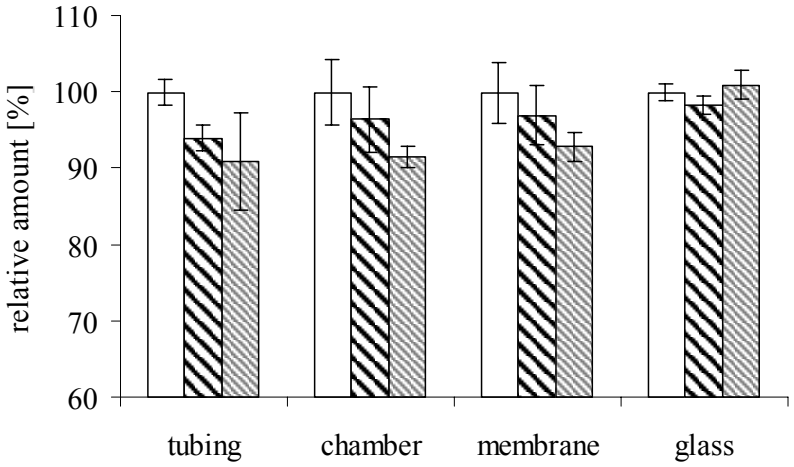


Fig. 3-73 Residual total content of a 60µg/ml ACT solution formulated in a 50mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188 during exposure to tubing material, chamber material, membrane material, and glass, respectively, measured by ELISA; left: control before exposure; centre: exposure for 18 hours, right: exposure for 75 hours;

Therefore, it can be concluded that during the release experiments in the static model ACT activity and total content is lost as well but to much lower extent compared to the dynamic model. Moreover, the loss of ACT seems to follow two mechanisms. The loss of total content is supposed to be rooted in absorption phenomena which result in ACT molecules remaining in the system. Besides, this mechanism also leads to reduced recovered activity values. However, the 2-fold higher values of lost activity compared to total content loss can solely be explained by an additional deactivation of non-absorbed ACT molecules.

Indeed, based on the results it is to be expected that during release experiments with ideally releasing formulations 75% to 90% of relative activity can be recovered at best. As well, a maximum of 90% of total content is likely to be recovered at most.

A further improvement of the release system, e.g. by using glass instead of plastic materials for the chamber or pre-treatment of the membrane, would have been a promising possibility to improve ACT recovery results but was not possible for technical reasons. As well, a simple mathematical adjustment with a correction factor is problematic because of the measurement uncertainty of the analytical

methods being amplified in such an operation. Thus, a result gained after correction would imply an unsatisfactory inaccuracy.
Moreover, the control experiments most notably on the tubing material have to be understood as worst case conditions because the surface of tubing presented to the protein containing medium is very low in the release model (Fig 3-65) compared to control experiments. For the latter, of course, a certain amount of solution (0.5ml) had to be filled in the tubing for technical and analytical reasons which also requires a certain tubing volume that naturally is correlated with a higher surface area compared to the small pieces of tubing used for the release chamber. This difference in the relation of solution amount and exposed tubing surface in control and actual release situation leads to an exaggerated impression about the influence of the tubing material. To conclude, a simple multiplication of the particular recovered activities and amounts, respectively, of Fig. 3-72 and Fig. 3-73 will not result in a correct overall value for the maximum recovered amount of ACT to be expected in release tests.
For those reasons, the uncorrected values are used for the comparison of the release results of the different formulations.

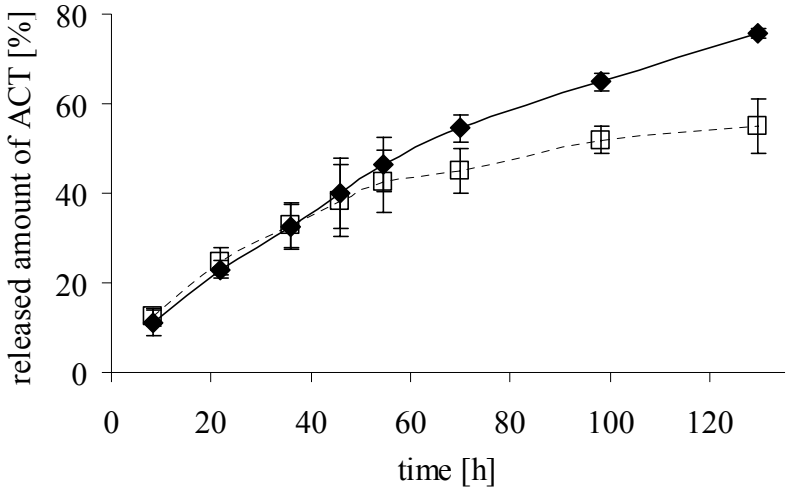


Fig. 3-74 Release diagram of ACT from a xerogel formulation in the static model; release profiles are given as active ACT gained by activity assay (□) and total ACT content measured by ELISA (◆); underlying hydrogel contains 60µg/ml ACT, 10mM potassium phosphate buffer pH 7.2, 10mM arginine, 0.1% Poloxamer® 188, 0.05% PVP 17, and 2.5% hydroxyethyl cellulose 100.000 in water – xerogel formulation (1) of Tab. 3-18; release medium is a 10mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188;

Fortunately, for the estimation of future in vivo experiments the situation is different. The constant recovered amounts in the glass samples for both analytical methods (Fig. 3-72, 3-73) indicate that the release system with its plastic

components is responsible for the losses. In an in vivo situation, however, these materials and surfaces are not present in the application site why it can be stated that the here detected losses are unlikely to occur.

After the control experiments actual release tests on ACT xerogel formulations are performed. In fact, in release tests of ACT in the static model the complete loss of activity detected in the dynamic model is not observed. However, the curves measured by activity assay flattens after about 50 hours of testing time in comparison to the total content curve obtained from ELISA measurements (Fig. 3-74).

This corresponds to the control experiments predicting a higher loss of active ACT compared to total ACT content. As well, the magnitude of recovered contents in the release test concur with the results gained from control experiments.

Nevertheless, when drawn in the square root of time plot both curves are linear up to 50 hours releasing time. In that period, a linear fitting can be conducted delivering slopes for measuring release rates (Fig. 3-75).

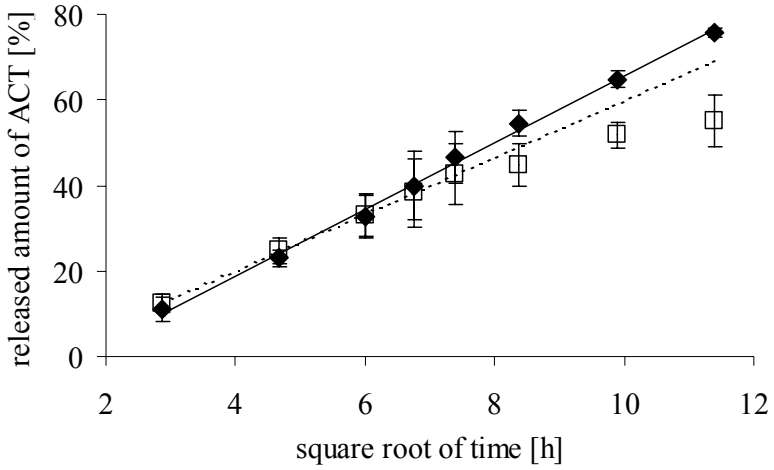


Fig. 3-75 Linearised release diagram of ACT from a xerogel formulation in the static model; release profiles are given as active ACT gained by activity assay (□, slope=6.6, $R^2$=0.98) and total ACT content measured by ELISA (◆, slope 7.8, $R^2$=0.99); underlying hydrogel contains 60µg/ml ACT, 10mM potassium phosphate buffer pH 7.2, 10mM arginine, 0.1% Poloxamer® 188, 0.05% PVP 17, and 2.5% hydroxyethyl cellulose 100.000 in water – xerogel formulation (1) of Tab. 3-18; release medium is a 10mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188;

For interpretation in the following, next to the slopes of the linearised release curves two values are presented: first, the total amount of ACT released within 120 hours is gained from ELISA. Second, the amount of bioactive ACT is delivered by activity assay in the same release period. Additionally, the bioactive fraction of liberated ACT calculated by division of the two latter values is used. The amount

values are read after 120 hours of releasing time because this represents the common change of dressing interval in clinical practice.

One may notice that slopes of release curves are lower in the static method – 6.6 vs. 10.8 for activity assay readout and 7.8 vs. 12.8 in the ELISA readout - and therefore the release is delayed in comparison with the studies on the dynamic model.

#### *3.5.6.2.2 Release of ACT from xerogel formulations*

For the release studies of ACT from xerogels a set of formulations taken over from the xerogel stability studies (section 3.4.2.2) is tested using the static model. Experiments are conducted over a releasing time of 120 hours (5 days) which corresponds to the usual change of dressing interval in clinical practice. Approximately every 12 hours, 24 hours in later phases of the release, a sample is taken and analysed by both activity assay and ELISA. Interpretation is carried out as given above. Below, the linearised release curves for each formulations are presented (Fig. 3-75 to 3-80), a summary of calculations is given in Tab. 3-18.

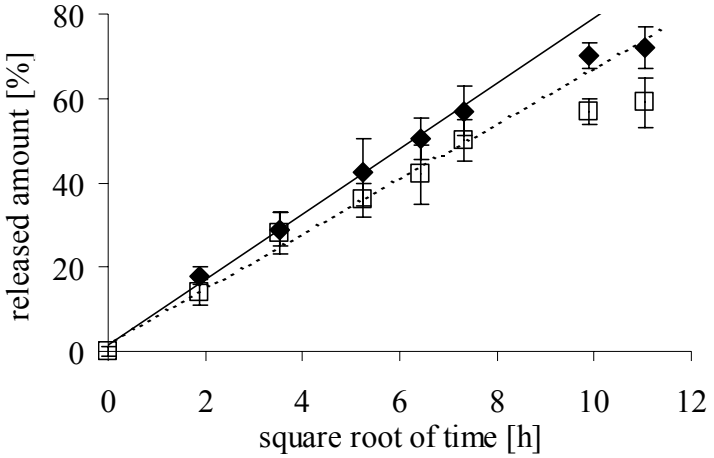


Fig. 3-76 Linearised release diagram of ACT from a xerogel formulation in the static model; release profiles are given as active ACT gained by activity assay (□, slope=6.5, $R^2$=0.99) and total ACT content measured by ELISA (◆, slope 7.8, $R^2$=0.98); underlying hydrogel contains 60µg/ml ACT, 10mM potassium phosphate buffer pH 7.2, 10mM arginine, 0.1% Tween® 80, 0.05% PVP 17, and 2.5% hydroxyethyl cellulose 100.000 in water – formulation (2); release medium is a 10mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188;

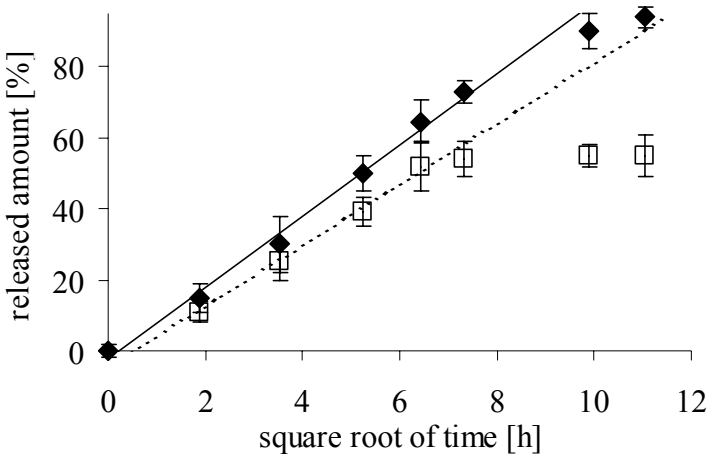


Fig. 3-77 Linearised release diagram of ACT from a xerogel formulation in the static model; release profiles are given as active ACT gained by activity assay (□, slope=8.6, $R^2$=0.98) and total ACT content measured by ELISA (◆, slope 10.2, $R^2$=0.99); underlying hydrogel contains 60µg/ml ACT, 10mM potassium phosphate buffer pH 7.2, 10mM arginine, 0.1% Poloxamer® 188, 0.05% PEG 2000, and 2.5% hydroxyethyl cellulose 100.000 in water – formulation (3); release medium is a 10mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188;

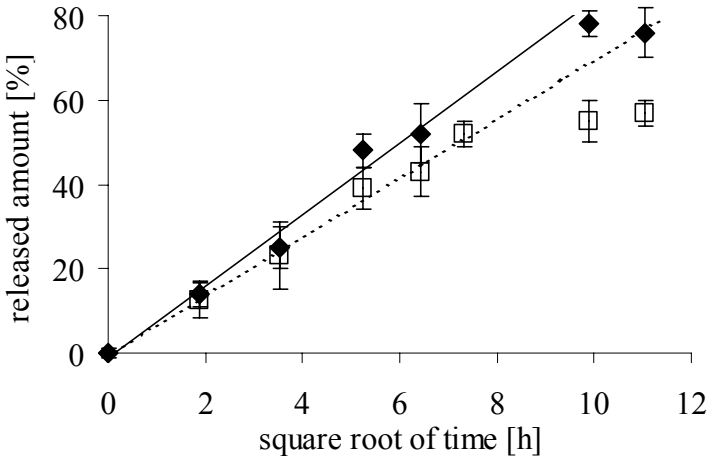


Fig. 3-78 Linearised release diagram of ACT from a xerogel formulation in the static model; release profiles are given as active ACT gained by activity assay (□, slope=7.0, $R^2$=0.99) and total ACT content measured by ELISA (◆, slope 8.5, $R^2$=0.99); underlying hydrogel contains 60µg/ml ACT, 10mM potassium phosphate buffer pH 7.2, 10mM arginine, 0.1% Poloxamer® 188, 0.05% PVP 17, 2.0% hydroxyethyl cellulose 100.000, and 1.0% gellan gum LT100 in water - formulation (4); release medium is a 10mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188;

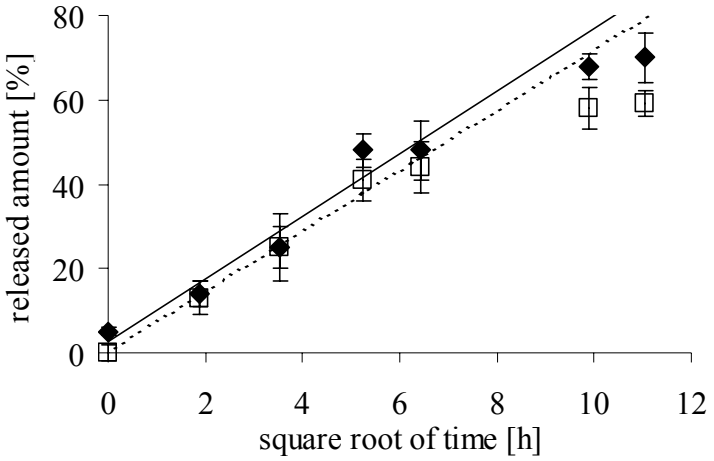


Fig. 3-79 Linearised release diagram of ACT from a xerogel formulation in the static model; release profiles are given as active ACT gained by activity assay (□, slope=7.2, $R^2$=0.98) and total ACT content measured by ELISA (◆, slope 7.4, $R^2$=0.98); underlying hydrogel contains 60µg/ml ACT, 10mM potassium phosphate buffer pH 7.2, 10mM arginine, 0.1% Poloxamer® 188, 0.05% PVP 17, 2.0% hydroxyethyl cellulose 100.000, and 1.0% gellan gum F in water - formulation (5); release medium is a 10mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188;

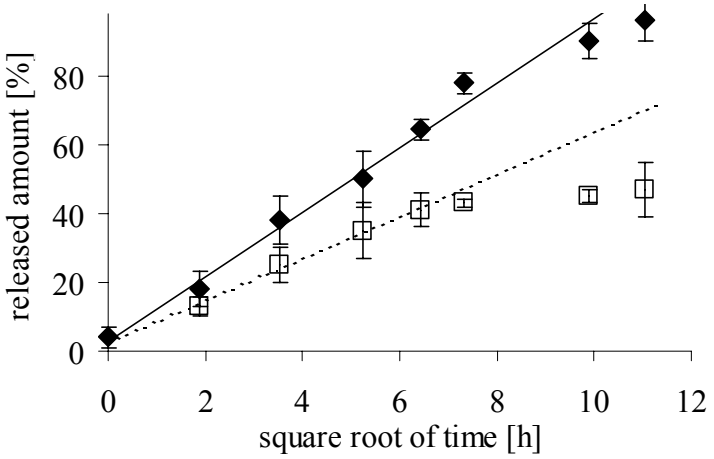


Fig. 3-80 Linearised release diagram of ACT from a xerogel formulation in the static model; release profiles are given as active ACT gained by activity assay (□, slope=6.1, $R^2$=0.99) and total ACT content measured by ELISA (◆, slope 9.5, $R^2$=0.99); underlying hydrogel contains 60µg/ml ACT, 10mM Tris hydrochloride buffer pH 7.2, 10mM arginine, 0.1% Poloxamer® 188, 0.05% PVP 17, and 2.5% hydroxyethyl cellulose 100.000 in water – formulation (6); release medium is a 10mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188;

Generally, the release kinetics of ACT from xerogels follow the theoretical predictions of the square root of time function (Equ. 3-15 , Figs. 3-75 to 3-80).

The total released amount of ACT values of all tested formulations vary in the range of 70% to 96%. Thereby, the lower values, also correlated with lower release

rates, are detected in Tween® 80 – formulation (2), Fig. 3-76 - and gellan gum containing samples – formulations (3), Fig. 3-77 and formulation (5), Fig. 3-79. However, a conclusion about specific influence of particular excipients is not significant because the released amount of bioactive ACT ranges only between 55% to 60%. Solely, the Tris hydrochloride containing samples – formulation (6), Fig. 3-80 - show a lower value. Slopes and released amounts of all formulations are summarised in Tab. 3-18.

The detected loss of activity and total amount exceed the predictions of the control experiments for the influence of the release system. Therefore, a certain amount of ACT should be lost by other means, e.g. due to an incomplete release or further degradation maybe within the matrix impeding the detection by both assays. Unfortunately, only minimal residual amount of ACT is detected in the donor gel so no data are available to support the speculations about missing ACT. As well, efforts to detect the absorbed amount of ACT by desorption with sodium dodecyl sulfate lead to no result due to incompatibility with assay methods.

Moreover, the bioactive fraction of the total released amount, being a quotient of the bioactive and total amount, varies in reciprocal means of the total amount. The highest bioactive fraction is found in the Tween® 80 samples – formulation (2), Fig. 3-76 – and in the gellan gum F sample – formulation (5), Fig. 3-79.

Furthermore, although slopes of ACT release curves tend to be slightly higher than in the curves of the model studies on FITC-Dextran70kDa, the differences are still too small to draw sound conclusions referring to interactions between diffusing substances and excipients (Tab. 3-18).

To conclude, although they do not provide the fastest release, formulation (2) and formulation (5) are to be assessed as best candidates because they offer the highest bioactive fraction released with acceptable release rates and sufficient total amounts liberated.

| | | | | |
|---|---|---|---|---|
| (1) | 60µg/ml | α1-antichymotrypsin | slope of ELISA curve | 7.8 |
| | 10mM | phosphate buffer | slope of activity curve | 6.6 |
| | 10mM | arginine | total amount released | 85% |
| | 0.1% | Poloxamer® 188 | bioactive amount released | 59% |
| | 0.05% | PVP 17 | bioactive fraction | 62% |
| | 2.5% | HEC 100.000 | | |
| (2) | 60µg/ml | α1-antichymotrypsin | slope of ELISA curve | 7.8 |
| | 10mM | phosphate buffer | slope of activity curve | 6.5 |
| | 10mM | arginine | total amount released | 72% |
| | 0.1% | Tween® 80 | bioactive amount released | 59% |
| | 0.05% | PVP 17 | bioactive fraction | 82% |
| | 2.5% | HEC 100.000 | | |
| (3) | 60µg/ml | α1-antichymotrypsin | slope of ELISA curve | 10.2 |
| | 10mM | phosphate buffer | slope of activity curve | 8.6 |
| | 10mM | arginine | total amount released | 94% |
| | 0.1% | Poloxamer® 188 | bioactive amount released | 55% |
| | 0.05% | PEG 2000 | bioactive fraction | 58% |
| | 2.5% | HEC 100.000 | | |
| (4) | 60µg/ml | α1-antichymotrypsin | slope of ELISA curve | 8.5 |
| | 10mM | phosphate buffer | slope of activity curve | 7.0 |
| | 10mM | arginine | total amount released | 76% |
| | 0.1% | Poloxamer® 188 | bioactive amount released | 57% |
| | 0.05% | PVP 17 | bioactive fraction | 75% |
| | 2.0% | HEC 100.000 | | |
| | 1.0% | gellan gum LT100 | | |
| (5) | 60µg/ml | α1-antichymotrypsin | slope of ELISA curve | 7.4 |
| | 10mM | phosphate buffer | slope of activity curve | 7.2 |
| | 10mM | arginine | total amount released | 70% |
| | 0.1% | Poloxamer® 188 | bioactive amount released | 59% |
| | 0.05% | PVP 17 | bioactive fraction | 84% |
| | 2.0% | HEC 100.000 | | |
| | 1.0% | gellan gum F | | |
| (6) | 60µg/ml | α1-antichymotrypsin | slope of ELISA curve | 9.5 |
| | 10mM | Tris HCl buffer | slope of activity curve | 6.1 |
| | 10mM | arginine | total amount released | 96% |
| | 0.1% | Poloxamer® 188 | bioactive amount released | 47% |
| | 0.05% | PVP 17 | bioactive fraction | 49% |
| | 2.5% | HEC 100.000 | | |

Tab. 3-18 Formulations of the release studies on xerogels; ingredients (left column), slopes of Linearised release curves for ELISA and activity assay detection, respectively, and content values after 120 hours release time; total released amount measured by ELISA, bioactive amount measured by activity assay, and the bioactive fraction released calculated by the quotient of bioactive amount and total amount;

### *3.5.6.2.3 Release of ACT from film formulations*

For the release studies of ACT from films a set of formulations taken over from the film stability studies (section 3.4.4.2) is tested using the static model. As given in the previous chapter dealing with the release from xerogels, experiments are

conducted over a releasing time of 120 hours (5 days) which corresponds to the usual change of dressing interval in clinical practice. Approximately every 12 hours, 24 hours in later phases of the release, a sample is taken and is analysed by both activity assay and ELISA. Interpretation is carried out as given above. Below, the linearised release curves for each formulations are presented (Fig. 3-81 to 3-85), a summary of calculations is given in Tab. 3-19.

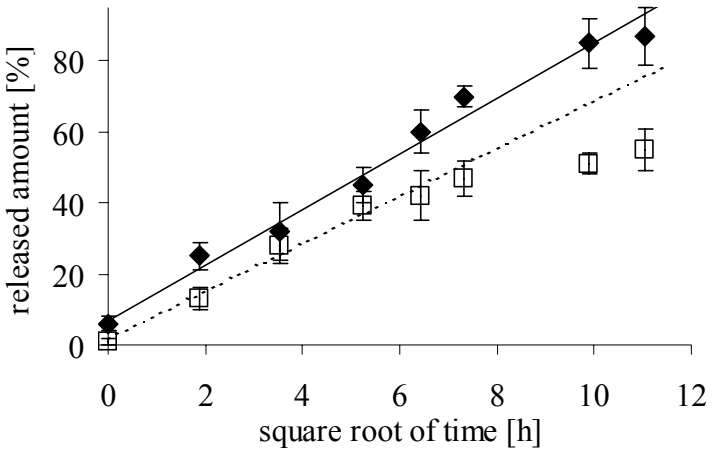


Fig. 3-81 Linearised release diagram of ACT from a film formulation in the static model; release profiles are given as active ACT gained by activity assay (□, slope=6.7, $R^2$=0.99) and total ACT content measured by ELISA (◆, slope 7.8, $R^2$=0.98); underlying hydrogel contains 60µg/ml ACT, 5mM potassium phosphate buffer pH 7.2, 10mM arginine, 0.1% Poloxamer® 188, 2.0% PVP 17, 2.0% PEG 400, and 2.5% hydroxyethyl cellulose 100.000 in water – formulation (1); release medium is a 10mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188;

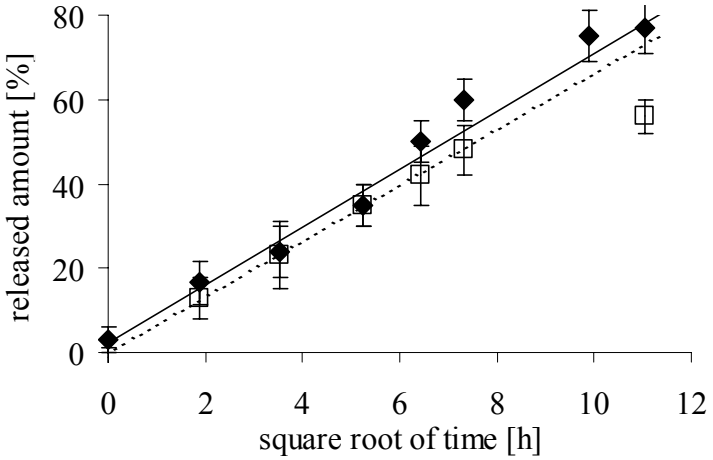


Fig. 3-82 Linearised release diagram of ACT from a film formulation in the static model; release profiles are given as active ACT gained by activity assay (□, slope=6.6, $R^2$=0.99) and total ACT content measured by ELISA (◆, slope 6.8, $R^2$=0.98); underlying hydrogel contains 60µg/ml ACT, 5mM potassium phosphate buffer pH 7.2, 10mM arginine, 0.1% Tween® 80, 2.0% PVP 17, 2.0% PEG 400, and 2.5% hydroxyethyl cellulose 100.000 in water – formulation (2); release medium is a 10mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188;

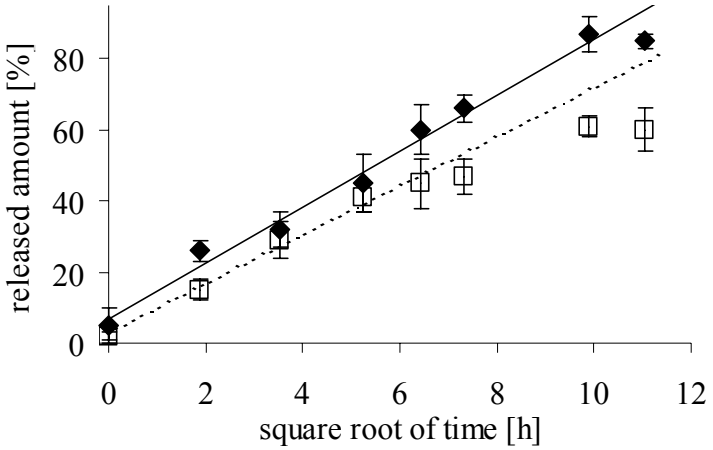


Fig. 3-83 Linearised release diagram of ACT from a film formulation in the static model; release profiles are given as active ACT gained by activity assay (□, slope=6.9, $R^2$=0.99) and total ACT content measured by ELISA (◆, slope 7.9, $R^2$=0.98); underlying hydrogel contains 60µg/ml ACT, 5mM potassium phosphate buffer pH 7.2, 10mM arginine, 0.1% Poloxamer® 188, 2.0% PVP 17, 2.0% PEG 400, 2.0% hydroxyethyl cellulose 100.000, and 1.0% gellan gum LT100 in water – formulation (3); release medium is a 10mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188;

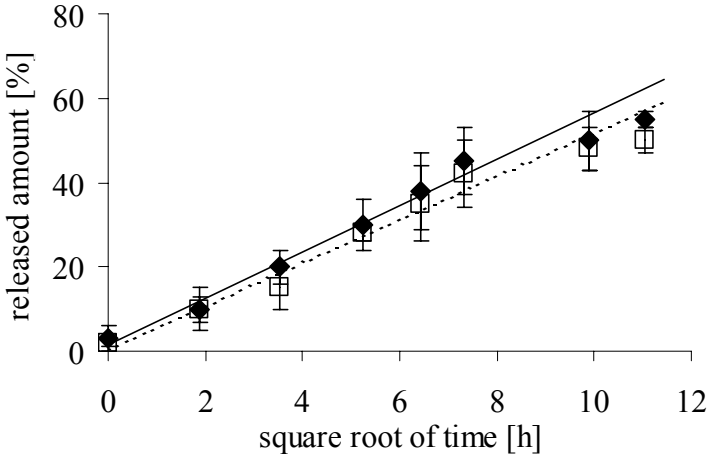


Fig. 3-84 Linearised release diagram of ACT from a film formulation in the static model; release profiles are given as active ACT gained by activity assay (□, slope=5.1, $R^2$=0.98) and total ACT content measured by ELISA (◆, slope 5.5, $R^2$=0.99); underlying hydrogel contains 60µg/ml ACT, 5mM potassium phosphate buffer pH 7.2, 10mM arginine, 0.1% Poloxamer® 188, 2.0% PVP 17, 2.0% PEG 400, 2.0% hydroxyethyl cellulose 100.000, and 1.0% gellan gum F in water – formulation (4); release medium is a 10mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188;

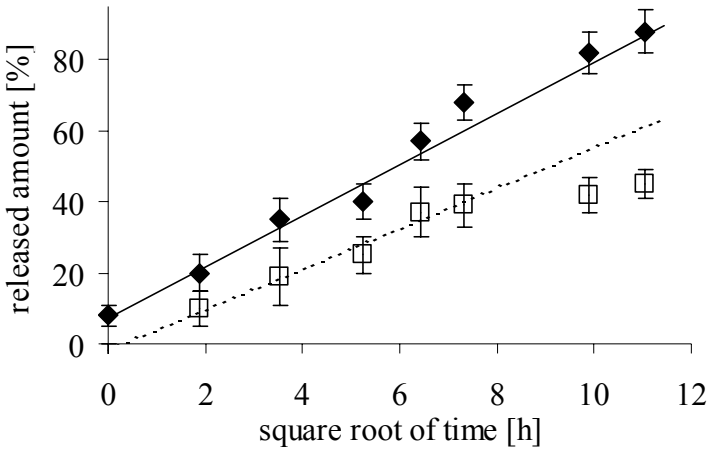


Fig. 3-85 Linearised release diagram of ACT from a film formulation in the static model; release profiles are given as active ACT gained by activity assay (□, slope=5.7, $R^2$=0.98) and total ACT content measured by ELISA (◆, slope 7.2, $R^2$=0.98); underlying hydrogel contains 60µg/ml ACT, 10mM Tris hydrochloride buffer pH 7.2, 10mM arginine, 0.1% Poloxamer® 188, 2.0% PVP 17, 2.0% PEG 400, and 2.5% hydroxyethyl cellulose 100.000 in water – formulation (5); release medium is a 10mM potassium phosphate buffer pH 7.2 with 0.1% Poloxamer® 188;

In all experiments films liberate about 55% of bioactive ACT within the given period. And, most of the corresponding total amounts of liberated ACT are detected between 77% and 87%.

But, differences between the particular film formulations can be seen in the different values for the bioactive fraction of released ACT. So, 73% of ACT molecules released by the Tween® 80 containing samples – film formulation (2), Fig. 3-82 – are active. Similar cases are seen with the gellan gum samples – film formulation (3), Fig. 3-83 and film formulation (4), Fig. 3-84 – which show bioactive fractions of 71% and 91%, respectively. Moreover, the Tris containing sample – film formulation (5), Fig. 3-85 – indeed releases a high amount of ACT in the given period, but only a fraction of 51% is bioactive.

Generally, these results correspond to the behaviour of particular xerogel formulations with similar excipient compositions. Besides, the amounts of non-recovered ACT are in the same range as in the xerogel experiments (Tab. 3-19).

The observation for films providing a faster release during model studies with FITC-Dextran can not be confirmed. This might be due to specific interactions of the protein structure with formulation excipients or experimental hardware superposing the small difference. Moreover, the uncertainty of results created by spreading of analytical read outs is also supposed to contribute to that.

| | | | | |
|---|---|---|---|---|
| (1) | 60µg/ml | α1-antichymotrypsin | slope of ELISA curve | 7.5 |
| | 5mM | phosphate buffer | slope of activity curve | 6.7 |
| | 10mM | arginine | total amount released | 87% |
| | 0.1% | Poloxamer® 188 | bioactive amount released | 55% |
| | 2.0% | PVP 17 | bioactive fraction | 63% |
| | 2.0% | PEG 400 | | |
| | 2.5% | HEC 100.000 | | |
| (2) | 60µg/ml | α1-antichymotrypsin | slope of ELISA curve | 6.8 |
| | 5mM | phosphate buffer | slope of activity curve | 6.6 |
| | 10mM | arginine | total amount released | 77% |
| | 0.1% | Tween® 80 | bioactive amount released | 56% |
| | 2.0% | PVP 17 | bioactive fraction | 73% |
| | 2.0% | PEG 400 | | |
| | 2.5% | HEC 100.000 | | |
| (3) | 60µg/ml | α1-antichymotrypsin | slope of ELISA curve | 7.9. |
| | 5mM | phosphate buffer | slope of activity curve | 6.9 |
| | 10mM | arginine | total amount released | 85% |
| | 0.1% | Poloxamer® 188 | bioactive amount released | 60% |
| | 2.0% | PVP 17 | bioactive fraction | 71% |
| | 2.0% | PEG 400 | | |
| | 2.0% | HEC 100.000 | | |
| | 1.0% | gellan gum LT100 | | |
| (4) | 60µg/ml | α1-antichymotrypsin | slope of ELISA curve | 5.5 |
| | 5mM | phosphate buffer | slope of activity curve | 5.1 |
| | 10mM | arginine | total amount released | 55% |
| | 0.1% | Poloxamer® 188 | bioactive amount released | 50% |
| | 2.0% | PVP 17 | bioactive fraction | 91% |
| | 2.0% | PEG 400 | | |
| | 2.0% | HEC 100.000 | | |
| | 1.0% | gellan gum F | | |
| (5) | 60µg/ml | α1-antichymotrypsin | slope of ELISA curve | 7.0 |
| | 10mM | Tris HCl buffer | slope of activity curve | 5.7 |
| | 10mM | arginine | total amount released | 88% |
| | 0.1% | Poloxamer® 188 | bioactive amount released | 45% |
| | 2.0% | PVP 17 | bioactive fraction | 51% |
| | 2.0% | PEG 400 | | |
| | 2.5% | HEC 100.000 | | |

Tab. 3-19 Formulations of the release studies on film formulations; ingredients (left column), slopes of Linearised release curves for ELISA and activity assay detection, respectively, and content values after 120 hours release time: total released amount measured by ELISA, bioactive amount measured by activity assay, and the bioactive fraction released calculated by the quotient of bioactive amount and total amount;

#### *3.5.6.2.4 Summary*

Generally, the release characteristics of ACT from gel matrices follow the theoretical predictions of a square root of time function. Moreover, both dried delivery systems – the xerogels and the films - are proven to be suitable for delivery of ACT in chronic wounds. In general, within five days 50% to 60% of initial ACT load is liberated in bioactive form. The total released content differs in broader range – 70% to 96% - within the different formulations. Thereby, the composition of ingredients has greater influence than the type of matrix. For instance, Tween® 80 and gellan gum F addition leads to lower total released amounts but provide high bioactive fractions in both xerogel and film matrices. The loss of activity is partly accredited to the influence of the release apparatus and therefore can be expected to be lower in an in vivo situation. However, a higher bioactive fraction released form a formulation in vitro is a measure for favourable properties of this particular formulation.

Though the non-liberated substance is not detected properly, speculations about it remaining in the gel seem appropriate since the phenomenon of incomplete release of protein drugs from gels has been described earlier[202].

As a result, for xerogels the formulations (2) and (5) appear as best choices, as is the case with formulations (2) and (4) for polymer films. The ingredients advantageous for release behaviour are as well found to be favourable for stabilisation in the hydrogel state (section 3.3.2.4.2). However, like with stability studies the most promising formulation is to be estimated for a combination of Tween® 80 and HEC/gellan gum F. But, this combination is as well not yet tested for its release behaviour and therefore cannot be directly recommended supported by data. But, in future studies, e.g. adjustment of the formulation for market launch, this can be taken into account. As well, due to the reduced amount of liberated and bioactive ACT an adaptation of the drug load in the carriers may be necessary to ensure the delivery of a sufficient dose of ACT into its site of action. However, the difficulty of in vitro in vivo correlation has to be kept in mind, especially against the background of the observed differences between the two release models as far as the release rate is concerned.

# 4 General summary, conclusions, and perspective

Despite wound treatment has gone through great revolutions during the last decades, the chronic cases, especially those associated with diabetes, are still difficult to treat. Indeed, about 25% of diabetes patients frequently develop chronic wounds with about half of them requiring very patient and persistent treatment. Therefore, there is strong desire for improved methods of therapy.
Besides, enormous progress was achieved in recombinant DNA techniques and methodology during the last decade. Hence, a multitude of biosynthetic, pharmaceutically relevant polypeptides and proteins have become available for a broad variety of diseases including chronic wounds and partly have been employed in pharmaceutical products already.
Yet, due to the proneness of proteins to degradation affecting pharmaceutical relevant features such as biological activity and immunogenicity, appropriate pharmaceutical formulation development of peptides and protein drugs is of utmost importance. Because, only in a well developed formulation the optimal therapeutic effects, harmlessness, and shelf life necessary for commercial success can be achieved and granted.

The delivery of $\alpha_1$-antichymotrypsin (ACT) was identified as one approach for new ways of chronic wound treatment by SWITCH BIOTECH AG, Neuried, Germany. Hence, to take the next step in development of ACT as drug candidate a collaboration between SWITCH and the Department Pharmaceutical Technology and Biopharmaceutics of the LMU Munich was contracted in terms of this Ph. D. study at the Department under the supervision of Prof. Dr. Winter.
So, it was the aim of this study to create formulations and drug carriers that stabilise and deliver ACT in bioactive state into the wound site.

The theoretical introduction encompasses the sections of **Chapter 1**, where first an overview over the physiology and pathophysiology of wound healing is described. This represents the medicinal context of this work and highlights the scientific rationale behind the delivery of ACT in chronic wounds. Moreover, the established methods for treatment of wounds including dressings and carriers are discussed. Hereby, the methodical context of a treatment with ACT as drug product with the

indication wound treatment is given. Subsequently, several aspects of protein delivery from hydrogels as application form are presented.

**Chapter 2** outlines materials and methods used during the studies.

In **Chapter 3** the results of this research work are presented and discussed.
In the course of the first section the stabilisation of ACT in **solution** is evaluated. Therefore, buffer systems at various pH levels and a multitude of stabilising ingredients were screened under temperature stress as well as freeze-thaw stress. By testing buffer conditions it turned out that a pH of 7.2 is the optimal value. However, the optimal range of pH values is very narrow. Especially, low pH levels can harmfully damage, fragment and inactivate ACT. So, for application on a wound site which can provide an acidic pH, a buffer system is recommended in the delivery device to guarantee suitable pH for non-liberated protein. For a phosphate buffer system the optimal content and relating osmolarity were examined. Moreover, from screened additives especially surfactants, cyclodextrins, and amino acids proved stabilising potential on ACT.
Except for acidic conditions, the main degradation pathways for ACT seem to be deactivation presumably by refolding and dimer formation and maybe later higher order aggregation.
Indeed, depending on the stress type two patterns for ingredients of stabilising solutions were identified relating to solutions to be kept in liquid state and frozen solutions, respectively. All ingredients are already approved for parenteral use by the FDA.
The following section is dealing with the formulation of ACT as **hydrogel**. After the evaluation of sterilisation and rheological properties of the gel carriers the stability studies on ACT were continued on the semi-solid hydrogel matrices. In a first approach the placebo hydrogel carriers were evaluated with regard to ability for sterilisation and rheological properties. After short term studies on the influence of polymers on ACT solution stability, consequently, ACT stabilising hydrogel formulations were created containing excipients being suggested by short term studies. Following, drug loading procedures into hydrogels were evaluated. After that, the named formulations were introduced in mid term stability studies and F/T studies. It turned out that a commercial formulation of ACT in a wet hydrogel is not possible due to insufficient shelf life. In contrast, all formulations can be applicated into the wound site as far as stability of ACT is concerned. As well, several formulations are applicative as early refrigerated formulation for the

purpose of animal experiment or early clinical trial where only limited shelf life is required.

For the inappropriateness of aqueous hydrogel and liquid formulations for long term stability of ACT, formulations with higher stabilising potential had to be developed that also have to fit the requirements of modern wound care. So, a dry form appears the best choice for improvement of drug stability, and hydrogels still are the state-of-the-art application form on wounds. As a result, dry forms had to be developed that form hydrogels after reconstitution. Based on the hydrogels reported in the prior chapter two alternatives were investigated to meet these aims - **xerogels** and **polymer films**.

For both forms carrier development was carried out by establishing production procedures concerning gel technology and drying techniques. Further, ingredient composition was optimised for both mechanical properties of the products and stabilising ACT in the matrices. To characterise these products their texture properties, swelling behaviour, residual moisture, ACT stability during drying, and during storage were examined.

Two xerogel formulations - based on hydroxyethyl cellulose and a mixture of hydroxyethyl cellulose and gellan gum, respectively - are identified that meet all the requirements. In fact, these matrices have a homogeneous and soft appearance, they swell spontaneously when hydrated to a hydrogel, and they deliver shelf lives for ACT exceeding 18 months.

For the films, as well, two formulations based on the same polymer components turned out to fulfil the aims. These matrices offer very convenient texture properties, as well form suitable hydrogels after hydration, and also provide shelf lives greater 18 months for ACT.

Thus, development of stabilising formulations for ACT suitable for application into wound sites was finished successfully.

In the following, the **release** of ACT from the preparations was studied in vitro. Generally, the release characteristics of ACT from gel matrices follow the theoretical predictions of square root of time functions. Moreover, both dried delivery systems – the xerogels and the films - are proven to be suitable for delivery of ACT in chronic wounds. In general, within five days about 60% of initial ACT load is liberated in bioactive form thereby being influenced to a higher degree by the composition of ingredients rather than the type of matrix.

Due to the reduced amount of liberated bioactive ACT an adaptation of the drug load in the carriers may be necessary to ensure the delivery of a sufficient dose of

ACT into its site of action. However, the difficulty of in vitro in vivo correlation has to be considered.

This thesis has shown that formulation of ACT for the topical delivery into wounds is possible. Thereby, two dry application systems – xerogels and films – that form hydrogels after reconstitution with aqueous solution have been identified being suitable for stabilisation and release of ACT.
Concerning stability the goal of a minimum shelf life of 18 months has been reached. Furthermore, the release period of ACT from the preparations is consistent with the common change of dressing intervals in clinical practice enabling an effective delivery.
It has also been shown that the tested dry gel based carriers are suitable for protein stabilisation and delivery which could be a kick off for further proteins to be developed on the basis of these systems.
During the whole development process great emphasis has been placed on the mechanical properties of the matrices to be suitable for wound treatment. Also, manufacturing procedures have always been evaluated with prospect of a future larger scale process for aseptic manufacture of a commercialised product. Concomitantly, ingredients have been reviewed for their status of approval to avoid unnecessary obstacles in a later process of approval by authorities.

But, it also has to be mentioned that the difficulties created by analytical tools used for characterisation of ACT have not been completely overcome. Hence, for a further development of this project another evaluation and validation of analytical tools might be appropriate. Moreover, the discussed results are exclusively based on in vitro research which might not be correlated with in vivo situations in an unconfined way.

The future milestones of this project should be the verification of these promising in vitro results in an animal experiment. Therefore, a study on excision wounds on diabetic rats has been evaluated. Furthermore, provided that these animal studies are finished successfully, Phase I clinical trials in man represent the next milestones to be aspired.

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# Curriculum Vitae

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