

Wound dressing compositions, especially for delivery of protease inhibitors

The present invention relates to dry and hydrated, i.e. wet wound dressings and delivery systems also suitable for active ingredients, their use for the treatment of wounds and skin diseases, preferably chronic wounds, and methods of preparing them. More particularly, the present invention relates to a xerogel or film comprising a mixture of a cellulose ether and a gellan gum. Such systems can be used directly as wound dressings or alternatively as dry, storage stable delivery systems for active ingredients, preferably proteins, in the field of cosmetics and medicine. Before use and/or during application in a moist environment, especially wounds, the dry composition is rehydrated, thus serving as a hydrogel loaded with active ingredients which are released at a controlled rate. Depending on the ionic strength of the hydrating solution the resulting gel can be immobilised and thus the composition cannot flow out of the wound, e.g. under the influence of gravity. Such a system can be used for moist wound healing or dermal delivery of therapeutic substances and for other medical or cosmetic purposes.

In an especially preferred embodiment, the wound dressing according to the invention comprises a cellulose ether, a gellan gum and alpha-1-antichymotrypsin (ACT). Such compositions are characterized by excellent release characteristics for ACT and exceptional storage stability regarding ACT when stored in dry form.

Background of the invention.

The present invention can be used for delivery of active ingredients, especially alpha-1-antichymotrypsin, into wounds. The growing number of patients with diabetes mellitus, venous insufficiency and other chronic diseases and injuries have resulted in an increased incidence of chronic non-healing soft-tissue wounds of the skin: Apart from causing great costs to the public health system, chronic wounds give rise to a very painful, distressing condition of the patients and may even lead to amputation. Therefore, adequate treatment and promotion of dermal wound healing is necessary.

The mechanisms of wound healing in general and characteristics of different wound healing phases are well known. Since 1962 moist wound healing has become a widely accepted treatment. Already many different moist bandages, like hydrocolloid, hydropolymer or alginate systems are on the market. These bandages shall ensure a moist environment in the wound. Sometimes they additionally take up wound secretions while they swell or in exchange with solutions that are incorporated into the bandages (for exampleringer solution). All these wound dressings consist of a swollen or swellable polymer and sometimes of a water resistant backing layer, but they usually do not contain any therapeutic substances.

Next to a moist environment adequate concentrations of growth factors are necessary to promote healing. In the 1970s the inductive effect of platelet derived factors and other cytokines was first described. The necessary balance and concentration of different growth factors for the promotion of healing is often disturbed, especially in elderly people and/or patients with diabetes or autoimmune disorders. Therefore, it was proposed to apply wound healing factors like PDGF, TGF- β , F XIII, KGF- or EGF, to enumerate just few, topically into wounds. Moreover, protease inhibitors have been proposed to promote healing of chronic wounds. Especially, alpha-1-antichymotrypsin has been described as being useful in stimulating the wound healing process, particularly in diabetic wounds (WO 02/088180).

A problem which has often been encountered is that the topical delivery system for active ingredient does not adequately stabilize or release the active ingredient and/or that it is not adequate regarding patient compliance. Especially for proteins as active ingredients, which are typically labile, the above prerequisites are difficult to meet.

Drug solutions with low viscosity evaporate fast and therefore have to be applied very often. Although solutions are easy to apply the persistent contact with the wound surface is not good because a solution does not adhere to the site of action on the wound surface. Moreover, growth factors, enzymes, and some inhibitors which promote wound healing in the first inflammatory phase are proteins and therefore quite unstable and sensitive molecules. When stored in aqueous media at room temperature many proteins do not remain stable, they may aggregate and lose activity rapidly. EGF in an aqueous formulation, for example, lost 40% of its activity within two weeks (Clanan, D.P. *et al.* Potency and stability of C-terminal truncated human epidermal growth factor, *Gut* 47, 622-627 (2000)). To achieve longer storage stabilities protein solutions have to be stored under defined conditions at deep temperatures (-20°C or 4-8°C). Therefore, these aqueous products have technical, economical and handling drawbacks. E.g. the solution Eurokinin,

a cleaned solution of the patient's own growth factors, must be stored in a freezer. This leads to additional problems as it has to be carefully thawed before application, which decreases patient and medical personnel compliance. An alternative to deep temperature storage is the stabilisation of a sensitive substance in dry products. It is a widespread method in pharmaceutical technology to embed sensitive substances like proteins in dry, amorphous matrices to ensure low degradation rates. Thus, there is a high need for the development of dry, storage stable active ingredient products. It is often not possible to design formulations based merely on the lyophilisation of the bulk solutions, as the amounts of active ingredient used in the formulation for a single dose will usually be very small and as during the lyophilisation process the drug may accidentally be pulled from the lyophilisation container by the vacuum employed in the process. Furthermore, many proteins are relatively unstable when lyophilised in small concentrations. They can adsorb to product packaging and lose activity. This may only be overcome by the use of a diluent or extender to increase the amount of solid present during the process.

Moreover, hydrogels are preferred over solutions with low viscosity as they keep the wound moist, do not evaporate fast and therefore have to be applied only once daily. The solution Eurokinin, for example, has to be applied continuously onto a compress on the wound. Regranex is a hydrogel which shows good wound healing and handling properties, but inadequate storage stabilities. Gels can provide a controlled delivery system for protein on a wound site. Controlled release refers to a drug release sufficient to maintain a therapeutic level over an extended period of time. This is an important advantage because it permits less frequent application of the formulation to the wound and thereby permits less disturbance of the wound. A variety of gels and ointments for application to wounds are described in the prior art for a variety of purposes. They may for example be used to clean wounds, to promote healing of the wound or to prevent infection. In certain circumstances, the gel may include an active ingredient which is administered to the patient by topical application of the gel. One example of a commercially available wound gel is Intracel[®] produced by Smith & Nephew Ltd. This gel contains hydrated carboxymethyl cellulose-Na as a main ingredient and is packaged and applied to wounds in gel form as a primary treatment in order to debride the wound.

Gels have the further advantage of having a high water content, easy application to a wound, and easy removal from the wound after application by washing. The gel may also assist in preventing the wound from drying out, thereby promoting the healing process. Since gels are mobile they offer the advantage of intimate contact with the often irregular surface of a wound, something that is often not achieved with a more rigid or liquid wound dressing. The advantage of good contact is, however, tempered by the conflicting

needs of making the gel sufficiently mobile that it can be applied to the wound but not so mobile that it runs out of the wound under the influence of gravity. Gels currently in use suffer from the disadvantage that they can run out of the wound.

However, other problems of existing topical products have to be faced as, for example, the absence of exact and reproducible dosage. All hydrogels on the market like Regranex® are dosed by the amount of the applied hydrogel strand, which is not sufficiently exact and reproducible.

An alternative to deep temperature storage of wet drug forms is the stabilization of a sensitive substance in dry products. It is a widespread method in pharmaceutical technology to embed sensitive substances like proteins in dry, amorphous matrices to ensure low degradation rates. Thus, there is a high need for the development of dry, storage stable active ingredient products. These dry products have to be rehydrated before or during use, as for wound healing the application of moist products, like preferably hydrogels, is necessary.

The drying procedure can be carried out by various methods. Freeze drying and warm air drying are preferred drying procedures for the present invention. Particularly preferred is freeze-drying. Drying methods using organic solvents are less preferred due to the unwanted interactions of labile active ingredients with organic solvents and the problems of residual solvent contents in the product that at least affords additional testing routines adding to production costs.

A dry storage system can either be hydrated before use with a suitable aqueous solution and/or during use in contact with aqueous body fluids, especially when applied topically to a wound. Preferred dry storage systems for hydrogels are films and xerogels, especially xerogels. The xerogel or film take up water upon hydration, swell and eventually form a hydrogel.

A desirable, optimal active ingredient product would have a dry, storage stable form and could be hydrated to a hydrogel before use. Such storage stable forms need not to be stored at very low temperatures and thus allow also easy and cheap transportation. Moreover, such products may also be stored by the patient himself without complications. Thereby, high costs which occur when treatment has to be effected at hospitals are avoided. It would be useful to have a dry, ready to use, single dose product covering a defined contact area, which can also be cut reproducibly into pieces in order to achieve defined doses. Moreover, it would be a great advantage if the patient to be treated could adapt the

medicament or cosmetic composition according to his needs, for example to immobilize the gel by hydration to prevent flowing out of the wound or to reconstitute it to a mobile gel that can insert in even cleft regions of a wound without gaps.

It is described in the prior art to mix an active medicament unstable to heat with a biodegradable protein carrier such as collagen, atelocollagen, or gelatin to form a carrier matrix having sustained release properties. The resultant mixture is then dried, and the dried material is formed into an appropriate shape, as described in US 4,774,091. Examples of active ingredients for this purpose are given as t-Pa, prostaglandines, prostacyclines biohormones, e.g. hGH, bGH, GRF, somatomedins, and calcitonin, interferons, interleukins, tumor necrosis factor and other cytokines such as macrophage activating factor, migration inhibitory factor, and colony stimulating factor. JP 2002143290 describes a freeze dried matrix made from a gel formed by a mixture of PLGA-copolymer and bovine atelocollagen I. Additionally, the matrix is cross-linked by glutaraldehyde vapour. EP 083491A2 discloses a polysaccharide sponge made by solvent drying. US 5,189,148 describes a stabilized FGF composition and production thereof. US 2003/0105007 discloses a solid formulation for growth factors, e.g. PDGF in fibronectin. WO 91/19480 describes a freeze-dried hydrogel preparation containing a wound healing medicament which is compressed after freeze drying. The polymer in this invention is Hydroxyethylcellulose (HEG), EP 0308238A1 describes stable lyophilised formulations containing growth factors. US 5,714,458 discloses a stable lyophilised formulation of FGF in a xerogel. DE 19503338 A1 describes a film for the release of collagenase. The film can consist of various polymers. Gellan gum is not mentioned. WO03/034993 and B1102118 describe collagen sponges as wound/tissue healing material. CA2246895 describes bioabsorbable solid materials including films and sponges.

Cellulose derivatives have been used to formulate therapeutic proteins or polypeptides for topical use. See, e.g. EP 267,015, EP 308,238, and EP 312 208, which disclose formulations of a polypeptide growth factor having mitogenic activity, such as TGF beta, in a polysaccharide such as methylcellulose. EP 261,599 discloses human topical applications such as TGF beta. EP 193,917 discloses a slow release composition of a carbohydrate polymer such as cellulose and a protein such as growth factor. GB 2,160,528 describes a formulation of a bioactive protein and a polysaccharide. US 4,609,640 describes a therapeutic agent and a polymer selected from polysaccharides, cellulose, starches, dextroses. DE4328329 claims a freeze dried biomatrix consisting of natural polysaccharides and modified polysaccharides. GB 2357765 describes an alginate foam crosslinked by di- and bivalent cations.

The above-described wound dressing materials provide important advantages. The materials are of natural, biological origin (albeit chemically modified), and consequently tend to have low antigenicity. Furthermore, some of these materials can have positive therapeutic effects on wound healing. In principle, they are suitable for the delivery of protein active ingredients.

However, some difficulties remain. For example, a drawback of some collagen- and gelatine-based wound dressing materials is that the collagen breaks down too fast in vivo, due to the action of collagenase enzymes in the wound. This can be countered to some extent by cross-linking the collagen/gelatine with a covalent cross-linking agent such as glutaraldehyde or dicyclohexylcarbodiimide. Residual contents of these agents, however, can cause unwanted interactions with loaded protein active ingredients and the wound milieu itself. Besides, by cross-linking a strong irreversible immobilization is effected. So depending on the type of wounds, the immobile sponge in the first period after application may not cover cleft wound grounds and in the later stages the sponge is liquefied by enzymatic degradation what as well does not provide good contact to the wound ground. Moreover, collagen as a component of wound dressings is prone to denaturation when it is sterilized by gamma-irradiation. Collagen and gelatine also are extracted from natural animal sources. So, the problems of contamination with pathogens for animal or human, like the bovine spongiform encephalopathy (BSE) and others are relevant. Moreover, it can be antigenic to certain patients unless stringent measures are taken to purify the collagen, which add to its cost.

Non cross-linkable materials like non-ionic cellulose ethers suffer from the rheological disadvantage that they can run out of the wound and so lose close contact with the wound surface. Materials that are covalently cross-linked are always endangered of having unwanted effects caused by residual contents of cross-linking agents antagonising wound healing. Di- and bivalent cations used e.g. for crosslinking alginate also can have antagonising effects on the wound healing process. Moreover, solutions of these cations in the needed high concentrations are not used in clinical practice. This complicates the possibility that the cross-linking is carried out by the applicant when it seems appropriate for the individual wound condition. Additionally, alginates are not suitable for steam sterilization.

Biodegradable polymers like PLGA, collagen, gelatine, and others in some cases are known to interact with labile drugs due to their fragmentation. So, fragments of PLGA, i.e. lactic acid and glycolic acid, can change pH and the osmotic pressure within the matrix to awkward proportions. The manufacture of matrices consisting of polymers unsuitable for

steam sterilization, e.g. hydroxypropyl cellulose, hydroxypropylmethyl cellulose, collagen, and gelatine, need a more complicated and expensive sterilization process, e.g. gamma irradiation, which adds to its costs. Sodium salts of polymers like carboxymethyl cellulose sodium or carboxymethyl starch sodium are unfavourable for lyophilisation because their combination with phosphate buffer, which is essential for many protein formulations, results in a pH-shift into the sour region, which is counterproductive for protein stability.

Therefore, there remains a need for drug delivery compositions for topical use, especially improved wound dressing materials exhibiting control of physical properties, therapeutic effects on wound healing, reduced costs, and material safety in terms of reduced antigenic response and circumvention of unwanted effects of fragments or counterions of matrix molecules.

It is an object of the present invention to provide improved drug delivery compositions for topical use, especially wound dressing materials suitable for delivery of active ingredients if necessary, to mammalian wounds, and especially to human chronic wounds, such as venous ulcers, decubitus ulcers and diabetic ulcers. We make a new combination of dressing materials available, which exhibit a mechanical, i.e. rheological, behaviour which can be controlled and adapted easily by the patient simply by choosing the hydration medium. Moreover, the wound dressing compositions of the invention are surprisingly suitable for stabilizing and releasing proteins, in particular alpha-1-antichymotrypsin.

The present invention relates a wound dressing composition comprising a cellulose ether and a gellan gum.

In a preferred embodiment the cellulose ether is hydroxyethyl cellulose (HEC).

As it can be taken from the examples, a wound dressing composition made out of gellan gum and HEC is especially advantageous since it is very soft and comfortable. The use of HEC is advantageous over e.g. the use of carboxymethyl cellulose (CMC), since CMC matrices generally are harder in texture due to higher density, more brittle and show reduced swelling in comparison to HEC. Furthermore, CMC is usually applied in the form of sodium CMC. However, the ionic groups of sodium CMC may interact with active, therapeutic ingredients, which may result in a destabilization or at least partial inactivation of the ingredient, especially if it is a protein.

In another preferred embodiment the gellan gum is a deacetylated gellan gum.

In an even more preferred embodiment, the gellan gum is available under the trade name Kelcogel F.

In an especially preferred embodiment, the composition comprises hydroxyethyl cellulose (HEC) and deacetylated gellan gum.

In a preferred embodiment, the cellulose ether and the gellan gum are homogeneously mixed.

In a preferred embodiment the composition is in dry form, particularly a xerogel or film.

In a preferred embodiment, the wound healing composition is a hydrogel. In another embodiment, the hydrogel is obtained by hydration of a dry storage form of the gel, in particular by hydration of a xerogel or film.

The composition may contain, if desired, one or more additional excipients like sugars, sugar alcohols, surfactants, amino acids, antioxidants, polyethylene glycols. In a preferred embodiment, the excipients comprise at least one non-ionic surface active component preferably selected from Poloxamer® 188, Tween 80 and Tween 20.

In a preferred embodiment, the excipients comprise at least one buffer agent, preferably selected from phosphate and Tris.

In a preferred embodiment, the excipients comprise at least one amino acid, preferably arginine.

In a preferred embodiment, the excipients comprise at least one polyethylene glycol, preferably selected from PEG 400 suitable as plastiziser and PEG 2000 suitable as pore former.

In a preferred embodiment, the excipients comprise at least one polyvinyl pyrrolidone, preferably Kollidon 17PF, which is suitable as strengthener or pore former or stabilizer. The wound dressing compositions according to the present invention preferably are a homogeneous mixture of gellan gum and hydroxyethyl cellulose.

In a preferred embodiment, the wound dressing composition comprising the homogeneous mixture of gellan gum and hydroxyethyl cellulose is in a suitable

vehicle, such as a solvent, in an even more preferred embodiment the solvent is aqueous and the composition is a hydrogel. In another embodiment the hydrogel is formed by hydration of a film, xerogel or sponge.

In another preferred embodiment, the wound dressing composition comprising the homogeneous mixture of gellan gum and hydroxyethyl cellulose is dry. In an even more preferred embodiment the dry composition is a film or a sponge or xerogel.

The mixture of the two components, the gellan gum and the hydroxyethyl cellulose, synergistically combine their benefits and complement one another to prevent their disadvantages observed when used alone.

Hydroxyethyl cellulose (HEC) can be obtained by the process described in US 4,084,060. HEC is a non-ionic water-soluble cellulose ether, formed by reaction of cellulose with ethylene oxide. It is widely used in pharmaceutical compositions and very well known in the art. It can be dispersed in cold or hot liquids, but is insoluble in most organic solvents. This material offers numerous advantages including the features that it is biocompatible, biostable, non-immunogenic and readily commercially available. It can be obtained in various degrees of molecular weight at high levels of purity. Preferably, the hydroxyethyl cellulose has a molecular weight of at least 500 kDa. Gels of hydroxyethyl cellulose can be steam sterilized.

Processes for producing gellan gum are well-known in the art, e.g., in US 4,326,052, US 4,326,053, US 4,377,636, US 4,385,126, and US 4,503,084. Particularly preferred is deacylated gellan gum, which is for example described in US 4,326,052. Gellan gum is a microbial polysaccharide derived from *Pseudomonas elodea*. It consists of a tetrasaccharide unit, α -D-glucose, α -D-glucuronic acid, α -D-glucose and α -L-rhamnose. $(\rightarrow 3)$ -b-D-Glcp-(1 $\rightarrow$ 4)-b-D-GlcpA-(1 $\rightarrow$ 4)-b-D-Glcp-(1 $\rightarrow$ 4) α -L-Rhap-(1 $\rightarrow$). Gellan is produced with two acyl substituents present on the 3-linked glucose, namely, L-glyceryl, positioned at O(2) and an acetyl substituent at O(6). The native polysaccharide is partially esterified; the 1,3-D-Glc residue can be linked to L-glycerate at C-2 and/or to acetate at C-6, and there is 1 mol of glycerate per repeating unit and 0.5 mol of acetate per repeating unit. Acyl substituents affect the rheology of gels, and deacylation of native gellan results in a change from soft, elastic, thermoreversible gels to harder, more brittle gels. Gellan gum has been suggested for use in wound healing as solution/gel in US 6,596,704 and fibers in EP 0454373 as well as in WO 95/05204. Gellan gum has the CAS No. 71010-52-1. Well known Gellan gum products are available under the tradenames Gelrite® and Kelcogel®.

"Deacetylated Gellan Gum" according to the present invention is to be understood as gellan Gum obtainable by partial or complete deacetylation of native Gellan Gum. Preferably, the deacetylated Gellan Gum contains less than 10%, more preferably less than 25%, in particular less than 50%, most preferably less than 75% of the acetyl groups present in native Gellan Gum. Native Gellan Gum can be obtained by aerobic fermentation using *Sphingomonas elodea*.

Deacetylated Gellan gum is readily soluble in water and is characterized by high viscosity at low concentration. The gum also has a high rheological yield point. Changes of pH in the range 3-11 do not substantially affect the viscosity of the gel. The viscosity of such gel is also stable in the range of 20°C – 70°C. Above this temperature it reversibly liquefies; i.e. the thermoreversibility is formed after heating and cooling. Following heating in the presence of various cations deacetylated gellan gum produces firm, non-elastic or brittle gels. Cations especially useful in the formation of gels with are those of sodium, potassium, magnesium, calcium. Gellan gum is used as a thickening, suspending and stabilizing agent in aqueous systems in various fields including nutrition.

The common advantages of gellan gum and hydroxyethyl cellulose are that both components can promote wound healing. Moreover, both gelling agents can be sterilized by steam sterilization, which strongly simplifies production processes. The swelling and thermoreversible behaviour of gellan gum also are activated by steam sterilization. Moreover, they can be lyophilised and they also form stable films when air dried. Also, a very low content of each gelling agent is needed for forming gels with high viscosities. As a result of the low concentrations of both HEC and gellan gum needed for the manufacture of a hydrogel of the invention, the pore sizes of freeze dried hydrogels of the invention (i.e.

xerogel) are very high and the thickness of dried hydrogels of the invention (i.e. films) are very low, respectively. Together with the high hydrophilicity of both HEC and gellan gum, dry wound healing compositions of the invention show a strong and fast hydration and swelling which make them suitable for application in wound care. Moreover, both HEC and gellan gum are commercially available with a range of defined and controllable properties offering the possibility to adjust and control the properties of the wound healing compositions of the present invention as needed to an exceptional degree. In particular, the mechanical texture, viscosity, the rate of hydration, porosity and density of the materials can be adjusted in a wide range as it seems appropriate.

However, hydroxyethyl cellulose has the disadvantage that it is a non-crosslinked gelling agent. Such HEC-gels not containing gellan gum show a pseudoplastic rheology. That means there is no rheologic yield point and it is therefore free-flowing under the influence of gravity. The free-flowing HEC gels without gellan gum will flow out of the wound site and so lose contact with the wound ground resulting in a loss of their therapeutic effects.

Moreover, deacetylated gellan gum in the absence of HEC has the disadvantage that it forms brittle gels in presence of cations that would not be suitable for administration to a highly sensitive region such as a wound. Moreover, it lacks of the flexibility and flow tendency to be able to be applied without gaps to uneven and cleft wound surfaces. This, however, is essential for a wound dressing because the suitability of such wound dressing is greatly dependent on the interaction between the hydrogel wound dressing and the wound fluid: the release from the wound dressing can only take place by diffusion of the active substances at the interface between hydrogel wound dressing and wound fluid. For accurate, controlled, and reproducible release of drugs, especially in case of highly potent substances at low doses, e.g. proteins, a close contact between wound dressing and the wound is a prerequisite.

The mixture of a HEC and a gellan gum has all the advantages of the single components alone and overcomes disadvantages of the single components, which makes the mixture exceptionally suitable for its use as wound healing composition. First, the rheology of the wound healing composition comprising a mixture of a gellan gum and HEC in the gel state can be adjusted as needed offering a broad range of advantageous wound dressing compositions:

Hydration of a xerogel/film comprising gellan gum and HEC, and optionally at least one therapeutic wound healing agent with water:

The resulting gel behaves more like a HEC gel than a gellan gum gel. It still has a good flowability and is very smooth. The low salt contents incorporated into the matrix for other purposes, e.g. production of raw materials, pH adjustment, buffering, etc., provide only a very lowly pronounced yield point and plastic rheology. It is superior to a pure HEC gel in terms of running out of the wound site, but has the necessary flowability to creep into clefts of wounds.

Hydration of a xerogel/film comprising Gellan gum and HEC, and optionally at least one therapeutic wound healing agent with electrolyte solutions, e.g. isotonic NaCl, Ringer, etc:

The resulting gel is immobilized and provides a higher yield point and a highly pronounced plastic rheology. Moreover, it still is flexible enough to adjust to any surface morphology. Moreover, the soft pressure of a bandage is enough for the gel pad to overcome the yield point and start to creep and to deform to the given shape of the wound site.

Split up of the films/xerogels comprising Gellan gum and HEC, and optionally at least one therapeutic wound healing agent to fill extremely deep wounds:

For some very deep wound caves the flowability of the immobilized gel could not be sufficient. In that case the film/xerogel can be cut into parts. The part(s) for the deep cave of the wound ground can be hydrated in water. They will start to flow and creep into the deepest wholes of the wound like a normal free flowing hydrogel. The second part(s) will be hydrated in electrolyte solutions and get immobilized, They are placed over the first non-immobilized part(s) of the gel into the wound and serve as a clot. So the wound is completely filled without gaps, but the gel is still immobile and will stay in the wound accurately as long as desired.

Preferably, the weight ratio of HEC to gellan gum is in the range from 1:5 to 50:1, preferably from 1:1 to 10:1 in the wound dressing compositions of the invention.

Preferably, the hydroxyethyl cellulose and gellan gum together make up at least 25% by weight of the material on a dry weight basis, more preferably at least 30% by weight of the material on a dry weight basis, even more preferably at least 40% by weight of the material on a dry weight basis, particularly at least 50% by weight of the material on a dry weight basis. In wet hydrogels the content of gelling agent mixture; i.e the sum of HEC and gellan gum is preferably between 1% and 10 %, preferably between 2 and 4%.

In one embodiment, the material essentially consists of the hydroxyethyl cellulose and gellan gum. In a preferred embodiment the composition is in form of a hydrogel or a dried or lyophilised form thereof, i.e. a film, xerogel or sponge. Such compositions may be used directly for topical applications in the field of cosmetics or medicine.

The composition according to the present invention may be in any convenient form, such as powder, microspheres, flakes, a mat, a sponge, a xerogel or a film.

In certain embodiments, the composition according to the present invention is in the form of a semisolid or gel ointment for topical application. In a preferred embodiment the wound dressing compositions of the present invention are in form of a hydrogel.

In another preferred embodiment, the wound dressing compositions of the present invention are in form of a freeze-dried or solvent-dried xerogel or sponge.

In yet other embodiments, the composition according to the present invention is in the form of a flexible film, which may be continuous or interrupted (e.g. perforated).

In a preferred embodiment, the flexible film preferably comprises a plasticiser to render it flexible, such as PEG 400, glycerol, or propylenglycol.

In another embodiment the invention relates to a wound dressing package comprising a sterile wound dressing composition of the invention, packaged in a microorganism-impermeable container. Such packages may usually represent single or multi unit dosage forms of the present compositions. These packages can be easily used by the patient himself, especially if the package of the invention contains a wound dressing composition in dry form, especially in form of a xerogel, sponge or film. Such packages are especially preferred embodiments of the invention,

In another embodiment of the present invention the wound dressing compositions are located on an inert support, preferably selected from adhesive strip, adhesive wrap, bandage, gauze bandage or compress system. Thus, the present invention also relates to wound dressing systems comprising an inert support and a wound dressing composition of the invention. Such wound dressing systems can also be packaged in a microorganism-impermeable container or foil and thus represent wound dressing packages with high patient compliance.

The wound dressing compositions, systems and packages of the present invention may be used for the treatment of wounds, especially badly healing wounds like chronic wounds, in particular for the treatment of diabetic, venous, decubitus or neuropathic ulcers or infected wounds.

Moreover, the present compositions may also be used for cosmetic or medical treatment of skin diseases and skin conditions or diseases of a mucosa or eye in general, especially when an active ingredient is part of the compositions and the compositions therefore serve as topical delivery system for the active ingredient. Dependent on the therapeutic or cosmetic aspect of the applied active ingredients the compositions suitable for cosmetic and/or medical use of the invention can be used, for example for treating and/or preventing ophthalmologic, nasal diseases, wounds or skin disorders, e.g. selected from psoriasis, dermatoses, eczema, urticaria, lupus erythematosus, vitiligo, pigmentation disorders, wrinkling, aged skin, ichthyoses, hyperkeratoses, contact dermatitis, hand eczema or atopic dermatitis. It may also be used, depending on the active ingredient, for transdermal delivery of active ingredients.

The invention therefore relates to a wound dressing composition according to the present invention comprising HEC and Gellan gum, wherein the composition further comprises one or more active ingredients. In another embodiment the invention relates to a wound dressing composition according to the present invention comprising HEC and Gellan gum, wherein the composition further comprises one or more active ingredients for use as a medicament. Also, the invention relates to the use of a wound dressing composition according to the present invention comprising HEC and Gellan gum for the preparation of a medicament for the treatment and/or prevention of ophthalmologic, nasal diseases, wounds and skin disorders.

Thus the present invention also relates to a wound dressing composition of the present invention, wherein the composition further comprises one or more active ingredients which are active in the treatment and/or prevention of ophthalmologic, nasal diseases or skin disorders, e.g. selected from psoriasis, dermatoses, eczema, urticaria, lupus erythematosus, vitiligo, pigmentation disorders, wrinkling, aged skin, ichthyoses, hyperkeratoses, contact dermatitis, hand eczema or atopic dermatitis.

Such active ingredients comprise steroids, like hydrocortisone and betamethasone, Calcineurin inhibitors like tacrolimus, Pimecrolimus and Cyclosporin A, clobetasol, vitamin A derivatives like retinoic acid and esters and amides thereof, Tazarotene, vitamin D derivatives like calcitriol and calcipotriol, to enumerate a few. Furthermore, the

invention thus relates to the use of a wound dressing composition of the present invention comprising at least one active ingredient for the preparation of the medicament for the treatment of mucosal diseases, ophthalmologic diseases, wounds and skin diseases,

The wound healing compositions can be used directly for the treatment of wounds, without any further active ingredient due to the beneficial effects of the components HEC and gellan gum on wound healing alone as well as their optimal rheologic and moist properties when mixed to homogeneity. The invention therefore relates to a wound dressing composition of the present invention for use as a medicament.

In a preferred embodiment, the invention thus relates to the use of a wound dressing composition of the present invention for the preparation of the medicament for the treatment of wounds, especially badly healing wounds like chronic wounds, in particular for the treatment of diabetic, venous or neuropathic ulcers or infected wounds.

If body fluid, for example wound secretion, nose secret or eye drops, exists at the place of use the wound dressing composition suitable for cosmetic and/or medical use can be applied directly, independent of whether it is in a "wet" state, especially as a hydrogel or in a dry storage form, especially as a xerogel or film.

Dry wound dressing compositions of the present invention can alternatively be reconstituted with water or other adequate solutions before application. This is necessary especially when compositions are to be applied to dry skin surfaces or if specific, especially rheologic, characteristics of the rehydrated hydrogel are needed. Thus, depending on the viscosity to be achieved, the hydration medium can be selected as needed.

In one embodiment the present invention relates to a method for treating wounds comprising the steps of:

- 1) Providing a dry wound dressing composition comprising a mixture of hydroxyethyl cellulose and gellan gum, and where necessary, one or more active ingredients, in an amount that the mixture will form a topical gel upon hydration of the mixture;
- 2) hydration of the mixture in a sufficient amount of water or liquid with low electrolyte content to form a free-flowing gel;
- 3) applying a therapeutically effective amount of the gel topically to a wound;
- 4) optionally covering the wound, for example with a plaster, compress or bandage.

In another embodiment the present invention relates to a method for treating wounds comprising the steps of:

- 1) Providing a dry wound dressing composition comprising a mixture of hydroxyethyl cellulose and gellan gum, and where necessary, one or more active ingredients, in an amount that the mixture will form a topical gel upon hydration of the mixture;
- 2) hydration of the mixture in a sufficient amount of liquid with a sufficiently high electrolyte content to form a immobilized gel;
- 3) applying a therapeutically effective amount of the gel topically on top of the free-flowing gel from the preceding method to the wound; in this case step 4 of the preceding method, i.e. covering the wound for example with a plaster, compress or bandage, may be performed after step 3 of the present method.

In another embodiment the present invention relates to a method for treating wounds comprising the steps of:

- 1) Providing a dry wound dressing composition comprising a mixture of hydroxyethyl cellulose and gellan gum, and where necessary, one or more active ingredients, in an amount that the mixture will form a topical gel upon hydration of the mixture;
- 2) hydration of the mixture in a sufficient amount of liquid with a sufficiently high electrolyte content to form a immobilized gel;
- 3) applying a therapeutically effective amount of the gel topically to a wound;
- 4) optionally covering the wound for example with a plaster, compress or bandage.

As described above, the wound dressing compositions comprising a gellan gum and HEC, especially in form of a hydrogel, are per se beneficial for wound healing. However, active substances beneficial for wound healing, in the following called wound healing therapeutic substances, may be added in order to further improve the beneficial effect of the compositions on the wound healing process.

Surprisingly, it has been found that the HEC/gellan gum wound dressing compositions of the invention have also an excellent ability to stabilize and deliver proteins in a controlled way, in particular when the protein is a protease inhibitor, most particularly when the protease inhibitor is alpha-1-antichymotrypsin. It was found that the wound dressing compositions of the present invention are suitable for stabilizing ACT in a storage form allowing easy storage, and which release ACT at a controlled rate (see example 7).

Thus the present invention also relates to wound dressing compositions of the present invention, wherein the compositions further comprise one or more wound healing therapeutic substances.

Such wound healing therapeutic substance may be present in a concentration of about up to about 10% by weight, preferably from about 0.001 to about 5% by weight, typically from about 0.1 to about 2% by weight of one or more wound healing therapeutic agents, such as non-steroidal anti-inflammatory drugs, e.g. acetaminophen, steroids, like hydrocortisone or betamethosone, local anaesthetics, antimicrobial agents, growth factors (e.g. fibroblast growth factors or platelet derived growth factor), or protease inhibitors, in particular alpha-1-antichymotrypsin (ACT) or alpha-1-antitrypsin (AAT). The antimicrobial agent may, for example, comprise an antiseptic, an antibiotic, or mixtures thereof. Preferred antibiotics include cephalosporins (cephalexin, cefoxitin, and others), penicillins (amoxycillin, ampicillin, phenoxymethylpenicillin, and others), tetracyclines (minocycline, doxycycline, and others), aminoglycosides (gentamicin, neomycin, and others), antifungals (isiconazole, clotrimazole, amphotericin, and others), sulphadiazine, chloramphenicol, erythromycin, vancomycin, trimethoprim, and others. Preferred antiseptics include silver, including colloidal silver, silver salts including one or more silver salts of one or more of the anionic polymers making up the material, silver sulfadiazine, chlorhexidine, povidone iodine, triclosan, sucralfate, quaternary ammonium salts and mixtures thereof. The concentrations refer to the concentration in the wet, hydrogel state of the wound dressing compositions of the invention.

In a preferred embodiment the wound healing therapeutic substance is a protein. In a more preferred embodiment, the wound healing therapeutic substance is a protease inhibitor belonging to the Serpin family. Preferred serpins are ACT, alpha-1-antitrypsin, antithrombin III, alpha-2-antiplasmin, C1 Inhibitor (C1INH), pancreatic trypsin inhibitor, plasminogen activator inhibitor-1 (PAI-1), Plasminogen activator inhibitor type-2 (PAI-2), Heparin Cofactor II, active protein C inhibitor, PN-1, Maspin, SERPINB12, Protease inhibitor 14, SERPINB3 and -4, SERPINB1. In an even more preferred embodiment, the protease is selected from ACT and alpha-1-antitrypsin. In the most preferred embodiment of the invention the wound healing therapeutic substance is ACT.

In another preferred embodiment, a wound healing composition in a wet, hydrogel state comprising a gellan gum, HEC and a wound healing therapeutic substance, particularly ACT, contains the wound healing therapeutic substance in a concentration of about ACT is between 10 µg/ml and 10 mg/ml in the wet, hydrogel state, preferably between 100 µg/ml and 10 mg/ml.

In another preferred embodiment, a dry wound healing composition comprising a gellan gum, HEC and a wound healing therapeutic substance, particularly ACT, contains the wound healing therapeutic substance in a concentration of about between $0,1 \mu\text{g}/\text{cm}^2$ and $1000 \mu\text{g}/\text{cm}^2$ of wound dressing surface.

In another aspect, the present invention makes available a method of treatment of a chronic wound in a mammal, such as a neuropathic ulcer decubitus ulcer, a venous ulcer or a diabetic ulcer or an infected wound. The method comprises applying a wound dressing according to the invention comprising a wound healing therapeutic substance to the wound.

Typically, the wound dressing according to the invention is applied to the chronic wound for a period of at least 1 hour, preferably at least 24 hours, more preferably at least 48 hours, and most preferably at least 72 hours.

The treatment may be extended for several days, weeks or months, with dressing changes as appropriate, if necessary for chronic wounds.

The production of sterile wound healing compositions can be performed as follows comprising the steps of:

HEC and gellan gum as dry powders are levigated in a mortar and thereby mixed. The mixture is dispersed in a suitable solvent, preferably an aqueous solution and is homogenised by stirring. The dispersion is then autoclaved, whereby sterility and the gelling notably of the gellan gum component, which is temperature dependent, is achieved at the same time. The preparation is poured at a temperature between 70°C to 90°C into a suitable dish or may get cast out with a scraper on a suitable base. Solvent is removed from the dispersion to leave a solid material comprising a homogeneous mixture of HEC and gellan gum.

The optional, additional components in the materials according to the present invention are preferably included in the dispersion prior to autoclavation. For materials that do not tolerate this procedure and/or for temperature-sensitive substances like proteins, comprising proteinogenic protease inhibitors, in particular alpha-1-antichymotrypsin, an additional working step may be added:

After the removal of solvent the solid is again hydrated by the addition of a suitable solution of the protein or other sensitive substance with suitable excipients. Then an additional step of removing the solvent is performed.

The solvent can be removed from the dispersion by evaporation, for example by evaporation from the dispersion in a tray resulting in a dry film. In other embodiments the solvent, preferably water, is removed by freeze-drying (lyophilising) or solvent-drying to obtain the material in the form of a xerogel or sponge. Preferably, the method of lyophilisation is carried out including an annealing step before primary drying. see example 9.

Therefore, the present invention also relates to a method for freeze-drying of an dispersion of gellan gum and HEC, characterized in that an annealing step is performed before the primary drying. Further preferred embodiments of the method of the invention can be taken from the attached claims.

The invention further relates to a dry wound dressing composition, obtainable by the method of the invention.

The invention further relates to the use of a gellan gum and HEC for the preparation of a wound dressing composition for topical administration. All embodiments discussed above for the wound dressing composition of the invention also apply to this use of the invention.

Especially, in a preferred embodiment, the use of the invention further comprises the use of an active ingredient. All embodiments discussed above with respect to the active ingredients of the wound dressing composition of the invention also apply to the use of the invention.

"Xerogel" according to the present invention is to be understood as porous, sponge-like matrix obtainable from a hydrogel e.g. by freeze-drying comprising at least one gelating substance wherein the matrix has the potential to swell and form hydrogels when in contact with aqueous solutions.

"Film" according to the present invention is to be understood as polymer-based foil of flat-shaped form of uniform thickness and consistency obtainable from a hydrogel by drying, e.g. evaporative drying or by casting from organic solutions. The matrix has the potential to swell and form hydrogels when in contact with aqueous solutions.

"Dry" according to the present invention is to be understood as containing a very low content of water, preferably less than 5% (w/w) moisture, more preferably less than 2% (w/w) moisture, especially preferably less than 1% (w/w) moisture. Moisture can be determined by coulometric Karl-Fischer titration, for example using KF 373 (Metrohm GmbH & Co, Filderstadt, Germany).

"Active ingredient" is to be understood as any substance which causes a biological effect, either directly or when released from its pro-drug form in vivo and which is thus beneficial for the medical treatment or prevention of diseases and /or disorders or for cosmetic treatment of conditions of the body.

"Wound" is to be understood as any injury of the skin resulting in a partial or complete destruction of the skin barrier function by partial or complete local destruction of the skin integrity. Such wounds may result from mechanical impact as slash wounds or stab wounds, or maceration and/or ulceration as observed in decubitus ulcers or diabetic ulcers, or burn wounds resulting from high temperature or chemicals. Preferred wounds according to the present invention are chronic wounds which heal slowly or badly or not at all under standard treatment, especially diabetic ulcer, venous ulcers, neuropathic ulcers and decubitus ulcers or infected wounds.

"Alpha-1-antichymotrypsin" or "ACT" according to the present invention is to be understood as an alpha-1-antichymotrypsin protein showing at least approximately 70%, in particular at least approx. 80%, especially at least approx. 90%, more preferred at least 95%, even more preferred at least 98% sequence identity to human wildtype alpha-1-antichymotrypsin and having at least 1%, more preferably at least 10%, even more preferably at least 50% inhibitory activity versus Cathepsin G compared to mature wildtype alpha-1-antichymotrypsin isolated from human blood serum. The inhibitory action can be determined by assays well known in the art. Such assays are, for example, described in Heidtmann et al., 1990, Clin Chem 36: 2077-2081 and in Example 7. An ACT protein according to the present invention may be isolated from a mammalian organism, preferably from human serum or may be produced recombinantly, for example by use of viral, bacterial, fungal or mammalian expression systems. Such ACT may be glycosylated, partially glycosylated or unglycosylated. In a preferred embodiment, ACT is mature human wildtype ACT. In another embodiment, the ACT lacks all or part of the N-terminal signal sequence, more particularly lacks the signal sequence.

"Sequence identity" is understood as degree of identity (% identity) of two sequences, that in the case of polypeptides can be determined by means of, for example, BlastP 2.0.1 and

in the case of nucleic acids by means of, for example, BLASTN 2.014, wherein the filter is set off and BLOSUM is 62 (Altschul et al., 1997, *Nucleic Acids Res.*, 25:3389-3402). In humans, one ACT gene encoding ACT is known, with polymorphism having been described, particularly in the signal peptide sequence (Rubin, 1989, database entry). The sequence of human wildtype ACT polypeptide sequence with signal peptide is shown in SEQ ID No. 1.

The sequence of a mature human ACT polypeptide sequence without signal peptide is shown in SEQ ID No. 2 (Lindmark et al., *Biochim. Biophys. Acta* 997:90-95(1989)). The sequence of another, in vivo predominant, mature ACT polypeptide is shown in SEQ ID No. 3. Both mature ACT polypeptides are obtained by cleavage of the signal peptide. ACT polypeptides which exhibit ACT activity comparable to ACT polypeptides according to SEQ ID No. 2 and 3 are polypeptides according to SEQ ID No. 4 and SEQ ID No. 5 which can be e.g. obtained by recombinant expression in yeast and which are characterized by an N-terminal Methionine followed by the sequence of the mature ACT polypeptides according to SEQ ID No. 7 and SEQ ID No. 10. Such N-terminal extensions, for example a Methionine extension which may be introduced in order to facilitate expression and/or purification do not affect ACT activity.

The polypeptides according to any of SEQ ID No. 1 to 5 and functional variants thereof are preferred ACT polypeptides according to the invention. Particularly preferred is the use of mature ACT polypeptides lacking the signal peptide according to SEQ ID No. 2 to 3 and functional variants thereof, especially functional variants according to SEQ ID No 4 to 5.

The term "functional variants" is to be understood as meaning variants of the ACT polypeptides, which can be used in accordance with the invention, which variants possess protease inhibitor specificity with regard to cathepsin G which is similar to that of the mature wildtype ACT polypeptide. For example, functional variants of ACT polypeptides according to SEQ ID No. 1 to 5 possess at least approximately 70%, in particular at least approx. 80%, especially at least approx. 90%, more preferred at least 95%, even more preferred at least 98% sequence identity with one of the sequences SEQ ID No. 1, SEQ ID No.: 3, SEQ ID No.: 4, or SEQ ID No, 5 and have the Cathepsin G protease inhibitory activity similar to that of the mature wildtype ACT polypeptide which can be determined as described in Example 7.

"Similar inhibitory activity" is to be understood as activity which is at least 1%, preferably at least 10%, even more preferably at least 50% of the activity of a mature human wildtype ACT polypeptide.

Functional variants of the polypeptides can also be parts of the polypeptides used in accordance with the invention which, when compared with the wildtype ACT polypeptides, do exhibit similar protease inhibitor activity. For example, the first amino acid, i.e. methionine, can be missing in a polypeptide without there being any significant change in the function of the polypeptide. Also, as mentioned above for SEQ ID No. 4 and 5, an N-terminal Methionine may be added to an ACT polypeptide or functional variants thereof, e.g. a methionine may be added to the N-terminus of a mature ACT polypeptide without there being any significant change in the function of the polypeptide. N- and/or C-terminal and/or internal deletions of the polypeptide in the range of approx. 1-60, preferably of approx. 1-30, in particular of approx. 1-15, especially of approx. 1-5, amino acids are also included provided the protease inhibitor specificity remains essentially unaltered as compared with that of the respective wildtype polypeptide. Particular preference is given to deletions which affect the signal peptide, or parts thereof, at the N terminus of an ACT polypeptide; e.g. it was shown that an ACT polypeptide having the last 4 amino acids of the signal peptides followed by the sequence of the mature ACT polypeptide according to SEQ ID No. 3 retains full ACT activity (US 5,367,064). Especially preferred are ACT polypeptides which are characterized in that all or part of the signal peptide of an ACT polypeptide according to SEQ ID No. 1 is missing, especially ACT polypeptides according to SEQ ID No. 2 and SEQ ID No. 3. ACT polypeptides having an additional N-terminal Methionine are also preferred ACT polypeptides according to the invention, especially ACT polypeptides according to SEQ ID No. 4 and SEQ ID No. 5.

Figure Legend:

Figure 1 shows the results of example 7.

Figure 2 shows a xerogel pad after freeze drying without annealing step

Picture 3 shows a xerogel pad after freeze drying with annealing step

Figure 4

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

Fig. 2-2 native gellan gum.

Fig. 2-3 deacetylated gellan gum.

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

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

Fig. 3-21 $\ln k$ values of carboxymethyl cellulose sodium 10.000 ($\square$), hydroxyethyl cellulose 100.000 ($\blacksquare$), and hydroxyethyl cellulose 10.000 ($\blacktriangle$) gels in dependence of the gelling agent content measured after steam sterilisation.

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 $\omega=10$ 1/s of 1.0% gellan gum in 0.9% sodium chloride with storage modulus G' ($\diamond$), loss modulus G'' ($\blacklozenge$), loss factor $\tan \delta_{\text{gellan}}$, yield point $\tau_{Y_{\text{gellan}}}$; 2.5% hydroxyethyl cellulose 100.000 with storage modulus G' ($\square$), loss modulus G'' ($\blacksquare$), loss factor $\tan \delta_{\text{HEC}}$

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' ($\diamond$), loss modulus G'' ($\blacklozenge$), loss factor $\tan \delta_{\text{phosphate buffer}}$, yield point $\tau_{Y_{\text{phosphate buffer}}}$; in water with storage modulus G' ($\square$), loss modulus G'' ($\blacksquare$), loss factor $\tan \delta_{\text{water}}$.

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.

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.

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

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.

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.

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.

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

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 (□).

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 .

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

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.

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

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.

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.

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

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.

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.

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.

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.

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.

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.

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.

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.

Fig. 3-47 Recovered relative activities of a xerogel formulation consisting of 60 μ g/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.

Fig. 3-48 Recovered relative activities of a xerogel formulation consisting of 60 μ g/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.

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.

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

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).

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

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

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.

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.

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.

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.

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.

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.

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(♦, $k=1.30 \cdot 10^{-4}$ 1/day) and 40°C(□, $k=7.43 \cdot 10^{-4}$ 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.

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.

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 0.45 μ m pore size; the system is sealed by a polyurethane rubber o-ring.

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

Fig. 3-66 Release diagram of Fluorescein Na (Δ), FITC-Dextran 19kDa ($\diamond$), FITC-Dextran 70kDa ($\square$), 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.

Fig. 3-67 Release diagram of Fluorescein Na (Δ , slope=34.4, $R^2=0.98$), FITC-Dextran 19kDa ($\diamond$, slope=12.3, $R^2=0.99$), FITC-Dextran 70kDa ($\square$, 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.

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; membrane pore size is given as 450nm, and ACT diameter is estimated as 5.4nm.

Fig. 3-69 Release diagram of FITC-Dextran 70kDa from hydrogel (Δ , slope=7.2, $R^2=0.99$), film ($\diamond$, slope=10.0, $R^2=0.98$), xerogel ($\square$, 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.

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 ($\square$, slope=10.8,

$R^2=0.98$) and total ACT content measured by ELISA ($\blacklozenge$, slope=12.8, $R^2=0.98$); underlying hydrogel contains 60 μ g/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.

Fig. 3-71 Decrease of relative activity of a 60 μ g/ml ACT solution (10mM potassium phosphate buffer pH 7.2, 0.1% Poloxamer[®] 188) during stirring in the acceptor surplus ($\square$) and during pumping through the tubes and stirring in the surplus ($\blacklozenge$).

Fig. 3-72 Residual activity of a 60 μ g/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.

Fig. 3-73 Residual total content of a 60 μ g/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.

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 ($\square$) and total ACT content measured by ELISA ($\blacklozenge$); underlying hydrogel contains 60 μ g/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.

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 ($\square$, slope=6.6, $R^2=0.98$) and total ACT content measured by ELISA ($\blacklozenge$, slope 7.8, $R^2=0.99$); underlying hydrogel contains 60 μ g/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.

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 ($\square$, slope=6.5, $R^2=0.99$) and total ACT content measured by ELISA ($\blacklozenge$, 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.

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 ($\square$, slope=6.7, $R^2=0.99$) and total ACT content measured by ELISA ($\blacklozenge$, slope 7.8, $R^2=0.98$); underlying hydrogel contains 60 μ g/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 ($\square$, slope=6.6, $R^2=0.99$) and total ACT content measured by ELISA ($\blacklozenge$, slope 6.8, $R^2=0.98$); underlying hydrogel contains 60 μ g/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 ($\square$, slope=6.9, $R^2=0.99$) and total ACT content measured by ELISA ($\blacklozenge$, slope 7.9, $R^2=0.98$); underlying hydrogel contains 60 μ g/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 ($\square$, slope=5.1, $R^2=0.98$) and total ACT content measured by ELISA ($\blacklozenge$, slope 5.5, $R^2=0.99$); underlying hydrogel contains 60 μ g/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 ($\square$, slope=5.7, $R^2=0.98$) and total ACT content measured by ELISA ($\blacklozenge$, slope 7.2, $R^2=0.98$); underlying hydrogel contains 60 μ g/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.

The following examples are not intended to limit the scope of the invention. Rather, they represent preferred embodiments of the present invention which could be used to further define the aspects of the invention as shown above.

Examples

Specific embodiments of the present invention will now be described further, by way of example.

Example 1: Preparation of xerogels comprising HEC, gellan gum and ACT in vials

- (1) 2g Hydroxyethyl cellulose (Natrosol® 250 HHX, purchased from Dow Chemicals) and 1g gellan gum (Kelcogel F, purchased from Kelco) as dry powders are levigated in a mortar and so intimately mixed.
- (2) The mixed powder is dispersed in 97g of demineralised water with a propeller mixer at high stirring speed. The dispersion is stirred several minutes until it appears homogeneous.
- (3) The semisolid mass is filled into infusion bottles and which is then sealed. In an autoclave a standard sterilisation program is carried out (121°C, 15 minutes, 2 bar). With this step sterility and the gelling notably of the gellan gum component (which is temperature dependent) is achieved at the same time.
- (4) Under sterile conditions the preparation is poured at a temperature between 70°C to 90°C into a petri dish to a height of 4 mm. Under these conditions the mass is freely flowing and forms a cylindrical shape with uniform surface and thickness in the dish.
- (5) Water is removed from the dispersion by drying at 25°C under a flow of warm (40°C) nitrogen gas. At the end of drying a uniform dry film is left in the dish which consists of homogeneously mixed HEC and gellan gum.

- (6) For ACT loading simply by hydration, a first solution without the protein containing a 10 mM potassium phosphate buffer, 0,1 % of Poloxamer 188 (BASF AG, Ludwigshafen, Germany) and 0,05% of Kollidon 17PF (BASF AG, Ludwigshafen, Germany) is prepared and sterilized by autoclavation. Under aseptic conditions the alpha-1-antichymotrypsin is dissolved and the solution is again filtered through a 0,22 μ m unit to provide sterility for the whole solution.
- (7) The film is cut in pieces that exactly cover the bottom of glass vials. The film is placed in the vial and is hydrated with the ACT containing solution of step (6) to a filling height of 4 mm. In a 2R vial the necessary amount is 0.4ml. The mixture is allowed to swell for at least 24 hours to again form a homogeneous hydrogel. By this form of drug loading almost no shear stress (which is a critical part in protein handling) is applied to the drug substance.
- (8) The vial with the gel inside is now placed into a freeze dryer at 20°C. The lyophilisation is carried out following the program in example 9:

The dry pad has a homogeneous appearance and its mechanical properties are very suitable for proper handling by both patients and personnel. It swells within minutes to form a hydrogel when put in contact with an aqueous solution. Once rehydrated, the gel is very soft and feels comfortable when put onto a wound. It also provides intimate contact with the wound ground to ensure the proper release of the active ingredient load.

Example 2: Preparation of xerogels comprising HEC, gellan gum and ACT in sheets of 2 mm height

The preparation of the xerogel in sheets follows the same procedure as the preparation of the xerogel in vials from step ACT containing solution of step (6) of example 1 is poured onto the placebo film in the dish to a height of 4 mm and is swollen for at least 24 h.

The hydrated gel is heated to 40°C to achieve good flowability and is filled into a scraper. The gel is now cast out on a glass base to produce a wet film with a height of 2 mm.

Step (9) is carried out like step (8) above in example 1.

The matrix is obtained in sheets and can be cut into pieces suitable for further packaging. This simplifies production processes.

Example 3: Preparation of films comprising HEC, gellan gum and ACT in vials

The preparation of the film follows the same procedure as the preparation of the xerogel from step (1) to (5).

- (6) Then for ACT loading simply by hydration a first solution without the protein containing a 5 mM potassium phosphate buffer, 0,1% of Poloxamer 188; 1,0% PEG 400, and 1,5% of Kollidon 17PF is made and gets sterilized by autoclavation. Under aseptic conditions the alpha-1-antichymotrypsin is dissolved and the solution is again filtered through a 0,22 μm unit to provide sterility for the whole solution.
- (7) The film is cut in pieces that exactly cover the bottom of glass vials. The film is placed in the vial and is hydrated with the ACT containing solution to a filling height of 4 mm. The mixture is allowed to swell for at least 24 hours to form a homogeneous hydrogel again. By this form of drug loading almost no shear stress which is a critical part in protein handling is applied to the drug substance.
- (8) Water is removed from the dispersion by drying at 25°C under a flow of warm (40°C) nitrogen gas.

The obtained film is very soft and elastic but provides the necessary robustness for good handling qualities. It swells when hydrated to form a clear gel within about 45 minutes. Once rehydrated the gel on the wound is very soft and comfortable. It also provides intimate contact with the wound ground to ensure the proper release of the active ingredient load.

Example 4: Preparation of films comprising HEC, gellan gum and ACT in sheets

The preparation of the film follows the same procedure as the preparation of the xerogel from step (1) to (5) in example 1.

- (6) For ACT loading simply by hydration a first solution without the protein containing a 5 mM potassium phosphate buffer, 0,1% of Poloxamer 188,0, 1,0% PEG 400, and 1,5% of Kollidon 17PF is prepared and sterilized by autoclavation. Under aseptic conditions the alpha-1-antichymotrypsin is dissolved and the solution is again filtered through a 0.22 μm unit to provide sterility for the whole solution.

- (7) The ACT containing solution is poured onto the placebo film in the dish to a height of 4 mm and is swollen for at least 24 h.
- (8) The hydrated gel is heated to 40°C to achieve good flowability and is filled into a scraper. The gel now is cast out on a glass base to produce a wet film with a height of 2 mm.
- (9) Water is removed from the dispersion by drying at 25 °C under a flow of warm (40°C) nitrogen gas.

The matrix is obtained in sheets and can be cut into pieces suitable for further packaging. This simplifies production processes.

Example 5: Preparation of HEC/Gellan gum xerogels without active ingredient

The xerogels are produced like in example 2 but without the steps (4) to (7). The product has the same properties than example 2. It can for example be used as a wound dressing.

Example 6: Preparation of HEC/Gellan gum films without active ingredient

The films are produced like in example 4 but without the steps (4) to (7). The product has the same properties than example 4. It can for example be used as a wound dressing.

Example 7: Release of ACT from wound dressing compositions comprising HEC, gellan gum and ACT and determination of stability

In order to determine the suitability of ACT-containing wound dressing compositions of the invention, release tests were carried out in vitro. Model chambers described in LothJH. & Holla-Benninger, A. (Studies on the drug release from ointments. Part 1. Development of an in vitro release model, *Pharmazeutische Industrie* 40, 256-261 (1978)) were modified in that way that the donor chamber has only 1.25 ml volume and is cylindrical in shape with a diameter of 2 cm. The dividing membrane between donor and acceptor is a cellulose acetate filter membrane of 0.45 µm pore size. Therefore, it does not simulate a physiological barrier like skin, but it separates the chambers sterically and thereby mimicks the situation in a wound that also lacks of a skin barrier. The pore size is chosen in a way that the membrane provides a barrier for the large gelling agent molecules, but does not remarkably influence the diffusion of ACT. The acceptor consists of 10 mM potassium

phosphate buffer pH 7.2. The reconstitution of the dried forms is done with phosphate-buffered saline (PBS).

In order to determine the ACT activity, an activity assay based on Cathepsin G binding was performed. 96-well plates were coated with BSA and subsequently with Cathepsin G. After washing, ACT probes were added and incubated for 30 min at 37°C. After washing with PBS-T 3 times, a rabbit anti human ACT antibody was added to the wells and was incubated for 30 min at 37°C. After washing three times with PBS-T buffer, a goat anti rabbit IgG antibody conjugated with horseradish peroxidase was added and was again incubated for 30 min at 37°C. The wells were washed three times with PBS-T buffer. Subsequently OPD (1,2-Diaminobenzene) substrate solution was prepared according to the manufacturer's protocol (Sigma), added to the wells and Incubated at room temperature in the dark. After 10 minutes the reaction was stopped by adding 100 µm 0,5 M H₂SO₄ per well. Immediately after stopping the reaction the absorption at 490 nm was determined.

The percentage of the bioactive fraction of the total ACT amount in each sample was determined and expressed in Figure 1 as percentage in comparison with untreated samples of ACT. The release maximum is determined 5 days after start of diffusion when an equilibrium is reached.

Before drying all gel formulations contain 0.1% Poloxamer 188®, 10 mM arginine, 10 mM phosphate buffer. The HEC/Gellan gum (Kelcogel) mixtures contain 2% HEC and 1% gellan gum in the wet state. HEC gels contain 2,5% of hydroxyethyl cellulose without gellan gum (Figure 1). HEC is hydroxyethyl cellulose (Natrosol® 250 HHXpharm, Dow Chemicals), two different types of Gellan gum were tested: Kelcogel F® (Kelco) as a deacetylated Gellan Gum, and Kelcogel LT100® (Kelco), as an acetylated Gellan gum.

It was observed that especially dry storage forms of homogeneous mixtures of deacetylated gellan gum and HEC result in wound dressing compositions which excellently stabilize and release ACT after manufacture. Especially xerogels comprising HEC and deacetylated Gellan gum provide both excellent stabilization and release of ACT. Moreover, the rheological characteristics of the wound dressing compositions are excellent. Thus, the compositions of the invention comprising ACT are especially suitable for use in wound healing.

Example 9: Freeze drying of hydrogels to form xerogels

The freeze drying operation is carried out in a Christ® Lab scale freeze dryer. The program is as follows:

Start	20 °C	1000 mbar
1 h	-15 °C	1000 mbar
1,5 h	-15 °C	1000 mbar
1 h	-1 °C	1000 mbar
2,5 h	-1 °C	1000 mbar
2 h	-40 °C	1000 mbar
1,5 h	-40 °C	1000 mbar
0,5 h	-25 °C	0,5 mbar
24 h	-25 °C	0,5 mbar
0,1 h	-25 °C	0,001 mbar
5 h	20 °C	0,001 mbar
12 h	20 °C	0,001 mbar

Surprisingly, we found out that the annealing step that again heats up to -1 °C after 2.5 h and the low freeze speed of 0.65 °C/min after 5 h before primary drying are essential for the homogeneous appearance of the xerogel pad (Figure 2 versus Figure 3). This is very important for patient and medical personnel compliance.

Example 10

2 Materials and Methods

2.1 Materials

2.1.1 α_1 -Antichymotrypsin (ACT)

α_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 188 μ l solution consisting of 1mg ACT, 150mM NaCl, 20mM Tris, pH 7.4) to be stored at -20°C .

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
Glycine, 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 C₆-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.

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 group¹³²:

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 H₂SO₄ 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 earlier¹⁶⁵ is used (Barkey GmbH & Co. KG, Leopoldshöhe, Germany) (Fig. 2-4).

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 chamber¹³⁴. 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 coulometric Karl Fischer titration with generating the iodine electrolytically to keep the reaction ongoing. Therefore, a coulometric 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 coulometric 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 volumes¹³⁵.

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 stability¹³⁶ 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 known¹³⁷. 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).

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.

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).

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.

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 [%] $\pm$ sd
50mM phosphate	40°C/9 days	78.2 $\pm$ 0.5
50mM TrisHCl	40°C/9 days	68.5 $\pm$ 5.1
25/25mM phosphate/citrate	40°C/9 days	80.2 $\pm$ 6.8
10mM phosphate	F/T/5 cycles	42.5 $\pm$ 7.2
10mM TrisHCl	F/T/5 cycles	41.0 $\pm$ 7.1
10/10mM phosphate/citrate	F/T/5 cycles	46.1 $\pm$ 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.

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).

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 3rd order degree, i.e. on four levels, is calculated by the Statistica 6.1 software. For interpretation a 2nd 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.

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.

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 described¹³⁷. In the course of this work, these 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 surfaces¹⁴², gas-liquid interfaces¹⁴³ and at the protein molecule itself mostly at hydrophobic areas¹⁴⁴. Therefore, mechanisms of protein protection by surfactants based on a competitive situation for adsorption on denaturing interfaces between protein and surfactant are discussed¹⁴⁵. 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 reported¹⁴⁶. But surfactants are also able to prevent chemical degradation in some cases¹⁴⁷. Ionic surfactants are usually avoided because of their ability to bind to polar as well as to unpolar groups and therefore denature proteins¹⁴⁸. 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%.

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).

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 injection¹⁴⁹, whereas Tween® 80 is approved also by FDA to a maximum potency of 0.2% for intralesional injection and 8% for intravenous injection¹⁴⁹. 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 considered¹⁵². 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.

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 injection¹⁴⁹. Sorbitol is approved also by FDA to a maximum potency of 45% for intralesional injection and 30% for intravenous injection¹⁴⁹.

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 60 μ g/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.

The non-destabilising cyclodextrins are further tested in F/T studies. The testing is conducted in 0.1ml ACT solution 60 μ g/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).

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.

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 investigated¹³⁷.

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.

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.

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 studies¹⁵⁵. 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.

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 injection¹⁴⁹.

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%.

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 be 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 alternative: 20mM potassium phosphate / 50mM potassium chloride pH 7.2 alternative: Tris HCl pH 7.2 alternative: potassium phosphate/citrate pH 7.2	10mM potassium phosphate pH 7.2
surfactant	0.1% Poloxamer [®] 188 alternative: 0.1% Tween [®] 80	0.1% Poloxamer [®] 188 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 60 μ g/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 polymers¹⁵⁶ 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 stability¹⁵⁷.

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 $\dot{\gamma}$ is drawn against the shear stress σ 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.

$$\sigma = k \cdot \dot{\gamma}^n$$

Equ. 3-1

$$\ln \sigma = \ln k + n \cdot \ln \dot{\gamma}$$

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/cm² = 10Pa], shear rate $\dot{\gamma}$ [1/s], consistency coefficient k [0,1Pa sⁿ], and flow behavior index or Power-law exponent n [-]; $n < 1$ meaning pseudoplastic and $n > 1$ shear thickening behaviour¹⁵⁸;

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).

		$\ln k$	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.

$$\ln k = A + B \cdot \text{concentration}(\text{gelling agent})$$

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

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 not 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).

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

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 $\tan \delta$; with loss angle δ , amplitude shear stress τ_A , and shear rate amplitude γ_A ;

Thereby, the storage modulus G' describes the elastic properties of the sample that is 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 $\tan \delta$ 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 $\tau_{Y \text{ gellan}}$ 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).

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 $\tan \delta$
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 $\tan \delta$ 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.

For interpretation, again the relative position of moduli is considered. For influencing the gel behaviour isotonic sodium chloride solution ($\tan \delta_{0.9\% \text{ NaCl}} = 0.18$) is nearly on a par with 50mM potassium phosphate buffer pH 7.2 ($\tan \delta_{\text{phosphate buffer}} = 0.15$) like it is developed for some ACT formulations. The gels also provide a yield point τ_Y which is slightly reduced by the hydroxyethyl cellulose surrogate ($\tau_{Y \text{ 0.9\% NaCl}} = 169.0\text{Pa}$, $\tau_{Y \text{ phosphate buffer}} = 168.7\text{Pa}$) compared to gellan gum ($\tau_{Y \text{ gellan gum}} = 195.2\text{Pa}$) 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 FDA¹⁴⁹.

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 proteins¹³⁷.

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 60 µg/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).

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).

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 delivery¹⁴⁹.

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 evaluated¹⁵⁹(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.

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 from 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 from¹⁶⁰). 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.

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:

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

$$\text{Equ. 3-8} \quad 1^{\text{st}} \text{ order} \quad \ln c(t) = \ln c(0) - k_1 \cdot t$$

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

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.

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.

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.

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 α_1 -antichymotrypsin 50mM phosphate buffer 10mM arginine 0.1% Tween® 80 0.05% PVP 17 2.5% HEC 100.000	reaction constant k (1°C)	$2.99 \cdot 10^{-4}$ 1/day
		reaction constant k (24°C)	$1.41 \cdot 10^{-3}$ 1/day
		reaction constant k (41°C)	$1.05 \cdot 10^{-2}$ 1/day
(2)	60µg/ml α_1 -antichymotrypsin 50mM phosphate buffer 10mM arginine 0.1% Poloxamer® 188 0.05% PVP 17 2.5% HEC 100.000	shelf life (6°C)	253.4 days
		shelf life (25°C)	50.1 days
		shelf life (32°C)	22.1 days
(3)	60µg/ml α_1 -antichymotrypsin 50/50mM phosphate/citrate buffer 10mM arginine 0.1% Poloxamer® 188 0.05% PVP 17 2.5% HEC 100.000	reaction constant k (1°C)	$7.19 \cdot 10^{-4}$ 1/day
		reaction constant k (24°C)	$1.05 \cdot 10^{-3}$ 1/day
		reaction constant k (41°C)	$3.63 \cdot 10^{-3}$ 1/day
(4)	60µg/ml α_1 -antichymotrypsin 50mM phosphate buffer 10mM arginine 0.1% Poloxamer® 188 0.05% PEG 2000 2.5% HEC 100.000	shelf life 6°C	138.0 days
		shelf life 25°C	67.4 days
		shelf life 32°C	47.0 days
(5)	60µg/ml α_1 -antichymotrypsin 50/50mM phosphate/citrate buffer 10mM arginine 0.1% Poloxamer® 188 0.05% PVP 17 2.5% HEC 100.000	reaction constant k (1°C)	$1.58 \cdot 10^{-3}$ 1/day
		reaction constant k (24°C)	$2.15 \cdot 10^{-3}$ 1/day
		reaction constant k (41°C)	$5.29 \cdot 10^{-3}$ 1/day
(6)	60µg/ml α_1 -antichymotrypsin 50mM phosphate buffer 10mM arginine 0.1% Poloxamer® 188 0.05% PVP 17 2.0% HEC 100.000 1.0% gellan gum LT100	shelf life 6°C	63.3 days
		shelf life 25°C	37.0 days
		shelf life 32°C	28.3 days
(7)	60µg/ml α_1 -antichymotrypsin 50mM phosphate buffer 10mM arginine 0.1% Poloxamer® 188 0.05% PVP 17 2.5% HEC 100.000	reaction constant k (1°C)	$1.70 \cdot 10^{-4}$ 1/day
		reaction constant k (24°C)	$8.16 \cdot 10^{-4}$ 1/day
		reaction constant k (41°C)	$4.61 \cdot 10^{-3}$ 1/day
(8)	60µg/ml α_1 -antichymotrypsin 50mM phosphate buffer 10mM arginine 0.1% Poloxamer® 188 0.05% PVP 17 2.0% HEC 100.000 1.0% gellan gum LT100	shelf life 6°C	439.3 days
		shelf life 25°C	96.8 days
		shelf life 32°C	45.1 days
(9)	60µg/ml α_1 -antichymotrypsin 50mM phosphate buffer 10mM arginine 0.1% Poloxamer® 188 0.05% PVP 17 2.0% HEC 100.000 1.0% gellan gum LT100	reaction constant k (1°C)	$9.31 \cdot 10^{-4}$ 1/day
		reaction constant k (24°C)	$1.44 \cdot 10^{-3}$ 1/day
		reaction constant k (41°C)	$6.25 \cdot 10^{-3}$ 1/day
(10)	60µg/ml α_1 -antichymotrypsin 50mM phosphate buffer 10mM arginine 0.1% Poloxamer® 188 0.05% PVP 17 2.0% HEC 100.000 1.0% gellan gum LT100	shelf life 6°C	105.7 days
		shelf life 25°C	45.6 days
		shelf life 32°C	29.8 days
(11)	60µg/ml α_1 -antichymotrypsin 50mM phosphate buffer 10mM arginine 0.1% Tween® 80 0.05% PVP 17 2.0% HEC 100.000 1.0% gellan gum LT100	reaction constant k (1°C)	$6.48 \cdot 10^{-3}$ 1/day
		reaction constant k (24°C)	$6.62 \cdot 10^{-3}$ 1/day
		reaction constant k (41°C)	$8.41 \cdot 10^{-3}$ 1/day
(12)	60µg/ml α_1 -antichymotrypsin 50mM phosphate buffer 10mM arginine 0.1% Tween® 80 0.05% PVP 17 2.0% HEC 100.000 1.0% gellan gum LT100	shelf life 6°C	16.3 days
		shelf life 25°C	14.5 days
		shelf life 32°C	13.7 days
(13)	60µg/ml α_1 -antichymotrypsin 50mM phosphate buffer 10mM arginine 0.1% Poloxamer® 188 1.5% PVP 17 1.0% PEG 400 2.0% HEC 100.000 1.0% gellan gum LT100	reaction constant k (1°C)	$1.36 \cdot 10^{-3}$ 1/day
		reaction constant k (24°C)	$3.50 \cdot 10^{-3}$ 1/day
		reaction constant k (41°C)	$1.05 \cdot 10^{-2}$ 1/day
(14)	60µg/ml α_1 -antichymotrypsin 50mM phosphate buffer 10mM arginine 0.1% Poloxamer® 188 1.5% PVP 17 1.0% PEG 400 2.0% HEC 100.000 1.0% gellan gum LT100	shelf life 6°C	63.1 days
		shelf life 25°C	24.8 days
		shelf life 32°C	15.4 days

(8)	60µg/ml α_1 -antichymotrypsin	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 α_1 -antichymotrypsin	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 α_1 -antichymotrypsin	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	10 mM phosphate	10 mM phosphate	10 mM phosphate
	10 mM arginine		10 mM arginine	10 mM arginine
	0.1% Poloxamer®	0.1% Poloxamer®	0.1% Tween® 80	0.1% Poloxamer®
	188	188		188
	0.05%PVP 17	0.05%PVP 17	0.05%PVP 17.	0.05% PEG 2000

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

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 used¹⁶³. 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 hydrogels¹⁶⁴.

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 solutions¹⁶⁵. 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 drugs¹⁶⁶⁻¹⁷⁶. 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.

3.4.1.1.2 *Lyophilisation program*

time [h]	plate temperature [$^{\circ}\text{C}$]	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).

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 time¹⁷⁷, 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.

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 [$^{\circ}\text{C}$]	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

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.

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).

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

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

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).

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).

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).

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).

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).

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.

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.

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 [%]	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 investigated.

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 drying¹⁷⁹. 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 polymers¹⁸⁰.

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.

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 dehydration¹⁸¹, 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 PEG¹⁸⁴, PVP^{185 186}, and PVA¹⁸⁷ 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.

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 varying 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 proteins¹⁸⁸. 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).

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.

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.

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 10mM phosphate buffer 10mM arginine 0.1% Poloxamer® 188 0.05% PVP 17 2.5% HEC 100.000	reaction constant k (24°C)	8.71·10 ⁻⁴ 1/day
		reaction constant k (40°C)	3.08·10 ⁻³ 1/day
		shelf life 25°C	121.0 days
		shelf life 40°C	34.3 days
(2)	60µg/ml α1-antichymotrypsin 10mM phosphate buffer 10mM arginine 0.1% Tween® 80 0.05% PVP 17 2.5% HEC 100.000	reaction constant k (24°C)	1.48·10 ⁻⁴ 1/day
		reaction constant k (40°C)	1.21·10 ⁻³ 1/day
		shelf life 25°C	711.3 days
		shelf life 40°C	87.3 days
(3)	60µg/ml α1-antichymotrypsin 10mM phosphate buffer 10mM arginine 0.1% Poloxamer® 188 0.05% PEG 2000 2.5% HEC 100.000	reaction constant k (24°C)	1.17·10 ⁻³ 1/day
		reaction constant k (40°C)	3.53·10 ⁻³ 1/day
		shelf life 25°C	89.9 days
		shelf life 40°C	29.9 days
(4)	60 µg/ml α1-antichymotrypsin 10mM phosphate buffer 10mM arginine 0.1% Poloxamer® 188 0.05% PVP 17 2.0% HEC 100.000 1.0% gellan gum LT100	reaction constant k (24°C)	8.68·10 ⁻⁴ 1/day
		reaction constant k (40°C)	3.47·10 ⁻³ 1/day
		shelf life 25°C	121.4 days
		shelf life 40°C	30.4 days
(5)	60µg/ml α1-antichymotrypsin 10mM phosphate buffer 10mM Arginine 0.1% Poloxamer® 188 0.05% PVP 17 2.0% HEC 100.000 1.0% gellan gum F	reaction constant k (24°C)	1.92·10 ⁻⁴ 1/day
		reaction constant k (40°C)	1.40·10 ⁻³ 1/day
		shelf life 25°C	547.7 days
		shelf life 40°C	75.5 days
(6)	60µg/ml α1-antichymotrypsin 10mM Tris HCl buffer 10mM Arginine 0.1% Poloxamer® 188 0.05% PVP 17 2.0% HEC 100.000	reaction constant k (24°C)	4.06·10 ⁻⁴ 1/day
		reaction constant k (40°C)	1.19·10 ⁻³ 1/day
		shelf life 25°C	259.3 days
		shelf life 40°C	88.4 days

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.

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-swellaable 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 swellaable film dressing materials¹⁸⁹. Some of these films also contain protein drugs¹⁹⁰. 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 swellaable 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 temperature¹⁹¹. The film forms on the bottom and side walls of the container (Fig. 3-50).

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.

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).

The tensile strength value is calculated from applied force at rupture and the cross section area of the film specimen¹⁹² (Equ. 3-12).

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

Equ. 3-12 Tensile strength TS [N/mm²]; F is applied force at rupture [N], A is cross-sectional area [mm²];

Moreover, the elastic modulus is tested in a puncture test where the sample is expanded and penetrated by a ball probe¹⁹² (Fig. 3-53).

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

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

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 3rd 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 $4.5 \text{ N}/\text{mm}^2$ 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 $10 \text{ N}/\text{mm}^2$ can be achieved a reasonable content should be between 2.0% to 3.5% leading to strength values of 8.0 to $9.0 \text{ N}/\text{mm}^2$ (Fig. 3-54).

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.

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).

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 gellan gum 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.

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.

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 apparatus¹⁶⁵ 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 previously¹⁵⁵ (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, hydroxypropyl-methyl 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).

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 .

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)	7.90·10 ⁻⁴ 1/day
	5 mM	phosphate buffer	reaction constant k (40°C)	8.76·10 ⁻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 months	2.0%
	2.5 %	HEC 100.000		
(2)	60 µg/ml	α1-antichymotrypsin	reaction constant k (24°C)	1.22·10 ⁻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 months	3.5%
	2.5 %	HEC 100.000		
(3)	60 µg/ml	α1-antichymotrypsin	reaction constant k (24°C)	1.30·10 ⁻⁴ 1/day
	5 mM	phosphate buffer	reaction constant k (40°C)	7.43·10 ⁻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 months	2.8%
	2.5 %	HEC 100.000		
(4)	60 µg/ml	α1-antichymotrypsin	reaction constant k (24°C)	not evaluable
	5 mM	phosphate buffer	reaction constant k (40°C)	2.52·10 ⁻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 months	3.5%
	2.0 %	HEC 100.000		
	1.0 %	gellan gum LT100		
(5)	60 µg/ml	α1-antichymotrypsin	reaction constant k (24°C)	1.04·10 ⁻⁴ 1/day
	5 mM	phosphate buffer	reaction constant k (40°C)	4.20·10 ⁻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 months	2.1%
	2.0 %	HEC 100.000		
	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.

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 release¹⁹⁵.

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 best¹⁹⁶ (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 ranges¹⁹⁷. 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 model¹³⁴.

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.

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 calculations¹⁹⁹.

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

Equ. 3-14

$$c_A = 2 \cdot c_0 \cdot \sqrt{\frac{D \cdot t}{\pi}}$$

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 [m²/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 $\lambda_{\text{ex}} = 493 \text{ nm}$ and $\lambda_{\text{in}} = 515 \text{ nm}$. The released amounts of the particular model drugs - given in percentage of total content - are drawn against the diffusion time (Fig. 3-66).

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.

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 diameters²⁰⁰. The diameter of ACT is calculated by an approximation according to Equ. 3-16²⁰¹.

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

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

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.

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 maximum 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.

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).

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).

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).

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.

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).

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.

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		
(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		
(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.

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%
			released	
	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 from 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 earlier²⁰².

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 α_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.

5 References

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CLAIMS

- 5 1. Wound dressing composition comprising a cellulose ether and a gellan gum and one or more wound healing therapeutic substances, characterized in that the wound healing therapeutic substance is a protease inhibitor.
2. Wound dressing composition according to claim 1, comprising a hydroxyethyl
10 cellulose and a gellan gum and one or more wound healing therapeutic substances.
3. Wound dressing composition according to claim 1 or 2, wherein the cellulose ether and the gellan gum are homogeneously mixed.
- 15 4. Wound dressing composition according to any of claims 1 to 3, wherein the gellan gum is deacetylated gellan gum.
5. Wound dressing composition according to any of claims 1 to 4, characterized in that the composition is dry.
- 20 6. Wound dressing composition according to claim 5, wherein the composition is a film.
7. Wound dressing composition according to claim 5, wherein the composition is a
25 xerogel or sponge.
8. Wound dressing composition according to any of claims 1 to 4, characterized in that the composition is a hydrogel.
- 30 9. Wound dressing composition according to claim 8, obtainable by rehydration of a dry wound dressing composition according to claims 5 to 7.
10. Wound dressing composition according to any of the claims 1 to 9 characterized in that the composition contains additional excipients.

11. Wound dressing composition according to any of the claims 1-10, wherein the hydroxyethyl cellulose and gellan gum together make up at least 25% by weight of the material on a dry weight basis, preferably at least 50% of the material on a dry weight basis.
12. Wound dressing composition according to any of the claims 1-11, wherein the weight ratio of hydroxyethyl cellulose to gellan gum is in the range of 1:5 to 50:1, preferably 1:1 to 10:1.
13. Wound dressing package comprising a sterile wound dressing composition according to any of the claims 1 to 9, packaged in a microorganism-impermeable container.
14. Wound dressing package according to claim 13, wherein the sterile wound dressing composition is a composition according to any of the claims 5 to 7.
15. Wound dressing package comprising a sterile wound dressing composition according to any of the claims 1-12 and an inert support, preferably selected from adhesive strip, adhesive wrap, bandage, gauze bandage and compress system.
16. Wound dressing composition or wound dressing package according to any of the claims 1-15 for use as a medicament.
17. Use of a wound dressing composition or wound dressing package according to any of the claims 1-15 for the preparation of a medicament for the treatment of wounds, especially chronic wounds.
18. Use according to claim 17, wherein the said chronic wound is selected from the group consisting of venous ulcers, decubitus ulcers, neuropathic ulcers, diabetic ulcers and infected wounds.
19. Wound dressing composition according to any of the claims 1 to 12, wherein the composition further comprises one or more active ingredients.
20. Use of a wound dressing composition according to claim 19 for the preparation of a medicament for the treatment and/or prevention of ophthalmologic, nasal diseases, wounds and skin disorders.

21. Wound dressing composition or wound dressing package according to any of claims 1 to 12, characterized in that the wound healing therapeutic substance is a serpin, preferably selected from the group consisting of alpha-1-antichymotrypsin, alpha-1-antitrypsin, antithrombin III, alpha-2-antiplasmin, C1 Inhibitor (C1INH),
5 pancreatic trypsin inhibitor, plasminogen activator inhibitor-1 (PAI-1), Plasminogen activator inhibitor type-2 (PAI-2), Heparin Cofactor II, active protein C inhibitor, PN-1, Maspin, SERPINB12, Protease inhibitor 14, SERPINB3 and -4, SERPINB1.
- 10 22. Wound dressing composition or wound dressing package according to claim 21, characterized in that the wound healing therapeutic substance is alpha-1-antichymotrypsin.
- 15 23. Wound dressing composition according to claim 22, characterized in that alpha-1-antichymotrypsin is present in a concentration of about between 1 $\mu\text{g/ml}$ and 10 mg/ml in the wet state, preferably between 100 $\mu\text{g/ml}$ and 10 mg/ml in the wet state.