This item is the archived peer-reviewed author-version of:

Optimisation of HPMC ophthalmic inserts with sustained release properties as a carrier for thermolabile therapeutics

Reference:

Everaert Arnout, Wouters Yannick, Melsbach Eline, Zakaria Nadia, Ludwig Annick, Kiekens Filip, Weyenberg Wim.- Optimisation of HPMC ophthalmic inserts with sustained release properties as a carrier for thermolabile therapeutics
International journal of pharmaceutics - ISSN 0378-5173 - 528:1-2(2017), p. 395-405
Full text (Publisher's DOI): <https://doi.org/10.1016/J.IJPHARM.2017.06.047>
To cite this reference: <http://hdl.handle.net/10067/1457280151162165141>

Accepted Manuscript

Title: Optimisation of HPMC ophthalmic inserts with sustained release properties as a carrier for thermolabile therapeutics

Authors: Arnout Everaert, Yannick Wouters, Eline Melsbach, Nadia Zakaria, Annick Ludwig, Filip Kiekens, Wim Weyenberg

PII: S0378-5173(17)30555-0
DOI: <http://dx.doi.org/doi:10.1016/j.ijpharm.2017.06.047>
Reference: IJP 16771

To appear in: *International Journal of Pharmaceutics*

Received date: 14-3-2017
Revised date: 6-6-2017
Accepted date: 13-6-2017

Please cite this article as: Everaert, Arnout, Wouters, Yannick, Melsbach, Eline, Zakaria, Nadia, Ludwig, Annick, Kiekens, Filip, Weyenberg, Wim, Optimisation of HPMC ophthalmic inserts with sustained release properties as a carrier for thermolabile therapeutics. *International Journal of Pharmaceutics* <http://dx.doi.org/10.1016/j.ijpharm.2017.06.047>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Optimisation of HPMC ophthalmic inserts with sustained release properties as a carrier for thermolabile therapeutics

Arnout Everaert^a, Yannick Wouters^a, Eline Melsbach^b, Nadia Zakaria^{b}, Annick Ludwig^{a**}, Filip Kiekens^{a***}, Wim Weyenberg^{a**}**

^a Laboratory of Pharmaceutical Technology and Biopharmacy, Department of Pharmaceutical Sciences, University of Antwerp, Wilrijk, Belgium

^b Department of Ophthalmology, Antwerp University Hospital, Edegem, Belgium

*Corresponding author. Tel.: +32 3 265 27 13, fax: +32 3 265 26 85

E-mail address: filip.kiekens@uantwerpen.be

**Equally contributing authors

Abstract

A methodology was developed and optimised for the preparation of a new drug delivery system (DDS) with sustained release properties to allow ocular protein delivery and to limit destructive production steps during manufacturing. Elevated temperatures, shear forces and an oxidative environment should be avoided in order to prevent denaturation or oxidation of proteins. An aqueous HPMC solution was prepared using heat and casted into small semi-rod-shaped PVC blisters. The polymer solution was allowed to cool down and was partially dehydrated at room temperature. A drug solution containing glycerol, drug and water was subsequently added to rehydrate the partially dehydrated polymer matrix at a temperature of 2 °C. Several parameters of the production process were varied to determine their influence on the release kinetics from HPMC inserts from three different molecules of different molecular weight. This production method was further optimised in order to shorten the rehydration time from weeks to days, while eliminating heat and shear forces on the selected drug molecules sodium fluorescein, lysozyme and albumin. Slow release kinetics were achieved for sodium fluorescein and lysozyme as model drug molecules. The higher molecular weight of albumin prevented a good penetration into the insert during the rehydration process resulting in predominantly burst release. The biocompatibility of a viscous HPMC solution was evaluated on SV40-human corneal epithelial cells with PrestoBlue[®] and no cytotoxic effects were observed.

Keywords: ocular, insert, HPMC, protein drug delivery, human corneal epithelium cells

1. Introduction

The most commonly applied pharmaceutical dosage form for topical drug delivery to the eye are eye drops (Lang, 1995). Although these classical drug delivery systems are generally well accepted by patients, they are well known to give a limited ocular availability of less than 5% of the total amount of administered drug due to the barrier function of the cornea and conjunctiva (Davies, 2000, Xinming et al., 2008, Gaudana et al., 2010). Upon instillation, sensory nerves of the cornea and conjunctiva trigger lachrymation and rapid eye blinking, with clearance of the drug from the ocular surface in a few minutes. Consequently, patient compliance may be reduced due to the frequent instillation required. A high instillation frequency and pulsed dosing of drugs may furthermore lead to undesirable side effects. (Lee and Robinson, 1986; Salminen, 1990)

Different efforts have been attempted to overcome these drawbacks. Strategies have been developed to either overcome the low permeability of the cornea or to prolong the residence time of drug molecules at the site of absorption. Several drug delivery systems have been designed such as muco/bioadhesive dosage forms, collagen shields, drug loaded contact lenses and ocular inserts to overcome these issues (Ebrahim et al., 2005; Ludwig, 2005; Saettone and Salminen, 1995; Baeyens et al., 1998; Zimmer and Kreuter, 1995; Kaur and Kanwar, 2002; Aburahma and Mahmoud, 2011; Achouri et al., 2013). One of the most important strategies involves the use of viscosity-enhancing agents as drug carriers in order to prolong the residence time of the medication at the site of absorption and thereby limiting the drainage of the drug (Urtti and Salminen, 1993). The improved residence time of the drug in the conjunctival sac enhances its ocular availability and thus leads to less side effects and fewer administrations required.

In recent years many research initiatives have been taken on the use of proteins for the treatment of ocular diseases such as wet age-related macular degeneration (Barakat and Kaiser, 2009), uveitis (Guly and Forrester, 2010) and corneal neovascularisation (Chang et al, 2012). Intra-ocular injections are an effective way of administering therapeutic proteins, such as antiangiogenic proteins. Protective ocular barriers prevent an easy access of drugs into the eye. However, direct intravitreal injections of therapeutic proteins allow for a targeted treatment of specific retinal diseases, such as the monthly intravitreal injection of Ranibizumab, an anti-VEGF monoclonal antibody fragment (Fab) for the treatment of wet age-related macular degeneration (Thakur et al, 2014; El Sanharawi et al, 2010; Barakat and Kaiser, 2009.).

Proteins are known to potentially denature when applying high temperatures, pH changes and mechanical forces but can also experience an influence from ionic strength and possible oxidation (e.g. due to air bubble entrapment) of the drug substance. Therefore, it is important to develop a method which takes these influences into account when formulating drug delivery systems loaded with thermolabile molecules such as proteins (Vogt, 1995; Di Stasio and De Cristofaro, 2010; Torosantucci et al., 2014). In this manuscript, the method developed is designed to incorporate peptides and proteins in general without need for high temperatures, high shear forces or air bubble entrapment while releasing the active substance in a slow release manner.

Conventional methods for the preparation of homogenous gel systems include blending of different excipients and drug molecules together in the same vessel under high shear forces and elevated temperatures. These conditions cannot be used to formulate thermolabile proteins because proteins can unfold, break down, aggregate or lose their activity during the formulation process (Vaishya et al., 2014, Jaenicke, 1967; Tsai et al., 1998). High shear forces during mixing can not only compromise the integrity of the protein structure but it can also create an oxidative

environment by air bubble entrapment (Maa and Hsu, 1996; Charm and Wong, 1981; Thomas and Dunnill, 1979; Virkar et al., 1981; Glatz et al., 1986; Caussette et al., 1997; Colombié et al., 2000). This situation can potentially cause oxidation of certain amino acids such as methionine and cysteine in the protein structure and with reduction or loss of its activity (Levine et al., 2000). The solvent casting method has been a commonly applied method for the preparation of ocular inserts (e.g. ocular films) (Siemann, 2005; Hermans et al., 2014). A low-viscous solution is formed by mixing the various components at room temperature and is poured into an appropriate mould. The solution comprising the drug is dried over an extended period of time at room temperature or above. As the drug molecules are exposed to air, chemical reactions such as oxidation might occur. This drying process can be carried out in an inert atmosphere and at low temperatures for drug stability reasons. But inherent to these low temperatures, the time required to fully dry the dispersion will extend and is therefore undesirable.

The previous methodology to produce rod shaped, ocular inserts consists of four phases (Everaert et al., 2015). In a first stage a hydrogel network was formed, in a second stage a dehydrated polymer cylinder was obtained at a temperature of 150 °C. In a third stage the rehydration of the polymer cylinder was performed with a homogenous drug solution consisting of drug, glycerol and purified water in a closed barrel. After obtaining a homogenous, semi-solid mass by means of passive diffusion, the content was extruded from the barrel in the fourth and last stage of the development process, producing rod shaped ocular inserts with a diameter of 2 mm.

The main focus of the present study was to evaluate and optimise the method and the conditions for the preparation of drug loaded inserts without a particular drug substance or pathology envisioned. As opposed to the previously developed method (Everaert et al., 2015) shear forces are eliminated entirely and the rehydration time has been shortened significantly.

2. MATERIALS AND METHOD

2.1. Materials

Sodium fluorescein, lysozyme from chicken egg white, bovine serum albumin and glycerol analytical grade (99.5% (V/V)) were obtained from Sigma-Aldrich (Steinheim, Germany). Hydroxypropyl methylcellulose (HPMC) type E10M Premium CR was provided by Colorcon (Dartford, UK). By specification, an aqueous 2% (w/w) E10M HPMC solution has an apparent viscosity of 10.000 mPa.s (at 20 °C). This type of HPMC was selected, based on experience from previous research (Everaert et al., 2015).

Phosphate-buffered saline solution (PBS, pH 7.4) was prepared using 8.2 g/l sodium chloride (NaCl), 0.300 g/l sodium dihydrogen phosphate dihydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), 1.540 g/l disodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) and purified water. All electrolytes of analytical grade were purchased from Merck (Darmstadt, Germany). PVC-blisters capsule size 4 were used as a mould to produce the ocular inserts and were supplied by Janssen Pharmaceutica (Beerse, Belgium).

SV40-immortalised human corneal epithelium cells were kindly gifted by the laboratory of Integrative Regenerative Medicine Centre, Linköping University, Linköping, Sweden. Keratinocyte-Serum-Free medium (K-SFM) enriched with bovine pituitary extract (BPE) and human recombinant epidermal growth factor type B 1-53 (EGF 1-53) nutrients were obtained from Gibco[®] by Life Technologies (Paisley, UK). PrestoBlue[®] cell viability reagent and trypan blue stain (0.4%) were purchased from Life Technologies (Frederick, USA).

2.2. Preparation of ocular inserts

In earlier research, a new method was developed in order to produce highly viscous ocular inserts loaded with the drug of interest (Everaert et al., 2015). Firstly, a polymer solution was prepared using high shear forces and high temperatures, but in the absence of the drug of interest. Subsequently, the resulting polymer solution was dehydrated at a temperature of 150 °C yielding polymer cylinders of high HPMC concentration. These partially dehydrated polymer cylinders were transferred to a closed barrel to which a drug solution was added. The drug solution consisted of drug molecules, glycerol and purified water allowing the polymer cylinder to rehydrate during several weeks. After rehydration, small ocular inserts were extruded from the barrel by means of mild extrusion forces. A major drawback of this method is the extended period required to fully rehydrate and load the polymer cylinder after which the inserts could be produced by means of extrusion. Furthermore, shear forces, although they can be considered mild, are still applied in this method during the extrusion step.

A disadvantage of this process is that it takes up to several weeks in order to obtain ocular inserts with a homogenous drug distribution. To minimise the duration of the production process and to eliminate any shear forces including the mild forces used during extrusion, an alternative method based on the same principles as the method described above was developed. The goal of the current research was to shorten the time required for the production process and at the same time to avoid any shear forces by preparing the rod shaped inserts individually in a PVC-package for capsule size 4 by shortening the rehydration path significantly. This alternative production method can be divided into three major phases. A schematic overview of the different stages of the development and production of the ocular inserts is given in figure 1.

Phase I: Polymer matrix-formation

A polymer solution of HPMC 20% (w/w) was prepared by heating purified water up to 90 °C and adding HPMC powder under continuous agitation. A small amount of glycerol was added to obtain an intermediate percentage of 0.80% (w/w) during this stage of the production process. The polymer dispersion was continuously stirred and heated on a magnetic heating and stirring device (VWR, VMS-C7-2, IKA, Staufen, Germany). Once a homogenous HPMC dispersion was obtained, the hot solution was gravimetrically casted into individual PVC-blisters using an analytical balance (Mettler Toledo AB135-S, Greifensee, Switzerland) and cooled down to room temperature.

Phase II: Dehydration of the polymer solution

The polymer solution was exposed to air to allow evaporation of water from the polymer matrix. The individual blisters were dried to the chosen HPMC concentrations according to the design of experiments. The percentage of HPMC in the matrix was determined by gravimetrical analysis using an analytical balance. The drying time would take up to several hours at room temperature in order to obtain dry percentages of 50% (w/w) HPMC and 75% (w/w) HPMC. The drying process took up to three days at room temperature to reach a constant weight yielding a theoretical HPMC percentage of 100% (w/w). In contrary to the previous method, the viscous polymer solution could not be exposed to high temperatures accelerating the evaporation process, due to the thermolabile nature of the PVC-blister packaging.

Phase III: Rehydration of the polymer matrix

In this phase, an aqueous drug solution was added to the dehydrated polymer matrix. The percentages of drug and glycerol in the aqueous solution added to the dried polymer matrix was

chosen accordingly to reach final percentages of 3% (w/w) drug, 1% (w/w) glycerol and 25% (w/w) HPMC. The ratio drug:polymer was not varied and was kept equal for all drugs incorporated. As being the smallest molecule of the various components, water migrates through the polymer matrix followed by the solvated drug by means of passive diffusion (Métayer et al., 1999). No active forces were applied on the drug substance to minimise the potential risk of protein denaturation.

A viscous drug solution comprising HPMC as a viscosity enhancing agent was also tested on a dehydrated polymer matrix of 100% (w/w). The viscous drug solution consisted of 1% (w/w) HPMC, drug, glycerol and purified water.

After addition of the drug solution, blisters were allowed to rehydrate at a temperature of 2 °C. This temperature was chosen in favour of temperature stability of thermolabile drugs. The rehydration time was varied between 24 or 72 hours as described in the design of experiments.

After phase III, the final inserts were carefully removed from the blister package. The shape and dimensions of the blister package is shown in more detail in figure 2. The miniaturisation of the inserts described in this work was not within the scope of the research study.

Factorial design and statistical analysis

Sodium fluorescein 0.376 kDa, lysozyme from chicken egg white 14.307 kDa and bovine serum albumin 66.430 kDa, three molecules with different molecular weights were used as model molecules to determine the influence of molecular weight on the release profiles of the inserts prepared. The dry percentage of HPMC and the rehydration time were selected as variable parameters based on experience from earlier research. A 2² full factorial design was set up for each of these molecules. Four inserts per formulation were prepared and analysed. Each

formulation was prepared in duplicate. An overview of the parameters and different levels is given in table 1 and an overview of the different formulations is given in table 2.

The influence of the dry percentage HPMC (50% (w/w) and 75% (w/w)) and rehydration time (24 hours and 72 h) on the release profiles of different molecules at time point 460 or 480 minutes was tested using ANOVA with Statistica[®] 12 (Statsoft, Tulsa, OK, USA). The p-value is considered statistically significant when $p < 0.05$. Each experiment was performed in duplicate with four inserts per formulation.

2.3. In vitro drug release studies

The release patterns of the different formulations were plotted and analysed. Four inserts per formulation were put into separate glass tubes to which 15 g PBS was added at a temperature of 32 °C. The glass tubes were placed in a non-oscillating water bath (Julabo 20B, Merck-Belgolabo, Overijse, Belgium) at a temperature of 32 ± 0.5 °C (Purslow and Wolffsohn, 2005). This temperature corresponds to the lowest eye surface temperature. At different time points, samples of 2 g release medium were withdrawn from each glass tube and replaced with an equal amount of PBS at 32 °C to ensure sink conditions. Sampling intervals of 10, 30, 60, 90, 120, 180, 240, 300, 360, 420 and 480 minutes were chosen for sodium fluorescein and lysozyme. Sampling intervals of inserts formulated with albumin were chosen at 10, 30, 60, 90, 120, 180, 240, 300, 390 and 460 minutes. Before and after sampling, the content of the glass tubes was homogenised by means of gentle stirring.

Samples were analysed spectrophotometrically at a wavelength of 484 nm, 280 nm, 278 nm for sodium fluorescein, lysozyme and albumin respectively. HPMC inserts without drug were also

released over time in order to take the influence of the excipients into account and were subsequently subtracted from the measured values as a blank. At the end of every series of measurements, the content of each glass tube was intensively vortexed to release the remaining drug from the insert and measured spectrophotometrically.

2.4. Drug release analysis

The in vitro drug release curves were analysed for zero order, first order or mixed order kinetics. The Korsmeyer-Peppas equation or power law is a semi-empirical equation to describe drug release from polymeric systems (Ritger and Peppas, 1987). The equation is valid in swellable systems when the system only swells to a moderate equilibrium degree and not more than 25% of its initial volume. The power law is given as follows:

$$M_t/M = Kt^n$$

M_t = amount of drug released at time t

M = total amount of drug in the dosage form

K = kinetic constant

n = release exponent indicating the drug release mechanism

Release profiles from rod shaped dosage forms which follow first order kinetics indicate the release of drug by means of Fickian diffusion. In such cases, the exponent n will have a value of 0.45. If $n \geq 0.89$ drug release follows zero order kinetics indicating the release of drug corresponding to case-II transport (Siepmann et al., 1999). Values of n between 0.45 and 0.89 are mixed zero and first order kinetics, which is also referred to as anomalous diffusion or non-Fickian diffusion.

2.5. Cytotoxicity and biocompatibility of ophthalmic inserts on human corneal epithelial cells

To assess the cytotoxicity and biocompatibility of the excipients used for the development of the ophthalmic inserts, SV40-immortalised human corneal epithelial cells (SV40-HCEC) were used. The human corneal epithelial cells were treated by adding two different dilutions of a blank HPMC-insert. These results were assessed and compared to two different dilutions of a reference polymer (HPC). The cell viability was measured using PrestoBlue[®] reagent. PrestoBlue[®] is a resazurin-based cell viability reagent which is reduced from resazurin, which is a blue compound with no intrinsic fluorescent value, to resorufin which is red in colour and highly fluorescent. This conversion is proportional to the number of metabolically active cells making quantitative measurements possible.

Two different dilutions of 0.555% (w/w) and 1.11% (w/w) HPMC were prepared by dissolving an adequate amount of HPMC-insert (containing 20% (w/w) HPMC and 1% (w/w) glycerol in purified water) in K-SFM. These percentages, determining the viscosity of the dilutions, were chosen to allow pipetting in 96-well plates. For the sake of reference, two different dilutions of 1.22% (w/w) and 2.44% (w/w) HPC were prepared by dissolving HPC-powder in medium, also comprising glycerol in a 1% (w/w) percentage. The amount of HPC-polymer was chosen based on comparable viscosities between the HPMC-solution and the HPC-solution. The viscosity was measured using an Anton Paar rheometer (MCR-102, Graz, Austria) using a parallel plate measuring device with a diameter of 50 mm, 1 mm gap and at a temperature of 32 ± 0.1 °C. The shear rate was set between 0.01 s^{-1} to 100 s^{-1} . To prevent the polymer from drying out during measurement, a solvent trap was used to cover the sample. All measurements were performed in

triplicate. Figure 3 shows the rheological profiles for HPMC in a percentage of 0.555% (w/w) and 1.11% (w/w) and for HPC in a percentage of 1.22% (w/w) and 2.44% (w/w).

The stock cell cultures were maintained in T-75 flasks in Keratinocyte-Serum-Free medium (K-SFM) enriched with bovine pituitary extract (BPE) and human recombinant epidermal growth factor type B 1-53 (EGF 1-53) at 37 °C in a 95% air and 5% CO₂ incubator (Galaxy R, Eppendorf, Hamburg, Germany). The human corneal epithelial cells were plated into 96-well plates with K-SFM at 37 °C in 5% CO₂ incubator overnight. During this time period, cells were allowed to attach. The amount of cells was determined at an average of 30.000 cells per well using an automated cell counter (ABX Micros 60 OT, Horiba, Montpellier, France). Both hydrogel types (HPMC and HPC) and both groups of dilutions (high and low viscosities) were added to different wells on the same plate. Benzalkonium chloride is known to be cytotoxic (Noecker, 2001) and was used in three different percentages, 1% (w/V), 0.01% (w/V) and 0.004% (w/V), as a positive control group. Untreated cells in medium and plain medium without cells were added as negative and blank control groups respectively.

All experiments were performed in triplicate and on different time points: 0, 2, 8, 24, 48 and 72 hours. For each time point, a separate 96-well plate was used. At every time point, all wells were rinsed four times with medium to remove the compound of interest. The wells with the highest percentage HPMC or HPC were rinsed up to seven times to remove the viscous polymer solution as much as possible. The viability of the cells was measured at every time point by adding 100 µl PrestoBlue[®] solution for exactly 30 minutes to all wells after rinsing. The PrestoBlue[®] solution was prepared by adding one part of pure PrestoBlue[®] reagent with nine parts of K-SFM medium. After transferring the PrestoBlue[®] solution to a black 96-well plate (Optiplate[®], Perkin Elmer, Shelton, USA) the fluorescence was measured using a microplate reader (Victor 3 1420

Multilabel Counter, Perkin Elmer, Shelton, USA) at a wavelength of 531/590 nm for excitation/emission respectively.

As it was practically not possible to assess the influence of the insert in its true shape and composition, a qualitative analysis of the HPMC inserts was set up and performed using microscopic visualisation. Inserts weighing 0.15 g of a 10% and 20% final percentage of HPMC were prepared and added to three wells of a 6-well plate for each percentage type in the centre of the well. These inserts were allowed to settle during 24 hours at 37 °C and 5% CO₂ in a humidified atmosphere. An amount of 400.000 cells per well was seeded and incubated using the same conditions. After seeding the cells (time point 0 hours) and after 5, 24, 29, 48, 53 and 72 hours, pictures were taken with a Sony ILCE-7Rm2 camera (Tokyo, Japan) using a Leica DM IL (Leica Microsystems, Wetzlar, Germany) microscope with 40x magnification. All measurements were carried out in triplicate. Additionally, at the 72 hours time point, a trypan blue staining was performed. Trypan blue stain is an aqueous solution which is not absorbed by healthy viable cells but can readily enter damaged or dead cells. Visibly blue cells are therefore indicative for damaged or dead cells.

3. RESULTS AND DISCUSSION

3.1. In vitro drug release studies

The main objective of this research was to optimise the method of preparing ocular HPMC inserts. The long rehydration time required in the previous method could be shortened by no longer rehydrating the polymer with drug solution in a barrel for multiple inserts at the same time, but allowing the rehydration process to complete in a sealed single-dose package. Not only is the contact area of the polymer matrix with the surrounding drug solution increased in such single-dose preparations, the penetration depth of the drug molecules is also reduced thereby

shortening the rehydration time required. Using even smaller dimensions of the single-dose package would accelerate the rehydration process even further. This alternative method to produce ocular HPMC inserts allows the manufacturing process to happen even without the use of mild extrusion forces.

This method was evaluated using three drug molecules with different molecular weights. The linearity, sensitivity, precision, accuracy and specificity of the method were assessed prior to the measurements.

3.1.1. Sodium fluorescein

The release rate of drug molecules is driven by diffusional forces of the drug and solvent molecules. As the prepared inserts already contain a certain amount of water, dissolved drug molecules can readily be released from the insert by means of passive diffusion. Upon contact with the release medium, inserts swell due to water uptake and as a result, the polymeric network disentangles. A total disentanglement of the unlinked polymeric network will eventually occur, slowly releasing the drug by means of erosion (Rajabi-Siahboomi et al., 1994; Bettini et al., 2001). Because of the dense packing of the polymer network, slower release rates are expected to occur, especially for larger molecules (Ghosal et al, 2012; Ghori et al., 2014).

The amount of sodium fluorescein released from HPMC inserts is shown in the cumulative release plots in figure 4. Each of the formulations in the design of experiments show a slow release profile with an average cumulative release amount of $68.59 \pm 1.22\%$ (w/w) on the total amount of drug after a time interval of 480 minutes. Statistical analysis revealed no significant differences when the dry percentage HPMC or rehydration time was altered. Sodium fluorescein has a relatively low molecule weight (0.376 kDa) compared to lysozyme (14.307 kDa) and albumin (66.430 kDa) and therefore experiences little resistance by the polymeric network upon

diffusion. An overview of the effect of the different parameters on the release of sodium fluorescein loaded inserts is given in table 3. Effect % represents the difference in total amount of drug released after 480 minutes when the according parameter is altered from the lower level to the upper level.

3.1.2. Lysozyme

Release profiles of inserts loaded with lysozyme from chicken egg white are shown in figure 4. The dry percentage of HPMC showed no significant influence upon statistical analysis. Opposed to the parameter “dry percentage”, increasing the rehydration time from 24 to 72 hours did have a statistical significant effect ($p=0.000$). The increased molecular weight of lysozyme lowered the total amount of drug released from the insert for a given time point compared to sodium fluorescein and was even further lowered by prolonging the rehydration period from 24 to 72 hours. A difference of 9.99% (w/w) over an interval of 480 minutes was calculated. This could be indicative for the influence of time on the diffusion process of drug molecules into the polymeric network. Chicken egg white lysozyme is larger (14.307 kDa) than sodium fluorescein and therefore experiences more resistance from the polymeric network upon diffusion out of the network as well as upon diffusion into the polymer matrix during preparation. After 24 hours, it was visually apparent that the lysozyme molecules did not fully penetrate the core of the insert as opposed to the situation after 72 hours. Consequently, a significant amount of drug resided on the surface of the inserts prepared in 24 hours resulting in a faster release. In table 3, an overview of the effect of the different parameters on the release of lysozyme loaded inserts is given.

3.1.3. Bovine serum albumin

In figure 4, the release curves of inserts loaded with bovine serum albumin are shown. Slow release curves of inserts loaded with bovine serum albumin could not be achieved with the chosen parameters. An amount of approximately 38 to 49% (w/w) albumin was released immediately by all formulation types after 10 minutes. After 30 minutes, about 80% (w/w) of the total amount of drug present in the insert was released into the surrounding medium. Varying either the parameter “dry percentage” HPMC or “rehydration time” in accordance to the design did not lower the release profiles. A small but significant decrease ($p=0.024$) of the total amount of albumin released after 460 minutes could be achieved by prolonging the rehydration time up to 72 hours. This effect was minimal with an average decrease of 3.30% (w/w) albumin released after 460 minutes when rehydration time was increased from 24 hours to 72 hours. An overview of the results and their significance is shown in table 3.

The molecular weight of the selected drug molecules influences the release profiles from the inserts formulated. Bovine serum albumin is a protein with the highest molecular weight of the three molecules tested. Its diffusion, using no active forces, into the matrix is limited due to the entanglement of the polymeric network. As larger molecules diffuse at a lower rate compared to smaller molecules, only a small amount of albumin is embedded deeply enough in the matrix. The majority of drug molecules is located at the surface of the insert. The size of the drug molecules on one hand and the relatively high diffusional rate of solvent molecules on the other hand attributed to this. Solvent molecules will diffuse rapidly into the matrix hydrating the surrounding HPMC chains. At the surface of the insert, the diffusion of the solvent molecules into the insert may result in an oversaturation of the drug molecules causing precipitation of albumin molecules.

3.1.4. Influence of molecular weight

An influence of the molecular weight was observed comparing the release profiles of sodium fluorescein (NaFlu) and chicken egg white lysozyme (LYS) in figure 4. Due to its larger hydrodynamic diameter, lysozyme was released slower than sodium fluorescein from the polymeric network. It was expected that inserts loaded with albumin (ALB) would yield inserts with an even slower release profile. But due to the high molecular weight, the resistance upon passive diffusion into the polymer during loading was too large to overcome in a period of 72 hours.

3.1.5. Viscous polymer drug solution

In order to produce slow release ocular inserts of molecules with the largest molecular weight tested, inserts were dehydrated at room temperature to a constant weight giving a theoretical dry percentage of 100% (w/w) HPMC. Instead of using an aqueous drug solution in which albumin molecules would precipitate on the surface of the insert, a viscous polymer drug solution was prepared and added instead of the aqueous drug solution. Because of the viscous character of the drug solution, the migration of water into the dehydrated polymer matrix is slowed and drug molecules are surrounded by polymer molecules from the beginning of the diffusion process (Masazumi and Nakagami, 2002). This results in a lower migration speed of water molecules towards the dehydrated polymer matrix which might prevent the occurrence of an oversaturated drug solution giving more time to solvated drug molecules to diffuse into the polymer matrix and being embedded in some viscous matrix straight from the beginning. The results as mentioned above indicate that time is an influential parameter. For this reason, a rehydration period of 72 hours was chosen instead of 24 hours. The release profiles of inserts loaded with bovine serum albumin with a rehydration time of 72 hours are shown in figure 5.

A significant decrease in release rate was observed when using a viscous HPMC drug solution, in which albumin molecules are surrounded by HPMC molecules, instead of an aqueous drug solution. The amount of protein released at every given time point is significantly lower when inserts are formulated with a viscous polymer drug solution. For instance, after an interval of 60 minutes, a total amount of $51.99 \pm 5.51\%$ albumin (w/w) was released from the inserts prepared with the viscous drug solution as opposed to $85.59 \pm 3.63\%$ (w/w) from inserts prepared with an aqueous drug solution.

3.2. Drug release analysis

3.2.1. Sodium fluorescein

The release profiles of ocular inserts formulated with sodium fluorescein and loaded during 24 hours and 72 hours are shown in figure 6A. The slope of the linear regression of the curve equals a value of 0.5176 and 0.4900 respectively, indicating the release of drug by means of diffusion as well as case-II transport. However, the slope n obtained for the inserts prepared with a loading time of 72 hours approaches more the value of 0.45 which could be indicative for a release of drug molecules predominantly characterised by diffusion.

In figure 6B, the same cumulative release profiles are shown after omitting the first measuring point (10 minutes). A very small but rapid release of drug molecules within the first 30 minutes of the release process was apparent. Thereafter, the drug molecules were released by diffusion indicated by the slope n being smaller than 0.45 after regression analysis. This observation might be attributed to drug molecules residing on the surface of the insert and being rapidly released in the release medium.

3.2.2. Chicken egg white lysozyme

Figure 7 shows the release profiles of inserts loaded with chicken egg white lysozyme and shows the release kinetics of inserts which were allowed to rehydrate over a period of 24 hours and the release kinetics of inserts which were released after a rehydration period of 72 hours. Both profiles indicate the release by means of first and mixed zero order release kinetics due to the value of the slope of their linear regression lines, 0.5086 and 0.5461 respectively. Inserts which were allowed to rehydrate over a period of 72 hours, show a value of n closer to 0.89 than inserts rehydrated over a period of 24 hours, indicating a more prominent role for case-II transport of the drug. This supports the hypothesis that longer rehydration periods allow certain molecules to penetrate deeper into the polymer matrix. Upon contact with the release medium, resistance by the polymeric network and increase in diffusional distance by swelling impair the diffusion of deeply penetrated drug molecules to the surrounding medium. These deeply penetrated molecules will be released when the unlinked polymer network disentangles while releasing the drug by means of erosion.

3.2.3. Bovine serum albumin

No release kinetic profiles of inserts loaded with albumin were examined because no slow release profiles were achieved by formulations of the design of experiments.

3.3. Cytotoxicity and biocompatibility

The viability of SV40-HCEC was evaluated by adding the chosen hydrogel solutions to these cells in a 96-well plate and was compared to cells in plain medium. Three different

concentrations of benzalkonium chloride were added to wells containing cells as a cytotoxic reference product. PrestoBlue[®] was subsequently added and the resulting fluorescence was measured. The results are shown in figure 8.

The results indicate that for all percentages tested (1% (w/V), 0.01% (w/V) and 0.004% (w/V)) benzalkonium chloride has a cytotoxic effect and the relative fluorescence falls to reflect complete absence of viable cells as present in the blank control samples by the second measurement at 2 h. All polymer solution treated cells, both HPC and HPMC in a high and low viscosity solution, yield excellent results with relative fluorescence values at least as high as the negative reference. After 2 hours, the relative fluorescence of the polymer solution treated cells increases progressively, indicating increasing cellular proliferation and metabolic activity reaching a plateau after 48 hours, indicative for confluency of the cells.

To evaluate the ability of SV40-HCEC to grow in the presence of an insert with a final E10M HPMC percentage of 10% (w/w) and 20% (w/w), an insert of about 150 mg was added to a 6-well plate and incubated overnight to allow the insert to spread out. Thereafter, 400.000 cells were added to each well. Each experiment was performed in triplicate. Cellular attachment and growth was evaluated using a light microscope and is shown in figure 9A and 9B.

After an incubation period of 5 hours, the cells show attachment to the surface of the well. The cells observed at the periphery appear somewhat flattened as the viscous mass of the insert forms a mechanical barrier for the growth of cells on the insert as the cells cannot grow where the insert is located. After an incubation period of 72 hours, the cells were near confluent over the surface of the well. At this time point, the insert has dissolved entirely. The cells still do not appear to attach to the bottom of the well where the insert was originally located which suggests a

continued presence of HPMC film on the bottom of the well. This observation does not come as a surprise as some researchers have suggested the possibility of using cellulose-derivatives as anti-adhesive substrates for cells (Velzenberger et al.). Following trypan blue staining, the number of dead cells can be visually evaluated as only the dead cells stain blue. The picture taken after trypan blue treatment is shown in figure 10.

Very few dead cells are observed sparsely dispersed throughout the culture. The edge of the HPMC insert is populated with viable cells indicating the biocompatibility of the HPMC inserts.

Conclusion

The ocular inserts prepared by the alternative preparation method as described in this research, allows the production of inserts with slow release properties. A drug can be loaded into the insert without exposing the drug to high temperatures or shear forces while minimising the risk of oxidation. When larger molecules such as lysozyme need to be incorporated, a significant influence of the rehydration time is observed on the release profiles of the prepared inserts. Compared to the previous method of preparing the inserts, the rehydration time required is significantly reduced from several weeks to a few days for sodium fluorescein and lysozyme. This optimised preparation method allows furthermore the rehydration to occur in the end package of the product even after being sealed. When molecules with a size such as albumin need to be formulated, adding an aqueous viscous drug solution seems to be more appropriate. A significant influence of the rehydration time was observed on inserts formulated with bovine serum albumin. The HPMC inserts described in the research work show biocompatibility with and do not have a negative influence on the growth of the SV40-human corneal epithelial cells.

References

Aburahma, M.H., Mahmoud, A.A., 2011. Biodegradable ocular inserts for sustained delivery of brimonidine tartarate: preparation and in vitro/in vivo evaluation. *AAPS PharmSciTech.* 12 (4), 1335-1347.

Achouri, D., Alhanout, K., Piccerelle, P., Andrieu, V., 2013. Recent advances in ocular drug delivery. *Drug Dev. Ind. Pharm.* 39 (11), 1599-1617.

Baeyens, V., Kalsatos, V., Boisramé, B., Varesio, E., Veuthey, J-L., Fathi, M., Balant, L.P., Gex-Fabry, M., Gurny, R., 1998. Optimized release of dexamethasone and gentamicin from a soluble ocular insert for the treatment of external ophthalmic infections. *J. Control. Release.* 52, 215-220.

Barakat, M.R., Kaiser, P.K., 2009. VEGF inhibitors for the treatment of neovascular age-related macular degeneration. *Expert Opin. Inv. Drug.* 15 (5), 637-646.

Bettini, R., Catellani, P.L., Santi, P., Massimo, G., Peppas, N.A., Colombo, P., 2001. Translocation of drug particles in HPMC matrix gel layer: effect of drug solubility and influence on release rate. *J. Control. Release.* 70, 383-391.

Caussette, M., Planche, H., Delepine, S., Monsan, M., Gaunand, A., Lindet, B., 1997. The self catalytic enzyme inactivation induced by solvent stirring: a new example of protein conformational change induction. *Protein Eng.* 10, 1235–1240.

Chang, J-H., Garg, N.K., Lunde, E., Han, K-Y., Jain, S., Azar, D.T., 2012. Corneal neovascularization: an anti-VEGF therapy review. *Surv. Ophthalmol.* 57 (5), 415-429.

Charm S.E., Wong B.L., 1981. Shear effects on enzymes. *Enzyme and Microbial Technology*, 3 (2), 111–118.

Colombié, S., Gaunand, A., Lindet, B., 2000. Stirring effect on lysozyme inactivation and aggregation: kinetic study and aggregates characterisation. *Biotechnol Lett.* 22, 277–283.

Davies, N.M., 2000. Biopharmaceutical considerations in topical ocular drug delivery. *Clin. Exp. Pharmacol. P.* 27, 558-562.

Di Stasio, E., De Cristofaro, R., 2010. The effect of shear stress on protein conformation: Physical forces operating on biochemical systems: The case of von Willebrand factor. *Biophys. Chem.* 153 (1), 1-8.

Ebrahim, S., Peyman, G.A., Lee, P.J., 2005. Applications of liposomes in ophthalmology. *Surv. Ophthalmol.* 50 (2), 167-182.

Everaert, A., Broeckx, G., Fransen, E., Ludwig, A., Kiekens, F., Weyenberg, W. Evaluation of a newly developed HPMC ophthalmic insert with sustained release properties as a carrier for thermolabile therapeutics. *Int. J. Pharm.* 481 (1–2), 37-46.

El Sanharawi, M., Kowalczyk, L., Touchard, E., Omri, S., de Kozak, Y., Behar-Cohen, F., 2010. Protein delivery for retinal diseases: From basic considerations to clinical applications. *Progress in Retinal and Eye Research*, 29 (6), 443-465.

Gaudana, R., Ananthula, H. K., Parenky, A., Mitra, A. K., 2010. Ocular Drug Delivery. *The AAPS Journal*, 12 (3), 348–360.

Ghori, M.H., Ginting, G., Smith, A.M., Conway, B.R., 2014. Simultaneous quantification of drug release and erosion from hypromellose hydrophilic matrices. *Int. J. Pharm.* 465, 405-412.

Ghosal, K., Chandra, A., Rajabalaya, R., Chakraborty, S., Nanda, A., 2012. Mathematical modeling of drug release profiles for modified hydrophobic HPMC based gels. *Pharmazie* 67 (2), 147-155.

Glatz, C.E., Hoare, M., Landa-Vertiz, J., 1986. The formation and growth of protein precipitates in a continuous stirred-tank reactor. *AIChE J.* 32, 1196–1204.

Guly, C.M., Forrester, J.V., 2010. Investigation and management of uveitis. *BMJ.* 341, c4976.

Hermans, K., Van den Plas, D., Kerimova, S., Carleer, R., Adriaenssens, P., Weyenberg, W., Ludwig, A., 2014. Development and characterization of mucoadhesive chitosan films for ophthalmic delivery of cyclosporine A. *Int. J. Pharm.* 472 (1-2), 10-19.

Jaenicke R., 1967. Intermolecular forces in the process of heat aggregation of globular proteins and the problem of correlation between aggregation and denaturation phenomena. *J. Polymer Sci.* 16, 2143-2160.

Kaur, I.P., Kanwar, M., 2002. Ocular preparations: the formulation approach. *Drug Dev. Ind. Pharm.* 28 (5), 473-493.

Kojima, M., Nakagami, H., 2002. Investigation of water mobility and diffusivity in hydrating micronized low-substituted hydroxypropyl cellulose, hydroxypropylmethyl cellulose, and hydroxypropyl cellulose matrix tablets by magnetic resonance imaging (MRI). *Chem Pharm Bull.* 50 (12), 1621-1624.

Lang, J.C., 1995. Ocular drug delivery conventional ocular formulations. *Advanced Drug Delivery Reviews.* 16 (1), 39-43.

Lee, V.H.L., Robinson, J.R., 1986. Topical ocular drug delivery: recent developments and future challenges. *J. Ocul. Pharmacol. Th.* 2, 67-108.

Levine R.L., Moskovitz J., Stadman E.R., 2000. Oxidation of methionine in proteins: roles in antioxidant defense and cellular regulation. *IUBMB Life*, 50 (4-5), 301-307.

Ludwig, A., 2005. The use of mucoadhesive polymers in ocular drug delivery. *Adv. Drug Deliv. Rev.* 57, 1595-1639.

Maa Y-F, Hsu CC, 1996. Effect of high shear on proteins. *Biotechnol Bioeng.* 51, 458–465.

Métayer, M., Labbé, M., Marais, S., Langevin, D., Chappey, C., Dreux, F., Brainville, M., Belliard, P., 1999. Diffusion of water through various polymer films: a new high performance method of characterization, *Polymer Testing*, 18 (7), 533-549.

Noecker, R., 2001. Effects of common ophthalmic preservatives on ocular health. *Adv. Ther.*, 18 (5), 205-215.

Purslow, C., Wolffsohn, J.S., 2005. Ocular surface temperature: a review. *Eye Contact Lens.* 31 (3), 117-123.

Rajabi-Siahboomi, A.R., Bowtell, R.W., Mansfield, P., Henderson, A., Davies, M.C., Melia, C.D., 1994. Structure and behaviour in hydrophilic matrix sustained release dosage forms: 2. NMR-imaging studies of dimensional changes in the gel layer and core of HPMC tablets undergoing hydration. *J. Control. Release.* 31, 121-128.

Ritger, P.L., Peppas, N.A., 1987. A simple equation for description of solute release II: Fickian and anomalous release from swellable devices. *J. Control. Release.* 5, 37-42.

Saettone, M.F., Salminen, L., 1995. Ocular inserts for topical delivery. *Adv. Drug Deliv. Rev.* 16, 95-106.

Salminen, L., 1990. Review: systemic absorption of topically applied ocular drugs in humans. *J. Ocul. Pharmacol.* 6, 243-249.

Siemann, U., 2005. Solvent cast technology - A versatile tool for thin film production. *Prog. Colloid Polym. Sci.* 130, 1-14.

Siepmann, J., Kranz, H., Bodmeier, R., Peppas, N.A., 1999. HPMC-matrices for controlled drug delivery: A new model combining diffusion, swelling, and dissolution mechanisms and predicting the release kinetics. *Pharm. Res.* 16 (11), 1748-1756.

Thakur, S.S., Barnett, N.L., Donaldson, M.J., Parekh, H.S., 2014. Intravitreal drug delivery in retinal disease: are we out of our depth? *Expert Opin. Drug Deliv.* 11 (10), 1575-1590.

Thomas, C.R., Dunnill, P., 1979. Action of shear on enzymes: studies with catalase and urease. *Biotechnol. Bioeng.* 21, 2279-2302.

Torosantucci, R., Schöneich, C., Jiskoot, W., 2014. Oxidation of therapeutic proteins and peptides: Structural and biological consequences. *Pharm. Res.* 31 (3), 541-553.

Tsai, A.M., van Zanten, J.H., Betenbaugh, M.J., 1998. Study of protein aggregation due to heat denaturation: a structural approach using circular dichroism spectroscopy, nuclear magnetic resonance, and static light scattering. *Biotechnol. Bioeng.* 59, 273-279.

Urtti, A., Salminen, L., 1993. Minimizing systemic absorption of topically administered ophthalmic drugs. *Surv. Ophthalmol.* 37 (6), 435-456.

Vaishya, R., Khurana, V., Patel, S., & Mitra, A. K., 2015. Long-Term Delivery of Protein Therapeutics. *Expert Opinion on Drug Delivery*, 12 (3), 415–440.

Velzenberger, E., Vayssade, M., Legeay, G., Nagel, M-D, 2008. Study of cell behaviour on a cellulose anti-adhesive substratum. *Cellulose*, 15 (2), 347-357.

Virkar, P.D., Narendranathan, T.J., Hoare, M., Dunnill, P., 1981. Studies of the effect of shear on globular proteins: extension to high shear fields and to pumps. *Biotechnol. Bioeng.* 23, 425–429.

Vogt, W., 1995. Oxidation of methionyl residues in proteins: Tools, targets, and reversal. *Free Radic. Biol. Med.* 18, 93-105.

Xinming, L., Yingde, C., Lloyd, A.W., Mikhalovsky, S.V., Sandeman, S.R., Howel, C.A., Liewen, L. Polymeric hydrogels for novel contact lens-based ophthalmic drug delivery systems: A review. *Contact Lens and Anterior Eye.* 31 (2), 57-64.

Zimmer, A., Kreuter, J., 1995. Microspheres and nanoparticles used in ocular delivery systems. *Adv. Drug Deliv. Rev.* 16, 61-73.

Fig. 1. General overview of the preparation method.

Fig. 2. Graphical representation of semi-rod-shaped inserts.

Table 1. Overview of the constant and variable parameters in the factorial design. The lower (-) and upper (+) levels of the chosen parameters are given.

Table 2. Overview of the different experiments and the chosen variables.

Fig. 3. Rheological profiles for HPMC in a percentage of 0.555% (w/w) (HPMC LV) and 1.11% (w/w) (HPMC HV) and for HPC in a percentage of 1.22% (w/w) (HPC LV) and 2.44% (w/w) (HPC HV).

Fig. 4. Cumulative release profiles of sodium fluorescein (NaFlu), lysozyme from chicken egg white (LYS) and bovine serum albumin (ALB) loaded HPMC inserts. Each graph is a representation of inserts formulated according to the parameters as shown in the legend. The drug release is compared to the total amount of drug present after full release of the drug from the matrix ($n = 8$).

Table 3. An overview of the effects of the different parameters and statistical effects on the release of sodium fluorescein, lysozyme and albumin from the HPMC inserts.

Fig. 5. Cumulative release profiles of inserts loaded with bovine serum albumin. Each graph is a representation of inserts formulated according to the parameters as shown in the legend. The drug release is compared to the total amount of drug present after full release of the drug from the matrix. Inserts are formulated with an aqueous drug solution for dry percentages 50% (w/w) and 75% (w/w) ($n = 8$), whereas a viscous drug solution (VS) is used for inserts with a theoretical dry

percentage of 100% (w/w), as well as an aqueous drug solution for a dry percentage of 100% (w/w) (n = 4).

Fig. 6A. Cumulative release profiles of inserts loaded with sodium fluorescein after 24 and 72 hours.

Fig. 6B. Cumulative release profiles of inserts loaded with sodium fluorescein after 24 and 72 hours after omitting the first measuring point (10 min).

Fig. 7. Cumulative release profiles of inserts loaded with chicken egg white lysozyme.

Fig. 8. Effect on cell viability by adding a solution of 2.44% (w/w) HPC (HPC HV) and 1.44% (w/w) HPC (HPC LV), 1.11% (w/w) HPMC (HPMC HV) and 0.555% (w/w) HPMC (HPMC LV) in medium. Benzalkonium chloride in concentrations of 1% (w/V), 0.01% (w/V) and 0.004% (w/V) dissolved in medium was added as a known cytotoxic reference. A negative control (medium + cells) and a blank group (medium – cells) were also included.

Fig. 9A. Light microscopic evaluation of SV40-HCEC in the presence of a 10% (w/w) HPMC insert after 5 hours incubation. On the bottom left hand side of the picture, a small part of the insert is visible. (40x magnification)

Fig. 9B. Visual evaluation of the growth of SV40-HCEC in the presence of a 10% (w/w) HPMC insert after 72 hours incubation. On the bottom left hand side of the picture, a small part of the area where the insert was located, is visible. (40x magnification)

Fig. 10. Visual evaluation of the growth of SV40-HCEC in the presence of a 10% (w/w) HPMC insert after 72 hours incubation and trypan blue staining. (40x magnification)

Fixed parameters		
Final concentration drug % (w/w)	3	
Final concentration glycerol % (w/w)	1	
Final concentration HPMC % (w/w)	25	
Variable parameters		
	Lower level (-)	Upper level (+)
Dry percentage HPMC % (w/w)	50	75
Rehydration time (h)	24	72

Formulation	Dry percentage HPMC % (w/w)	Rehydration time (h)
1	+	+
2	+	-
3	-	+
4	-	-

Parameter	Sodium fluorescein		Lysozyme		Albumin	
	Effect %	p-value	Effect %	p-value	Effect %	p-value
Dry percentage HPMC	-0.19	0.145	1.79	0.177	1.10	0.432
Rehydration time	0.60	0.643	-9.99	0.000	-3.30	0.024

◆ ALB - 72 h - 75% (w/w) ◆ ALB - 72 h - 50% (w/w) ▲ NaFlu - 24 h - 50% (w/w) ● NaFlu - 24 h - 75% (w/w) ▲ NaFlu - 72 h - 50% (w/w)
◆ NaFlu - 72 h - 75% (w/w) ▲ LYS - 24 h - 75% (w/w) ▲ LYS - 24 h - 50% (w/w) ▲ LYS - 72 h - 75% (w/w) ▲ LYS - 72 h - 50% (w/w)

Log cumul. rel. sodium fluorescein (%)

■ sodium fluorescein loaded inserts 24 h rehydration

◆ sodium fluorescein loaded inserts 72 h rehydration