Hydrogen-bonded complexes and blends of poly(acrylic acid) and methylcellulose: nanoparticles and mucoadhesive films for Ocular Delivery of Riboflavin

Hydrogen-Bonded Complexes and Blends of Poly(acrylic acid) and Methylcellulose: Nanoparticles and Mucoadhesive Films for Ocular Delivery of Riboflavin[a]

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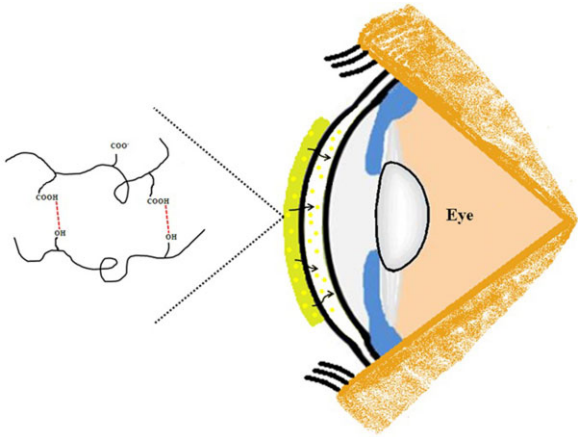
Poly(acrylic acid) (PAA) and methylcellulose (MC) are able to form hydrogen-bonded interpolymer complexes (IPCs) in aqueous solutions. In this study, the complexation between PAA and MC is explored in dilute aqueous solutions under acidic conditions. The formation of stable nanoparticles is established, whose size and colloidal stability are greatly dependent on solution pH and polymers ratio in the mixture. Poly(acrylic acid) and methylcellulose are also used to prepare polymeric films by casting from aqueous solutions. It is established that uniform films can be prepared by casting from polymer mixture solutions at pH 3.4–4.5. At lower pHs (pH < 3.0) the films have inhomogeneous morphology resulting from strong interpolymer complexation and precipitation of polycomplexes, whereas at higher pHs (pH 8.3) the polymers form fully immiscible blends because of the lack of interpolymer hydrogen-bonding. The PAA/MC films cast at pH 4 are shown to be non-irritant to mucosal surfaces. These films provide a platform for ocular formulation of riboflavin, a drug used for corneal cross-linking in the treatment of keratoconus. An in vitro release of riboflavin as well as an in vivo retention of the films on corneal surfaces can be controlled by adjusting PAA/MC ratio in the formulations.



1. Introduction

Hydrogen-bonded interpolymer complexes (IPCs) formed between poly(carboxylic acids) and different water-soluble non-ionic cellulose ethers such as methylcellulose (MC), hydroxypropylcellulose, hydroxypropylmethylcellulose, and hydroxyethylcellulose are of growing interest because of various applications of these polymers in pharmaceutical and biomedical areas.[1–3] Poly(acrylic acid) (PAA) and its weakly cross-linked derivatives (Carbopols) are widely used as mucoadhesives in various pharmaceutical formulations due to their excellent adhesion to biological surfaces.[4–6] The presence of a mucoadhesive polymer in a formulation often helps to retain a dosage form on the site of

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[a]**Supporting Information** is available online from the Wiley Online Library or from the author.

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administration and improves drug bioavailability. Excellent mucoadhesive properties of poly(acrylic acid) are related to its ability to bind to mucins through hydrogen-bonding.[7] However, the applicability of PAA and its derivatives in drug delivery via transmucosal routes is limited because these polymers are often too hydrophilic (resulting in quick dissolution or excessive swelling), have relatively poor film forming and mechanical characteristics, and sometimes cause irritation to sensitive mucosal surfaces.[8] Non-ionic cellulose ethers have numerous pharmaceutical applications ranging from liquid ophthalmic and nasal formulations to emulsifiers and excipients used for manufacturing tablets.[9,10] They are typically less mucoadhesive than polycarboxylate polymers but have excellent biocompatibility profiles and film forming properties.[11] A combination of PAA with cellulose ethers is expected to provide materials with improved physicochemical and biological characteristics.

Previously, we have studied the complexation between PAA and MC in aqueous solutions by turbidimetric titration, isothermal titration calorimetry, transmission electron microscopy, and surface plasmon resonance (Biacore) and evaluated the effects of pH and solution concentration on the intensity of interactions and structure of IPCs formed.[12–16] Rheological properties of PAA–MC complexes in aqueous solutions were also reported by other authors.[17] It was established that the complexes formed have non-stoichiometric nature and contain excessive molar quantities of PAA. Formation of insoluble complexes was observed at pHs below the critical pH of complexation (pH_{crit}), which values depended on polymer concentrations. Polymer solutions of 0.01 unit-base mol^{-1} exhibited the pH_{crit} of 3.10 ± 0.05, whereas at 0.05 unit-base mol the pH_{crit} was observed at 3.30 ± 0.05.[13] Multilayered ultrathin hydrogel films and coatings have been developed from PAA–MC using layer-by-layer deposition of IPC on the surface of unmodified[14,15] and chemically modified microscopy glass slides,[16] respectively.

In this study, we have further investigated complexation between PAA and MC in aqueous solutions with an emphasis on the structure and aggregation stability of IPC nanoparticles as a function of PAA–MC ratios in the mixture and solution pH using dynamic light scattering. We have also established optimal conditions for developing uniform films by casting PAA–MC solution mixtures and demonstrated the importance of balancing the strength of hydrogen bonding between the polymers to avoid intensive complexation/aggregation at low pHs and complete immiscibility at relatively high pHs. Optimized conditions for casting homogeneous PAA/MC blends were used to develop mucoadhesive films for ocular delivery of riboflavin.

2. Experimental Section

2.1. Materials

PAA ($\overline{M}_n$ 450 kDa) was supplied by Sigma–Aldrich (UK). Methylcellulose (Methocel 60 HG; 93 kDa) with 28–30% methoxyl content was purchased from Fluka (UK). The viscosity of 2 wt% methylcellulose (MC) in water at 20 °C was 35–55 mPa · s. Both polymers were used without further purification. All other chemicals such as hydrochloric acid, sodium hydroxide, riboflavin, and buffer solutions were purchased from Sigma–Aldrich (UK) and used without further purification.

2.2. Preparation of Solutions and Adjustment of pH

Polymer solutions were prepared by dissolving the required amounts of MC or PAA in deionized water at defined pH and leaving it stirring overnight at room temperature. The pH of the solutions was then further adjusted by adding small amounts of 0.1 M HCl or NaOH and was measured using a digital pH-meter (Metrohm, Switzerland).

2.3. Dynamic Light Scattering

The size of IPC particles formed in aqueous solutions at various polymer ratios and solution pHs was studied by dynamic light scattering (DLS) at 25 °C using a Malvern Zetasizer Nano-S (Malvern Instruments, UK). Each DLS experiment was repeated in triplicate by preparing and analyzing solutions of each polymer sample separately.

2.4. Turbidimetric Measurements

Turbidity of aqueous mixtures of PAA and MC was examined at 400 nm with a V-530PC spectrophotometer (Jasco, UK). In these experiments, samples were prepared by mixing PAA and MC solutions of different concentrations (0.2, 1.0, and 2.0 wt%) and pH (1, 2, 3, and 4) and were left stirring overnight before turbidity readings were taken.

2.5. Preparation of Films

Homogeneous drug-loaded films were prepared using 3 wt% PAA and MC solutions in ultrapure water (18.2 Ω). 200 mL of each solution was stirred overnight and 16 mg riboflavin added, shielded with aluminium foil and again stirred overnight. The solution was adjusted to pH 4.5 using HCl (0.1 M) or NaOH (0.1 M), stirred for a further 2 h and pH adjusted as necessary. Solutions were mixed in different proportions. Similar protocol was used to prepare drug-free films without addition of riboflavin. These solutions were used to cast films by pouring 50 mL into 100 mm × 100 mm Petri dishes, which were subsequently placed in a ventilated chamber, shielded from light for 5 d. Each film appeared uniform in thickness and color, they were relatively easy to detach from the Petri dishes. Care was taken to minimize exposure to light during handling due to the photosensitivity of riboflavin.

2.6. Scanning Electron Microscopy (SEM)

SEM experiments used an FEI Quanta FEG 600 Environmental Scanning Electron Microscope with an acceleration voltage of 20 kV. The surface of PAA–MC films was sputtered with gold before analysis.

2.7. Slug Mucosal Irritation Test

The slug mucosal irritation test (SMIT) was carried out with the drug-free films as described in our previous publication.[18] *Limax flavus* and *Arion lusitanicus* slugs weighing 3–8 g were sourced locally (Reading, UK). Individual slugs were kept in 2.5 L glass beakers lined with a paper towel moistened with 20 mL PBS solution and left at room temperature for 2 d before the start of an experiment. In both positive and negative controls Whatman filter paper moistened with 2 mL 1% benzalkonium chloride in PBS and 2 mL PBS solution, respectively, were used to line 90 mm Petri dishes. The samples of films were moistened with 2 mL PBS solution just before the experiments with slugs were carried out.

Each slug was individually weighed before the experiment and then placed in Petri dishes containing either polymeric sample, or positive/negative controls. After 1 h contact period slugs were taken out, rinsed with 10 mL PBS, gently wiped with a tissue paper and re-weighed. The mucus production (MP) was estimated as a slug body weight loss and calculated by the following formula:

$$\mathrm{MP} = \frac{(m_b - m_a)}{m_b} \times 100\% \quad (1)$$

where m_b and m_a are the weights of a slug before and after experiment, respectively. Each experiment was repeated 4–6 times using different slugs and the results were statistically treated, calculating the mean values ± standard deviations.

2.8. In Vitro Drug Release Studies

10 mm discs containing ≈40 μg riboflavin were cut from films to be used for release studies. The thickness of these films measured using digital micrometer was around 0.15 mm. It was noticed that films with a high PAA content were substantially more brittle than those of lower PAA content. The average weight of the films was 1.5 g and the average weight of each disc was ≈15 mg. Riboflavin-loaded discs were placed in 50 mL plastic vials and positioned in a water bath with a shaker stage. The temperature was set to 34 °C and the stage set to reciprocate at 100 times per minute. 5 mL of simulated tear fluid was added and 100 μL samples were taken at 10 min intervals for 1 h. Each aliquot was placed in a 1.8 mL auto sample vial and 900 μL of ultrapure water added to dilute it for analysis. Analysis was carried out using HPLC (Perkin Elmer) with a C18 reversed phase column. Each experiment was carried out in triplicate.

2.9. In Vivo Experiments

In vivo experiments on ocular administration of riboflavin films were conducted on chinchilla rabbits (2.5–3.0 kg). These experiments were approved by Semey State Medical University ethics committee and were conducted following the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. Prior to experiments, rabbits were housed in standard cages and allowed free access to food and water. During the experiments rabbits were placed in restraining boxes, where their eye and eye-lid movements were not restricted. Polymeric discs containing riboflavin (10 mm in diameter) were quickly soaked (10 s) in a saline solution (0.9% NaCl) and carefully placed on rabbit left eye's cornea; their right eye always served as a control. The behavior of each polymeric disc on the eye was monitored visually and images were taken at regular time intervals with a high resolution digital camera. Each type of polymeric film was tested on three rabbits and each experiment was conducted for 60 min.

2.10. Statistical Analysis

The results on slug mucosal irritation and drug release data were analysed by using one-way analysis of variance (ANOVA) using Minitab 16 software (version 16.1.1).

3. Results and Discussion

3.1. Structure and Colloidal Stability of Interpolymer Complexes

All our previous studies of complexation between PAA and MC in aqueous solutions were focused on determination of the stoichiometry of complexation,[12,13] probing the complexation strength and thermodynamics,[14] evaluation of pH and concentration effects on the formation, structural organization, and stability of IPCs,[12–14] and the fabrication of PAA–MC based multilayered materials.[14–16] We have established the possibility of forming PAA–MC complexes as nanoparticles when 0.2 wt% polymer solutions were mixed at 30:70 wt% ratio at pHs 1.4, 2.4, and 3.2,[14] however, did not evaluate the stability of these colloidal suspensions to aggregation. In this study, we applied dynamic light scattering (DLS) to look at the effects of polymer ratio and pH on the size, size distribution and colloidal stability of IPC nanoparticles. Figure 1a presents DLS data on the complexes formed by PAA and MC at different polymer ratios at pH 3.0. It can be clearly seen that IPCs formed by mixing polymers at 50:50 wt% ratio have the smallest size of approximately 100–120 nm and these nanoparticles have a relatively narrow size distribution (Figure 1b). This polymer ratio is consistent with the IPC stoichiometry established earlier.[14] When the composition of a polymer mixture deviates from the stoichiometric ratio the size of particles increases. For example, the size of nanoparticles formed by mixing 10 wt% of PAA with 90 wt% MC is nearly doubled in comparison with the dimensions of IPCs formed at 50:50 wt% polymers ratio. It can be expected that this increase is associated with

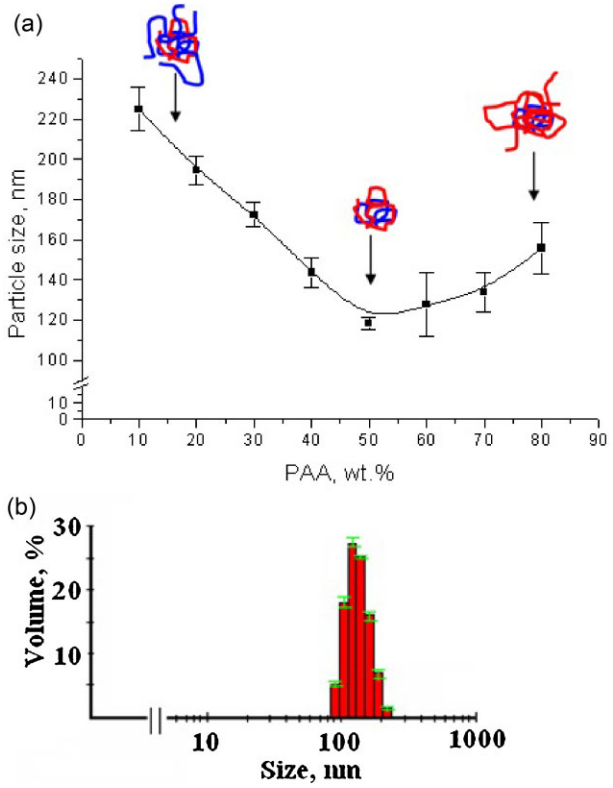

Figure 1. a) Size of IPCs formed by mixing 0.2 wt% solutions PAA and MC at different ratios at pH 3.0. b) Size distribution of PAA–MC (50:50 wt%) complexes.

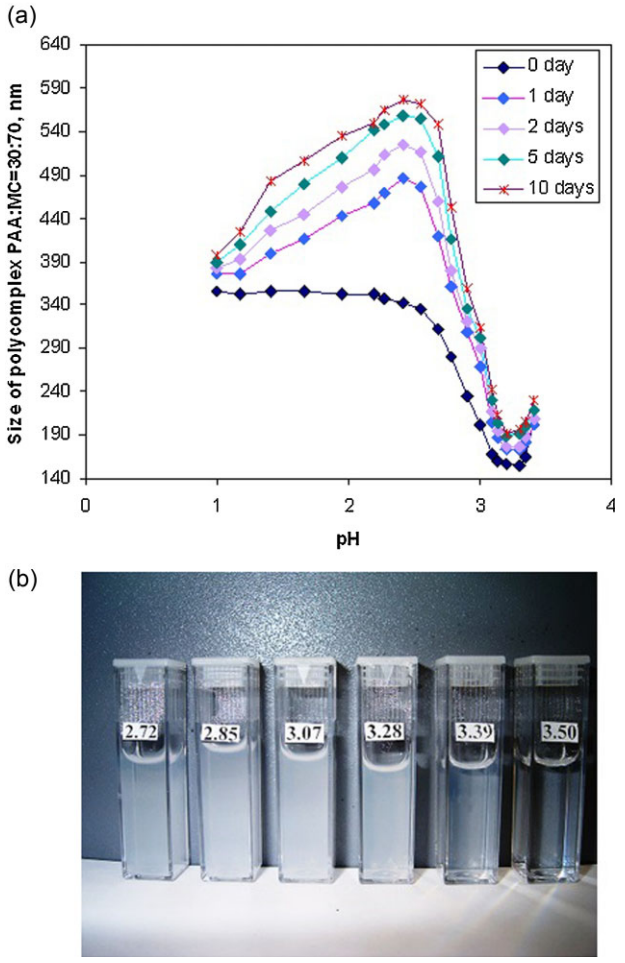

Figure 2. a) Effect of pH and time on the particle size for interpolymer complexes formed by mixing 0.2% solutions of PAA and MC (30:70 wt%). b) Physical appearance of IPC solutions at different pHs.

formation of larger and looser structures incorporating fragments of uncomplexed macromolecules present in the mixture in excess.

The growth in nanoparticles size is more pronounced in the presence of excessive quantities of MC, which is possibly related to its non-ionic nature having poorer ability to stabilize colloidal structures against aggregation. It should be noted that the colloidal stability of IPC nanoparticles improves when the polymers are mixed at ratios deviating from the stoichiometic one (50:50 wt%), therefore in further work on the complexation we have used 30:70 wt% PAA–MC ratios.

In our earlier study,[14] we applied transmission electron microscopy (TEM) and observed an interesting structural transformation of IPC nanoparticles when solution pH was changed from 1.4 to 3.2. IPCs formed at pH 1.4 and 2.4 had spherical and dense structure with a particle size ranging from 80 to 200 nm, whereas nanoparticles formed at pH 3.2 were much smaller (20–30 nm) and were apparently stabilized by a network of uncomplexed macromolecules. DLS study of pH effect on the size of IPCs performed in the current work confirms the presence of this interesting structural reorganization (Figure 2a), however, there is a discrepancy in the nanoparticle sizes determined by TEM and DLS. In the present study nanoparticles formed at pHs 3.2–3.6 have the smallest dimensions of approximately 160 nm. It possibly corresponds to a situation when nanoparticles are stabilized by a network of uncomplexed loops and tails, therefore they are less prone to aggregation and maintain small sizes and good colloidal stability. A further decrease in pH down to 2.5 results in a dramatic increase in particle size up to 340 nm, which is likely to be due to the involvement of uncomplexed macromolecular loops and tails in the complexation leading to a loss of surface hydrophilicity/steric stabilization and subsequent aggregation. Reduction in solution pH below 2.5 does not result in major changes in particle size. When pH is above 3.6 the size of nanoparticles increases again; this may be associated with a gradual disruption of hydrogen bonding leading to formation of looser and more swollen structures. However, unfortunately, the

applicability of our DLS instrument for characterization of changes with the complexes at higher pH was limited possibly due to less dense structures of the associates and their inability to scatter laser light efficiently.

Data on the effect of pH on the IPC particle sizes are consistent with visual observations (Figure 2b). At pHs 3.39 and 3.5 the IPC solutions show only minor cloudiness, whereas at lower pHs (3.28, 3.07, 2.85, and 2.72) they form notably cloudy systems.

The discrepancy between particle sizes determined by DLS in the present study and TEM data from our previously published work[14] is likely related to differences in sample hydration. Indeed, the sizes of IPCs determined by DLS are significantly larger as the nanoparticles are being measured in solutions in their fully hydrated and swollen form. When nanoparticles were studied by TEM their sizes tend to be much smaller because of the sample preparation technique involving drying (de-hydration and contraction of nanoparticles).

To get a further insight into the colloidal stability of IPCs formed at different pHs we continued our DLS experiments by leaving samples undisturbed at room temperature and periodically measuring their particle size. As it is seen from the results shown in Figure 2 IPCs show a tendency to increase their particle size with time, i.e. they gradually undergo aggregation. At pHs 3.2–3.6 aggregation is not very pronounced and nanoparticles increase their size from 160 to 190 nm within 10 d. This is possibly explained by steric stabilization of IPC by uncomplexed loops and tails. At lower pHs aggregation is more notable with its peak observed at pH 2.4–2.5: particle size increases from 340 nm on day 0 to 570 nm on day 10. Upon further decrease in pH from 2.5 to 1.0 the nanoparticles gradually become more stable to aggregation again. These observations are consistent with data reported by Usaitis et al.,[19] who studied the aggregation kinetics of complexes formed by poly(methacrylic acid) and polyvinylpyrrolidone. They also established that fastest and most intensive particle size growth is observed at pH 3.2, whereas a slower aggregation is taking place at pHs 3.4 and 3.6.

It is important to note that an intensive growth in the IPCs particle size observed at pH below 3.2–3.3 agrees well with our previously reported turbidimetric determination of pH_{crit}.[12,13]

The understanding of the complex formation between PAA and MC in aqueous solutions at different pHs is of significant interest for practical applications, especially for the development of novel dosage forms. Nanoparticles formed by PAA–MC complexes can be used to formulate drug delivery systems, e.g., as nano-containers for poorly soluble drugs because of presence of hydrophobic domains in the IPC structure. In this case, their colloidal stability in aqueous systems is very important. On the other hand the formation, structure and stability of IPC nanoparticles may affect the possibility for preparation of solid dosage forms for drug delivery. In the next sections, the importance of controlling the complexation by selecting the optimal pH conditions will be demonstrated for the preparation of polymeric films based on PAA–MC blends.

3.2. Optimization of Films Casting Conditions and Assessment of their Physical Properties

Formation of IPC between PAA and MC accompanied by their aggregation and precipitation can be considered as a limitation for preparing homogeneous polymeric films by casting from solution mixtures. Indeed, the films, prepared from solutions with strong aggregation and precipitation of complexes, are not uniform, not fully transparent and have poor mechanical properties. In order to optimize the properties of films we have studied the extent of complexation between the polymers as a function of solution pH and polymer concentrations using simple turbidimetric measurements (Figure 3). The mixtures of PAA and MC at $pH < 4.0$ display high values of solution turbidity indicating their poor suitability for casting films. At pH 4.0 and above the solution mixtures show good transparency and no signs of phase separation.

To examine the effect of solution pH on the properties of polymeric films we have prepared a series of samples cast at various pHs (2.5, 3.0, 3.4, 4.0, 4.4, and 8.3) and probed their surface morphology by scanning electron microscopy (Figure 4). The scanning electron microscopy (SEM) images of samples cast at pHs 2.5 and 3.0 indicate high degree of sample irregularity likely related to the formation of IPC aggregates as pH conditions for these samples favor intensive complex formation between the polymers. Indeed, the presence of IPC nanoparticles incorporated in the structure of the film can clearly be seen in the sample prepared at pH 3.0. Films prepared at pHs 3.4, 4.0, and 4.4 show completely uniform morphology and miscibility between PAA and MC, whereas the sample cast at 8.3 displays the presence of a phase separation. The irregular morphology of the latter sample is related to immiscibility between MC and PAA, whose carboxylic groups are fully ionized. The immiscible nature of this blend is likely to be due to the lack of intermacromolecular hydrogen bonding ensuring compatibility between the polymers.[20] Similar pH-induced complexation, miscibility, immiscibility transitions in the polymeric blends have been previously reported by us for PAA and poly(vinyl alcohol),[21] polyethylene oxide[22] and hydroxypropylcellulose.[23]

3.3. Mucosal Irritancy

Polymeric materials serving as vehicles for drug delivery via mucosal routes of administration should satisfy a number

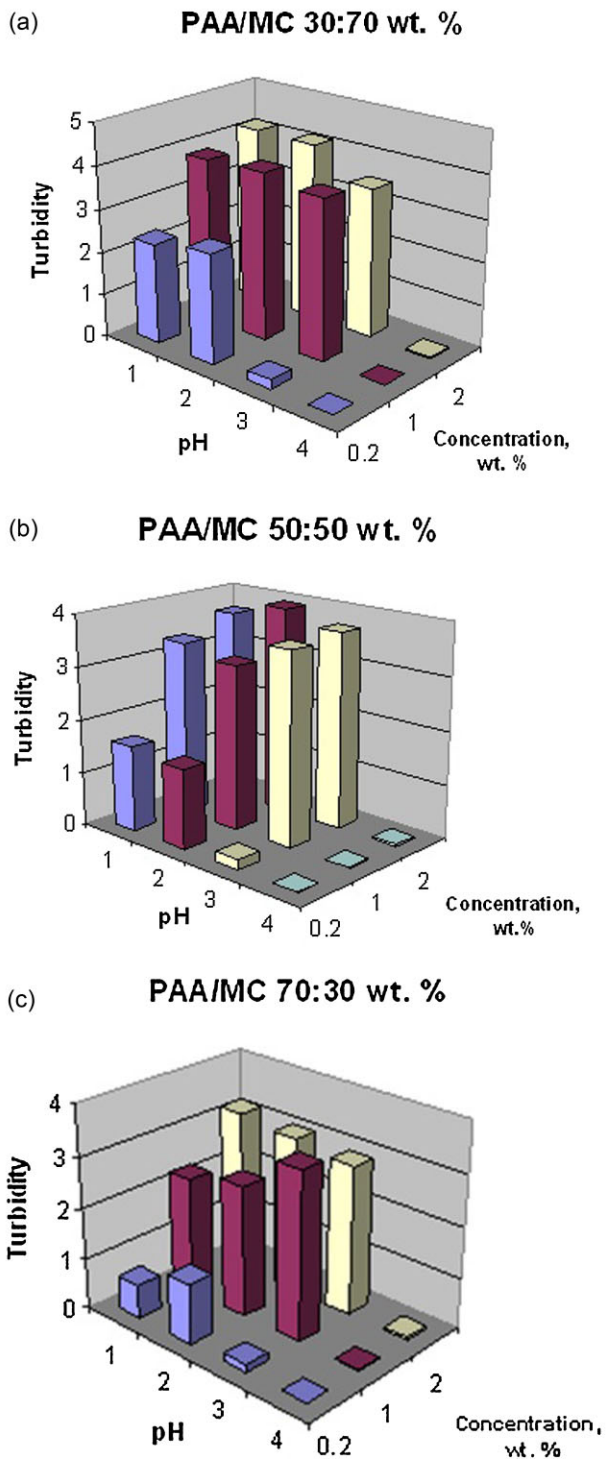

Figure 3. Turbidity of PAA–MC solution mixtures a) 30:70, b) 50:50, and c) 70:30 wt% at different polymer concentrations and pH.

of criteria and one of the most important requirements is their biocompatibility. Biocompatibility of polymeric excipients is of paramount importance for ocular drug delivery as the human eye is a very sensitive organ that can be easily irritated. Traditionally testing for ocular irritancy has been done using so-called Draize test, where an excipient is administered into the conjuctival sac of one eye of an albino rabbit and the other eye serves as a control.[24] However, prior to in vivo experiments on rabbits it would be useful to have a preliminary screening for the ocular irritancy using some alternative testing methodologies.

Alternatives to the Draize test are continuously being sought and one of the methodologies has been proposed by Adriaens and Remon.[25–29] Their test involves the use of invertebrate organisms such as terrestrial slugs. Slugs have a very sensitive mucosal surface, which releases mucus to aid locomotion and in response to irritation. Adriaens and Remon have demonstrated that amount of the mucus released is directly related to the toxicity of chemicals and can be used as a quantitative measure for mucosal irritancy of various excipients. SMIT was also validated in rabbits and compared against libraries of reference substances.

Previously we reported the use of SMIT for assessing mucosal irritancy of random copolymers based on 2-hydroxyethylmethacrylate and 2-hydroxyethylacrylate[18] and in the present work we have used the same methodology for evaluating the biocompatibility of PAA–MC films. Two species of slugs (*Limax flavus* and *Arion lusitanicus*) were used in our experiments to provide a comparative assessment. Figure 5 presents data on mucus production by slugs exposed to 1 h contact with films containing various quantities of MC. 2% solution of benzalkonium chloride was used as a positive control as it causes a severe irritation to slugs with the production of yellow mucus reaching $33 \pm 12\%$ and $40 \pm 13\%$ for *Limax flavus* and *Arion lusitanicus*, respectively (see Figure 1S in Supporting Information for the images of slugs exposed to various materials). Slugs exposed to filter paper surfaces soaked with phosphate buffer saline (used as a negative control) did show low levels of mucus production: $3 \pm 2\%$ and $4 \pm 2\%$ for *Limax flavus* and *Arion lusitanicus*, respectively.

Films consisting of pure PAA displayed significantly higher irritancy ($p < 0.001$) compared to negative controls, with the levels of mucus production reaching $15 \pm 7\%$ and $8 \pm 2\%$ for *Limax flavus* and *Arion lusitanicus*, respectively. This result is consistent with the literature data[8] and our previous observations,[18] confirming that PAA and its derivatives have some tendency to cause irritation to mucosal tissues. However, the mucosal irritation caused by exposure of slugs to 100% PAA films is still significantly lower than the effect of positive controls used ($p < 0.05$ and $p < 0.001$ for *Limax flavus* and *Arion lusitanicus*, respectively).

Films containing increasing quantities of MC showed a reduction in their irritation potential as no significant differences were observed between PAA–MC blend films

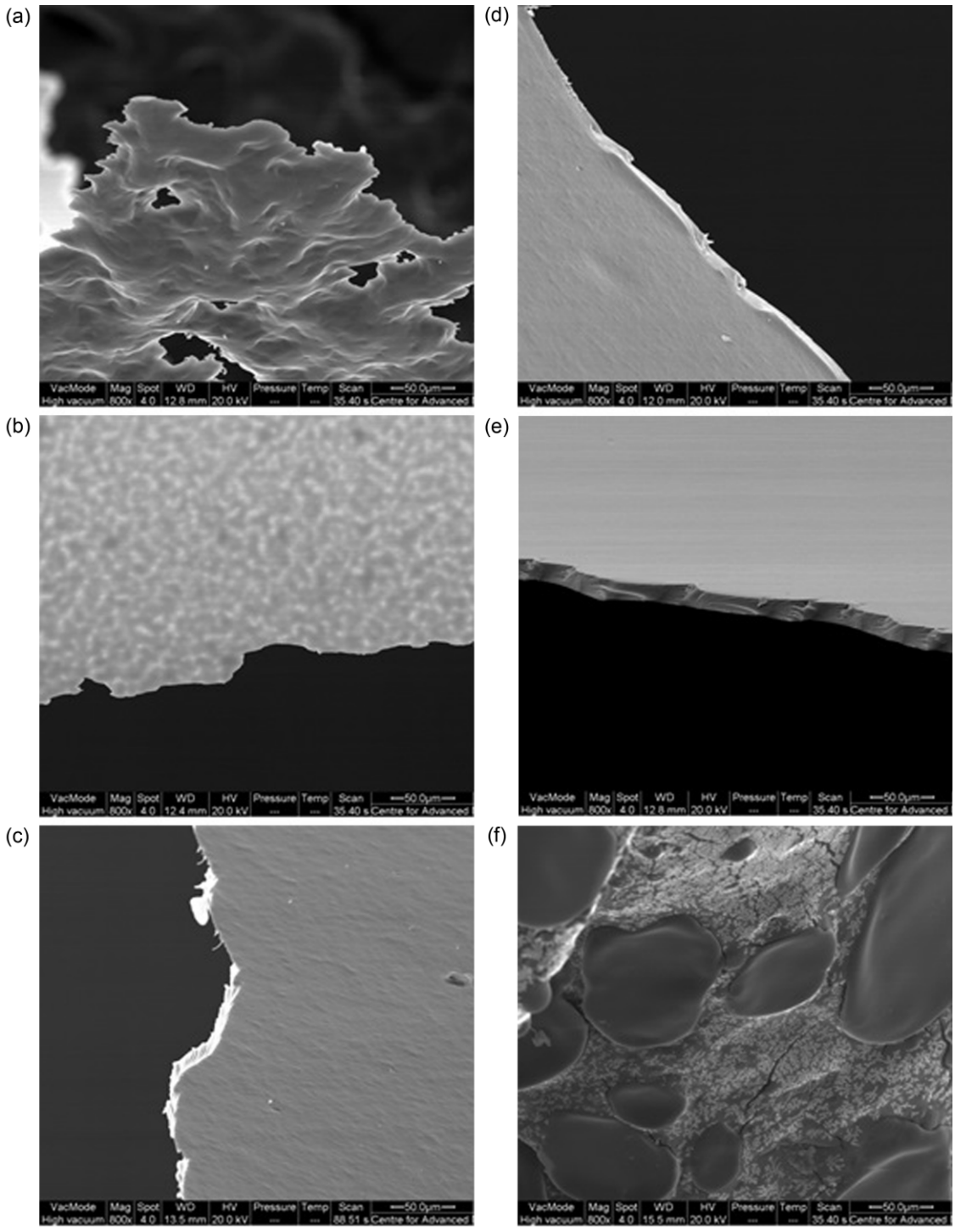

Figure 4. Morphology of PAA – MC complexes and blends (50:50 wt%) at different pH: a) 2.5, b) 3.0, c) 3.4, d) 4.0, e) 4.4, and f) 8.3.

and the negative control, when *Arion lusitanicus* slugs were used ($p > 0.1$). In the case of *Limax flavus*, the mucus production by slugs in response to contact with PAA–MC films was significantly higher than the negative control ($p < 0.05$), but still lower compared to 100% PAA ($p < 0.05$).

Lenoir et al.,[29] reported that mucus production (MP) of 3% observed in *Arion lusitanicus* species should correspond to the compounds that do not cause any ocular discomfort and MP > 15% should be associated with the chemicals causing severe stinging, itching and burning sensation.

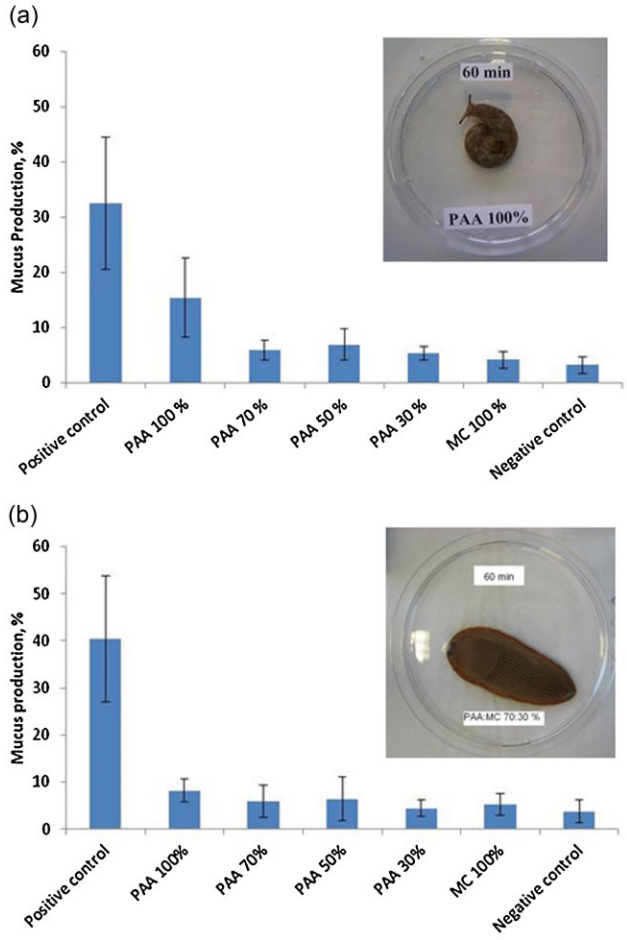

Figure 5. Biocompatibility of PAA–MC films assessed using slug mucosa irritation test: mucus production by a) *Limax flavus* and b) *Arion lusitanicus* in response to 60 min exposure to PAA–MC films as well as positive and negative controls

Despite the difference in the experimental protocols used by Lenoir et al.[29] and in our study, these data are broadly comparable with our results. It allows us to conclude that mucosal irritancy of PAA films can be reduced significantly upon its blending with MC.

3.4. In Vitro Release of Riboflavin from PAA–MC Films

Riboflavin is a drug that is widely used for treatment of keratoconus, a degenerative condition of the cornea in the eye, leading to serious vision distortion.[30] Wollensak et al.[31] established a novel treatment method for keratoconus with the use of riboflavin as a photosensitive compound for UV-mediated collagen cross-linking in the diseased cornea. This procedure involves physical removal of the epithelium to allow riboflavin to saturate the corneal stroma. Once cornea of a patient is saturated with riboflavin the eye is irradiated with UV-light to cause cross-linking of stromal collagen. Although the application of this method leads to successful restoration of the shape and mechanical integrity of the cornea, physical removal of corneal epithelium is a highly unpleasant procedure and a patient would be uncomfortable during recovery. Therefore, formulation approaches that could potentially lead to development of efficient riboflavin delivery to the cornea without recourse to physical removal of corneal epithelium are of significant interest.[32,33]

Two forms of this drug could potentially be used for corneal cross-linking procedure: riboflavin-5-phosphate and riboflavin base. 0.1% riboflavin 5-phosphate is currently used in clinical applications in aqueous mixtures with dextran. This compound is hydrophilic and has a better affinity to the corneal stroma compared to its hydrophobic counterpart.[33] However, the transepithelial permeability of riboflavin base is expected to be greater as the corneal epithelium is a lipophilic tissue.

In the present study, we have used more hydrophobic riboflavin derivative to formulate ocular PAA–MC films (see Figure 2S in Supporting Information for exemplary image of these films). In vitro experiments were performed to evaluate the films dissolution and riboflavin release into simulated tear fluid (Figure 6). The quickest release of riboflavin was observed from pure PAA-based films reaching $94 \pm 9\%$ drug liberation in 40 min and resulting in a complete dissolution of polymeric material. This is expected as PAA is a highly hydrophilic polymer that typically undergoes a relatively quick dissolution.[23] Films based on pure MC and PAA–MC (30:70 wt%) gave the slowest dissolution with approximately $69 \pm 6\%$ of riboflavin released in 40 min (no significant difference in the release profiles was observed between pure MC and PAA–MC (30:70 wt%), $p > 0.5$). MC as a semi-rigid polymer takes longer to dissolve, which substantially slows down the release of the riboflavin. Films containing 30 wt% MC showed a significantly slower release of riboflavin compared to pure PAA ($p < 0.05$), but no significant difference with pure MC ($p > 0.5$). Films with 50 wt% of MC gave a significantly faster release compared to pure MC ($p < 0.05$), but insignificant difference from PAA ($p > 0.05$).

3.5. In Vivo Retention of the Films

In vivo experiments on ocular administration of riboflavin-loaded PAA–MC films were performed on rabbits. In each experiment, the films were quickly soaked in 0.9% NaCl solution (10 s) to make them more flexible and adhesive, and then were administered directly on rabbits' cornea. A distinct yellow colour of riboflavin was very helpful to monitor the behavior of the films visually and using digital photography (Figure 4S in Supporting Information for exemplary image). During these experiments it was

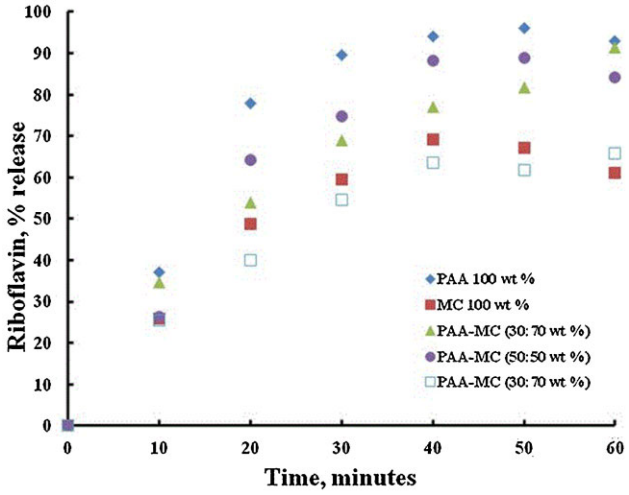

Figure 6. In Vitro release of riboflavin from PAAMC films. Error bars are not shown on the figure to avoid overcrowding. Individual release curves with error bars can be found in Supporting Information (Figure 3S).

concluded that the three main parameters are important for making polymeric films efficient for ocular delivery of riboflavin: 1) film flexibility that helps easier fitting to provide intimate contact with the cornea; 2) a balanced film mucoadhesivity (completely non-adhesive films are difficult to fit as they do not attach to the cornea, whereas excessively adhesive materials cause problems because of their sticking to eyelids); 3) film dissolution time in tear fluid as it affects the drug retention characteristics.

Table 1 summarizes the results of in vivo experiments with different PAA–MC formulations. It should be noted that because of the experimental technique used these data provide only a rough estimate of the retention time for the films on the corneal surface. Films composed of pure MC were found to retain for up to 50 min on the corneal surface. The extended residence of MC-based films on the cornea is believed to be related to lower chain flexibility and lower hydrophilicity of MC compared to PAA, which provides the cellulose ether better retention ability due to its longer dissolution. Indeed, the films remained on the corneal surface in the form of hydrated gel that gradually dissolved. Additionally, the excellent biocompatibility of MC ensured that there was no excessive tear production observed during these experiments. However, the disadvantage of pure MC-films was related to the difficulties of initial administration/attachment to the cornea related to their poor mucoadhesive characteristics. Pure PAA-based films, on the contrary, demonstrated a very poor retention on the cornea (≈10 min), which is related to their high hydrophilicity and some irritation properties that caused excessive tear production and more efficient film removal from the cornea. Although excellent mucoadhesive properties of PAA were noticed during these experiments upon initial administration, they were found to be detrimental for this application because of unwanted sticking of the films to eyelid mucosa. Films consisting of a combination of PAA and MC exhibited a retention capability, which ranged within 30–60 min. These films were also found to have good balance of mucoadhesive characteristics to ensure easier fitting/attachment to corneal surface and relatively good retention.

Table 1. In vivo experiments on retention of PAA–MC films on rabbits' cornea.

Polymeric film: PAA/MC [wt%]	Retention timeon the cornea[min]
0/100	≈50
30/70	≈30
50/50	≈60
70/30	≈30
100/0	≈10

All rabbit eyes were visually examined following the use of these films and no signs for adverse irritation were observed (no redness of conjunctiva; details of iris were clearly visible). Higher tear production was observed in the case of pure PAA, indicating some minor irritation potential, which is consistent with the evaluation of this material biocompatibility using SMIT.

The application of riboflavin-loaded ocular films can be considered as a new strategy to deliver this drug to the cornea for subsequent UV-mediated corneal cross-linking. Compared to conventional riboflavin eye drops the use of ocular films will provide improved pre-corneal retention. Once the film is completely dissolved and riboflavin saturated the cornea the UV-irradiation can be commenced to induce cross-linking. It should be noted that the current levels of riboflavin delivered to the eye using our films are relatively low (maximum content of the drug in the film was around 40 μg) and not fully comparable with 0.1% solution of riboflavin 5-phosphate typically used in clinical settings. Further experiments will be required to formulate riboflavin films with higher drug content.

4. Conclusion

This study demonstrates the effect of pH on hydrogen bonding between poly(acrylic acid) and methylcellulose in aqueous solutions. Mixing dilute aqueous solutions of these polymers at pHs < 3.4 results in formation of IPC as nanoparticles. These nanoparticles may undergo further aggregation resulting in the formation of larger species. Polymeric films prepared by casting solutions mixed at

these pHs have poor transparency and are not fully uniform. When poly(acrylic acid) and methylcellulose are mixed and cast at $3.4 < pH < 4.5$ the films are uniform, transparent, and show a complete miscibility between the polymers. Hydrogen bonding between the polymers is still present under these pH conditions, but it is not sufficient to form IPC. When hydrogen bonding is completely prevented, for example, at pH 8.3, the films are inhomogeneous again because of immiscibility between the polymers.

Films loaded with riboflavin were prepared by casting polymer solutions mixed with the drug at pH 4.5. The release of riboflavin from these films was studied in vitro into artificial tear fluid. It was established that films enriched with methylcellulose show a significantly slower release of riboflavin compared to samples containing high quantities of poly(acrylic acid).

In vivo retention of riboflavin-loaded films was studied in rabbits. Pure methylcellulose films exhibited retention up to 50 min but were not sufficiently adhesive for their successful application. Pure poly(acrylic acid) films showed excessive adhesiveness and poor retention (up to 10 min). Films composed of both poly(acrylic acid) and methylcellulose exhibited relatively good adhesiveness and retention for 30–60 min. In principle this is a good improvement compared to simple riboflavin eye drops; however, according to our recent diffusion studies,[31] riboflavin takes longer time to penetrate into the cornea. A lag time of at least 90 min was observed for riboflavin penetrating freshly excised bovine cornea. Further optimization of the properties of these films will still be required to improve their characteristics and to achieve at least 90 min retention. This improvement can potentially be achieved via a partial cross-linking of these materials. Additionally more complex film formulations could be designed to incorporate permeability enhancers or nanoemulsions, whose function will be to facilitate transepithelial permeation of riboflavin.[32,33]

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[1] a) Z. S. Nurkeeva, G. A. Mun, V. V. Khutoryanskiy, *Macromol. Biosci.* **2003**, *3*, 283; b) V. V. Khutoryanskiy, *Int. J. Pharm.* **2007**, *334*, 15; c) V. V. Khutoryanskiy, G. Staikos, "*Hydrogen-bonded Interpolymer complexes: Formation, Structure and Applications*", World Scientific, New Jersey **2009**.
[2] G. G. Bumbu, B. S. Munteanu, J. Eckelt, C. Vasile, *eXPRESS Polym. Lett.* **2009**, *3*, 286.
[3] a) Ş. Anlar, Y. úapan, O. Güven, A. Göğüş, T. Dalkara, A. Hincal, *Pharm. Res.* **1994**, *11*, 231; b) K. Satoh, K. Takayama, Y. Machida, Y. Suzuki, M. Nakagaki, T. Nagai, *Chem. Pharm. Bull.* **1989**, *37*, 1366.
[4] H. Park, J. R. Robinson, *Pharm. Res.* **1987**, *4*, 457.
[5] M. K. Marschütz, A. Bernkop-Schnürch, *Eur. J. Pharm. Sci.* **2002**, *15*, 387.
[6] B. S. Lele, A. S. Hoffman, *J. Controlled Release* **2000**, *69*, 237.
[7] M. M. Patel, J. D. Smart, T. G. Nevell, R. J. Ewen, P. J. Eaton, J. Tsibouklis, *Biomacromolecules* **2003**, *4*, 1184.
[8] S. Geresh, G. Y. Gdalevsky, I. Gilboa, J. Voorspoels, J. P. Remon, J. Kost, *J. Controlled Release* **2004**, *94*, 391.
[9] M. E. Aulton, "*Pharmaceutics: The Science of Dosage Form Design*", 2nd edition, Churchill Livingstone, Edinburgh **2002**.
[10] A. T. Florence, D. Attwood, "*Physicochemical Principles of Pharmacy*", 3rd editon, Palgrave, Basingstoke **1998**.
[11] V. V. Khutoryanskiy, *Macromol. Biosci.* **2011**, *11*, 748.
[12] Z. S. Nurkeeva, G. A. Mun, V. V. Khutoryanskiy, R. A. Mangazbaeva, *Polym. Int.* **2000**, *49*, 867.
[13] G. A. Mun, Z. S. Nurkeeva, V. V. Khutoryanskiy, R. A. Mangazbaeva, *Polym. Sci. Ser. B* **2001**, *43*, 73.
[14] O. V. Khutoryanskaya, A. C. Williams, V. V. Khutoryanskiy, *Macromolecules* **2007**, *40*, 7707.
[15] S. C. Bizley, A. C. Williams, F. Kemp, V. V. Khutoryanskiy, *Soft Matter* **2012**, *8*, 6782.
[16] O. V. Khutoryanskaya, M. Potgieter, V. V. Khutoryanskiy, *Soft Matter* **2010**, *6*, 551.
[17] O. Nikolaeva, T. Budtova, Yu. Brestkin, Z. Zoolshoev, S. Frenkel, *J. Appl. Polym. Sci.* **1999**, *72*, 1523.
[18] O. V. Khutoryanskaya, Z. A. Mayeva, G. A. Mun, V. V. Khutoryanskiy, *Biomacromolecules* **2008**, *9*, 3353.
[19] A. Usaitis, L. S. Maunu, H. Tenhu, *Eur. Polym. J.* **1997**, *33*, 219.
[20] M. Jiang, M. Li, M. Xiang, H. Zhou, *Adv. Polym. Sci.* **1999**, *146*, 121.
[21] Z. S. Nurkeeva, G. A. Mun, A. V. Dubolazov, V. V. Khutoryanskiy, *Macromol. Biosci.* **2005**, *5*, 424.
[22] V. V. Khutoryanskiy, A. V. Dubolazov, Z. S. Nurkeeva, G. A. Mun, *Langmuir* **2004**, *20*, 3785.
[23] A. V. Dubolazov, Z. S. Nurkeeva, G. A. Mun, V. V. Khutoryanskiy, *Biomacromolecules* **2006**, *7*, 1637.
[24] J. H. Draize, G. Woodard, H. O. Calvery, *J. Pharmacol. Exp. Ther.* **1944**, *82*, 377.
[25] E. Adriaens, J. P. Remon, *Pharm. Res.* **1999**, *16*, 1240.
[26] E. Adriaens, J. P. Remon, *Tox. Appl. Pharmacol.* **2002**, *182*, 169.
[27] M. M. M. Dhondt, E. Adriaens, J. V. Roey, J. P. Remon, *Eur. J. Pharm. Biopharm.* **2005**, *60*, 419.
[28] M. M. M. Dhondt, E. Adriaens, J. Pinceel, K. Jordaens, T. Backeljau, J. P. Remon, *Toxicol. In Vitro* **2006**, *20*, 448.
[29] J. Lenoir, I. Claerhout, P. Kestelyn, A. Klomp, J.-P. Remon, E. Adriaens, *Toxicol. In Vitro* **2011**, *25*, 1919.
[30] N. Lee, *Int. Ophthalmol. Clin.* **1997**, *37*, 51.
[31] G. Wollensak, E. Spoerl, T. Seiler, *Am. J. Ophthalmol.* **2003**, *135*, 620.
[32] P. W. J. Morrison, C. J. Connon, V. V. Khutoryanskiy, *Mol. Pharm.* **2013**, *10*, 756.
[33] K. M. Bottos, A. G. Oliveira, P. A. Bersanetti, R. F. Nogueira, A. A. S. Lima-Filho, J. A. Cardillo, P. Schor, W. Chamon, *PLoS One* **2013**, *8*, e66408.