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Research Article

Development of an *Ex Vivo* Method for Evaluation of Precorneal Residence of Topical Ophthalmic Formulations

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Abstract. This paper describes the development of an *ex vivo* perfusion method for the evaluation of topical ophthalmic formulations. The perfusion system developed consisted of a perfusion chamber, two precision pumps to control the in/out flow rates to simulate the tear flow rate, and a fluorescence microscope imager. Freshly excised rat cornea was used as a biomembrane. Fluorescein (FITC) was used as a marker. Residence time was determined by measuring fluorescence intensity over time after application of the formulation to the cornea. In addition, viscoelastic properties of the formulations were measured and correlated to the retention times. The perfusion method easily differentiated formulations based on the retention time on the cornea: For example, a 0.3% hydroxypropyl methylcellulose formulation had a short retention time of <10 min. Addition of 0.25% carboxymethylcellulose increased the retention time from less than 10 min to over 16 min, and addition of 0.1% Carbopol further increased retention time to over 40 min. For alginate formulations, the retention time was significantly longer in the presence of 0.06% calcium chloride than that of 0.006% calcium chloride. The longer residence time at a higher Ca++ concentration can be attributed to the greater elastic modulus associated with the gel. Interestingly, however, a hyaluronate formulation displayed a very long retention time but has low viscoelastic moduli. This suggests that the mucoadhesive properties may not always be discernable by the rheological properties. The *ex vivo* perfusion method may in fact provide more meaningful information with regard to retention times of formulations.

KEY WORDS: *ex vivo*; ophthalmic formulation; perfusion; residence; retention.

INTRODUCTION

Though easily accessible, the eye is actually a difficult organ as a drug delivery site. The difficulty arises from the effective protective mechanisms the eye poses. The protective mechanisms include lacrimal secretion/drainage and blinking reflex, both of which cause rapid loss of drug after topical administration. These self-protective actions of the eye reduce the precorneal drug concentration by more than tenfold within the first 4–20 min after instillation (1). The short precorneal residence time invariably results in low bioavailability. Frequent dosing is usually required to compensate for the decreased precorneal drug concentration. Improving precorneal residence time thus should be one of the most important attributes to be considered when designing topical ophthalmic formulations.

Several approaches have been proposed for ophthalmic use to prolong the contact time of the dosage form on the ocular surface and to slow down drug elimination (2,3). Successful results have been obtained with inserts (3,4), collagen shields (5), temperature sensitive *in situ*-forming gel (6–8), liposome (9–11), and microsphere and nanosphere (12,13). However, taking into consideration of patients' compliance, manufacturing cost, dosage adherence, and blurring effect these dosage forms may have, a simple aqueous solution with moderate viscosity is still preferred. Since the surface of the eye ball is covered by a layer of mucus, formulations containing polymers which can interact with mucin via mucoadhesion would have increased residence time. Indeed, several mucoadhesive polymers such as hydroxypropyl methylcellulose (HPMC), carboxymethylcellulose (CMC), polyethylene oxide, carrageenan (14), Carbopol (15), Gerlite (16), xanthan gum (17), alginate, and hyaluronic acid (18) have been introduced into ocular delivery systems.

On the human ocular surface, there exist several mucin types: MUC1, MUC4, and one large gel-forming mucin, MUC5AC. MUC5AC plays an important role in the formation of the gelled mucus layer of the tear film (19). However, because ocular mucin is not commercially available, porcine gastric mucin has often been employed (16,20). Although mucin secreted by the porcine gastric cells has different glycosylation pattern, the molecular conformation of mucin in the eye can be simulated by dispersing the porcine gastric type 2 mucin in saline. A review published in 2001 (21) supports the use of gastric mucin when investigating the interaction between mucin and ocular drug delivery systems.

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Table I. Formulation Composition

Formulation	HPMC K4M (*w*/*w*)	Na-CMC (*w*/*w*)	Carbopol 974P (*w*/*w*)	SA (*w*/*w*)	$CaCl_2$ (*w*/*w*)
1	0.3%	–	–	–	–
2	–	0.25%	–	–	–
3	–	–	0.1%	–	–
4	0.3%	0.25%	–	–	–
5	0.3%	–	0.1%	–	–
6				0.5%	0.006%
7				0.5%	0.06%

Determination of bioadhesive properties is of great interest because it can guide the selection of polymer and polymer concentration during formulation development. Limited number of *in vitro* methods have been used to study the bioadhesive properties of polymers. One technique for studying bioadhesion is tensiometric measurement (22). It measures the force necessary to separate the two surfaces after the bioadhesive bond has been formed. The method has been used to determine the bioadhesive strength of polymers on solid or semisolid substrate (23). It also is useful in classification of polymers for adhesion. However, it is not suitable for aqueous solution with low concentrations of polymer. Another *ex vivo* method tracks the movement of black carbon mixed in the polymeric solution and placed on animal tissues such as bovine tracheal explants using stereomicroscope (24). This method is suitable for tissues of ciliated epithelium.

The purpose of the present study was to develop an *ex vivo* perfusion model suitable for evaluation of residence times of topical ophthalmic solution formulations. To simulate *in vivo* conditions, we used the freshly excised rat cornea as the biomembrane, onto which freshly prepared mucin solution was applied prior to applying formulation. Fluorescein (FITC), spiked in the formulation, was used as a marker. The perfusion system consisted of a perfusion chamber, two precision pumps to control the in/out flow rates to simulate the tear flow rate, and a fluorescence microscope imager. The perfusion method easily differentiated formulations based on the residence time on the cornea.

An additional objective was to determine the viscoelastic properties of the formulations and to examine the relationship between viscoelastic properties and residence time obtained from the *ex vivo* perfusion model.

It has been reported that viscoelastic properties, the storage modulus, G', and loss modulus, G'', can affect the contact time of ophthalmic formulations (25) and are used to assess mucoadhesion characteristics. The storage (elastic) modulus G' is a measure of the energy stored and recovered per cycle of deformation and reflects the solid-like component of a viscoelastic material, while the loss (viscous) modulus is a measure of the energy lost per cycle and reflects the liquid-like component (14). The rheological analysis can indicate the rigidity and elasticity of the polymer network and its interaction with mucin mixture. This technique is based on a principle theory that if there is an adhesion component within a mixture system, then the viscoelastic properties of the mixture (especially the storage modulus) should be higher than any of the component under the same testing condition (26). Higher storage modulus generally indicates greater adhesion in the system. Thus, by examining the relationship between the storage modulus and residence time, we could indirectly validate the perfusion model.

MATERIALS AND METHODS

Materials

Carbopol 974P was purchased from Noveon Inc. (Cleveland, OH, USA). Hydroxypropyl methylcellulose (HPMC E4M, Dow Chemical Company, Midland, MI, USA) and sodium carboxymethylcellulose (Aqualon 7H3SXF PH, Hercules, Wilmington, DE, USA) were also purchased. Sodium alginate (SA) and sodium hyaluronate were supplied by Acros Organics (Morris Plains, NJ, USA). Porcine gastric mucin type 2 and fluorescein (FITC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium phosphate

Fig. 1. *Ex vivo* perfusion system

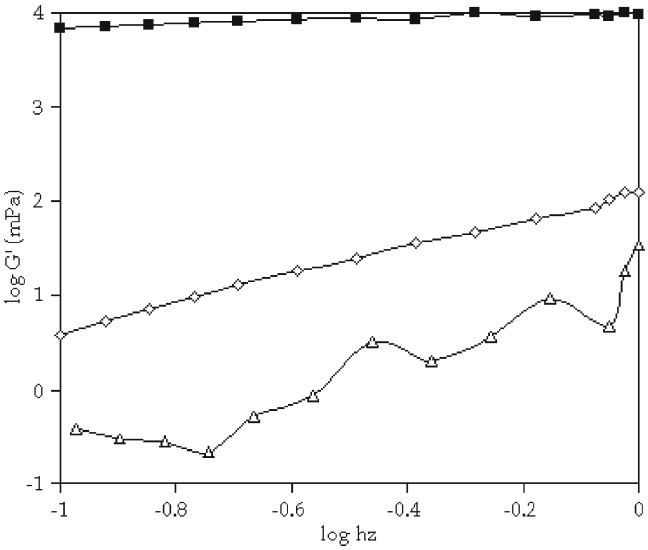

Fig. 2. Frequency sweeps of polymers at 25°C: HPMC 0.3%/CMC 0.25% (*diamond*), HPMC 0.3%/Carbopol 0.1% (*square*), and HPMC 0.3% (*triangle*; constant stress of 0.1 pa)

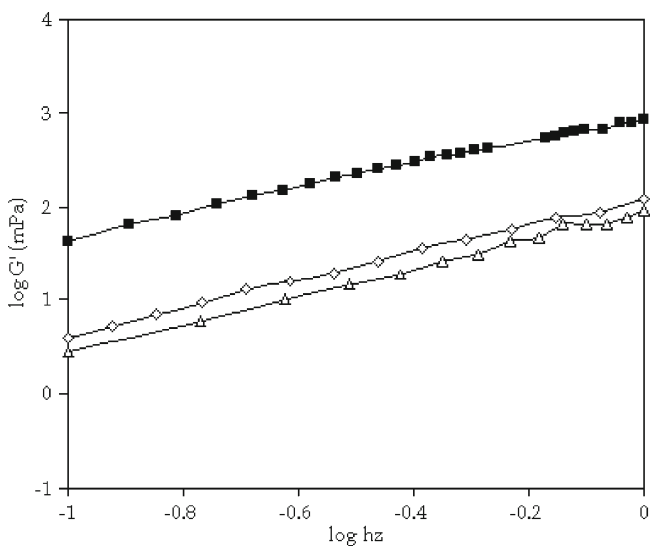

Fig. 4. Frequency sweeps of mucin and HPMC/CMC at 25°C: HPMC 0.3%/CMC 0.25% (*diamond*), HPMC 0.3%/CMC 0.25%/mucin 4% (*square*), and mucin 4% (*triangle*; constant stress of 0.1 pa)

monobasic (NaH_2PO_4), calcium chloride ($CaCl_2$), and sodium chloride (NaCl) and mannitol were all purchased of analytical reagent, or equivalent quality.

Sample Preparation

The composition of each formulation in this study is listed in Table I. Polymer was added under stirring. For HPMC containing formulations, distilled water was heated up to 90–94°C before addition of HPMC. Other components were added in after the solution was cooled. Mannitol was used to adjust osmolarity for Carbopol containing formulations. For other polymeric formulations, NaCl and NaH_2PO_4 were used. The final solutions were brought to their final volume after the osmolarity and pH adjustments. The pH and osmolarity of all formations were around 7.4 and 280 mOsm, respectively.

Polymer Mucin Mixture

Mucin type 2 was gradually added into distilled water while stirring. After mucin was totally dispersed, the mucin dispersion was added gradually into the polymer solutions shown in Table I. The final concentration of mucin in all mixture was 4% *w*/*w*. The pH of the mixtures was adjusted to about 7.4.

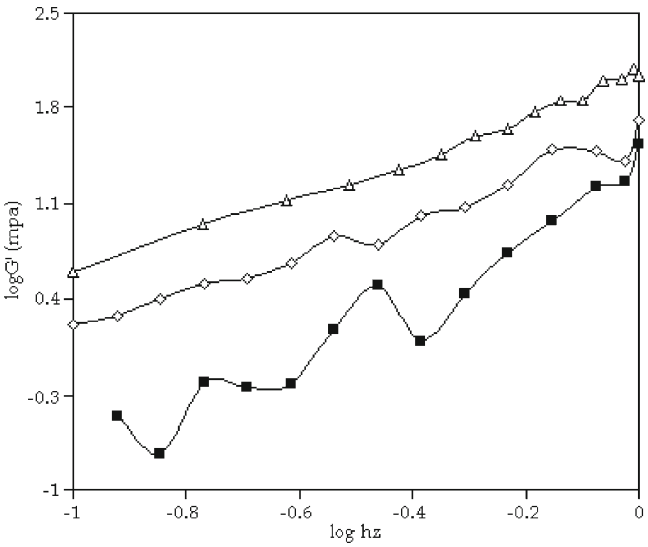

Fig. 3. Frequency sweeps of mucin and HPMC at 25°C: HPMC 0.3%/mucin 4% (*diamond*), HPMC 0.3% (*square*), and mucin 4% (*triangle*; constant stress of 0.1 pa)

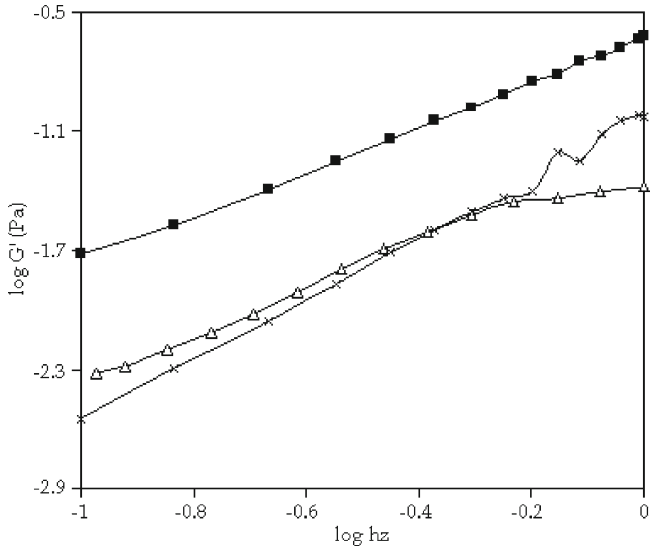

Fig. 5. Frequency sweeps of mucin and HPMC/Carbopol at 25°C: HPMC 0.3%/Carbopol 0.1%/mucin 4% (*square*), mucin 4% (*triangle*), and HPMC 0.3%/Carbopol 0.1% control (the HPMC/Carbopol control solution has the same osmolarity as that of the mixture of HPMC, mucin, and Carbopol; *multiplication symbol*; constant stress of 0.1 pa)

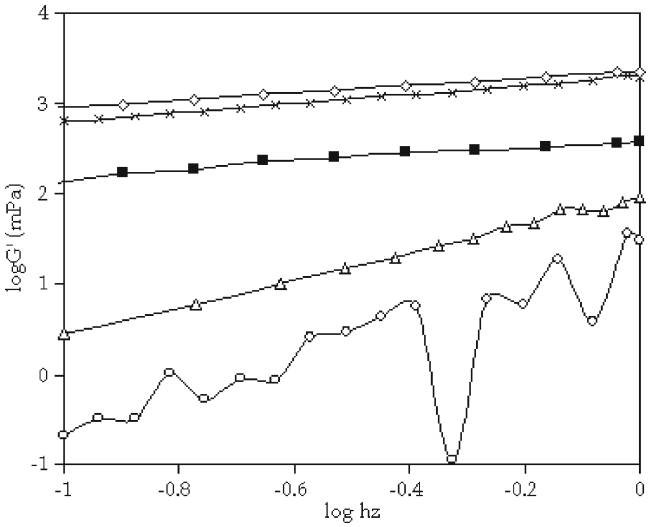

Fig. 6. Frequency sweeps of mucin and alginate–Na 0.5% at 25°C: alginate–Na 0.5%/Ca^{2+} 0.06%/mucin 4% (*diamond*), alginate–Na 0.5%/Ca^{2+} 0.006%/mucin 4% (*square*), mucin 4% (*triangle*), alginate–Na 0.5%/Ca^{2+} 0.06% (*multiplication symbol*), and alginate–Na 0.5%/Ca^{2+} 0.006% (*circle*; constant stress of 0.1 pa)

Rheological Measurement

Rheological measurements were carried out using a Bohlin rheometer C-10 (Malvern instrument Ltd, Westborough, MA, USA) with a Double Gap (DG 40/50) measuring system. For all samples, strain sweep measurements were made to determine the linear viscoelastic region of each sample. The frequency sweep followed was performed within the linear region below the maximum strain. Frequency sweep measurements were performed over a frequency range from 0.01 to 1 Hz. These frequency sweeps were used to determine the storage (elastic) modulus (G') and the loss (viscous) modulus (G'') of the samples. All samples, both polymer solutions and polymer–mucin mixtures, were measured in triplicates at 25°C and 35°C.

Perfusion Study

The perfusion system (Fig. 1) consisted of a perfusion chamber (CoverWell perfusion chambers, diameter 2.5× 20 mm, Sigma-Aldrich, St. Louis, MO, USA), two precision pumps (Bioscience Tools, San Diego, CA, USA) to control in/out flow rates, and a fluorescence microscope (Imager M1, Zeiss, Thornwood, NY, USA). The perfusion medium was normal saline.

A freshly excised rat cornea was used as a biomembrane. Five (5) microliters of 4% mucin was added onto the surface

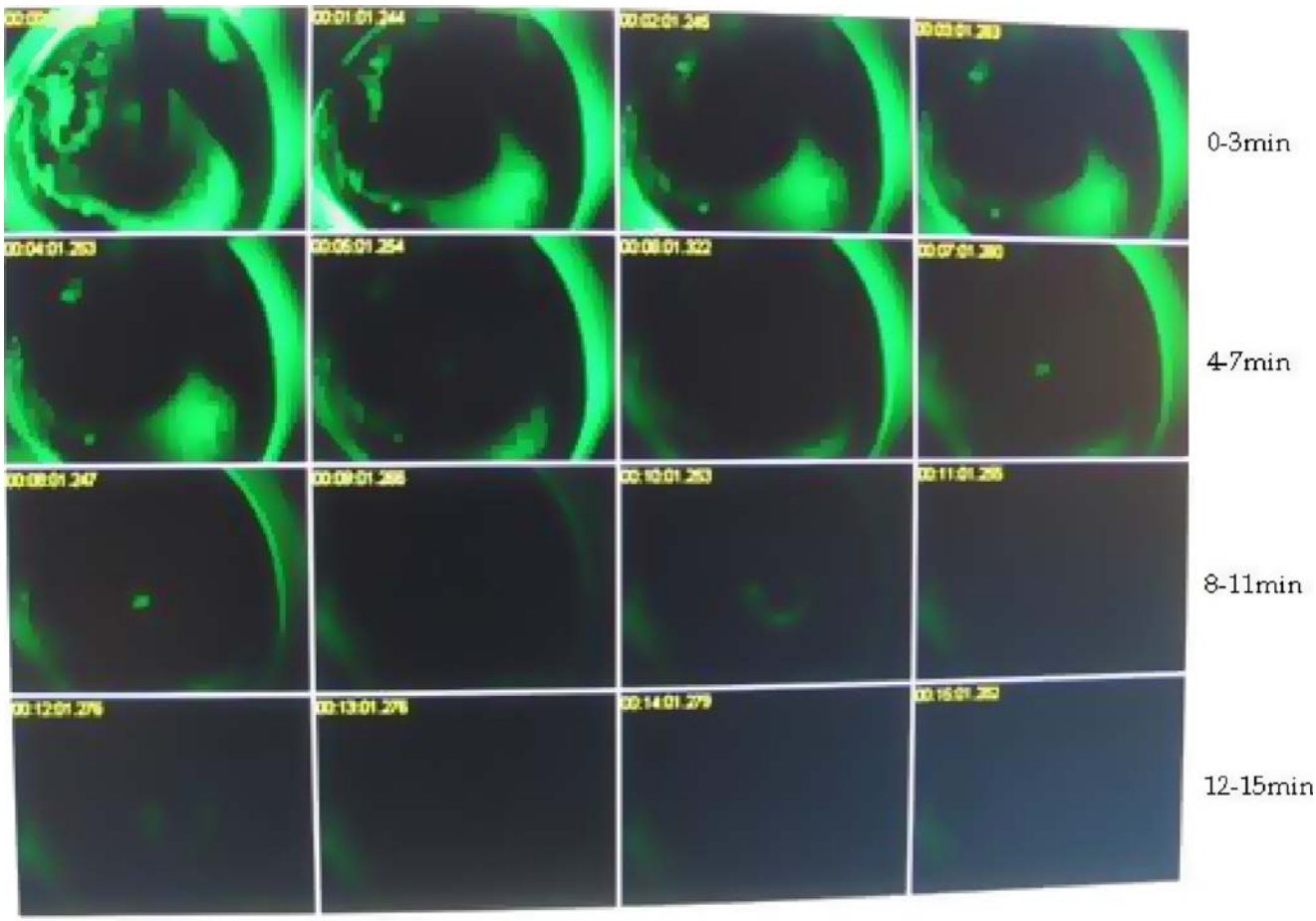

Fig. 7. Imaging of HPMC 0.3% *w/w* retention as a function of time (slides are ordered from *left* to *right* in 1 min intervals)

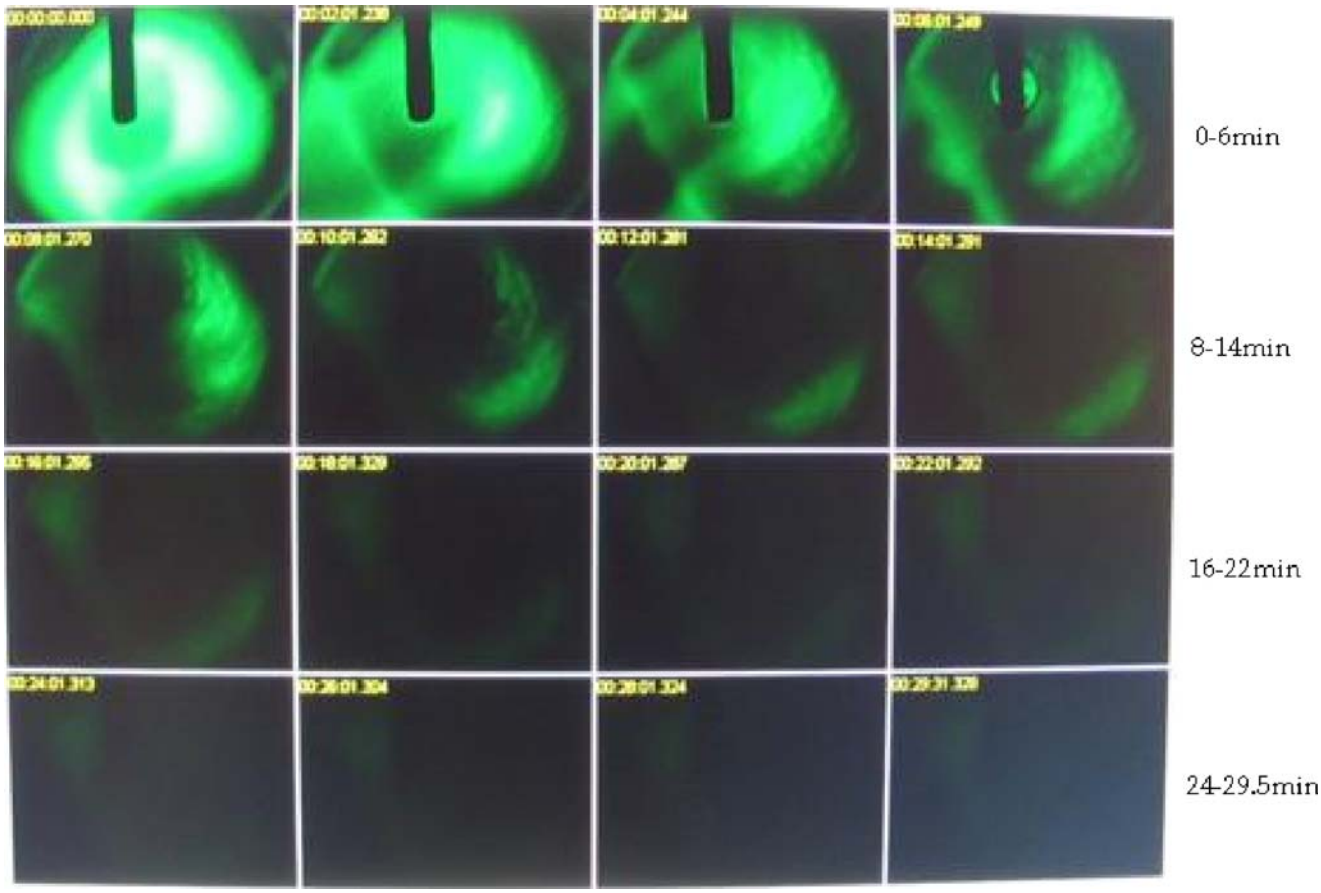

Fig. 8. Imaging of HPMC/CMC formulation retention as a function of time (slides are ordered from *left* to *right* in 2 min intervals)

of the cornea via pipette. Then, 5 μl of formulation spiked with FITC (30 ppm) was applied. Immediately, the perfusion pumps were turned on to allow perfusion medium to flow over the cornea surface at a rate of ~4 μl/min. The fluorescence image of the cornea was taken at 30 s intervals for up to 60 min. The fluorescence intensity of the cornea was determined using AxioImager software (Zeiss, Thornwood, NY, USA). To correct for photobleaching due to repeated exposures and to the environmental light, a control experiment was done in the same manner as the perfusion experiment except that the perfusion pumps were turned off. Both the perfusion and control experiments were done in triplicate for each formulation.

RESULT AND DISCUSSION

Rheological Study

Aqueous solutions containing HPMC 0.3% or HPMC 0.3%/CMC 0.25% did not form a gel, and their linear viscoelastic regions from stress sweep (data no shown) were very narrow in the low stress range. When these solutions were subjected to frequency sweep (Fig. 2), both the elastic modulus (G') and viscous modulus (G''; data not shown) showed frequency-dependent profile indicating that the polymers in each of these solutions existed as entangled polymer dispersion (27). The elastic modulus of HPMC 0.3%/CMC 0.25% solution under constant stress at every frequency was higher than that of HPMC 0.3% solution indicating a stronger structure formed via interactions between CMC and HPMC. Since HPMC is an unionizable polymer, hydrogen bonding or hydrophobic interaction may be involved in the interaction between CMC and HPMC. With the solution containing HPMC 0.3% and Carbopol 974P 0.1%, it showed a much longer linear viscoelastic region in stress sweep test (data not shown). When the solution was subjected to frequency sweep (Fig. 2), both of its elastic modulus and viscous modulus (data not shown) showed a frequency independent pattern and the value of elastic modulus was higher than that of viscous modulus at each frequency. The frequency sweep profile of HPMC and Carbopol 974P containing formulation indicated a relatively strong gel structure.

Rheological study is usually carried out to investigate mucoadhesion by comparing the change in elastic modulus between a polymer alone and the mixture of the polymer and mucin. A higher elastic modulus of the mixture than that of the polymer alone at the same testing condition would suggest a mucoadhesive component. To evaluate the interaction between mucin and polymers in concentration normally employed in topical formulations for dry eyes, frequency sweep was performed on the mixture of mucin and polymer solution, and the change in elastic modulus was determined. The sweep frequency profiles of the mixture of mucin 4% with HPMC 0.3%, HPMC 0.3%/CMC 0.25%, or HPMC 0.3%/Carbopol 0.1% are depicted in Figs. 3, 4, and 5,

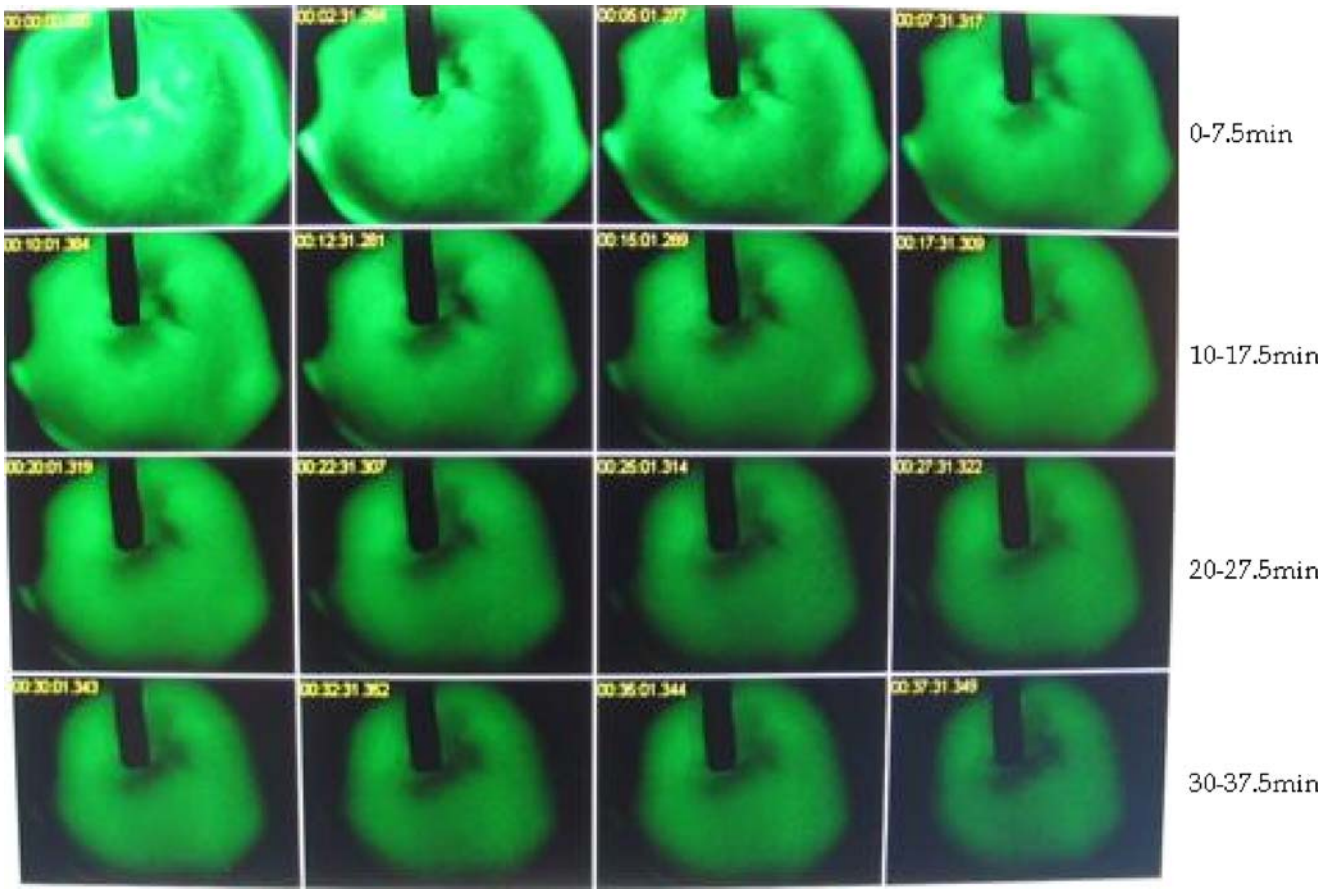

Fig. 9. Imaging of HPMC/Carbopol formulation retention as a function of time (slides are ordered from *left* to *right* in 2.5 min intervals)

respectively. For comparison purposes, the frequency sweep profiles of corresponding polymer solution are also shown. For the HPMC 0.3% solution (Fig. 3), the elastic modulus curve of the polymer mucin mixture went below that of the mucin solution. According to the theory proposed by Tamburic and Craig (26), this pattern indicates a negative interaction between HPMC and mucin. For the HPMC 0.3%/CMC 0.25% solution (Fig. 4), the elevated curve of elastic modulus from the mixture suggests a positive mucin and polymer interaction.

For the HPMC 0.3% and Carbopol 0.1% solution (Fig. 5), the elastic modulus (×) was found to be similar to

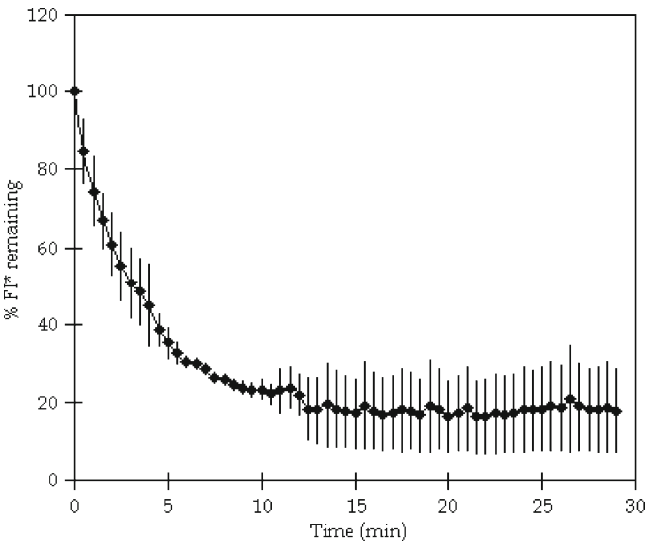

Fig. 10. Perfusion data as the mean percent of fluorescence intensity *versus.* time for HPMC 0.3% *w/w* (same data as shown in Fig. 8; SD expressed as error bar and $n=3$). **FI* fluorescence intensity

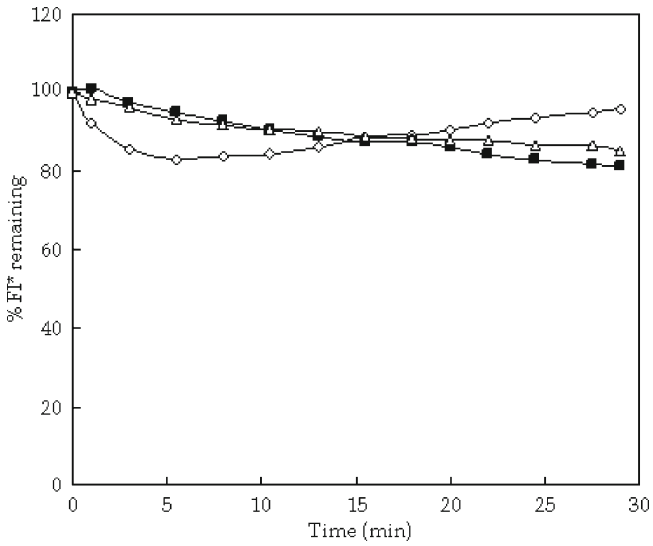

Fig. 11. Photo bleaching of HPMC 0.3%: run 1 (*diamond*), run 2 (*square*), and run 3 (*triangle*). **FI* fluorescence intensity

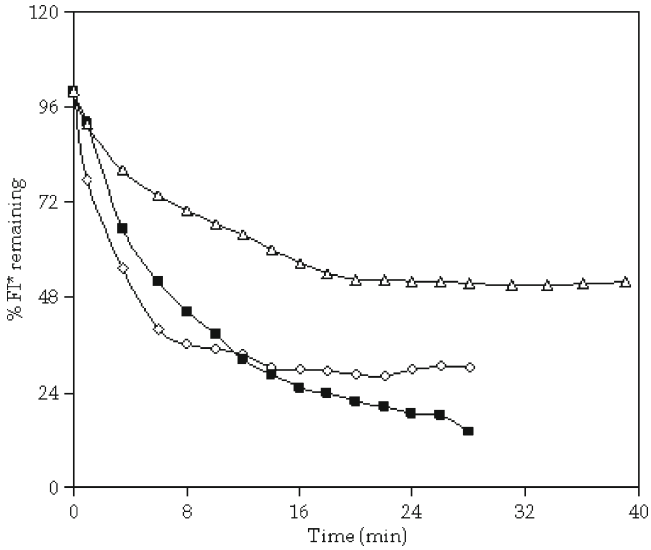

Fig. 12. Comparison of perfusion data from different polymer combination: HPMC 0.3% (*diamond*), HPMC 0.3%/CMC 0.25% (*square*), and HPMC 0.3%/Carbopol 0.1% (*triangle*)

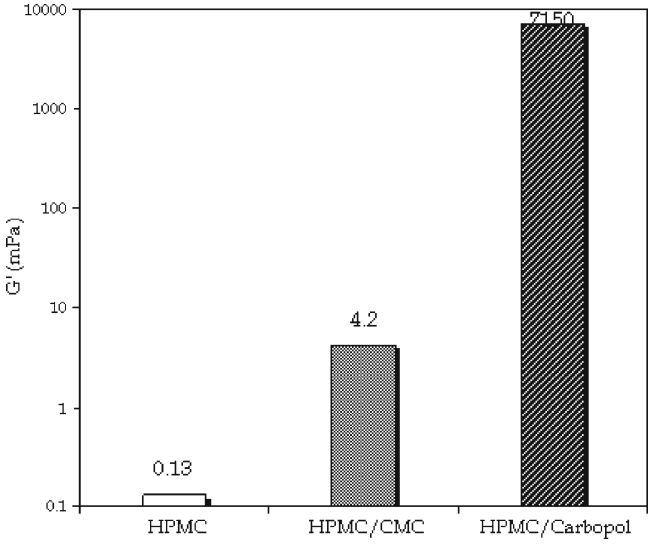

Fig. 14. G' at 0.1 Hz for HPMC 0.3%, HPMC 0.3%/CMC 0.25%, and HPMC 0.3%/Carbopol 0.1% (constant stress is 0.1 pa)

that of the mucin solution (Δ). Both the polymer solution and the mucin solution had lower elastic modulus than that of the polymer and mucin mixture. The result again suggests a positive interaction between HPMC/Carbopol 974P and mucin. Given what was observed for HPMC (Fig. 3), the positive interaction of the HPMC/Carbopol with mucin is clearly attributed to Carbopol. This is consistent with the common knowledge that Carbopol is a mucoadhesive polymer.

Alginate is another interesting mucoadhesive polymer. Chemically, alginic acid is a linear copolymer with homopolymeric blocks of (1–4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, respectively, covalently linked together in different sequences or blocks. Divalent ion (e.g., Ca^{2+}, Ba^{2+}, Sr^{2+}, among others) facilitates the formation of alginate gel via the ionic bridge. Thus, the effect of Ca^{2+} concentration on the gelling properties of alginate was evaluated by rheological technique. Ca^{2+} of two concentrations (0.06% and 0.006% *w*/*w*) was studied. Frequency sweeps of alginate solution and its mixture with mucin are illustrated in Fig. 6. Alginate–Na 0.5% *w*/*w* with Ca^{2+} 0.006% did not form a gel, while after being mixed with 4% mucin, a strong gel formed and there was a huge increase in elastic modulus. Two factors may contribute to this phenomenon: One is due to the residue electrolytes present in mucin since during purification of mucin, electrolytes are added and the removal of electrolytes is incomplete; another is due to the interaction of the polymer with mucin. For alginate–Na 0.5% *w*/*w* with Ca^{2+} 0.06%, there was a small increase in elastic modulus before and after mixing with mucin. This could be due to the fact that at the high Ca ion concentration, ionic bridges were completely formed with the available alginate molecules bridging sites

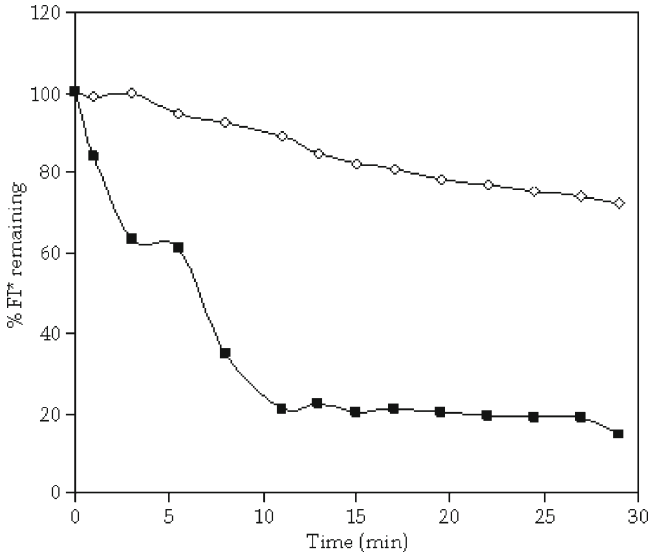

Fig. 13. Perfusion of alginate 0.5% with Ca^{2+} in different concentration: alginate–Na 0.06% (*diamond*) and alginate–Na 0.006% (*square*)

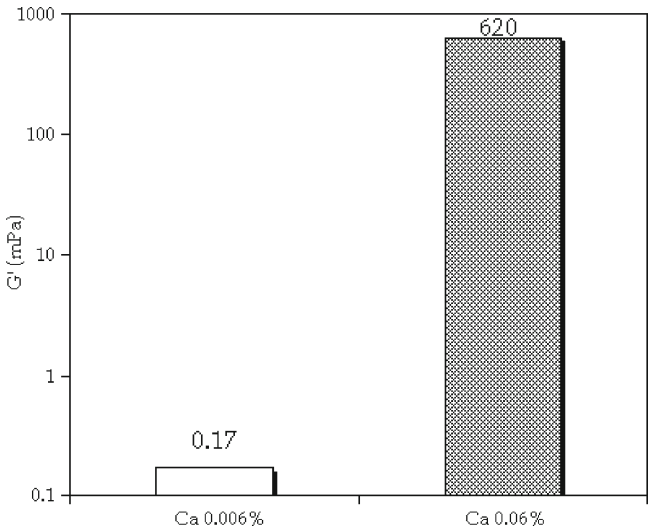

Fig. 15. G' at 0.1 Hz of alginate–Na 0.5% with Ca^{2+} in different concentration (constant stress is 0.1 pa)

Table II. Relationship Between $t_{0.5}$ from Perfusion Study and Elastic Modulus at 0.1 Hz of Different Polymer Combination

Formulation	HPMC	HPMC/CMC	HPMC/Car
$t_{0.5}$ (min)	4–4.5	6–6.5	>30
G' (mPa)	0.13	4.2	7,150

and the small increase in elastic modulus is due to interaction with mucin.

Ex Vivo Residence Time

In the *ex vivo* perfusion study, a freshly excised rat cornea was used as the biomembrane. To simulate the physiological condition, 5 μl of 4% mucin was added on the cornea surface before instillation of a formulation to compensate for the mucus layer lost during operation and cornea storage. The other reason to add mucin was to investigate the potential interaction of mucin and polymer and its effect on residence time. The very low perfusion rate (~4 μl/min) was chosen to simulate the rate of tear flow. Normal saline was used as perfusion medium. The perfusion medium was kept at 25°C since preliminary study showed no difference between 25°C and 35°C. Figure 7 shows a typical fluorescence intensity decay as a function of time for HPMC 0.3%. The dark stick shown on each picture is the image of the inlet needle. The residence of the formulation on the cornea surface is clearly reflected by the fluorescence intensity change. As can be expected, the fluorescence intensity decreased with the time of flushing. At around 11 min, the intensity decreased to that of the background (Fig. 7) suggesting that the majority of the formulation (dye) had been washed away. Figure 8 shows the time series images of HPMC 0.3%/CMC 0.25% formulation. The retention time was obviously longer than that of the HPMC 0.3% formulation. Figure 9 shows the time series images of HPMC 0.3%/Carbopol 0.1% formulation. This formulation had a retention time of more than 40 min.

To analyze the data more quantitatively, the fluorescence intensity of each image was integrated using the AxioImage software (Zeiss).

The mean percent of fluorescence intensity remaining on the cornea *versus* time for the HPMC 0.3% formulation is displayed in Fig. 10. Good reproducibility and low variance were demonstrated with the triplicate assay. It should be noted that there are potential error contributing factors in this *ex vivo* perfusion method. The first error contributing factor is the physical shape of the cornea. Although rat corneas were stored in nutritional media to maintain the viability of cells, it was observed that when the storage time was more than 5 h, the corneas lost their perfect dome shape. Ridges and valleys can form on the cornea surface. When these happen, the dye molecules in the valleys would have a longer residence time than those on the ridges with the same perfusion rate. As a result, the residence time of the same formulation from deformed cornea and perfect cornea could have large variances. To minimize the error contributed from cornea deformation, the *ex vivo* perfusion experiments need to be conducted immediately after cornea harvesting. The second error contributor is the location of the inlet needle. In order to mimic the real situation, the distance between the exit of the inlet needle should not be too far from the cornea surface; otherwise, the perfusion media would drip down onto the cornea surface. The inlet needle should not be pressed onto the cornea either which would cause the deformation of cornea. The optimal solution is to fix the position of inlet needle so that the distance is far less than the droplet size. This way, the perfusion medium would flow to the cornea surface due to surface tension. Lastly, the position of outlet needle could contribute to measurement error. The outlet needle should be placed close to the bottom of cornea so that the dye flushed down from the cornea surface can be drained away efficiently. Since the cornea was placed in the middle of the perfusion chamber with some space around, the error contributed by outlet needle position is small compared to those from cornea deformation and improper inlet needle position.

Because some perfusion experiments can last for as long as 60 min, the decrease in fluorescence intensity with time could be partially attributed to photo bleaching in addition to perfusion. To correct for the potential photo bleaching effect, photo bleaching experiment was done in the same manner as the perfusion experiment except that the perfusion pumps were turned off. Figure 11 shows the photo bleaching curve where the percentage of fluorescence intensity remaining was plotted against time for the HPMC 0.3% solution.

The difference between the percent of intensity remaining from the perfusion experiment and that from the bleaching experiment at each time point is the net intensity decrease from perfusion. The residence profile of each formulation is thus obtained by plotting the net intensity decrease *versus* time. Figure 12 shows the residence profiles for the HPMC containing formulations. As expected, the order of residence time was HPMC/Carbopol > HPMC/CMC > HPMC. Figure 13 shows the residence time of the alginate formulations. Again as expected, the alginate/Ca 0.06% formulation had a longer residence time than that of the alginate/Ca 0.006% formulation.

Relationship Between Viscoelastic Properties and Residence Time

To investigate the relationship between the viscoelastic properties and the *ex vivo* residence time, we first determined the elastic modulus of each formulation at 0.1 Hz (Figs. 14 and 15). We then defined a new parameter, $t_{0.5}$ (or $t_{0.8}$ for alginate formulations), as a time at which the fluorescence intensity decreased to 50% (or 80%) of fluorescence intensity at $t=0$. The reason for using $t_{0.8}$ for the alginate formulations was that these formulations stayed so long on the cornea that only about 30–40% of the instilled dose was eliminated at the end of the perfusion study (40 min). The results of the elastic modulus and residence time are shown in Table II for the

Table III. Relationship Between $t_{0.8}$ from Perfusion Study and Elastic Modulus at 0.1 Hz of Alginate 0.5% with Ca^{2+} in Different Concentration

Ca^{2+} concentration	0.006%	0.06%
$t_{0.8}$ (min)	1–1.5	~16
G' (mPa)	0.17	620

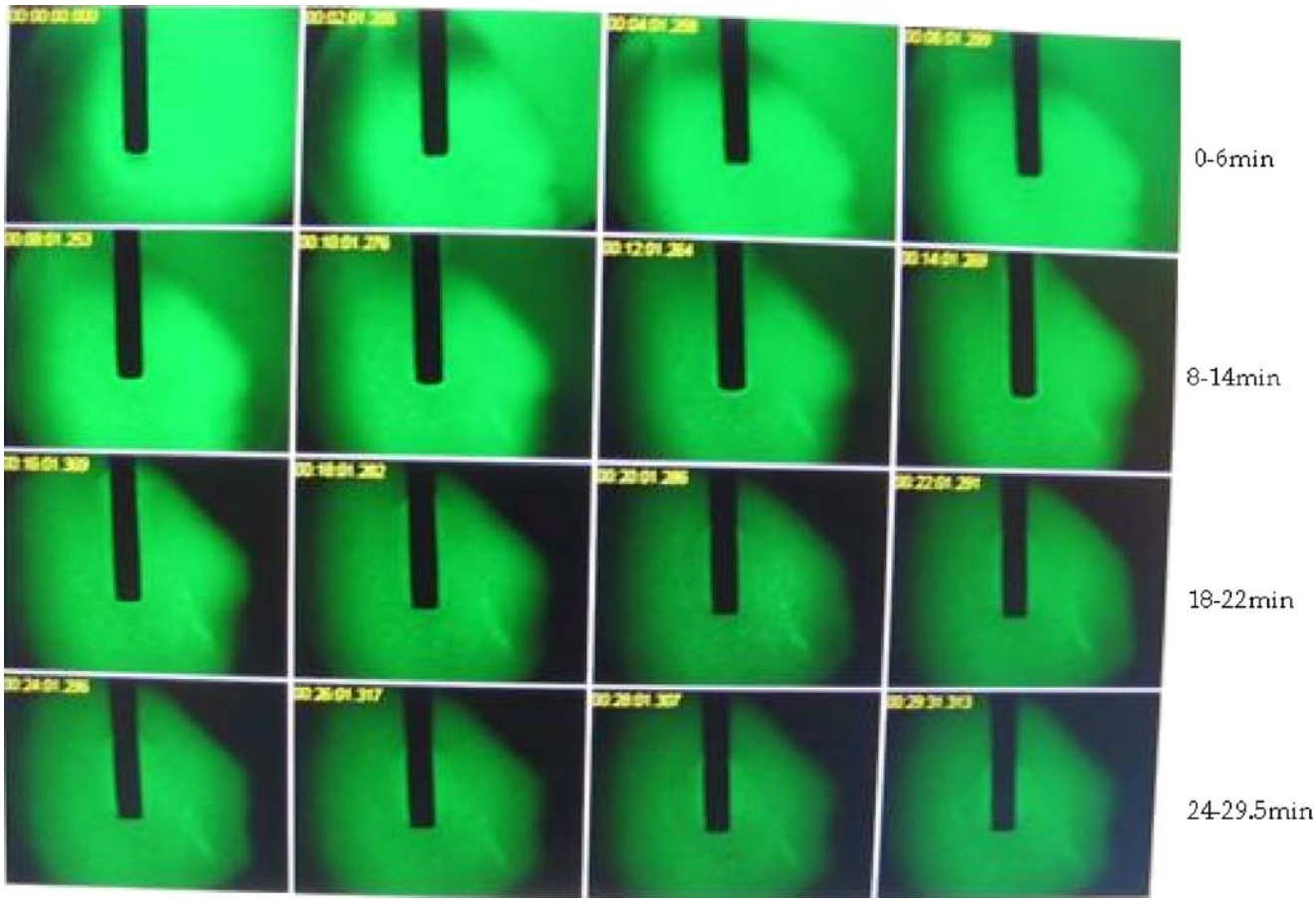

Fig. 16. Imaging of HA formulation (HPMC 0.3%/HA 0.125%) retention as a function of time (slides are ordered from *left* to *right* in 2 min intervals)

HPMC formulations and in Table III for the alginate formulations. It is apparent that formulations with a higher elastic modulus at 0.1 Hz also had a longer residence time. Interestingly, however, a hyaluronate formulation displayed a very long retention time despite its low viscoelastic moduli (Figs. 16 and 17). HPMC/HA had similar G' (Fig. 17) to that of HPMC/CMC but lower G'' (Fig. 18), yet HPMC/HA (Fig. 16) had a much longer retention time than that of HPMC/CMC (Fig. 8). We do not have a good explanation for this except to suggest that the mucoadhesive properties may

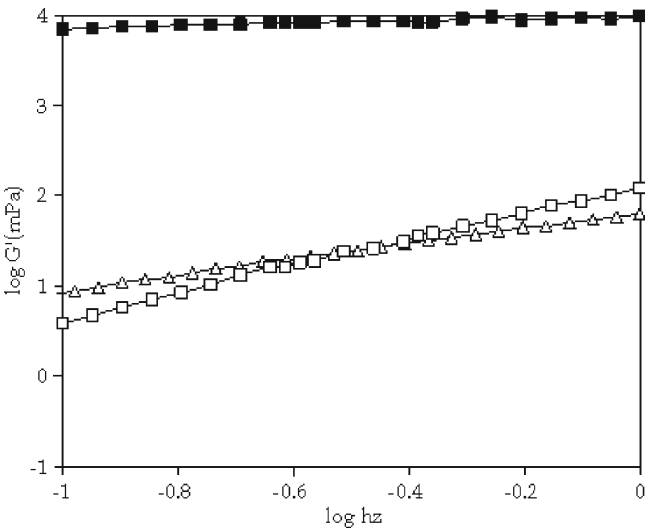

Fig. 17. Comparison of elastic modulus of polymer mixture containing formulations: HPMC/HA (*open triangle*), HPMC/CMC (*open square*), and HPMC/Carbopol (*closed square*; frequency sweeps, constant stress at 0.1 pa)

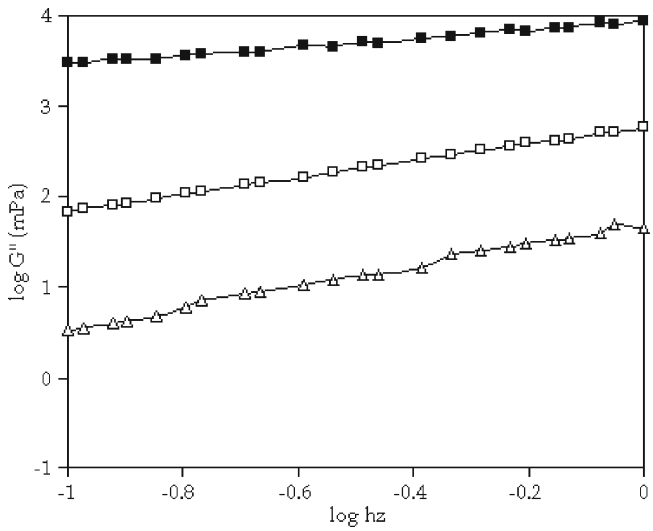

Fig. 18. Comparison of viscous modulus of polymer mixture containing formulations: HPMC/HA (*open triangle*), HPMC/CMC (*open square*), and HPMC/Carbopol (*closed square*; frequency sweeps, constant stress at 0.1 pa)

not always be discernable by the rheological properties of the formulation. The *ex vivo* perfusion methodology may in fact provide more meaningful information with regard to retention times of formulations.

CONCLUSION

A perfusion methodology was developed and evaluated for the determination of retention time of topical ophthalmic formulations. The perfusion system is easy to set up and produces reproducible results. The method easily differentiated formulations based on the retention time on the cornea. It can be a useful tool to screen ophthalmic formulations to optimize the retention times of formulations.

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