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# Comparison of ion-activated *in situ* gelling systems for ocular drug delivery. Part 2: Precorneal retention and *in vivo* pharmacodynamic study

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ABSTRACT

*In situ* gelling systems are viscous polymer-based solutions that exhibit a sol-to-gel phase transition upon change in a physicochemical parameter such as ionic strength, temperature or pH, therefore prolonging the formulations' residence time on the ocular surface. Ion-activated *in situ* gelling systems, that are able to crosslink with the cations in the tear fluid, have previously been evaluated in terms of their rheological, textural and *in vitro* release characteristics. The present study describes the ocular irritancy, precorneal retention time and *in vivo* release characteristics of the same formulations. It was shown that all tested polymer systems were non-irritant. Precorneal retention studies revealed a biphasic rapid release for the solution with less than 40% radioactivity left on the ocular surface after 15 min, while formulations based on gellan gum, xanthan gum and carrageenan seemed to drain at an almost constant rate with more than 80% radioactivity remaining. This was in agreement with the *in vivo* miotic studies, which demonstrated that the area under the curve and the miotic response at 120 min after administration for gellan gum, xanthan gum and carrageenan formulations of pilocarpine were increased by 2.5-fold compared to an aqueous solution, which demonstrates their potential use in ophthalmic formulations.



## 1. Introduction

Ion-activated *in situ* gelling systems are able to crosslink with the cations present in the tear fluid in order to form a gel on the ocular surface. This is thought to prolong the precorneal retention time and therefore lead to increased bioavailability of the drug. The rapid turnover rate of the lacrimal fluid, which generally leads to a dilution of common viscous eye drops, may therefore further enhance the viscosity of these formulations due to an increase in the amount of cations present (Greaves et al., 1990).

Various ion-activated *in situ* gelling systems have previously been evaluated for their gelling behaviour, rheological and textural properties, gel microstructure, spreading ability and *in vitro* release characteristics (Rupenthal et al., 2011). Although these studies provide a good insight into the physicochemical characteristics of the formulations, they cannot predict their behaviour *in vivo*, as the interaction between the properties of the formulation and the ocular pharmacokinetics is likely to determine the final performance of the system (Urtti and Salminen, 1993).

Gamma scintigraphy is a non-invasive technique that allows monitoring of the corneal residence without disturbing normal physiological functions (Wilson, 1999). It was first described by Rossomondo et al. (1972) and has since been widely used, i.e. to assess the precorneal drainage of various artificial tear products containing HPMC, PVA or sodium hyaluronate (Snibson et al., 1992), ophthalmic ointments (Greaves et al., 1993), liposomal formulations (Nagarsenker et al., 1999), w/o microemulsions (Alany et al., 2006), poloxamer gels (Wei et al., 2002), an ion-activated *in situ* gelling system based on alginate and HMPC (Liu et al., 2006) and an ion- and pH-activated *in situ* gelling system based on gellan gum and chitosan (Gupta et al., 2010). Increased bioavailability can generally be measured by assaying the drug concentration in the aqueous humor or by monitoring a pharmacodynamic response. As the former requires the sacrifice of large numbers of animals, non-invasive monitoring of a pharmacological response such as the pupil diameter or the intraocular pressure after application of a model drug is generally preferred (Urtti and Salminen, 1993). Pilocarpine hydrochloride is a parasympathomimetic alkaloid used in the treatment of chronic open-angle glaucoma. It increases ciliary muscle contraction, which results in the opening of the trabecular meshwork, but also acts on the iris

*Abbreviations:* HPMC, hydroxypropyl methylcellulose; PHCl, pilocarpine hydrochloride; HET-CAM, hen's egg test on chorioallantoic membrane; $^{99m}$Tc-DTPA, technetium-99m labelled diethylene triamine pentaacetic acid; ROI, region of interest; AUC, area under the curve.

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sphincter muscle, causing a miotic response which can easily be monitored.

This study assessed the ocular irritation potential of various ion-activated *in situ* gelling systems using a HET-CAM, before evaluating their precorneal retention and *in vivo* performance in rabbits. As in previous studies (Rupenthal et al., 2011), 0.5% (w/v) anionic polysaccharide solutions were compared to an uncharged as well as a positively charged polymer formulation of the same polymer concentration.

## 2. Materials and methods

### 2.1. Materials

Gellan gum (Kelcogel F) and xanthan gum (Keltrol F) were a generous gift from CP Kelco (Atlanta, GA, USA). Carrageenan (Genulacta L-100, κ-carrageenan) was a free sample from CP Kelco (Lille Skensved, Denmark). Sodium alginate (medium viscosity) was obtained from Acros Organics (Morris Plains, NJ, USA) and hydroxypropyl methylcellulose (HPMC, Methocel K4M) was purchased from Colorcon (Gao, India). Chitosan (Protasan UP CL 213) was obtained from Novamatrix (Sanvika, Norway). Pilocarpine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fertilised hen's eggs were ordered from Bromley Park Hatcheries Ltd. (Tuakau, New Zealand). $^{99m}$Tc-DTPA, freshly prepared for each experiment, was obtained from the Department of Radiology and Nuclear Medicine, Auckland Hospital (Auckland, New Zealand). Water used for preparation of the formulations was ion exchanged, distilled and passed through a Milli-Q water purification system (Millipore, Bedford, MA, USA).

### 2.2. Ocular irritation potential (HET-CAM test)

Freshly collected fertile hen's eggs were incubated at 37 ± 0.5 °C and 55 ± 5% humidity for three days. They were then cracked open into a growing chamber prepared according to the method described by Alany et al. (2006) and viable eggs with intact CAM and yolk were further incubated. On day ten, 0.2 ml of each formulation was dropped onto the membrane, with 0.1 M sodium hydroxide (NaOH) solution and 70% isopropyl alcohol serving as positive controls, while a saline solution was used as a negative control. Blood vessels were examined for irritant effects such as hyperaemia, haemorrhage or coagulation (Fig. 1) and formulations were scored according to the time point when these effects were initially detected (Luepke, 1985) (Table 1). The sum of the time-dependent numerical scores for all three responses gives a single

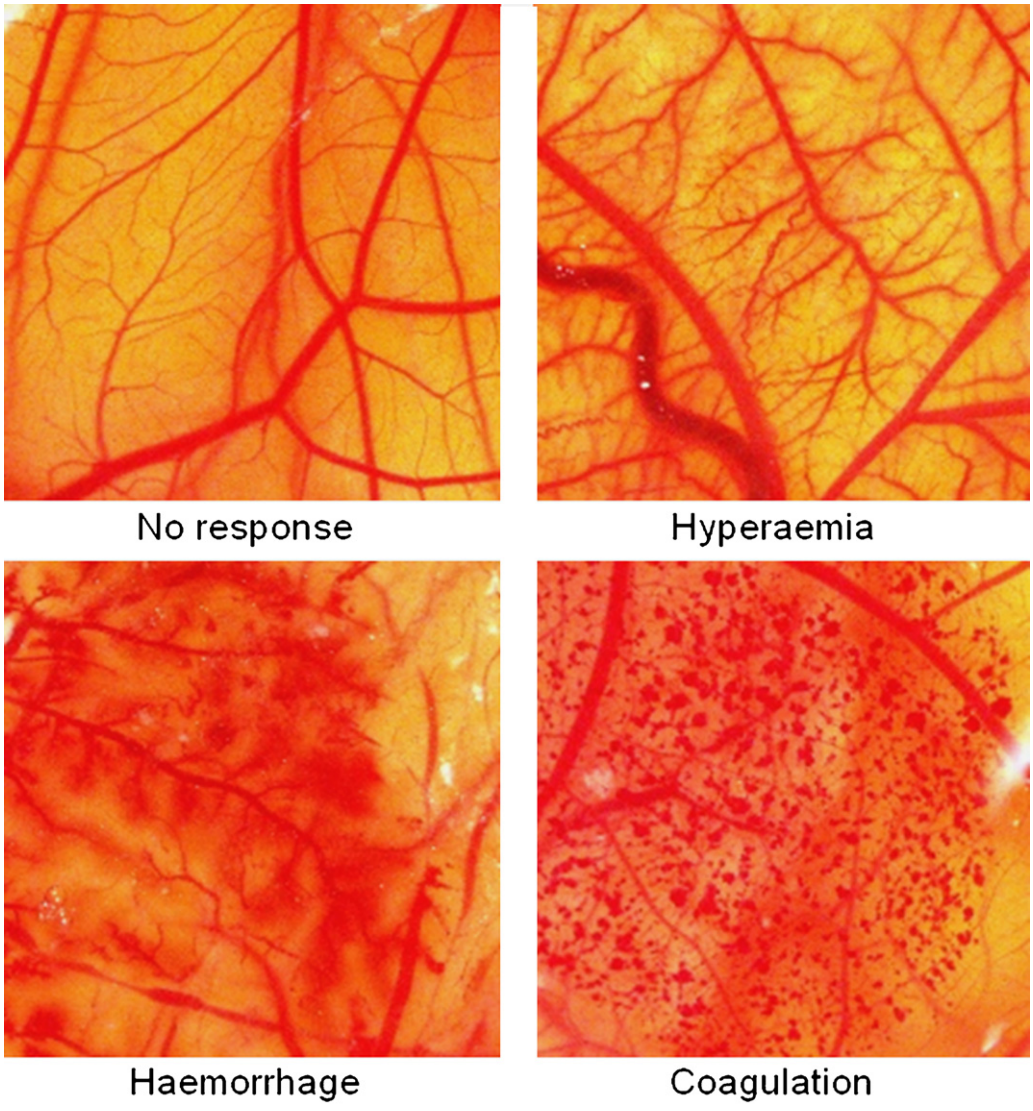


**Fig. 1.** Vascular responses used to score the irritation potential of the test formulations.

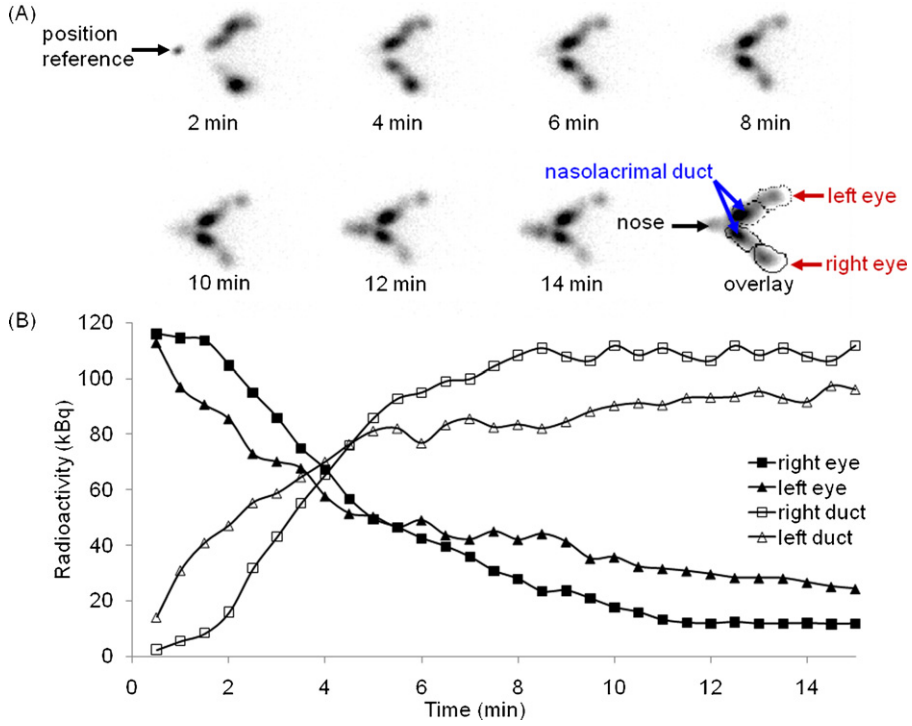


**Fig. 2.** Representative gamma scintigram of an aqueous solution instilled into both eyes of a New Zealand Albino rabbit showing the nasolacrimal drainage over time (A) and radioactivity versus time plots for the formulation drainage out of both eyes and into the ducts (B).

**Table 1**
Numerical time-dependent scores based on the initial detection of the three irritation responses (Luepke, 1985).

| Effect | Time (min) | | |
|---|---|---|---|
| | 0.5 | 2.0 | 5.0 |
| Hyperaemia | 5 | 3 | 1 |
| Haemorrhage | 7 | 5 | 3 |
| Coagulation | 9 | 7 | 5 |

numerical value, which allows classification of the tested formulations analogous to the Draize categories (Table 2).

### 2.3. Precorneal retention study

Six male New Zealand Albino rabbits weighing 3.0 ± 0.5 kg were restrained by wrapping them into a towel and were positioned on the head of the gamma camera, which allowed for simultaneous evaluation of the drainage in both eyes. A volume of 20 μl of each formulation, containing 0.5% (w/v) of polymer and 4 MBq of $^{99m}$Tc-DTPA, was instilled directly into the lower fornix of the conjunctival sac of both eyes using a positive displacement pipette (Finnpipette, Labsystem, Finland). Both eyes were manually blinked five times in order to distribute the formulation across the cornea. Dynamic images were collected at a rate of 30 s per image over 15 min using a GE Starcam 4000i XR/T Gamma Camera (GE Healthcare Global Diagnostic Imaging, USA) fitted with a low energy and high resolution parallel hole collimator. After correction for movement of the rabbits, regions of interest (ROI) were created around the precorneal and the nasolacrimal duct area. Time–activity-curves (Fig. 2) were generated for both regions and the area under the 'percentage-activity-remaining-versus-time' profile ($AUC_{0-15min}$) and the percentage activity remaining in the precorneal area after 15 min ($a_{15min}$) were calculated. After excluding any animal effect using a two-way ANOVA at a 95% confidence interval, a one-way ANOVA followed by Tukey's pairwise comparison at a 99% confidence interval was performed to test for significant differences between formulations. A minimum washout period of 48 h was allowed between experiments.

**Table 2**
Relationship between cumulative scores and ocular irritation potential (Luepke, 1985).

| Cumulative score | Irritation potential |
|---|---|
| 0.0–0.9 | Practically none |
| 1.0–4.9 | Slight |
| 5.0–8.9 | Moderate |
| 9.0–21.0 | Strong |

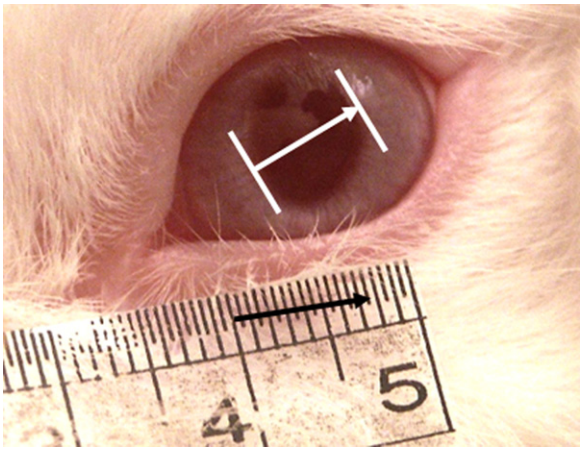

**Fig. 3.** Digital camera image showing the miotic response being measured with the ruler serving as a calibration scale.

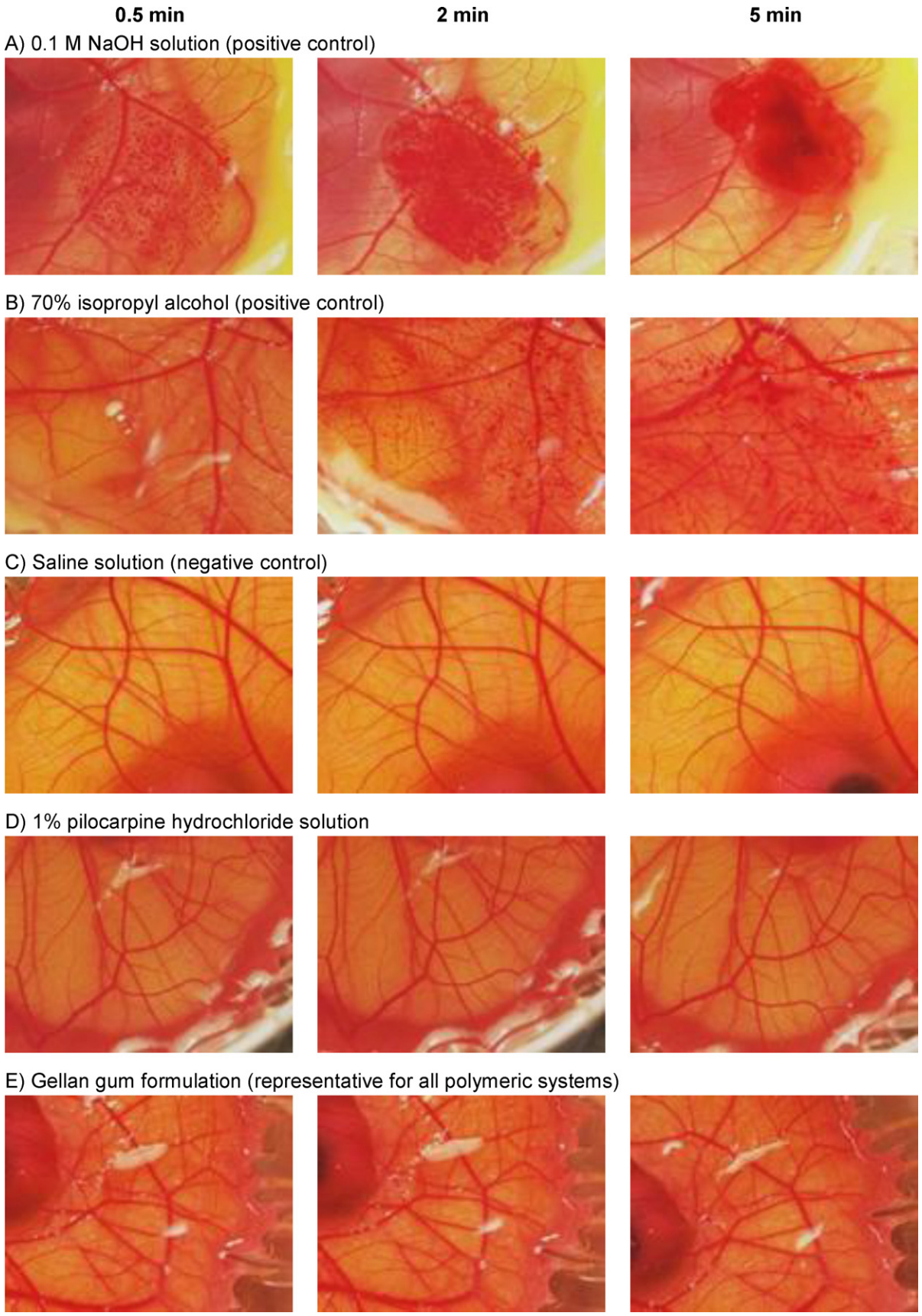


**Fig. 4.** Vascular responses seen on the CAMs at 0.5 (left column), 2 (middle column) and 5 min (right column) after application of the various formulations.

### 2.4. In vivo miotic study

A non-invasive miotic study was performed on six New Zealand Albino rabbits weighing 4.5 ± 0.5 kg. Tests were performed in the same room under standard lighting conditions. Light intensities were kept constant at 20 ± 1 Lux and were monitored using a photometer (Lux Meter Q-1400, DSE). After 30 min of acclimatization a ruler with 0.5 mm increments was placed under the left eye of each rabbit and baseline measurements were taken using a digital camera (Minolta Dimage 7, 5.2MPixel). A volume of 20 μl of each formulation containing 1% (w/v) of PHCl was then instilled into the cul-de-sac of the left eye using a positive displacement pipette (Finnpipette, Labsystems, Helsinki). The miotic response was monitored at 10 min intervals over 180 min with the right eye serving as a control. The pupil diameter was determined from the acquired images using the ruler as a calibration scale (Fig. 3). The miotic response was expressed as the change-in-pupil-diameter-over-time curve and the AUC was calculated using the trapezoidal method. Statistical analysis was performed using a two-way ANOVA followed by a one-way ANOVA with Tukey's pairwise comparison at a 95% confidence interval. A minimum washout period of 48 h was allowed between experiments.

## 3. Results and discussion

### 3.1. Ocular irritation potential (HET-CAM test)

Upon applying 0.1 M NaOH, all three signs of vascular response were observed after only 0.5 min (Fig. 4A), grading this solution as a very strong irritant to the ocular tissues (cumulative score of 21.0, $n = 3$). Administering 70% isopropyl alcohol to the CAM, no vascular responses were noticeable within 30 s, while hyperaemia and small areas of haemorrhage were visible after 2 min, increasing further within 5 min (Fig. 4B), giving this rather weak positive control a cumulative score of 8.0 ($n = 3$; moderately irritant). Saline solution (negative control), 1% PHCl solution and all tested *in situ* gelling systems showed no signs of vascular response (Fig. 4C–E), classifying them as practically non-irritant (cumulative score 0.0; $n = 3$). Therefore, all selected *in situ* gelling systems were perceived as being suitable for *in vivo* use.

### 3.2. Precorneal retention study

The percentage radioactivity remaining on the ocular surface was calculated assuming that the first activity value recorded in the precorneal ROI equalled 100%. As can be seen in Fig. 5 drainage of the aqueous solution was quite rapid with less than 40% radioactivity remaining in the precorneal ROI at the end of the study, while most of the polymer systems (apart from alginate and HPMC) remained in the precorneal region for a substantially longer time period, with more than twice as much radioactivity still remaining after 15 min. The solution exhibited a biphasic clearance pattern, with a rapid initial clearance due to tear fluid turnover, followed by a slower basal drainage phase. This was in agreement with the previously reported biphasic clearance process of a solution (Durrani et al., 1995). In fact, the drainage of the solution was so fast that it was detectable in the stomach of the rabbit at the end of the monitoring period (Fig. 6). This was not the case for any of the polymer formulations, which seemed to drain at a much slower rate.

Unlike the biphasic clearance pattern of the solution, the polymer formulations based on gellan gum, xanthan gum, carrageenan and chitosan seemed to drain at an almost constant rate. This clearance pattern was previously reported for formulations based on Pluronic F127 and F68 (Wei et al., 2002), which exhibited a more

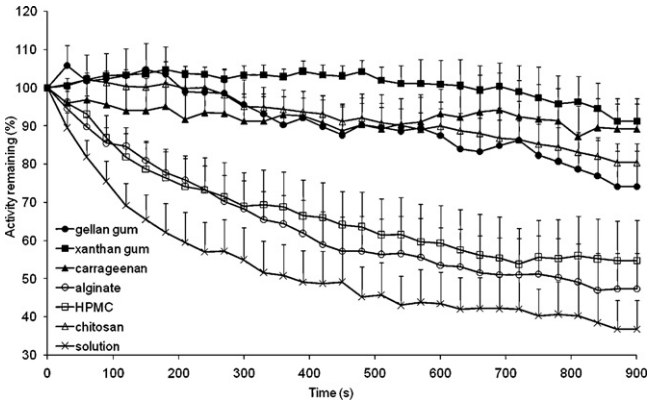


**Fig. 5.** Precorneal drainage of $^{99m}$Tc-DTPA incorporated into the various formulations illustrating the reduced drainage rate of gellan gum, xanthan gum carrageenan and chitosan formulations compared to an aqueous solution (results represent mean values + SE, $n = 12$).

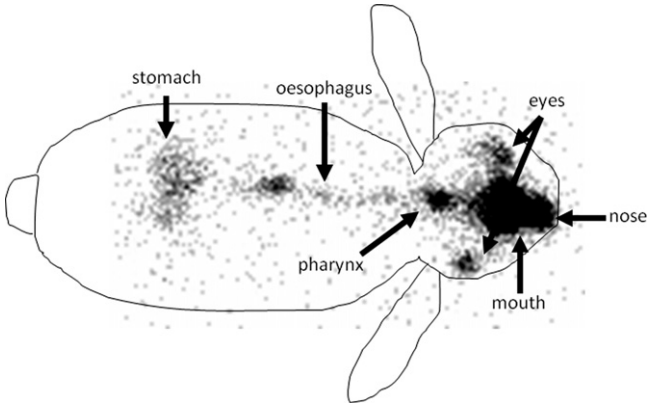


**Fig. 6.** Radio-image of the whole rabbit illustrating the fast drainage rate of an aqueous solution containing 4 MBq of $^{99m}$Tc-DTPA by showing the presence of radioactivity in the stomach 20 min after instillation.

than three-fold increase in the corneal residence time compared to a phosphate buffered solution. The results were also in agreement with studies performed by Meseguer et al. (1993), who found a significantly prolonged contact time for formulations containing Gelrite® ($P < 0.01$), xanthan gum ($P < 0.01$) or hydroxyethyl cellulose ($P < 0.05$), when compared to an aqueous solution. The drainage profiles for the HPMC and alginate systems were similar to the biphasic pattern of the solution, but with a much less rapid initial clearance. Similar drainage profiles were previously reported by Liu et al. (2006), who studied an alginate/HPMC mixture as *in situ* gelling ophthalmic delivery system for gatifloxacin and found much better ocular retention for the combination of the two polymers than for the individuals.

Calculated $AUC_{0-15min}$ values and percentages activity remaining at the end of the study ($a_{15min}$) are summarised in Table 3.

**Table 3**
Area under the curve ($AUC_{0-15min}$) and percentage activity remaining after 15 min ($a_{15min}$) (results represent mean values ± SE, $n = 12$).

| Formulation | $AUC_{0-15min}$ ± SE (% min) | $a_{15min}$ ± SE (%) |
|---|---|---|
| Gellan gum | 81,644 ± 6168 | 76.71 ± 9.35 |
| Xanthan gum | 87,897 ± 2728 | 91.81 ± 4.65 |
| Carrageenan | 78,400 ± 5426 | 85.53 ± 7.98 |
| Alginate | 60,838 ± 6054 | 54.06 ± 8.07 |
| HPMC | 57,726 ± 6488 | 51.40 ± 10.54 |
| Chitosan | 80,234 ± 3056 | 79.75 ± 5.10 |
| Solution | 45,605 ± 6153 | 38.67 ± 7.67 |

**Table 4**
Tukey's pairwise comparison of the area under the curve ($AUC_{0-15min}$) and the activity remaining after 15 min ($a_{15min}$); S = significant difference ($P \leq 0.01$), NS = no significant difference ($P > 0.01$).

| | Solution | Gellan gum | Xanthan gum | Carrageenan | Alginate | HPMC |
|---|---|---|---|---|---|---|
| Gellan gum | S; *S* | – | – | – | – | – |
| Xanthan gum | S; *S* | NS; *NS* | – | – | – | – |
| Carrageenan | S; *S* | NS; *NS* | NS; *NS* | – | – | – |
| Alginate | NS; *NS* | NS; *NS* | S; *S* | NS; *NS* | – | – |
| HPMC | NS; *NS* | NS; *NS* | S; *S* | NS; *NS* | NS; *NS* | – |
| Chitosan | S; *S* | NS; *NS* | NS; *NS* | NS; *NS* | NS; *NS* | NS; *NS* |

Results confirmed the prolonged retention of the gellan gum, xanthan gum, carrageenan and chitosan formulations, with the values for the $AUC_{0-15min}$ and $a_{15min}$ being about twice as high as those of the solution. Since formulations were equi-viscous (15 ± 1 mPa s) before instillation, this could be attributed to two different phenomena: the increased viscosity of the formulations due to gelling once in contact with the cations present in the tear fluid, and the mucoadhesive effect of the polymers.

Mucoadhesive properties of cationic polymers such as chitosan have previously been reported (Lehr et al., 1992). These arise from ionic interactions between the positively charged amino groups of the polymer and the negatively charged sialic acid groups of the mucus in the tear film. It was suggested that cationic polymers would exhibit better mucoadhesive properties than anionic or neutral ones. However, a study performed by Leung and Robinson (1988) also demonstrated good mucoadhesive properties for anionic polymers such as polyacrylic acid (PAA), based on unspecific interactions with mucin, such as electrostatic and hydrophobic interactions, hydrogen-bonding and inter-diffusion of the mucin and the polymer networks. In general, both anionic and cationic charged polymers seem to demonstrate better mucoadhesive properties than non-ionic polymers, such as cellulose derivates or PVA (Meseguer et al., 1993). When testing the mucoadhesive properties of the polymers (1% (w/v)) used throughout this study employing the rheological assessment using the method described by Hassan and Gallo (1990), the anionic polysaccharides (gellan gum, xanthan gum, carrageenan and alginate) showed significantly ($P < 0.05$) higher mucoadhesive properties than non-ionic HPMC (data not shown). This could explain the relatively fast drainage of the non-ionic HPMC system compared to the anionic polysaccharide systems.

As for the poor performance of the alginate formulation, the total amount of cations in the tear fluid may have played a role. As was seen in the partial phase diagram for alginate with $Ca^{2+}$ (Rupenthal et al., 2011), the gel formed upon addition of divalent cations may result in precipitation of the polymer, if the cation concentration is too high. Therefore, the rapid tear turnover, that may have further enhanced the viscosity of the formulations based on gellan gum, xanthan gum and carrageenan, may have resulted in precipitation of alginate in front of the eye. This would have resulted in a low viscosity aqueous medium containing the radioactive material, which would exhibit a similar drainage profile to that of the solution.

Performing a two-way ANOVA on the data presented in Table 3, no significant difference was detected between the 12 eyes (six rabbits). Therefore, a one-way ANOVA followed by Tukey's pairwise comparison at a 99% confidence interval was conducted. As can be seen in Table 4, formulations based on gellan gum, xanthan gum, carrageenan and chitosan were significantly different ($P < 0.01$) from the aqueous solution in terms of the $AUC_{0-15min}$ as well as the radioactivity remaining after 15 min ($a_{15min}$). The system based on xanthan gum achieved the best ocular retention, with $AUC_{0-15min}$ and $a_{15min}$ being significantly different not only from the aqueous solution, but also from the formulations based on alginate and HPMC.

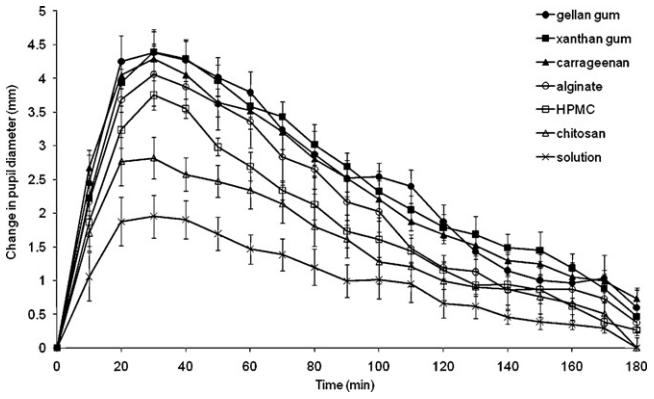


**Fig. 7.** Miotic response profiles displayed as change in pupil diameter (mm) versus time (min) curves illustrating the favourable delivery characteristics of the anionic polysaccharide formulations compared to an aqueous solution (data points represent mean values ± SE, $n = 6$).

### 3.3. *In vivo* miotic study

The miotic response profiles for PHCl released from the various formulations over a period of 3 h are shown in Fig. 7. In accordance with the *in vitro* release profiles (Rupenthal et al., 2011) the polymeric systems exhibited more favourable delivery characteristics than the aqueous solution. Comparing the calculated pharmacokinetic parameters listed in Table 5 by means of a one-way ANOVA followed by Tukey's pairwise comparison (Table 6), it was found that the area under the curve ($AUC_{0-180min}$) and the miotic response at 120 min after administration ($action_{120}$) for gellan gum, xanthan gum and carrageenan formulations were increased by 2.5-fold compared to an aqueous solution. While the significant difference concerning the $AUC_{0-180min}$ was the same for all three formulations when compared to the aqueous solution ($P < 0.001$), the significance

**Table 5**
Area under the 'change-in-pupil-diameter-versus-time' curve ($AUC_{0-180min}$), time required to achieve peak miotic response ($t_{max}$) and miotic response at 120 min after administration ($action_{120}$) (results represent mean values ± SE, $n = 6$).

| Formulation | $AUC_{0-180min}$ ± SE (mm min) | $t_{max}$ ± SE (min) | $Action_{120}$ ± SE (mm) |
|---|---|---|---|
| Gellan gum | 445.0 ± 17.5 | 35.0 ± 1.8 | 1.9 ± 0.1 |
| Xanthan gum | 446.5 ± 13.8 | 33.3 ± 0.9 | 1.8 ± 0.1 |
| Carrageenan | 430.2 ± 8.4 | 30.0 ± 1.0 | 1.7 ± 0.1 |
| Alginate | 378.0 ± 19.0 | 33.3 ± 2.3 | 1.2 ± 0.1 |
| HPMC | 339.1 ± 16.8 | 30.8 ± 1.5 | 1.1 ± 0.1 |
| Chitosan | 276.3 ± 18.4 | 25.0 ± 0.9 | 1.0 ± 0.1 |
| Solution | 183.0 ± 14.8 | 28.3 ± 1.3 | 0.7 ± 0.1 |

**Table 6**
Tukey's pairwise comparison of the area under the curve ($AUC_{0-180min}$), the time required to achieve peak miotic response ($t_{max}$) and the miotic response at 120 min after administration (**action$_{120}$**); S = significant difference ($P \leq 0.05$), NS = no significant difference ($P > 0.05$) unless otherwise stated.

| | Solution | Gellan gum | Xanthan gum | Carrageenan | Alginate | HPMC |
|---|---|---|---|---|---|---|
| Gellan gum | S$^{***}$; *NS*; **S**$^{**}$ | – | – | – | – | – |
| Xanthan gum | S$^{***}$; *NS*; **S**$^{*}$ | NS; *NS*; **NS** | – | – | – | – |
| Carrageenan | S$^{***}$; *NS*; **S** | NS; *NS*; **NS** | NS; *NS*; **NS** | – | – | – |
| Alginate | S; *NS*; **NS** | NS; *NS*; **NS** | NS; *NS*; **NS** | NS; *NS*; **NS** | – | – |
| HPMC | NS; *NS*; **NS** | NS; *NS*; **NS** | NS; *NS*; **NS** | NS; *NS*; **NS** | NS; *NS*; **NS** | – |
| Chitosan | NS; *NS*; **NS** | S; *NS*; **NS** | S; *NS*; **NS** | NS; *NS*; **NS** | NS; *NS*; **NS** | NS; *NS*; **NS** |

$^{*}$ $P < 0.01$.
$^{**}$ $P < 0.005$.
$^{***}$ $P < 0.001$.

in terms of the miotic response at 120 min after administration ($action_{120}$) was in the order of gellan gum ($P < 0.005$) > xanthan gum ($P < 0.01$) > carrageenan ($P < 0.05$), suggesting that the gellan gum formulation formed the strongest gel and was therefore retained in the precorneal area the longest. This was in agreement with the results for the precorneal retention (Fig. 5) as well as the *in vitro* release studies (Rupenthal et al., 2011), where formulations based in these three polymers also exhibited the best performance. Moreover, it supports previous data by Meseguer et al. (1993) which illustrated that gellan and xanthan gum formulations were significantly different from a reference solution in terms of the prolongation of the miotic response, while a hydroxy ethylcellulose solution was not. Chan et al. (2007) compared the miotic responses after topical application of a PHCl-containing microemulsion, liquid crystalline system and coarse emulsion to that of an aqueous solution and found that the pharmacokinetic parameters depended highly on the viscosity of the formulation and therefore the precorneal retention. The increase in viscosity due to interaction of the anionic polysaccharide polymer chains with the ions present in the rabbits' tear fluid, resulting in an increase of the precorneal retention, therefore seems to be the pivotal factor for the improved pharmacodynamic response.

No significant difference ($P > 0.05$) was detected between the formulations regarding the time required to achieve peak miotic response ($t_{max}$), indicating that the drug is initially available at the same time from all formulations. However, the chitosan formulation exhibited a rather low value for $t_{max}$ ($25.0 \pm 0.91$ min) compared to the other formulations, indicating earlier onset of the drug's pharmacodynamic response. This could be attributed to the repulsion between the positively charged drug and the cationic polymer backbone, facilitating the initial drug release from the chitosan formulation, while the ionic interactions of PHCl with the anionic polymer systems would lead to a slightly higher $t_{max}$ value due to increased precorneal retention of PHCl resulting from the charge–charge interactions.

## 4. Conclusion

All chosen polymer formulations were found to be non-irritant according to the HET-CAM and were therefore classified as safe for *in vivo* use. Systems based on gellan gum, xanthan gum and carrageenan exhibited the most favourable characteristics in terms of precorneal retention as well as delayed release of the model hydrophilic drug (PHCl) *in vivo*. This was mostly attributed to the *in situ* gelling effect of these polymer systems once in contact with the cations of the tear fluid and the therefore increased precorneal retention time. While many studies have been published on the use of gellan and xanthan gum in topical eye drops, which can also be found in marketed products (Timoptic XE and Timoptic GFS respectively), little has been known about the application of carrageenan in ophthalmic formulation. This paper compared the various anionic polysaccharides in terms of their *in situ* gelling performance *in vivo* and highlighted the potential use of carrageenan in ocular formulations.

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