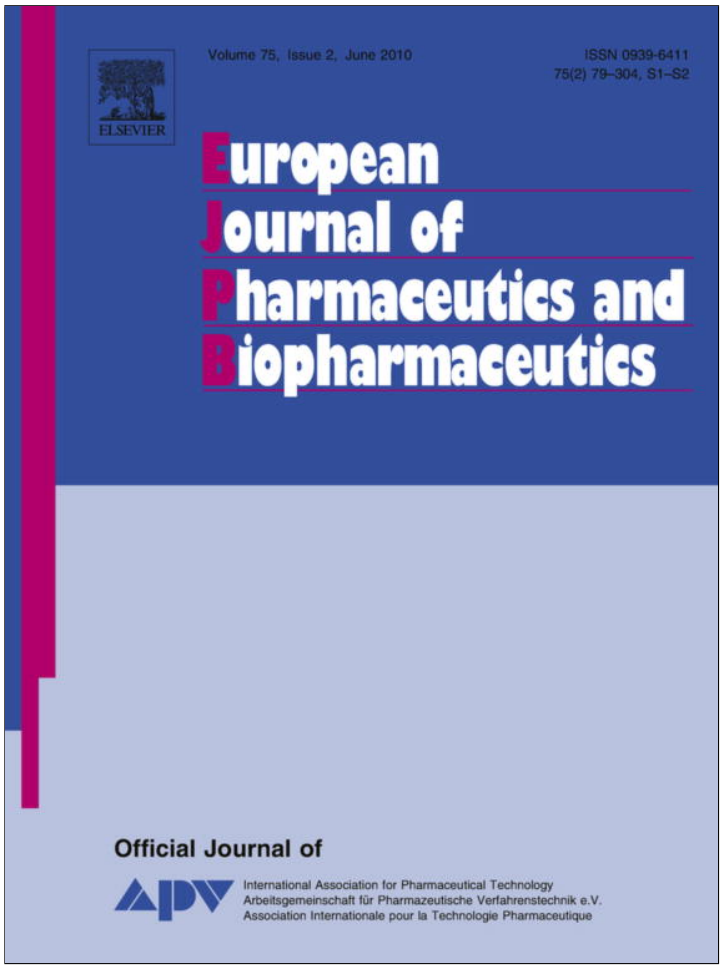

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Research paper

A poloxamer/chitosan *in situ* forming gel with prolonged retention time for ocular delivery

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ABSTRACT

The aim of the present work was to obtain an ophthalmic delivery system with improved mechanical and mucoadhesive properties that could provide prolonged retention time for the treatment of ocular diseases. For this, an *in situ* forming gel comprised of the combination of a thermosetting polymer, poly (ethylene oxide)–poly (propylene oxide)–poly (ethylene oxide) (PEO–PPO–PEO, poloxamer), with a mucoadhesive agent (chitosan) was developed. Different polymer ratios were evaluated by oscillatory rheology, texture and mucoadhesive profiles. Scintigraphy studies in humans were conduced to verify the retention time of the formulations developed. The results showed that chitosan improves the mechanical strength and texture properties of poloxamer formulations and also confers mucoadhesive properties in a concentration-dependent manner. After a 10-min instillation of the poloxamer/chitosan 16:1 formulation in human eyes, 50–60% of the gel was still in contact with the cornea surface, which represents a fourfold increased retention in comparison with a conventional solution. Therefore, the developed formulation presented adequate mechanical and sensorial properties and remained in contact with the eye surface for a prolonged time. In conclusion, the *in situ* forming gel comprised of poloxamer/chitosan is a promising tool for the topical treatment of ocular diseases.

1. Introduction

The ideal treatment of ocular diseases, especially when the drug must display a localized action (e.g., the cornea and/or anterior chamber), would be the topical administration of an eye drop solution. Unfortunately, in several cases, topical treatment is not effective due to protective mechanisms of the human eye. For example, lacrimal secretion and the blinking reflex cause rapid drainage of the formulation. The short pre-corneal contact time combined with corneal impermeability results in low bioavailability, and as a result, frequent dosing is usually needed [1]. In order to avoid the rapid dilution, formulations with an increased viscosity have been evaluated. Among them, the *in situ* gel-forming formulations, which undergo phase transition from liquid to semisolid gel upon exposure to physiological environments, seem to be a promising tool. These formulations should be a free-flowing liquid at room temperature to allow easily reproducible administration into the eye as a drop. They also should undergo *in situ* phase transition to form a strong gel that is capable of withstanding shear forces in the cul-de-sac and of sustaining drug release at physiological conditions [2]. Thermosensitive amphiphilic block copolymers, namely poly (ethylene oxide)–poly (propylene oxide)–poly (ethylene oxide) (PEO–PPO–PEO, poloxamers), have been extensively investigated as *in situ* forming gels [3–7]. The most accepted mechanism to explain the thermogelification of poloxamers is that it results from interactions between different segments of the copolymer [8,9]. The poloxamer copolymer molecules aggregate into micelles. These micelles are spherical with a dehydrated polyoxypropylene (PPO) core with an outer shell of hydrated swollen polyoxyethylene (PEO) chains [10]. An increase in the temperature leads to dehydration and conformational changes at the hydrophobic chains regions, increasing chain friction and entanglement of the polymeric network [11,12]. More unbound water is available at the hydrophilic regions of the gel [13]; therefore, the external PEO chains interpenetrate extensively in the gel. At this point, gelation has occurred, and the micelles remain apparently intact and orderly packed, which has been described as "hard-sphere crystallization" [14].

Though poloxamers are widely employed, they suffer from a major drawback of having weak mechanical strength, which leads to rapid erosion [15]. One interesting approach, however, focuses on blends of poloxamers with other polymers like carbopol [16]

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and alginate [17]. Carbopol, a mucoadhesive polymer, increases the formulation's mechanical strength, but also increases surface interaction with the ocular tissue and, consequently, contact time. Carbopol shows a solid-to-gel transition in aqueous solution as the pH is raised above its pK_a of about 5.5; therefore, to have an easy administration, an acidic pH would be needed before carbopol's phase transition. This formulation could stimulate the eye tissue to increase the lacrimal secretion and blinking reflex, causing the formulation to drain easily [17].

Chitosan is a biodegradable polymer that has demonstrated excellent ocular compatibility [18–20]. It presents positively charged amine groups in its chemical structure that could interact with the negatively charged mucous layer, conferring a mucoadhesive characteristic [21,22]. Chitosan solutions have been successfully used in prolonging contact time with the ocular surface [23]. Therefore, a combination of this polymer with poloxamer would be very promising for ocular administration, as the *in situ* mechanical strength of the formulation would be higher than that of both polymers alone. The result could be a prolonging of the contact time. In fact, specific blends of poloxamer and chitosan for the ocular delivery of timolol maleate were already studied [24]. It was shown that these polymers can be used in combination to produce clear, sterile and non-irritating ophthalmic formulations. Nonetheless, the polymer and chitosan concentrations used by in this case were very small (from 7% to 14% w/v of poloxamer and only 0.25% and 0.5% w/v of chitosan) [24]. Chitosan was not used to improve the mechanical strength or mucoadhesive properties of the gel. Also, essential determinations of the exact gelation temperature, mechanical and mucoadhesive properties as a function of chitosan concentration and *in vivo* drug permeation were not performed. Therefore, further studies are necessary in order to obtain an ophthalmic delivery system that could make the topical treatment of ocular diseases feasible.

The aim of the present work was to obtain an ophthalmic delivery system with improved mechanical and mucoadhesive properties and improved retention time for the treatment of ocular diseases. For this work, poloxamer and chitosan were used to prepare *in situ* forming gels, with the former used as a gelling agent and the latter used as a mucoadhesive agent. Concentrations from 14% to 20% and 0.5% to 1.5% w/w of poloxamer and chitosan, respectively, were evaluated by oscillatory rheology, with the purpose of obtaining an optimal gelation temperature. The rheological and mechanical properties, as well as the mucoadhesive ability of the poloxamer gels as a function of chitosan concentration, were evaluated. These results were used as a screening process to select the most suitable polymer concentration for the *in vivo* studies, where gamma scintigraphy was used in human eyes to evaluate the retention time of the formulation.

2. Materials and methods

2.1. Chemicals

Chitosan MMW (190,000–310,000 Da; 75–85% deacetylated – information provided by the manufacturer) and mucin type III were purchased from Sigma Aldrich (Steinheim, Germany). Poloxamer 407 was purchased from Embrafarma (São Paulo, Brazil). All other reagents were BDH or HPLC reagents. Deionized water (Milli-Q Millipore Simplicity 185, Bedford, MA, USA) was used to prepare all solutions.

2.2. Preparation of gels

All poloxamer solutions used in this study (14–20% w/w) were prepared by weighing the polymer in cold ultrapure water. The solutions were kept in a refrigerator for at least 24 h to ensure complete dissolution. In cases where chitosan (0.5–1.5% w/w) was used, it was initially dissolved in a solution of acetic acid 0.5% v/w. The chitosan solution was then refrigerated and used as a solvent for the poloxamer dispersion. All formulations had a pH between 6.0 and 6.5. The osmolality of the final formulation (poloxamer 16% chitosan 1.0% w/w) was 295 ± 5.7 mOsm kg^{-1}. This value was achieved by the addition of 68 mM of NaCl to the formulation and was determined by the freezing point depression method using a Semi-Micro Osmometer Model K-7400 (Knauer, Berlin, Germany).

2.3. Measurement of gelation temperature

The solid–gel transition temperature ($T_{sol/gel}$) of each formulation under examination was measured using a Carri-med CSL-100 rheometer (T.A. Instruments New Castle, DE) with a stainless steel cone and plate geometry (4 cm diameter and 1° angle and a gap of 55 μm between the cone and plate) and temperature ramp step oscillation procedure. Samples were carefully applied to the lower plate of the rheometer, ensuring that formulation shearing was minimized, and allowed to equilibrate for at least 5 min prior to analysis. Silicone oil was added to the surface of the sample to prevent evaporation of solvent. In all oscillation experiments, the strain amplitude value was obtained from the linear viscoelastic region of the samples analyzed at 15 and 50 °C, in which they had lower and higher strength, respectively. The linear viscoelastic region was identified as the region where stress was directly proportional to strain, while the storage modulus (G') remained constant. Following application of a constant stress, frequency of 1.0 Hz and maximum strain amplitude of 0.1 Pa, a temperature sweep analysis was performed over the temperature range of 15–50 °C, with the temperature being increased at 10 °C/min. The storage modulus (G') and loss modulus (G'') were then determined using Rheology Advantage software provided by T.A. Instruments. The analyses were performed on at least three replicates of each formulation. The $T_{sol/gel}$ was considered to be the temperature at which the two moduli were equal (G' and G'' crossover), as proposed by others [25,26].

Samples that had an adequate $T_{sol/gel}$ were also submitted to frequency sweep analysis at 25 °C and 35 °C over the frequency range of 0.1–10.0 Hz after application of a constant stress. All other parameters were the same as described above. The storage (G') and loss (G'') modulus were used as measurements for the rheological behavior.

Furthermore, in order to evaluate the formulation performance after ocular application in the worst case scenario, i.e., when all applied polymer solution (50 μl) would be immediately mixed with all available tear fluid (7 μl) [27], the polymeric solution was mixed with simulated tear fluid at a ratio of 50:7, and this mixture was submitted to frequency sweep analysis at 35 °C. Simulated tear fluid consisted of: NaCl 0.67 g, NaHCO 0.20 g, $CaCl{\cdot}2H_2O$ 0.008 g and water up to 100 g.

2.4. Texture profile

Texture profile analysis (TPA) was performed using a TA-XT2 Texture Analyzer (Stable Micro Systems, Surrey, England) in TPA mode, as previously described [28,29]. Formulations (35 g) were transferred into 50-ml bottles, taking care to avoid the introduction of air into the samples. A cylindrical analytical probe (35 mm diameter) was forced down into each sample at a defined rate (1 mm/s) and to a defined depth (10 mm). At least five replicate analyses of each sample were performed at temperatures of 25 °C and 35 °C. From the resulting force–time plots, the hardness (the force required to attain a given deformation), compressibility (the work required to deform the product during the first pass of the probe) and adhesiveness (the work necessary to overcome

the attractive forces between the surface of the sample and the surface of the probe) were derived [29].

2.5. In vitro mucoadhesive strength

The mucoadhesive strength of the formulations under investigation was evaluated *in vitro* by measuring the force required to detach the formulation from a mucin disc [28,30] using an Instron® universal testing machine [2]. Mucin discs were prepared by compression of a known weight of crude porcine mucin (250 mg), using a ring-press with a 9 mm diameter. These discs were then horizontally attached to the lower end of the cylindrical probe (1 cm diameter) by using double-sided adhesive tape. Prior to mucoadhesion testing, the mucin disc was hydrated by submersion in a 5% solution of mucin for 30 s. Excess surface liquid was removed by gentle blotting. The analytical probe was then lowered until the mucin disc was in contact with the surface of the sample, which had been packed into shallow cylindrical vessels. The probe and the disc remained in contact for 30 s [5]. The probe was then moved upwards at a constant speed of 1.0 mm/s, and the force required to detach the mucin disc from the surface of each formulation was determined from the resulting force–time plot. All measurements were performed at 35 °C, and at least three replicates were carried out.

2.6. In vivo scintigraphy studies

The retention time of the formulation developed was evaluated through gamma scintigraphy on four healthy volunteers with age range 24–35 years old and no evidence of eye infection or nasal pathology. The saline solution used as control was analyzed on the same volunteers after a washout period of 1 week. The research followed the tenets of the Declaration of Helsinki, and informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study. This research was approved by the ethical committee of the University of São Paulo ("HCRP Comitê de ética em pesquisa do HCRP e da FMRP" – Protocol No. 12190/2007).

Each subject was seated in front of the gamma camera (Orbiter Stand 6603, Siemens gamma sonics), with their eyes positioned at a distance of 5 cm from the pinhole collimator, and their head was supported on a modified ophthalmic table. The subject was then instructed to remain in this position throughout the imaging period. The developed hydrogel (50 μl, poloxamer/chitosan, respectively 16% and 1.0% w/w) containing Tc^{99} at 1 mCi was placed in the eye by gently pulling down the lower eyelid and inserting it into the cul-de-sac with a micro pipette. A series of dynamic images of 15 s duration was then acquired for 10 min (40 frames). The exam images were analyzed using an Image J freeware (available at: http://rsbweb.nih.gov/ij/). Regions of interest (ROIs) were clarified to avoid mistakes due to positioning. Data are expressed as a percentage of total administered doses and as a function of time.

2.7. Statistical analysis

The data obtained from all experiments were submitted to unpaired *t*-test. Values with $P < 0.05$ were considered statistically different (Prisma, Graphpad Software, La Jolla, US).

3. Results and discussion

3.1. Influence of chitosan on the gelation temperature and dynamic properties

Phase transition temperature ($T_{sol/gel}$), i.e., the temperature at which the liquid phase makes a transition to a gel, is obviously an important parameter for *in situ* gel-forming systems. The ideal $T_{sol/gel}$ should be between 25 °C, the average ambient temperature, and 35 °C, the eye temperature. Based on the suitable range of gelation temperature (25–35 °C), poloxamer was chosen as the gelling agent. Moreover, poloxamer 407 is reported to be the less toxic of the commercially available poloxamers [31].

The $T_{sol/gel}$ obtained for different poloxamer concentrations (14%, 16%, 18% and 20% w/w) (Table 1) is in accordance with the results obtained in the literature [3,9,25,32] and confirms that the $T_{sol/gel}$ is dependent on polymer concentration. Hence, according to the results, the solution that presented an adequate $T_{sol/gel}$ contained 16% of poloxamer (32 ± 1.2 °C). Nonetheless, the main goal of the present work is to combine poloxamer with another polymer, chitosan, in order to obtain an *in situ* gel with increased mucoadhesiveness. Because the mechanism of gelation of poloxamer is based on micelles packing and entanglements [33], the inclusion of drugs or additives may interfere in micelle formation and, consequently, cause a $T_{sol/gel}$ modification [27,32]. The effect of different chitosan concentrations (0.5–1.5% w/w) on the gelation temperature of the *in situ* gel containing 16% poloxamer was evaluated.

The results indicated that chitosan did not significantly interfere with the formulation $T_{sol/gel}$ (Table 1) in all concentration ranges studied. Chitosan is a polysaccharide (MW = 190,000–310,000 Da), and therefore, a big molecule that was expected to interfere in poloxamer gelation. In addition, it forms a viscous solution that could contribute to the formulation's elasticity, although at the range of concentrations used it was not able to shift the $T_{sol/gel}$. Thus, further investigation of the dynamic and textural properties of the formulation and extrapolations to physiological conditions were performed.

The dynamic properties of the formulation, such as elasticity, or storage modulus (G'), and viscosity, or loss modulus (G''), can provide information about the inherent characteristics of the formulation at room and physiological temperatures. The G' is an inherent characteristic of a solid material, and a higher G' value means that under a shearing force, the material is able to store the energy and not deform or flow. Meanwhile, the opposite is true for the G'' [1]. As explained above, at room temperature, the formulation should be a free-flowing liquid to allow easily reproducible administration into the eye as a drop. Thus, the G'' should be higher than the storage modulus (G') and dependent on the frequency. After administration, however, the formulation is expected to form a gel ($G' > G''$) and, in this way, withstands the shearing forces expected in the eye during and between blinking. Moreover, the formulation's characteristics should also bear the tear dilution [34].

The range of shear rates experienced during relative movements of the eyelids and globe is extremely wide. At the interval between blinking, while the eyes are open, the shear rate only depends on the gravitation and, in this case, it ranges from 0.03 to 0.1 s^{-1} [35,36]. While blinking, however, the shear rate can be calculated from the tear film thickness, that is 7–8 μm [37], and from the blinking speed, that is 10 cm s^{-1} [38]. In this case, the shear

Table 1
Phase transition temperature for poloxamer formulations.

Poloxamer (% w/w)	Chitosan (% w/w)	$T_{sol/gel}$ (°C)[a]
14	–	42 ± 2.3
16	–	32 ± 1.2
18	–	25 ± 0.9
20	–	23 ± 0.4
16	0.5	33 ± 0.8
16	1.0	32 ± 1.7
16	1.5	31 ± 1.3

[a] Mean (±SD) of at least three replicate measurements.

rate is approximately 10,000 s^{-1} [35]. Other authors reported a shear rate during blinking, ranging from 4250 to 40,000 s^{-1} [36,39]. In this way, after the application of the *in situ* forming gel, because this formulation is more viscous and consequently will form a thicker film than the tears, the shear force during blinking is expected to be even higher.

At the present work, oscillatory rheology analysis was employed to evaluate the formulation's properties at low shear force, which is the condition expected between blinks. The small oscillating amplitude applied in this methodology avoids destroying the gel structure, which usually occurs when high-speed shear is used in rotation measurements [1]. The final ability of the formulation to withstand the high shear force during blinking was indirectly evaluated in humans during the scintigraphy studies, as it is related to the formulation's residence time.

Regarding the ability on withstanding tear dilution, rheological analyses were performed in two extreme conditions: (a) without dilution, presuming immediate gelation in the eyes after administration and (b) after complete dilution with available tear fluid (50:7) [27]. We presumed the actual physiological behavior would lie between these two extreme scenarios.

The results of the rheological analyses confirm the viscoelastic characteristics of the formulation at 25 °C, where $G'' > G'$ and a frequency dependence is observed (Fig. 1A). At this temperature, the loss modulus is predominant, revealing that the poloxamer's micelles are not orderly packed and that the polymer chains are most probably in a relaxed conformation. However, without dilution at 35 °C (Fig. 1B), the rapid increase in the storage modulus reflects the formation of a strong gel network with $G' > G''$, independent of the oscillatory frequency.

After a 50:7 dilution with simulated tear fluid, the formulation contained 14% w/w of poloxamer. As a result, there was a shift on the $T_{sol/gel}$, and the formulation was still a viscoelastic solution at 35 °C and decreased frequencies (Fig. 2). Also, the salt content of the simulated tear fluid may have hindered micelle packing and entanglements, a process that was facilitated by the shear stress frequency. Fig. 2 shows that, as is expected for viscoelastic solutions, the storage and the loss modulus were frequency dependent and, with frequency increase, gelation ($G' > G''$) occurred.

Surprisingly, the chitosan addition influenced this process, and the frequency at which it occurred was dependent on the chitosan concentration. With an increase in the chitosan content, the elastic characteristic was higher; therefore, the frequency needed for gelation was lower. Although it has been previously shown that the addition of chitosan from 0.5% to 1.5% w/w did not produce marked changes in the $T_{sol/gel}$ of poloxamer aqueous solutions, it probably had an effect on microscopic diffusion coefficients within the gel structure, facilitating the accommodation of unbound water derived from micelle core dehydration [11–13]; therefore, facilitating molecular entangling and packaging and, in this way, contributing to the increase in the elastic characteristic of the system. The reported increment of the elasticity characteristic can be clearly observed with 1.5% w/w of chitosan (Fig. 2D).

In clinical conditions, the act of blinking could help the gel maintain its properties and prevent rapid drainage and flow caused by tear dilution. As the physiological conditions will be between the two extreme cases, it is expected that the formulation containing poloxamer 16% w/w and chitosan (0.5–1.5% w/w) becomes a gel *in situ* that is able to withstand tear dilution and blinking without network disruption, thus prolonging the contact time. Further texture analysis, discussed below, would corroborate these observations.

3.2. Texture profile

Textural analyses provide information on mechanical properties of samples, namely hardness, compressibility and adhesiveness. These properties can be directly correlated with sensory parameters *in vivo* and, therefore, are valuable in the development of a product with desirable attributes that contribute to patient acceptability and compliance [40]. A formulation designed for ophthalmic use should be, for example, easily removed from the package, present a good spreadability on the corneal surface and adhere to the mucous layer without disintegrating, in order to prolong retention time.

Formulations containing poloxamer/chitosan in different ratios were characterized using the texture analyzer in TPA mode, and the results are presented in Fig. 3.

A hardness test was performed to measure the force required to produce deformation of the gels, while compressibility measured the work required to achieve compression of the product along a definite distance [41]. Products possessing low hardness and compressibility properties will be easy to remove from the package and administer and, as a result, may be perceived favorably by the patient [40]. This property scenario was true for all formulations studied at 25 °C, the average temperature at which the administration will be performed. The observed increases in compressibility as a function of the polymer concentration are in accordance with results obtained with other polymers [5,41,42]. The formulation containing poloxamer and chitosan 1.5% w/w presented the greatest hardness and compressibility values, corroborating the results obtained by oscillatory rheology, i.e., there is increased elastic behavior (represented by G') with increased chitosan concentration. In this way, it is possible to predict that in an *in vivo* situation, it would be more difficult to administer the formulation containing 1.5% w/w of chitosan than it would be to administer other formulations with less chitosan.

At 35 °C, the formulation has already been administered and is a gel in the eye. In this case, it is desirable that the formulation possess a certain level of hardness in order to withstand drainage, be-

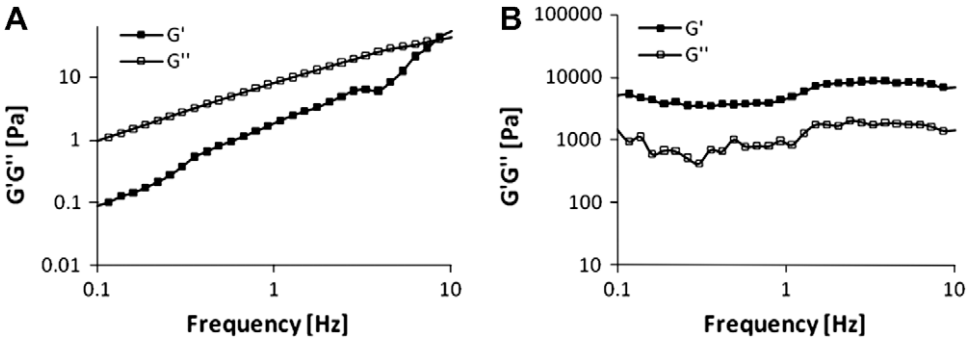

Fig. 1. Elastic (G') and viscous (G'') modulus as a function of frequency (Hz) of a formulation comprised of poloxamer/chitosan (16% and 1.0% w/w, respectively) at (A) 25 °C and (B) 35 °C.

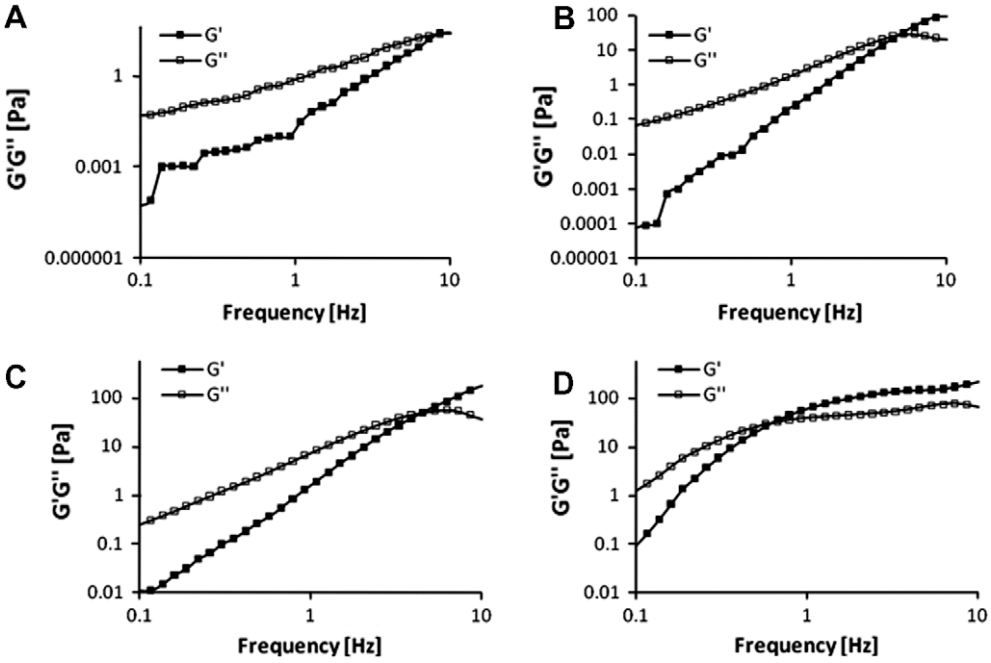

Fig. 2. Elastic (G') and viscous (G'') modulus as a function of frequency of formulations comprised of poloxamer (16% w/w) and chitosan in the following concentrations: (A) 0%, (B) 0.5%, (C) 1.0% and (D) 1.5% w/w at 35 °C. Samples were previously diluted with simulated tear fluid in a ratio of 50:7.

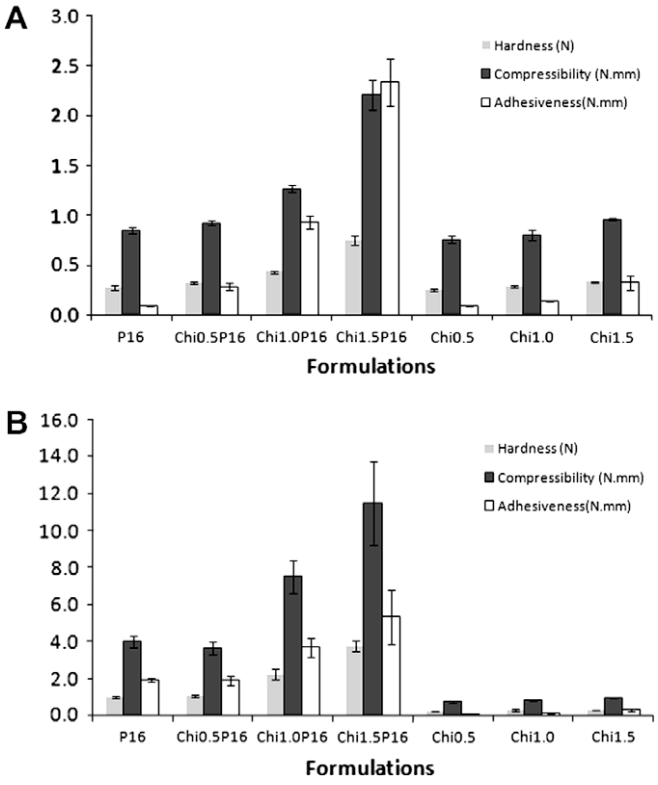

Fig. 3. The mechanical properties (hardness, compressibility and adhesiveness) of poloxamer (P) and chitosan (Chi) formulations, separately or together, determined using texture profile analysis at (A) 25 °C and (B) 35 °C. Data represent mean ± SD ($n = 5$). The numbers following polymer abbreviations P and Chi represent the amount of polymer expressed in % w/w.

cause an easy to flow formulation would be rapidly diluted by the tear and drained. Some authors have reported the relationship between hardness (strength) and formulation retention time [40,43]. As can be observed, increasing the content of chitosan (from 1.0% to 1.5% w/w) in the formulations containing 16% poloxamer significantly increased formulation hardness, compressibility and adhesiveness at both ambient (25 °C) and eye (35 °C) temperatures. The effects were clearly more pronounced in the eye temperature, with the exception of 0.5% w/w chitosan addition, which did not alter these properties at 35 °C. Therefore, formulations containing 1.0% or 1.5% w/w of chitosan are expected to hold for a prolonged time on the corneal surface before drainage.

Compressibility can also be related to the work required to spread the product over a certain surface. After phase transition at eye temperature, it is desirable for the gel to form a homogeneous layer on the corneal surface, thereby avoiding patient discomfort and blurred vision while facilitating drug diffusion. The formulations containing 1.0% or 1.5% w/w chitosan showed the highest compressibility values. These results, taken together with the higher hardness and compressibility of the 1.5% chitosan formulation at 25 °C, discourage its use in further *in vivo* studies.

Adhesiveness is commonly defined as the work necessary to overcome the attractive forces between the surface of the sample and the surface of the probe [42]. It is a desirable characteristic, as a higher adhesiveness value could imply greater adhesion at the tissue surface and increase the retention time [41]. Based on the results presented, formulations with at least 1.0% w/w chitosan could confer these desirable properties. We should note that although some authors reported this relationship between adhesiveness and efficacy [28,44], it has also been suggested that adhesiveness would be more appropriately applied as a comparative measure of adhesive affinity for non-mucous surfaces, e.g., the skin [40]. Thus, as the proposed formulation is intended for ophthalmic delivery, further *in vitro* mucoadhesive tests were performed in order to confirm the maintenance of chitosan mucoadhesive properties in poloxamer gels and to verify the influence of the chitosan concentration.

For all chitosan solutions alone, we observed a concentration dependence of the mechanical properties, but the differences between 0.5% and 1.5% w/w chitosan solutions were not as pronounced as when poloxamer was present. One possible

explanation for this fact is that chitosan may have increased poloxamer entanglements, thereby dramatically increasing the hardness and elastic modulus values of the formulation when this polymer is in its organized state at 35 °C.

3.3. In vitro mucoadhesive strength

Ocular mucoadhesion relies on the interaction of a polymer and a mucin coat covering the conjunctiva and corneal surfaces of the eye [16]. Chitosan was reported to be a linear polycation that readily adheres to negatively charged surfaces [45]. Interactions with mucin appear to be both electrostatic, via positively charged amino groups on the chitosan and negatively charged sialic acid residues of mucus glycoproteins or mucins, and/or hydrophobic, via methyl groups on acetylated chitosan residues with methyl groups on mucin side chains [46]. Nevertheless, factors such as polymer chain conformation and concentration can influence mucoadhesive performance [47,48]. In order to evaluate whether the chitosan mucoadhesive properties would be maintained even after incorporation into the poloxamer gels, which present a high mechanical strength at the eye temperature and to evaluate whether there is a chitosan concentration-dependent relationship, *in vitro* mucoadhesion tests were performed, with mucoadhesive force meaning the force required to detach the formulation from a mucin disc.

When formulations containing 16% w/w of poloxamer were used, fragments of the formulations were encountered on the mucin disc after detachment, indicating that the cohesive bounds within the sample were weaker than the mucoadhesive force [40,49]. For this reason, in order to verify the mucoadhesive property of chitosan as a function of chitosan concentration in poloxamer gels, formulations containing 18% w/w of poloxamer were used.

The *in vitro* mucoadhesion tests confirmed that the chitosan mucoadhesive properties were maintained even in a stronger gel (poloxamer 18% w/w) (Table 2). Furthermore, a concentration dependence on the force required to overcome the gel/mucin adhesive bounds was observed, which is in accordance with chitosan properties reported by others [50,51]. Statistical differences were observed for formulations containing 0.5%, 1.0% and 1.5% w/w of chitosan. Hence, chitosan confers mucoadhesive properties to the *in situ* gel.

3.4. In vivo scintigraph studies

Based on the results, it is possible to conclude that chitosan was able to form, in combination with poloxamer, an *in situ* gel. The formulation containing 1.5% w/w chitosan showed the highest mucoadhesive force and was able to form a gel at a lower frequency than the one containing 1.0% w/w after dilution with simulated tear fluid (Fig. 2). This finding could imply faster gelling under *in vivo* conditions, which could make drainage more difficult and prolong the retention time. On the other hand, the higher hardness and compressibility values presented by this formulation (Fig. 3) could make the administration and spreadability of the gel on the corneal surface difficult at 35 °C, leading to patient discomfort and possible blurred vision. The use of 1.5% w/w of chitosan is discouraged, and the combination that better fits the requirements for an acceptable ophthalmic delivery system is the one containing poloxamer/chitosan 16/1.0% w/w. This formulation presented an adequate $T_{sol/gel}$, and it was able to withstand a low shearing force at 35 °C. It also presented higher hardness (especially at 35 °C), adhesiveness and mucoadhesive force than did the other formulations, except the one containing 1.5% w/w. For those reasons, the formulation containing poloxamer/chitosan 16/1.0% w/w was chosen to be evaluated *in vivo*.

Gamma scintigraphy is a well-established technique for *in vivo* evaluation of ophthalmic retention time [52–54]. Although the rabbit is the traditional animal model for ophthalmic formulations evaluation [1,55,56], human volunteers are preferred for this study due to physiological differences between rabbits and humans, especially the blinking rate [57]. The ocular clearance profile of ^{99m}Tc labeled formulation is shown in Fig. 4. The curves of the remaining activities on the corneal surface as a function of time were plotted and are shown in Fig. 5.

No adverse or irritant effects (blinking, conjunctival redness or discharge) in the short or long term were observed. In Fig. 4, we notice that the gel formulation remained on the cornea surface (ROI) longer than did the saline solution; nevertheless, the distribution was not homogeneous. This *in vivo* performance, i.e., a difficulty in spreadability and prolonged retention time, confirms the relation of these factors with mechanical properties evaluated by texture analyses, i.e., compressibility and adhesivity (Fig. 3). For both gel and control, the activity remaining as a function of time (Fig. 5.) consisted of a rapid initial clearance phase followed by a slower basal drainage phase. After 2 min, approximately 65% of the gel formulation was still in contact with the cornea versus only 27% of the saline solution. At the end of the experiment, these val-

Table 2
The effect of chitosan concentration on the mucoadhesive property of poloxamer gels.

Concentration (% w/w)		Force (N)[a]
Poloxamer	Chitosan	
18	–	0.0836 ± 0.015
18	0.5	0.1007 ± 0.008
18	0.75	0.1045 ± 0.021
18	1.0	0.1052 ± 0.016
18	1.25	0.1111 ± 0.022
18	1.5	0.1275 ± 0.010

[a] Mean (±SD) of at least three replicate measurements.

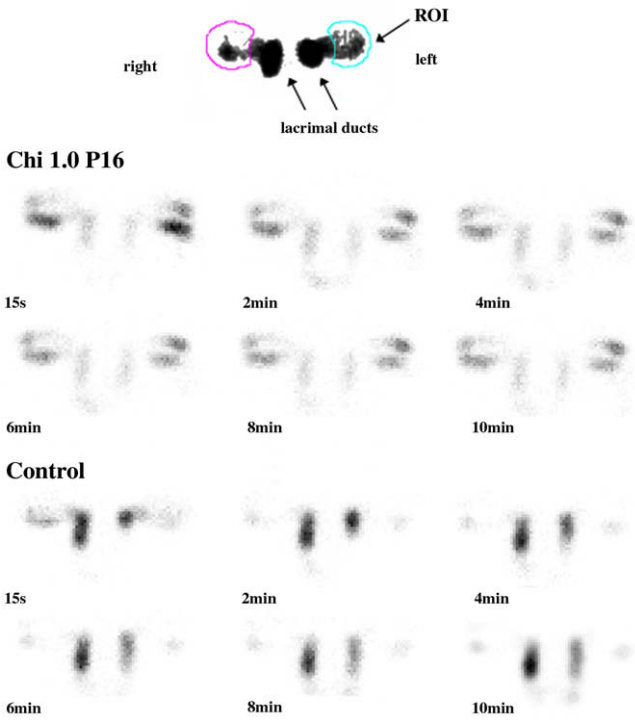

Fig. 4. Dynamic scintigraphic images of volunteers' eyes up to 10 min after administration of labeled poloxamer/chitosan *in situ* forming gel (Chi1.0P16) and saline control (control). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

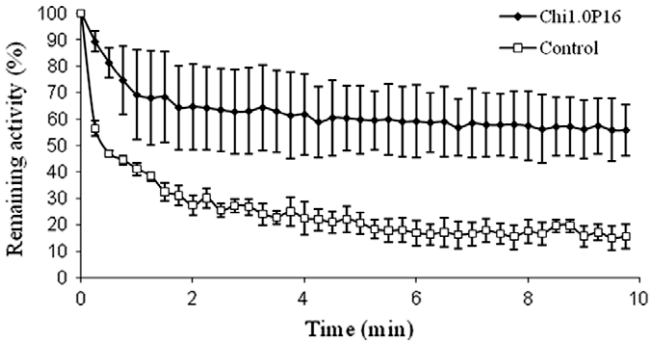

Fig. 5. Pre-corneal clearance of the poloxamer/chitosan *in situ* forming gel (Chi1.0P16) containing ^{99m}Tc. Labeled saline solution was used as control. Data show mean ± SD (n = 4).

ues were 50–60% for the gel and 15% for the saline formulation. The saline solution clearance is also evidenced by the high amount of radioactive tracer in the lacrimal ducts after the first few minutes (Fig. 4). The $AUC_{0\rightarrow 10min}$ was significantly greater when the gel was administered, compared to that of the aqueous control, with values of 610.50 ± 127.99 and 242.40 ± 35.36, respectively. The patients were allowed to freely move after the first 10 min and, after 30 min of instillation, another image was made. It was then possible to observe that the tracer was still present when the gel was applied (approximately 50%), but not when the solution was applied.

The human study confirmed the adequate $T_{sol/gel}$ of the formulation. It remained liquid when instilled, and no spillage was observed after the administration of 50 μl using a calibrated pipette. This volume corresponded to approximately one drop. After administration, the phase transition was observed in the conjunctival sac, which was triggered by eye temperature. The formulation seemed to be able to withstand the shearing forces in the eye, which probably contributed to the reduced lacrimal drainage observed. In addition, the electrostatic interactions between chitosan and the mucous layer at the eye surface may have contributed to longer retention time of the formulation. Thereby, this formulation could diminish the administration frequency of ophthalmic topical applied solutions.

Moreover, another aspect to be considered is that chitosan has been described as a penetration enhancer [58–60]. This property would be extremely useful in an ophthalmic formulation that already has a prolonged retention time in the eye. The resulting increased bioavailability and reduced need for frequent administration of the drug could lead to improved patient compliance [61]. In this way, further studies applying the *in situ* forming gel developed on drug delivery studies are currently under evaluation in our laboratory.

4. Conclusion

In this study, an *in situ* forming gel with improved mechanical and mucoadhesive properties, as well as improved retention time, was obtained by the combination of poloxamer and chitosan. We demonstrated that poloxamer/chitosan formulation in a concentration of 16/1.0% w/w showed an optimal gelation temperature (32 °C) and was able to withstand low shearing forces at 35 °C. The mechanical properties indicated that the formulation has a high hardness value (especially at 35 °C) and a high adhesiveness that may contribute to the retainment of the formulation at the administered site. The gels proved to possess a mucoadhesive ability that is influenced by chitosan concentration. Finally, gamma scintigraphy in humans confirmed a prolonged retention time of this formulation. Therefore, the developed delivery system seems to be a promising tool for ophthalmic use, as it is easily administered and shows a prolonged ocular contact time.

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