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Research paper

A novel poloxamers/hyaluronic acid *in situ* forming hydrogel for drug delivery: Rheological, mucoadhesive and *in vitro* release properties

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Abstract

The influence of hyaluronic acid (HA) on the gelation properties of poloxamers blends has been studied with the aim of engineering thermosensitive and mucoadhesive polymeric platforms for drug delivery. The gelation temperature (T_{gel}), viscoelastic properties and mucoadhesive force of the systems were investigated and optimised by means of rheological analyses. Poloxamers micellar diameter was evaluated by Photon Correlation Spectroscopy (PCS). Moreover in order to explore the feasibility of these platforms for drug delivery, the optimised systems were loaded with acyclovir and its release properties studied *in vitro*.

By formulating poloxamers/HA platforms, at specific concentrations, it was possible to obtain a thermoreversible gel with a T_{gel} close to body temperature. The addition of HA did not hamper the self assembling process of poloxamers just delaying the gelation temperature of few Celsius degrees. Furthermore, HA presence led to a strong increase of the poloxamer rheological properties thus indicating possible HA interactions with micelles through secondary bonds, such as hydrogen ones, which reinforce the gel structure. These interactions could also explain PCS results which show, in systems containing HA, aggregates with hydrodynamic diameters much higher than those of poloxamer micelles. Mucoadhesion experiments showed a rheological synergism between poloxamers/HA gels and mucin dispersion which led to a change of the flow behaviour from a quite Newtonian one of the separate solutions to a pseudoplastic one of their mixture. *In vitro* release experiments indicated that the optimised platform was able to prolong and control acyclovir release for more than 6 h.

Keywords: Poloxamer; Hyaluronic acid; Viscoelastic properties; *In situ* forming gel; Drug delivery; Thermosensitive gel; Mucoadhesion

1. Introduction

Several efforts in drug delivery field have been devoted to the development of semisolid carriers, particularly to viscoelastic hydrogels, which display micro and macroscopic properties suitably designed to ensure long residence times and controlled delivery of the active molecule(s). In this context, the use of carriers for the release of therapeutically active principles based on polymeric solution able to gel *in situ,* at temperatures close to the physiological one, appears to be very attractive. In fact, such a preparation is liquid at room temperature and can be easily administered or injected. Once the gelation occurs, high resistance to flow and prolonged permanence of the drug at the site of administration may be obtained. In general, the gelation of a polymeric solution can be triggered by a number of factors [1] such as variations of temperature, as for poloxamers [2] and ethyl/hydroxyethyl cellulose [3], pH, as for cellulose acetophthalate [4] and Carbopol [5], or the presence of cations, as for alginates [6]. Among them, a promising strategy appears to be a gelation triggered by a temperature change since platforms' properties can be easily tuned as a function of therapeutic needs and administration routes. In this con-

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text, thermosensitive amphiphilic block copolymers, namely poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO, Poloxamers), have been widely used in biomedical field, thanks to their ability to undergo phase reverse thermal gelation. Their self-assembling process occurs through micellization, which is characterized by two key parameters, i.e. critical micellization concentration (CMC) and critical micellization temperature (CMT). These parameters, which depend on poloxamer block composition (PEO/PPO ratio) and molecular weight (PEO and PPO block length), can be tailored to obtain materials with final properties suitable for a wide range of applications. Poloxamer gels have been widely investigated for drug delivery since they are relatively easy to manufacture and already widely employed in pharmaceutical fields being "generally regarded as safe" (GRAS) excipients. Nevertheless, the main drawbacks associated to poloxamer gels for drug delivery applications include a limited stability, poor mechanical properties and short residence times due to a rapid dissolution once placed in biological environments [7]. These drawbacks limit the suitability of these systems for most biomedical applications, so that many attempts were done to chemically modify poloxamer gels [8–11] to obtain gel with appropriate mechanical properties. Another interesting approach focuses on blends of poloxamers with mucoadhesive polymers [12–14] which are able to form entanglements or non-covalent bonds with the mucus covering epithelial tissues so prolonging platform *in vivo* residence time. In the literature, the use of various mucoadhesive polymers intended for different application sites such as ocular, oral cavity, vagina, stomach and intestinal mucosa has been reported [15–19]. However, modern research turned a very special interest to hyaluronic acid (HA), a naturally occurring mucoadhesive polymer which, besides being biodegradable, proved to be highly biocompatible [20]. HA is a natural polysaccharide that is a primary component of the extracellular matrix of the connective tissue, which regulates and controls several tissue physiological functions *in vivo*.

In this context, we have been designing novel thermosensitive drug delivery platforms with optimised mechanical properties and mucoadhesive force by blending two different poloxamers (F127 and F68) with HA. Platforms viscoelastic properties, gelation temperature and bioadhesive force were investigated and optimised by means of rheological analysis. Micelles dimensions of formulations with and without HA were measured by photon correlation spectroscopy. Then, in order to test the feasibility of these platforms for ocular drug delivery, the optimised formulations were loaded with acyclovir and its release studied *in vitro* under sink conditions in simulated tear fluid.

2. Material and methods

2.1. Materials

The series of different poloxamers (PEOa–PPOb–PEOa) are constituted through varying numbers of oxyethylene (a) and oxypropylene (b) units. Poloxamer F127 (a = 100 and b = 65) and F68 (a = 76 and b = 29) were purchased by Lutrol. Low molecular weight (150 kDa) HA was supplied by Fab (Abano Terme, Italy). Mucin type II was purchased from Sigma. Acyclovir was a gift from RECORDATI S.p.A (Milano, Italy).

2.2. Methods

2.2.1. Platform preparation

The formulations were prepared by dissolving different amounts of poloxamers F127 and F68 in water by mixing under continuous stirring at 4 °C until a clear solution was obtained. HA-containing formulations were prepared by adding HA to poloxamer blends, at room temperature, in different amounts to obtain concentration varying between 0.4% and 2% w/w. The compositions and the acronyms of the formulations tested are summarised in table 1.

2.2.2. Rheological characterization

The viscoelastic properties of the gels were assessed by small-amplitude oscillatory shear experiments using a rotational rheometer (Bohlin GEMINI). The experiments were performed at different temperatures (i.e. 25 and 43 °C well under and above TMC) with oscillation frequency ranging from 0.1 up to 10 Hz, and a strain amplitude at which linear viscoelastity is attained. As measuring systems, a couette geometry (SSC25) was used to test the samples below TMC, whereas a plate–plate geometry (PP15) was used for temperature above TMC. In the latter case, the samples were allowed to gelify between the rheometer plates. Thus, the shear storage or elastic modulus (G') as well as the shear loss or viscous modulus (G'') was measured as a function of frequency. G' gives information about the elasticity or the energy stored in the material during deformation, whereas G'' describes the viscous character or the energy dissipated as heat.

The gelification temperature of the polymeric systems was investigated by monitoring the variation of the elastic and viscous moduli with the temperature in the range from 10 to 40 °C, at a fixed frequency of 0.01 Hz in order to prevent any influence on the gelation process and at a strain amplitude where linear viscoelasticity is valid. Gelification temperature was identified as the temperature at which the sample exhibited a switch from a prevalently viscous

Table 1
Formulations prepared and tested*

Acronyms	F68 (% w/w)	F127 (% w/w)	HA (% w/w)
Pol-1	10	15	–
Pol-2	15	15	–
Pol-1/H05	10	15	0.5
Pol-1/H08	10	15	0.8
Pol-1/H1	10	15	1
Pol-1/H2	10	15	2
Pol-1/H1	15	15	1

* Compositions are referred as % w/w in water of each component.

behaviour ($G'' > G'$) to a prevalently elastic one ($G' > G''$). The analysis was repeated after mixing the optimised formulations with simulated tear fluid (STF) [21] prepared as reported below at 40:7 and 80:7 volume ratio to mimic an ocular administration through a conventional eye drop. During all the tests, the samples were placed into a chamber properly designed to avoid solvent evaporation.

2.2.3. Photon correlation spectroscopy

Photon correlation spectroscopy (PCS) was utilized to determine the micelle hydrodynamic diameter (DH) by means of a N5 Submicron Particle Size Analyzer (Beckman–Coulter). Polymeric blends were suitably diluted in Milli-Q water to achieve the optimal intensity (about 5×10^5 counts/s). Measurements were performed at 20° C on 90° angle. Results are reported as mean of three measurements ± standard deviation.

2.2.4. Mucoadhesion analysis

Mucoadhesive characteristics of the polymeric systems were evaluated through a method reported in the literature [22]. This method is based on the evaluation of the "Rheological synergism" existing between the mucoadhesive polymer and mucin. In particular, it represents a more than additive growth of the mixture viscosity that occurs when mucoadhesive polymers are mixed with mucin dispersions, depending on the interactions between the chains of the two macromolecular species. Viscosity of porcine gastric mucin dispersions (15% w/w) in 0.1 N HCl (pH 1) or 0.1 N acetate buffer (pH 5.5) was measured in absence (η_m) or presence (η_t) of polymeric mixtures in order to evaluate the mucoadhesive characteristics of our formulations in the presence of non-ionized or ionized hyaluronic acid (pH 1.0 and 5.5, respectively). Viscosity component of bioadhesion (η_b) was calculated from the equation

$$\eta_b = \eta_t - (\eta_m + \eta_p)$$

where η_p represents the viscosity of corresponding pure polymer solutions. The bioadhesive force can be calculated as follows:

$$F_b = \eta_b \times \gamma'$$

where γ' represents the shear rate at which viscosity value was calculated (10 s^{-1}).

2.2.5. In vitro release study

The polymeric platforms were loaded with acyclovir (3% w/w) by direct dispersion of the drug in the polymeric blend. Drug release profiles were evaluated in STF ($NaHCO_3$ 0.218 g, NaCl 0.678 g, $CaCl_2.2H_2O$ 0.0084 g, KCl 0.138 g in 100 ml of water) by means of cells designed to assess drug release from a gel ensuring sink conditions (fig. 1). In detail, the loaded polymeric platform was placed in a separate pan present at the bottom of a glass cell and covered by a lid. Then the cell was filled with STF and immersed in a thermostatic bath at 37 °C. When the temperature inside the cell reached 37 °C and the polymeric platform had gelified, the inner lid pan was removed to allow the contact between the gel and the STF. A magnetic

Fig. 1. Cells designed for drug release study.

stirrer into the cell provided a continuous agitation. At regular time intervals, 1 ml of solution was withdrawn from the cell and replaced with fresh STF. Acyclovir was quantified by HPLC using a Luna NH_2 column (250 × 4.6 mm) (Phenomenex) and a mobile phase composed of CH_3CN/H_2O (70:30, v/v). The flow rate and UV wavelength were 1 ml min^{-1} and 255 nm, respectively. Acyclovir retention time, expressed as $k' = (t_r - t_0)/t_0$, was 1,87 min.

3. Results

3.1. Rheological characterization

The gelation process of the formulations was evaluated by monitoring the variation of the viscoelastic parameters upon temperature. The elastic and viscous moduli of Pol-1 and Pol-2 as a function of temperature, at a frequency value of 0.01 Hz, are shown in figs. 2 and 3, respectively. The gelation temperature (T_{gel}) was identified as the temperature at which G' and G'' curves intersect each other.

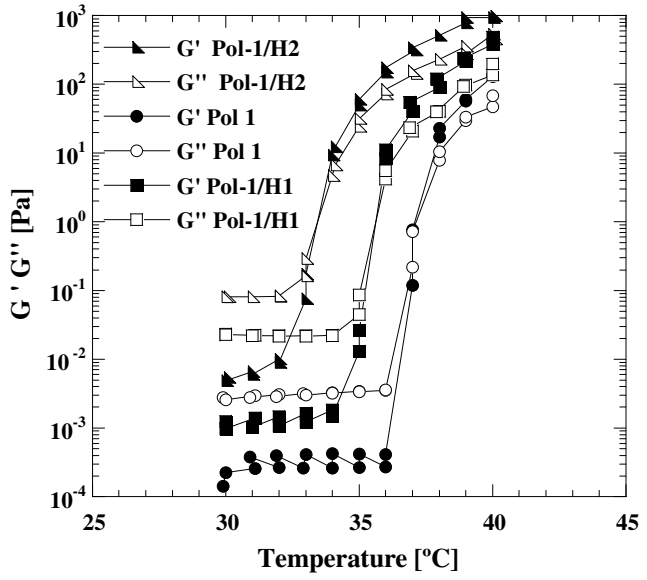

Fig. 2. Elastic and viscous moduli as a function of temperature of Pol-1, Pol-1/H1 and Pol-1/H2 at a frequency value of 0.01 Hz. Results are the means of three measurements. SD was always lower than 10%. Error bars are omitted for clarity purpose.

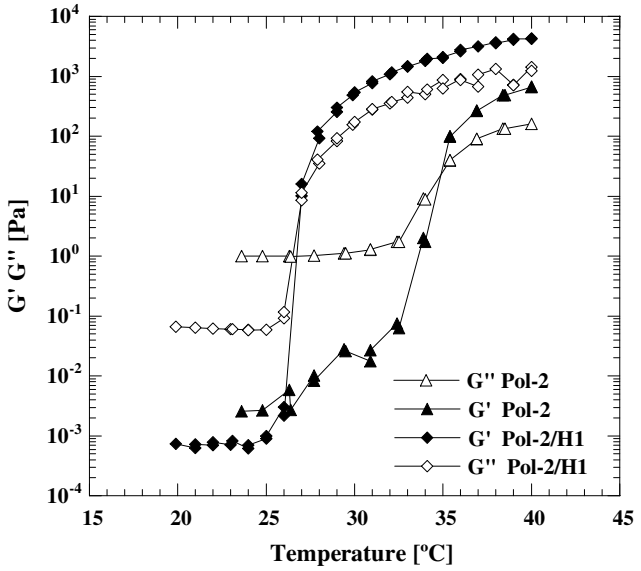

Fig. 3. Elastic and viscous moduli as a function of temperature of Pol-2 and Pol-2/H1 at a frequency value of 0.01 Hz. Results are the means of three measurements. SD was always lower than 10%. Error bars are omitted for clarity purpose.

Such a temperature corresponds to 35 °C for Pol-2 and 37 °C for Pol-1.

To formulate a drug delivery platform which is both thermosensitive and mucoadhesive, the thermosensitive properties of poloxamers were combined to the mucoadhesive skills of HA and its influence on poloxamers gelification process was evaluated on formulations containing 1% and 2% w/w of HA as reported in figs. 2 and 3. The presence of HA does not hamper the poloxamer gelation process and it only leads to a decrease of the gelation temperature of both Pol-1 and Pol-2. In particular, the influence of HA on gelation temperature is stronger on Pol-2 than on Pol-1 indeed, T_{gel} of Pol-1 decreased from 37 to 36 °C by adding 1% w/w of HA and to 34 °C by adding 2% w/w (fig. 2), while T_{gel} decreased from 35 to 27 °C by adding 1% w/w of HA to Pol-2 (fig. 3). The addition of HA to Pol-2 leads to a platform gelifying at about room temperature (24–28 °C) so that it was considered not suitable for drug delivery purposes, and only Pol-1 with different amounts of HA was considered for further investigations.

The mechanical properties of formulations were studied through small amplitude shear tests at temperature both below (25 °C) and above (43 °C) T_{gel}. The mechanical spectra, that is G' and G'' as a function of frequency, of Pol-1, at 25 and 43 °C, are shown in fig. 4. At 25 °C (fig. 4A), the rheological behaviour of the formulations is that typical of a viscous fluid, that is G'' is always higher than G' in all the frequency range analysed and the frequency dependence of the moduli is well described by a power law $G' \propto \omega^{1.7}$, $G'' \propto \omega^{0.98}$ with exponent close to that predicted for the viscous solution, i.e. 2 and 1 for G' and G'', respectively. Otherwise, at 43 °C (fig. 4B), Pol-1 elastic modulus is always higher than the viscous one and both the viscoelastic moduli are quite frequency-independent showing therefore a rheological behaviour characteristic of a gel-like material.

To investigate the influence of HA on the poloxamers viscoelastic properties, the mechanical properties of Pol-1 containing different amounts of HA (0.5%, 0.8%, 1% and 2% w/w) were studied. The elastic modulus values as a function of frequency of those samples at 43 °C are compared in fig. 5 whereas in table 2 the values of the elastic and viscous moduli at 0.5 Hz are reported. The addition of HA into the poloxamers formulation does not qualitatively alter the rheological behaviour of the system, which still appears typical of a gel-like material, even if a significant increase of both viscoelastic moduli is apparent. In particular, up to the addition of 0.5% w/w, the gel mechanical properties remain quite unchanged, while the addition of 0.8, 1 and 2% w/w leads to a 10, 30 and 50 time increase of elastic modulus values (table 2). Pol-1/H2 resulted to be

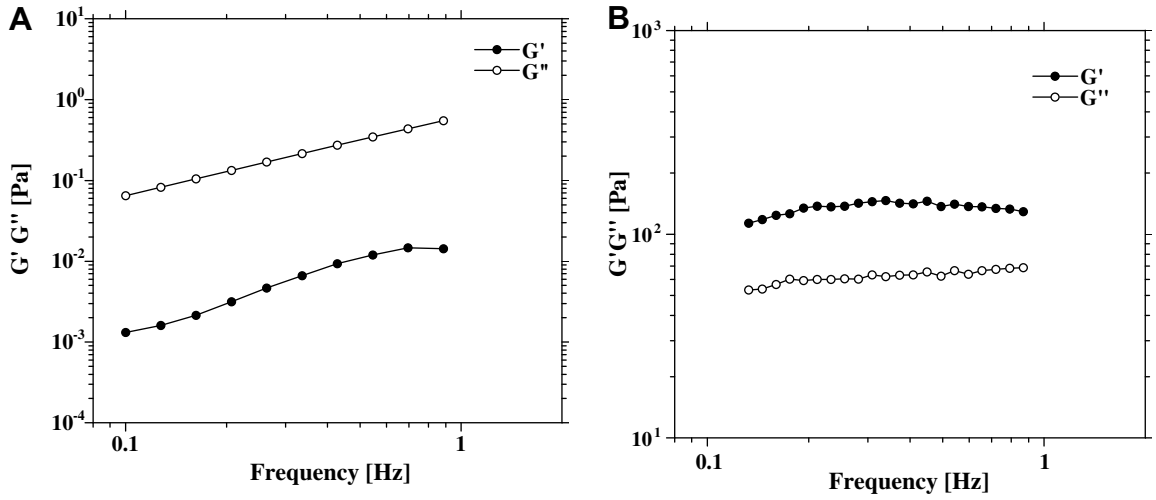

Fig. 4. Mechanical spectra of Pol-1 at 25 °C (A) and 43 °C (B). Results are the means of three measurements. SD was always lower than 10%.

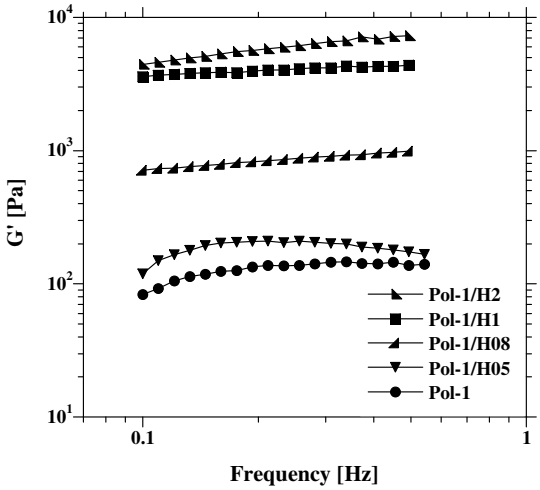

Fig. 5. Elastic modulus as a function of frequency of Pol-1 containing different amount of HA at 43 °C. Results are the mean of three measurements. SD was always lower than 10%. Error bars are omitted for clarity purpose.

Table 2
Hydrodynamic diameters, elastic and viscous moduli values (at 0.5 Hz and 43 °C) of Pol-1 containing 0.5%, 0.8%, 1% and 2% w/w of HA

	G' (Pa)	G'' (Pa)	DH (nm)
Pol-1	137	62	19.3
Pol-1/H05	174	71	168.7
Pol-1/H08	990	173	203.9
Pol-1/H1	4300	661	208.2
Pol-1/H2	7300	2720	551.9

Results are the means of three measurements. SD was always lower than 10%.

the best candidate as drug delivery platform since it presented optimised mechanical properties associated to a suitable T_{gel} close to body temperature. For this reason, Pol-1/H2 was chosen for mucoadhesion and drug delivery study.

3.2. Photon correlation spectroscopy

As it was shown by PCS data (Table 2), at 20 °C, Pol-1 forms micelles having a hydrodynamic diameter of 19.3 ± 1.5 nm, whereas the addition of HA to poloxamers blends, at 1% and 2% w/w, leads to the formation of aggregates with higher hydrodynamic diameters.

3.3. Mucoadhesion analysis

The flow curves, that is viscosity as a function of shear rate, of mucin dispersion, Pol-1/H2 and their mixture at pH values equal to 1 and 5.5 and $T = 37$ °C are shown in fig. 6. At both pH values, the presence of mucin in Pol-1/H2 strongly affected its shear flow behaviour. The mucus and Pol-1/H2 viscosity values indeed smoothly decrease with shear rate, while their blend shows a pseudoplastic behaviour being viscosity constant at low shear rates (Newtonian plateau) and decreasing sharply as the shear rate increases with a slope in a log/log scale of 0.04 at pH 1 and 0.09 at pH 5.5.

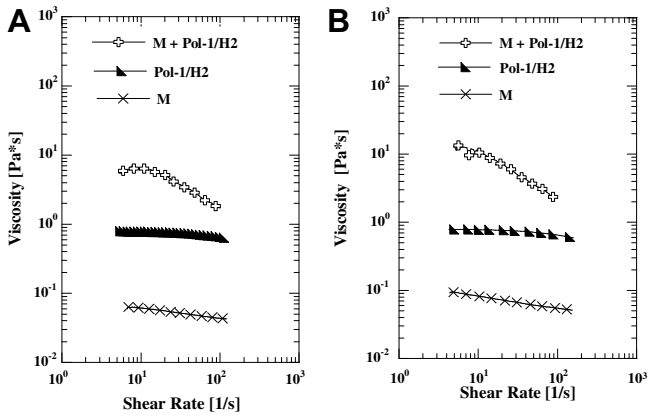

Fig. 6. Flow curves of mucin dispersion at 15% w/w (M), Pol-1/H2 and their mixture (M + Pol-1/H2) at pH 1 (A) and 5.5 (B); measurement were performed at 37 °C. Results are the mean of three measurements. SD was always lower than 15%. Error bars are omitted for clarity purpose.

The η_b and bioadhesive force values, calculated at a shear rate value equal to 10 s^{-1}, are summarised in table 3. As it can be noted, the viscosity values of the mixture are higher than the sum of the corresponding values of separate components at all the shear rates investigated. The bioadhesive bond between the formulation and mucin dispersion became stronger by increasing temperature and pH. In particular, at a shear rate equal to 10 s^{-1}, F value increases from 6.93 to 57.80 Pa passing from 25 to 37 °C at pH 1 and from 30.50 to 104.36 Pa at pH 5.5.

3.4. Platforms feasibility for ocular drug delivery

The effect of dilution in simulated tear fluid was investigated by analysing the thermogelation process of mixture made up of Pol-1/H2 with STF. In the temperature range investigated (from 20 to 40 °C) the gelation was not observed at 40:7 volume ratio, while T_{gel} was delayed only of 1 °C at 80:7 volume ratio (data not shown).

Drug release curves of acyclovir from Pol-1, Pol-1/H1 and Pol-1/H2 are depicted in fig. 7. As it can be seen, all the formulations are able to sustain acyclovir release for more than 6 h which is a time scale of interest for an ocular administration. Anyway, the release of the drug from these

Table 3
Viscosity (η_p, η_m, η_t, η_b) and F values calculated at a shear rate value equal to 10 s^{-1}

pH	Temperature (°C)	η_p (Pa*s)	η_m (Pa*s)	η_t (Pa*s)	F (Pa)
1	25	1.18	0.08	2.05	6.93
1	37	0.78	0.06	6.16	57.80
5.5	25	1.17	0.13	4.08	30.50
5.5	37	0.78	0.081	10.54	104.36

Results are the means of three measurements. SD was always lower than 15%.

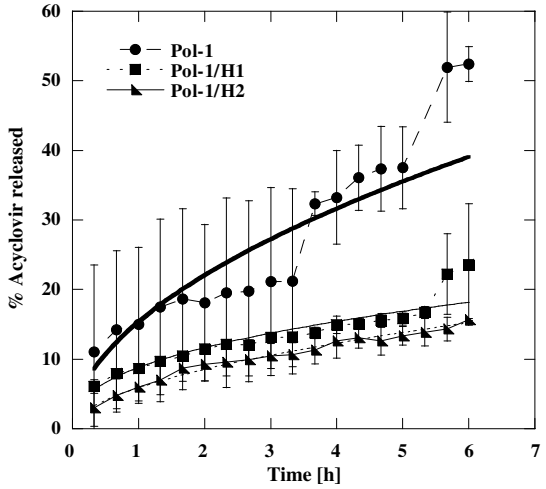

Fig. 7. Acyclovir release from Pol-1, Pol-1/H1 and Pol-1/H2 in simulated tear fluid at 37 °C. Results are the means of three measurements ± SD.

gels is complete within 100 h (data not shown). In particular, the release kinetic of acyclovir from Pol-1 is the fastest and, above all, poorly controlled. By adding HA from 1% to 2% w/w into the poloxamer blend it was possible to achieve more reproducible drug release kinetics and so a more accurate control over drug release from the polymeric platform.

4. Discussion

The aim of this work was to formulate thermosensitive and mucoadhesive polymeric platforms made up of poloxamer blends (F68 and F127) and low molecular weight HA with appropriate T_{gel}, viscoelastic properties and bioadhesive force. A possible application of these systems concerns drug delivery systems which are able to increase the drug residence time and bioavailability, thus overcoming the drawbacks associated to the use of conventional dosage forms. In particular, the feasibility of these platforms for an ocular drug delivery was explored by loading Acyclovir and studying its *in vitro* release in simulated tear fluid.

Separate poloxamer F127 or F68 solutions do not possess the appropriate gelling temperature for their application as drug delivery system since, in the concentration range from 10% up to 30% w/w, they present gelling temperatures higher than 40 °C or lower than 25 °C, respectively. By selecting poloxamer mixtures at specific concentrations, it is possible to modulate the gelation temperature and to obtain a T_{gel} suitable for this kind of application, i.e. near to body temperature (≈37 °C). In particular, the results collected here show that Pol-1 and Pol-2 present a T_{gel} of 37 and 35 °C, respectively.

The addition of mucoadhesive polymers to poloxamers gels is a widely explored strategy to improve drug residence time into the administration site and, among them, a special interest is devoted to naturally occurring polysaccharides due to their high biocompatibility. Our results show that the addition of low molecular weight HA into poloxamers blends leads to an improvement of gel mechanical properties with an increase of poloxamer gels strength which is related, not linearly anyway, to the amount of HA added. This indicates that some interactions between HA and poloxamers could occur during poloxamers gelification process which is ascribed to a significant microstructural change of the sample [23,24]. First, individual block copolymer molecules self-assemble into micelles in aqueous solution at block copolymer concentrations above CMC. By increasing the temperature, polymeric micelles overlap each other and pack together in ordered lattice domains by means of hydrophobic interactions of PPO blocks. Any solution component residing at the interface between micelles and water may affect the hydration of the PEO groups at the outer edge of the micelle corona and thus change the gelation temperature or even the formation of the gel [25]. Low molecular weight HA, in the concentration range considered in this study, behaves as a viscous solution so that the molecules can be considered as individual flow units having a reduced steric hindrance. In these conditions, HA molecules allow micelle movements and packing and could interact with micelles through secondary bonds, such as hydrogen ones, thus reinforcing the structure and so the mechanical properties of the final physical gel. The possibility of interactions between HA random coils and poloxamers micelles was confirmed by PCS results which show, in formulations containing HA, aggregates with hydrodynamic diameters values much higher than those of poloxamers micelles. A recent study showed that the addition of high molecular weight HA into a poloxamer blend caused a decrease of the final gel strength, so leading to a faster release of the drug from the gel [26]. We found that the improvement of the viscoelastic properties of poloxamers gel is related, not linearly anyway, to the amount of HA added. The opposite results found in this study can be explained considering that high molecular weight HA solution at 1% to 2% w/w behaves as an entangled network in which topological interactions exist among the molecules [27,28]. In these circumstances, micelles movements and packing are hampered and so the poloxamer gelation process is compromised. A similar finding was observed for fibrillogenesis of collagen gels that was altered by the presence of high molecular weight HA, while it was not in the case of low molecular weight HA [29].

The bioadhesion experiments show a rheological synergism between the formulation and a mucin dispersion which leads to a change of the flow behaviour from a quite Newtonian one of the separate solutions (mucin and Pol-1/H2) to a pseudoplastic one of their mixture. This indicates the formation of both molecular entanglements and secondary chemical bonds between the mucoadhesive polymer and mucus glycoproteins. In particular, the bioadhesive force of Pol-1/H2 increases by increasing pH thus showing that the ionization of HA improves the bioadhesive characteristics of Pol-1/H2. As a general rule, when dealing with

mucoadhesive polymer solutions, the kinetic energy of polymer chains increases as temperature does, giving rise to a decrease of viscosity and so to a weaker bioadhesive force. In the case of the formulation developed, this effect is compensated by the gelation of the system. As a matter of fact, at body temperature, namely the one at which the system must work, the bioadhesive force is higher than that at ambient temperature.

Envisaging an application of the platform to ocular drug delivery, the dilution of Pol-1/H2 with a simulated tear fluid volume usually held into the eye, was demonstrated to not affect the gelation of the system. One of the main drawbacks that limits the use of poloxamer gels as platforms for drug delivery is that they undergo a rapid dilution when exposed to large volumes of aqueous solutions [30,31]. This causes a poor control over drug release rate which is, generally, too fast. The *in vitro* drug delivery experiments designed to assess release kinetics from a thermosensitive gel, showed that Pol-1/H2 was able to prolong and control acyclovir release for more than 6 h while drug release kinetics from the starting poloxamers platform (Pol-1) result too fast and not reproducible. Drug release from these thermosensitive gels takes place through a combined diffusion/dissolution mechanism and the ability of low molecular weight HA to reinforce the poloxamers gel structure is reflected in gel ability to better control and prolong acyclovir release. Finally, the release kinetic of the drug from the system could be further improved *in vivo* by mucoadhesive interactions of the formulation with ocular tissue. These encouraging results, that need to be further assessed by *in vivo* experiments, lead to foresee for a sustained ocular drug delivery through this system.

5. Conclusions

This study demonstrates that the addition of low molecular weight HA into poloxamers blends can be considered an useful tool to engineer thermosensitive and mucoadhesive polymeric platforms for sustained drug delivery. On the basis of their viscoelastic properties, mucoadhesive force, gelation behaviour in simulated tear fluid and *in vitro* release properties, poloxamer/HA gels are expected to be considered for a wide range of applications in ocular delivery.

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