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Synergistic interaction between TS-polysaccharide and hyaluronic acid: Implications in the formulation of eye drops

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ABSTRACT

An interaction between tamarind seed polysaccharide (TSP) and hyaluronic acid (HA) in aqueous solution has been ascertained. Various TSP/HA mixtures have been studied as the basis for the development of a potential excipient for eye drops synergistically improved over those of the separate polymers. Information about the nature of interpolymer interactions, and their dependence on TSP/HA ratios were obtained by NMR spectroscopy in solution. Superior mucin affinity of TSP/HA mixtures with respect to the single polysaccharides was assessed by NMR proton selective relaxation rate measurements. The mucoadhesivity of the TSP/HA (3/2) mixture, evaluated *in vitro* by NMR or viscometry, and *in vivo* by its mean and maximum residence time in rabbit precorneal area, is stronger than that of the component polysaccharides or the TSP/HA mixtures of different composition. TSP/HA (3/2) is little viscous and well tolerated by rabbit eyes. It stabilizes the tear film, thereby prolonging the residence of ketotifen fumarate and diclofenac sodium in tear fluid, but is unable to permeabilize the cornea. In conclusion, mucoadhesivity is responsible for the TSP/HA (3/2) synergistic enhancement of either extra- or intra-ocular drug bioavailability.

1. Introduction

Topical treatment of extra- or intra-ocular diseases, especially by eye drops, is the best accepted option by the patients. Treatment with medicated eye drops, however, poses the issue of a poor bioavailability because the precorneal area, i.e., the site of drug action/absorption, is rapidly cleared of drugs by protective mechanisms of the eye, such as blinking, basal and reflex tearing, and nasolacrimal drainage. This implies the need of frequent instillations, and hence, the risk of side effects associated with the drugs administered by eye drops. Increasing ocular bioavailability remains a stimulating challenge for the formulators of topical systems. An approach to the task has been the reduction of drainage rate by increasing the viscosity of the preparation or resorting to mucoadhesive polymers (Chrai and Robinson, 1974; Patton and Robinson, 1975; Trueblood et al., 1975; Saettone et al., 1982b, 1984; Hui and Robinson, 1985; Lee and Robinson, 1986; Wilson, 2004; Ali and Lehmussaari, 2006). The ability of a polymer to prolong the drug contact time with the ocular surface by adhering to it and concurrently binding the drug is a more promising property than the polymer viscosity-enhancing power (Di Colo et al., 2009). Indeed, fluid solutions are better tolerated than viscous ones (Winfield et al., 1990). A prolonged precorneal residence can result in improved drug intra- or extra-ocular bioavailability, depending on whether the drug is absorbed into the anterior chamber or not. The intra-ocular bioavailability can be enhanced whichever the drug absorption pathway, i.e., paracellular or transcellular. Recently two polysaccharides of natural origin, namely, tamarind seed polysaccharide (TSP) and hyaluronic acid (HA), have been shown by both *in vitro* and *in vivo* tests to be mucoadhesive at the ocular surface of rabbits, thereby stabilizing the tear film and prolonging the retention of ophthalmic drugs of different chemical nature, such as ketotifen fumarate (KT) and diclofenac sodium (DS), in the precorneal area (Di Colo et al., 2009). As shown in Fig. 1 TSP is a non-ionic, neutral, branched polysaccharide consisting of a cellulose-like backbone carrying xylose and galactoxylose substituents (Gidley et al., 1991; Burgalassi et al., 2000; Rolando and Valente, 2007), while HA is known to be a polyanion alternating 2-acetamide-2-deoxy-β-D-glucopyranose and β-D-glucopyranuronic acid residues.

A possible interpolymer TSP–HA non-covalent interaction may generate an excipient for eye drops, composed of a mixture of these polysaccharides, having synergistically improved properties over those of the separate polymers. Ascertaining the above possibility

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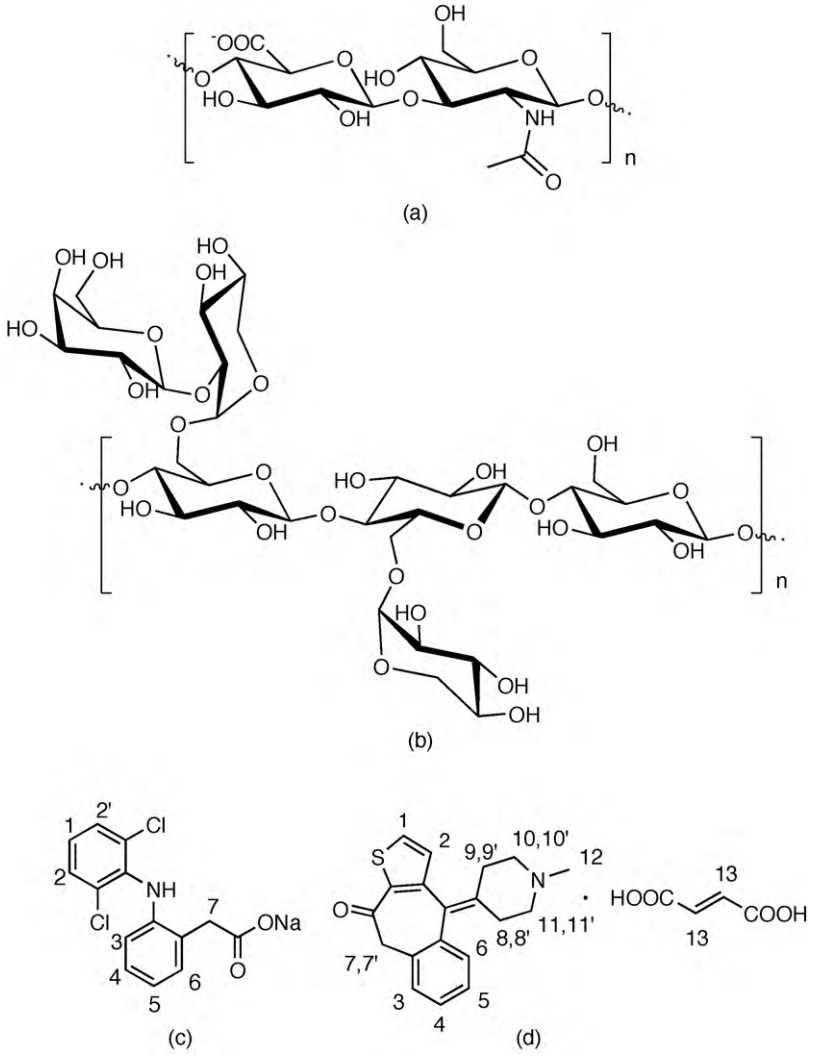

Fig. 1. Chemical structures of: (a) hyaluronic acid (HA); (b) tamarind seed polysaccharide (TSP); (c) diclofenac sodium (DS); and (d) ketotifen fumarate (KT);

and evaluating the composition of the mixture corresponding to the strongest interaction and optimal biopharmaceutical properties have been the fundamental purposes of the present work.

NMR spectroscopy is a very powerful technique to monitor structural transformations of macromolecules affecting their motional properties. Relaxation features of the NMR active nuclei are highly sensitive indicators of such transformations (Hills et al., 1991; Cowman et al., 1996). Longitudinal selective relaxation rates (R_1^{ms}) and cross-relaxation rates (σ_{ij}), which describe the magnetization transfer between proton pairs *ij*, are highly sensitive to slowing down of fast moving small molecules when they interact with slowly moving macromolecules (Neuhaus and Williamson, 1989; Valensin et al., 1986). σ_{ij} is a function of the reorientational correlation time τ_c of the vector connecting the two spins *i* and *j* and of their distance r_{ij}. Hence, σ_{ij} values for proton pairs at fixed distances can be usefully correlated to τ_c, which is a parameter very sensitive to drug–macromolecule interaction. The mucoadhesive properties of polymers can be analyzed by evaluating their interaction with mucin. Drugs having high affinity with mucin can be used as probes to detect such an interaction because they are sensitive to any variation in solution due to interaction phenomena between polysaccharides and mucin. In this work, two model drug compounds were selected, namely, KT, which interacts with TSP with good affinity, whereas its interaction with HA is significantly weaker (Uccello-Barretta et al., 2008), and DS (Di Colo et al., 2009).

Accordingly, R_1^{ms} and σ_{ij} values of drug protons were measured in the presence of bovine submaxillary mucin (BSM) and different TSP/HA ratios, in order to detect any variation in the drug–BSM affinity due to the presence of polysaccharides. Furthermore, longitudinal or transversal relaxation parameters of selected TSP or HA proton nuclei have been used to evidence the interaction between these polysaccharides and the dependence of this interaction on the composition of their mixtures. Also solvent (water) molecules have been employed as a probe for studying the above interactions. In fact, following interaction with polysaccharides water undergoes significant slowing down of its molecular motion.

The connection between the results of the above NMR studies and the potential of the TSP/HA mixtures as excipients for ophthalmic formulations was investigated *in vivo* by determining and comparing the time of residence of each polysaccharide mixture in the precorneal area of albino rabbit eyes. The precorneal retention time is influenced by the effects of environmental factors, such as, e.g., reflex tearing possibly induced by the polymers, on the mucoadhesion strength. This makes the comparisons more significant, from a practical standpoint, than those based on *in vitro* or *ex vivo* mucoadhesivity measurements. However, comparisons based on precorneal clearance may be biased by the viscosity of the applied polymer solution which may not be correlated with the polymer mucoadhesivity while still influencing the clearance. Therefore care was taken to distinguish mucoadhesion from vis-

cosity effects. To this purpose the rank order of polymer precorneal clearance, resulting from *in vivo* tests, was compared with that of mucin interactivity with TSP/HA mixtures. The latter was determined by an *in vitro* method based on the measurement of the effect of mucin interaction with polymers on the viscosity of a system of porcine gastric mucin and polysaccharides in solution (Hassan and Gallo, 1990). Such a method suffers from several limitations, as highlighted by Hägerström and Edsman (2003), nevertheless it has been used for its inherent simplicity and also because it has been profitably adopted by other authors in recent years (Burgalassi et al., 2007; Di Colo et al., 2009). The results from this *in vitro* procedure, in conjunction with those from the *in vivo* tests and the NMR studies were expected to provide reliable indications on the most suitable, of the TSP/HA mixtures under study, to be introduced into eye drops as an extra-ocular drug bioavailability-enhancing excipient. In order to materialize such indications the rabbit model has been used to assess the effects of the polysaccharide mixtures on the time of residence of KT or DS in tear fluid. Each of these drugs is the active ingredient of commercial eye drops, the former for the treatment of allergic conjunctivitis, the latter to treat inflammation and swelling of the eye following cataract surgery. Molecular drug–polymer binding is of great relevance to the drug residence time in tear fluid. Such a binding has been quantified for the actual drug–polymer proportions in the ophthalmic formulations by the dynamic dialysis method. This basically consists in measuring the effect of polymers on the drug permeation rate across a membrane permeable to the drug, impermeable to the polymer (Bottari et al., 1975).

It must be recognized that the precorneal clearance determined in rabbits is not representative of that in humans, mainly due to differences in blinking frequency (Saettone et al., 1982a,b; Zaki et al., 1986; Greaves and Wilson, 1993). Such differences may be reflected in differences in shear-thinning of tear film, mucoadhesion of polymer and ultimately in drainage of drugs. Nevertheless, the effects of blinking can be considered to be similar for the different preparations tested, then the rabbit model is deemed robust for the comparative purposes of the present work. Also the possible effect of a selected TSP/HA mixture to modify the corneal permeability to either KT or DS has been investigated *ex vivo* using the excised rabbit corneas, by a previously described procedure (Zambito et al., 2007).

2. Materials and methods

2.1. Materials

Ketotifen fumarate (Sifavitor S.p.A., Lodi, Italy), diclofenac sodium (Corden Pharmachem NV, Landen, Belgium), tamarind seed polysaccharide, MW 700 kDa (Opocrin S.p.A., Modena, Italy), hyaluronic acid, MW 950 kDa (Contipro, Dolní Dobrouč, Czech Republic) all were kindly gifted by Farmigea S.p.A. (Pisa, Italy). Fluorescein isothiocyanate (FITC) was purchased from Fluka. Porcine gastric mucin type III and bovine submaxillary mucin type I-S were purchased from Sigma. All other chemicals and solvents were of reagent grade.

2.2. NMR studies

^{1}H NMR measurements were performed on a spectrometer operating at 600 MHz. The temperature was controlled to $25 \pm 0.1\,^{\circ}$C. The longitudinal selective relaxation rates were measured in the initial rate approximation (Freeman and Wittekoek, 1969) using the inversion recovery sequence with a selective 180-pulse at the selected frequency. Transverse proton relaxation rates were measured by using the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence using a pulse spacing of 1 ms for polysaccharide signals and 10 ms for water signal.

The solutions for the NMR studies were prepared by mixing different volumes of stock solutions of the appropriate amounts of macromolecules and/or the drug in D_2O. The drug concentration was 2 mM in all samples.

2.3. FITC-labelling of polysaccharides

The following procedure was used for labelling polysaccharides with FITC (Clausen and Bernkop-Schnürch, 2000, 2001; Di Colo et al., 2009). A solution of FITC in dimethyl sulfoxide (1 mL, 2 mg mL^{-1}) was added to an aqueous solution of TSP, or HA (20 mL, 2 mg mL^{-1}) and the mixture was incubated at 4 °C for 8 h. The solution was then passed through a column of Sephadex G15 in order to clear the labelled polymer of non-reacted FTIC, then it was lyophilized. In no case did the Sephadex column retain any fluorescence, in all cases indicating the absence of non-reacted FITC and the complete labelling of polymer. Hence the fluorophore bound to the polymer could be calculated at 5% of the total mass (0.13 mmol g^{-1}).

2.4. Preparation of ophthalmic drops

For comparing the polysaccharides for their ability to adhere to the ocular surface non-medicated ophthalmic drops containing FITC-labelled TSP–HA mixtures at the total concentration of 5 mg mL^{-1} in isotonic phosphate buffered (0.0375 M) saline pH 7.4 (PBS) were prepared. The polymers were in the following wt ratios:

a. TSP/HA (1/4);
b. TSP/HA (2/3);
c. TSP/HA (3/2);
d. TSP/HA (4/1).

Medicated ophthalmic drops were also prepared, containing 0.7 mg mL^{-1} KT or 1 mg mL^{-1} DS in the above non-medicated ophthalmic drops prepared with non-FITC-labelled TSP/HA mixtures. The drugs were dissolved in the vehicle before adding the polymers. For control, polymer-free ophthalmic drops containing 0.7 mg mL^{-1} KT or 1 mg mL^{-1} DS in PBS were prepared. The isotonicity of ophthalmic drops was checked by a microosmometer (Hermann Roebling, Berlin, Germany).

2.5. Viscosity measurements

Rheograms of non-medicated ophthalmic drops prepared as described above with non-FITC-labelled polymers were recorded at 35 °C with a Haake RS1 rheometer equipped with coaxial cylinders Z40 (rotor) and Z41 (stator). Data were acquired and analyzed using Rheo Win Pro software (Haake). Means of at least three measurements are reported in Table 7. The coefficient of variation of measurements never exceeded 0.4%. All solutions showed a pseudoplastic behaviour (rheograms not shown). For these systems the viscosity values were measured at the shear rate of 200 s^{-1} because at rates of this magnitude the dependence of viscosity on shear rate was minimal in all cases tested. Viscosity measurements showed insignificant differences between FITC-labelled and unlabelled polymers.

2.6. In vitro comparative evaluation of mucoadhesivity of polysaccharide mixtures

According to Hassan and Gallo (1990) the viscosity coefficient, η, of a hydrophilic dispersion of mucin and a mucoadhesive polymer results from the additive contributions of the viscosity coefficients of mucin, η_m, and polymer(s), η_p, and a viscosity component due

to mucin interaction with polymer(s), η_{mp}. After measuring η, η_m and η_p the interactive component was calculated as:

$$\eta_{mp} = \eta - \eta_m - \eta_p \quad (1)$$

η_{mp} values, determined at the rate of shear of 200 s^{-1}, were used for a comparative evaluation of the mucoadhesivity of polysaccharide mixtures.

Dispersions containing 15% (w/v) mucin and the polymer mixtures TSP/HA (1/4), TSP/HA (2/3), TSP/HA (3/2) or TSP/HA (4/1), already described in Section 2.4, were tested. The dispersions were prepared by adding 2 mL of polymer solution, at fourfold the final concentration, to 6 mL of a 20% (w/v) mucin dispersion in the same solvent. The viscosity of each polymer mixture in the absence of mucin (η_p) was measured at polymer concentrations corresponding to those in the mucin–polymers system. The mucin–polymers systems and the dispersion of mucin alone showed pseudoplastic rheological behaviour. Their viscosity coefficients were measured at 35 °C by the Haake RS1 rheometer at the shear rate of 200 s^{-1}, i.e., the same as for the pseudoplastic polymers alone.

2.7. Measurement of elimination kinetics from tear fluid of rabbits

Male New Zealand albino rabbits weighing 3.0–3.5 kg were used. They were treated as prescribed in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 92-93, revised 1985). Prior to the experiments the animals were housed in standard cages in a light-controlled room at 19 ± 1 °C and 50 ± 5% relative humidity, with no restriction of food or water. During the experiments the rabbits were placed in restraining boxes, where they could move their heads and eyes freely. All experiments were carried out under veterinary supervision, and the protocols were approved by the Ethical Scientific Committee of the University of Pisa. For studying the elimination kinetics of KT, or DS, or the non-medicated polysaccharide mixtures from the tear fluid of rabbits following instillation of ophthalmic drops both eyes of a total of 12 animals were used. For each case studied six elimination curves were obtained, each determined in a single eye of different animals following the relevant procedure described below. Overall, each eye was used for determining 3–4 elimination curves at intervals of at least three days between successive determinations. The irritation caused in the rabbit eyes by the ophthalmic drops was evaluated according to the modified Draize test described by Lallemand et al. (2005).

2.7.1. Elimination kinetics of polysaccharides

The non-medicated ophthalmic drops TSP/HA (1/4), TSP/HA (2/3), TSP/HA (3/2) or TSP/HA (4/1), described in Section 2.4, were tested. One drop of each solution (50 μL) was instilled into the lower conjunctival sac by a Gilson pipette with care to avoid spillage. Tear fluid samples were collected at various intervals from the lower marginal tear strip using 1.0-μL disposable glass capillaries (Microcaps, Drummond Scientific Co., Broomall, PA), which were flushed with 1.0 μL of water. After further dilution with 100 μL of water the samples were analyzed for the polymers. For each polymer mixture six elimination curves were obtained, each determined in a single eye of different animals by withdrawing tear fluid samples at 2, 5, 10, 15, 20, 25, 30, 45, 60, 90, 120 min from instillation. The analysis of samples was carried out by a fluorimetric method (spectrophotofluorimeter Perkin-Elmer LS 45). The excitation was at 494 nm, the emission at 510 nm. For each polymer mixture a calibration curve was constructed using six standards in the concentration range of 0.05–0.5 μg mL^{-1}. In all cases the fluorescence *vs.* concentration plot was linear in the concentration range of the standards ($r^2 > 0.99$).

2.7.2. Elimination pharmacokinetics in the presence of polysaccharides

The ophthalmic drops medicated with KT or DS, as described in Section 2.4, were tested following the procedure described above for the non-medicated ones. The withdrawn tear fluid samples were diluted with 50 instead of 100 μL of water because the HPLC methods used for the analysis of the drugs have detection limits higher than the fluorimetric method used for the polysaccharides. The HPLC methods were described in a previous report (Di Colo et al., 2009). Tear fluid samples were withdrawn at 2, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35 min from instillation.

2.8. Elimination data treatment

The concentration in tear fluid (C_{TF}) *vs.* time data, obtained with the non-medicated and the medicated ophthalmic drops as described in the above sections, were used to calculate the mean residence time of each polysaccharide mixture, or drug, in tear fluid (MRT) according to the relevant non-compartmental technique (Gibaldi and Perrier, 1982). This parameter resulted from the ratio, AUMC/AUC, between the area under the $C_{TF}t$ *vs.* t curve, i.e., area under momentum curve (AUMC) and the area under the C_{TF} *vs.* t curve, i.e., area under curve (AUC). AUMC and AUC were calculated by the linear trapezoidal rule, between time 0 and the time when C_{TF} dropped below the minimum quantifiable value. For each elimination curve the corresponding MRT was calculated, thus, for each case studied 6 MRT values were obtained, of which mean and SE were calculated. Difference significance between two means was evaluated by the Student's t-test ($P < 0.05$).

With the medicated ophthalmic drops also the maximum residence time of the drug at quantifiable concentrations in tear fluid (RT_{max}) was reported. This time corresponded to the last point of the C_{TF} *vs.* time plot for the drug. In this plot for each time interval the mean of 6 C_{TF} values obtained with different animals was reported. The minimum quantifiable C_{TF} value was 1.1 μg mL^{-1} for KT, 1.5 μg mL^{-1} for DS, considering the necessity to dilute the withdrawn samples at least 1:50 (v/v).

2.9. Measurement of drug–polymer binding in the ophthalmic drops

A previously described method, based on the dynamic dialysis (Bottari et al., 1975; Uccello-Barretta et al., 2008), was used to determine the binding of each drug to each polymer mixture in the ophthalmic drops. Drug flux through a porous cellulose membrane permeable to the drug, impermeable to the polymers (Spectra/Por®, cut-off 3500 Da, Spectrum Laboratories Inc., Rancho Dominguez, CA) under quasi-steady-state conditions was measured at 35 °C in the presence or absence of the polymers in the donor phase. The polymer mixture and initial drug concentrations in the donor were equal to those used in the *in vivo* tests for the determination of the elimination kinetics of drugs in the presence of the polysaccharide mixtures. The composition of the donor medium was the same as that of the ophthalmic drops under study, with an ionic strength similar to that of the lachrymal fluid. Sink conditions were ensured in the receptor medium, which contained the same solutes at the same concentrations as the donor, except for the permeant and the polymers, in order to prevent volume variations due to osmosis. The receptor was spectrophotometrically analyzed for KT, at 301 nm, or DS, at 276 nm. The regression for the fitting of dialysis data, expressed as permeant concentration in the donor *vs.* time, to 1st order kinetics was always significant ($r^2 \geq 0.99$, $n \geq 8$). This allowed calculation of the dialysis rate constant. Under the above experimental conditions, a reduction of the dialysis rate constant caused by a polymer mixture was considered a sign and a measure of drug binding to such a mixture. The fraction

of bound permeant, f_B, was expressed by the following equation (Uccello-Barretta et al., 2008):

$$f_B = 1 - \frac{k_p}{k_a} \quad (2)$$

where k_p and k_a represent the dialysis constants in the presence and in the absence of polymers, respectively. Values of f_B were calculated, by Eq. (2), only for those cases where k_p and k_a were significantly different on the basis of the Student's t-test ($P < 0.05$).

2.10. Measurements of drug permeation across excised rabbit cornea

These measurements were carried out following a previously described procedure (Zambito et al., 2007). An account of the animals used and their treatment is given in Section 2.7. The rabbits were euthanized with intravenous pentobarbital (Pentothal sodium, Farmaceutici Gellini, Aprilia, Italy). The eyes were proptosed, and the corneas, with a 2 mm ring of sclera, were immediately excised and mounted in perfusion cells fabricated according to Camber (1985). The corneal area available for diffusion was 0.78 cm^2. The cell was maintained at 35 ± 1 °C. Preheated glutathione bicarbonate Ringer buffer pH 6.8 (GBR) was added to both donor (1.0 mL) and receptor (3.0 mL) compartments. To ensure oxygenation and agitation, an O_2–CO_2 (95:5) mixture was bubbled through each compartment at a rate of 3–4 bubble s^{-1}. After 10-min equilibration, the solution in the donor side was replaced with 1.0 mL of a solution of the test substance, either KT (0.7 mg mL^{-1}) or DS (1 mg mL^{-1}) in GBR (control), or of such a solution containing the TSP/HA (3/2) mixture (5 mg mL^{-1}). At appropriate time intervals, 100 μL of the receptor solution was withdrawn for analysis, and replaced with an equal volume of fresh preheated buffer. The analysis for KT or DS in the withdrawn samples was carried out by the HPLC methods described by Di Colo et al. (2009). Each experiment had a 4.0-h duration, and was repeated at least six times. At the end of each permeation run the cornea was removed from the perfusion apparatus and the percent corneal hydration level was evaluated by measuring the total water content of the cornea by desiccation. After carefully removing the remaining sclera the trimmed cornea was gently blotted dry and the wet corneal weight (W_w) was determined (10^{-5} g). The sample was then desiccated in an oven at 100 °C for 6 h after which it attained a constant weight (dry corneal weight, W_d). The percent corneal hydration level (HL) was calculated as $[1 - (W_d/W_w)]100$. Each of the HL values listed in Table 9 is the mean of 6 corneas (three rabbits). All data in the table were obtained with the corneas from the 12 rabbits used for the *in vivo* tests described in Section 2.7. The corneas were excised at least two weeks after the last test.

2.11. Permeation data treatment

For each permeation run a value of the apparent permeability coefficient, P^*_{app}, of permeant across the cornea was calculated from the following equation, assuming passive diffusion under steady-state conditions:

$$P^*_{app} = \frac{dM}{dt}\frac{1}{AC_0 f_F} \quad (3)$$

where dM/dt (1/A), the permeation flux, is the slope of the linear portion of the cumulative amount permeated per unit surface area *vs*. time plot, C_0 is the initial concentration of the permeant dissolved in the donor solution, and f_F is the drug fraction free from binding, determined by dynamic dialysis as described in Section 2.9 and calculated after Eq. (1) as follows:

$$f_F = \frac{k_p}{k_a}$$

Table 1
Selective relaxation rates (R_1^{ms}, s^{-1}; 600 MHz; 25 °C; D_2O) of H_1, H_2, H_3 protons of KT (see Fig. 1) alone (2 mM), or in the presence of BSM (10 mg mL^{-1}); or TSP (8 mg mL^{-1}); or HA (8 mg mL^{-1}); or BSM (10 mg mL^{-1}) and TSP (8 mg mL^{-1}); or BSM (10 mg mL^{-1}) and HA (8 mg mL^{-1}); or BSM (10 mg mL^{-1}) and different TSP/HA mixtures (each at the total concentration of 8 mg mL^{-1}).

	H_1	H_2	H_3
KT	0.26	0.56	0.38
KT + BSM	5.15	5.21	5.84
KT + TSP	2.37	3.02	3.41
KT + HA	0.22	0.45	0.44
KT + BSM + TSP	6.18	5.66	6.63
KT + BSM + HA	4.75	4.87	5.26
KT + BSM + TSP/HA (4/1)	3.65	3.69	4.07
KT + BSM + TSP/HA (3/2)	3.01	3.44	3.86
KT + BSM + TSP/HA (2/3)	3.04	2.88	3.17

In each dynamic dialysis run used to determine k_p the composition of the donor phase (medium, initial drug concentration, polymer mixture) corresponded to that of the solution used in the permeation run for determining P^*_{app}, described in Section 2.10.

For each permeation plot (not reported), the linear regression analysis was extended to the set of data points that gave the best fit, as judged from the r^2 value. This, in all of the cases investigated, exceeded 0.9. The single P^*_{app} values were averaged to calculate the mean apparent permeability, P_{app} ($n = 6$). The significance of the difference between two P_{app} values was assessed by the Student's t-test ($P < 0.05$).

3. Results and discussion

3.1. NMR studies

The proton selective relaxation rate (R_1^{ms}) values for KT, listed in Table 1, increased in the presence of TSP, whereas in the presence of HA only very low variations were observed. This shows that KT interacts strongly with TSP, whereas interaction processes between KT and HA are negligible in these experimental conditions. Higher variations were obtained in the mixture containing BSM, which indicates a very high affinity between KT and BSM. The analysis of ternary mixtures (KT, BSM and TSP or HA) allowed us to detect any variations of the KT–BSM affinity in the presence of the polysaccharides that could be attributed to the polysaccharide–mucin interaction. Table 1 shows that in the mixture containing KT 2 mM, BSM 10 mg mL^{-1} and TSP 8 mg mL^{-1} the enhancements of R_1^{ms} are lower than the sum of the contributions from the single macromolecules. On the other hand, the R_1^{ms} enhancements would correspond to such a sum if the two polymers interacted with the drug independently from each other. It should be pointed out that viscosity increases due to the presence of both biomacromolecules should produce a further increase, rather than a decrease in R_1^{ms}. These results suggest that interaction processes occurred in solution between TSP and BSM.

In Table 1 small decreases in relaxation rates of KT are observed in the ternary mixture, KT/BSM/HA, compared to the binary mixture, KT/BSM. Considering that HA did not interact with KT, these results indicate that HA interacted with BSM to a very low extent. For the quaternary mixtures KT/BSM/TSP/HA very high decreases in R_1^{ms} of KT protons are shown in Fig. 2 and Table 1 with respect to the values measured in the KT/BSM mixture, indicating that the TSP/HA mixtures formed stable adducts with mucin, effectively displacing KT from it. Fig. 2 shows that the magnitude of the effects was strongly dependent on the TSP/HA wt ratio. In particular TSP/HA ratios between 2/3 and 3/2 seemed to lead to the formation of supramolecular aggregates, the ability of which to bind mucin is superior to that of the single polysaccharides.

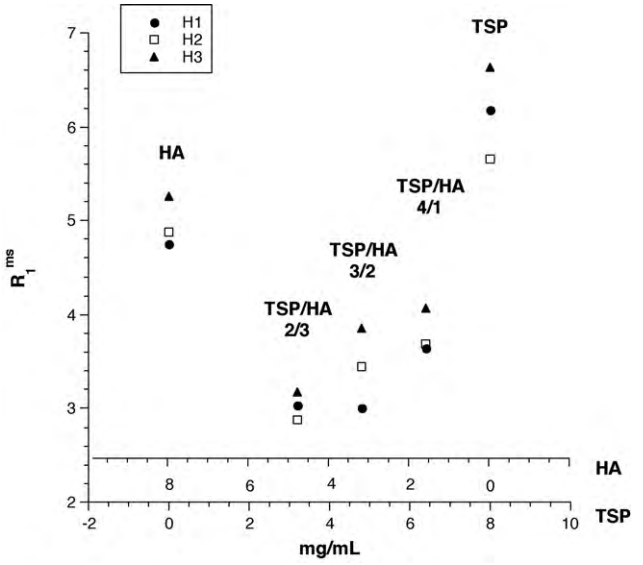

Fig. 2. Dependence of selective relaxation rates of KT (2 mM) protons on TSP and HA concentration, in the presence of BSM (10 mg mL^{-1}).

The cross-relaxation rate, σ_{ij}, is a very useful parameter as the occurrence of drug–macromolecule interaction can easily be verified by comparing the sign of the σ_{ij} value in the absence and in the presence of the macromolecule (Neuhaus and Williamson, 1989; Valensin et al., 1986). In fact, small molecules are in the fast motion conditions giving positive values of σ_{ij}, expressed by Eq. (4), whereas in the case of ligands bound to macromolecules the ligand experiences slow motion conditions and a negative value of σ_{ij} is expected, as in Eq. (5).

$$\sigma_{ij} = 0.5\gamma^4\hbar^2 r_{ij}^{-6}\tau_c \quad (4)$$

$$\sigma_{ij} = -0.1\gamma^4\hbar^2 r_{ij}^{-6}\tau_c \quad (5)$$

In Eqs. (4) and (5) γ, $\hbar$, r_{ij}, and τ_c represent the gyromagnetic ratio, the reduced Planck's constant, the distance between i and j, and the reorientational correlation time, respectively.

Therefore, if the macromolecule binds the drug a decrease in σ_{ij} with respect to the free value must be expected, the extent of which depends on the bound molar fraction. σ_{ij} values can be obtained by measuring the proton mono- (R_1^{ms}) and biselective (R_1^{bs}) relaxation rates (Valensin et al., 1986) of proton pair ij, and subtracting R_{1i}^{ms} from R_{1ij}^{bs}. For the proton pair H_1–H_2 of KT at a fixed distance of 2.94 Å, seen in Fig. 1, we measured a σ_{12} value of 0.04 s^{-1} for pure KT and a value of −1.83 s^{-1} for the mixture KT (2 mM)/BSM (10 mg mL^{-1}), which indicates that the drug experienced slow motions into which it was forced by the interaction with mucin. In the cases of the quaternary mixtures KT/BSM/TSP/HA the σ_{12} absolute value decreased to −0.94 s^{-1} (TSP/HA (4/1)), or −0.61 s^{-1} (TSP/HA (3/2)), or −0.59 s^{-1} (TSP/HA (2/3)). From these values we evaluated the average τ_c of the drug using Eq. (5). The averaged τ_c value calculated for the mixture KT/BSM was 20.7 ns, i.e. particularly high, which confirmed a high value of the molar fraction of KT bound to BSM. In the presence of the higher excess of TSP (TSP/HA (4/1)) the averaged τ_c decreased to 10.6 ns. Still lower τ_c values were obtained for lower TSP/HA ratios, namely, 6.9 ns for TSP/HA (3/2) and 6.7 ns for TSP/HA (2/3), which confirms the considerations made in the foregoing discussion about the formation of supramolecular aggregates between TSP and HA. The averaged molecular motion of KT being faster in the presence of polysaccharides and mucin than in the presence of only mucin may be explained by higher concentrations of free drug, in the former case, due to the formation of a TSP–HA adduct which would interact with BSM thus displacing KT from it. The ability of KT to act as a probe to reveal interactions between TSP and HA was confirmed also in the absence of mucin. Thus, we detected KT–TSP or KT–HA or KT–TSP–HA interactions. As data in Table 2 show, adding HA to TSP/KT mixtures caused a significant decrease in KT relaxation rates, which confirms the formation of HA adducts with TSP displacing KT from the latter.

Table 2
Selective relaxation rates (R_1^{ms}, s^{-1}; 600 MHz; 25 °C; D_2O) of H_1, H_2, H_3 protons of KT (see Fig. 1) (2 mM) in the presence of TSP or the mixture TSP/HA (3/2).

	H_1	H_2	H_3
TSP (8 mg mL^{-1})	2.37	3.02	2.61
TSP (4.8 mg mL^{-1})	1.77	2.42	1.84
TSP/HA (3/2) (4.8/3.2 mg mL^{-1})	1.28	1.84	1.96

DS showed no tendency to bind to neither TSP nor HA, probably due to electrostatic repulsive interactions originated by DS carboxylic function; indeed negligible variations of its R_1^{ms} are shown by data in Table 3 for its mixtures with these polysaccharides. Highly enhanced values of the NMR parameter are seen in Table 3 for the mixture DS/BSM. This indicates a strong interaction between mucin and this drug. In the same table the quaternary mixtures containing BSM (11 mg mL^{-1})/DS (2 mM) and variable ratios of the mixture TSP/HA at the total concentration of 8 mg mL^{-1} show R_1^{ms} values of drug protons lower than the respective ones measured for the mixture DS/BSM in the absence of polysaccharides. These measurements confirm the ability of the mixture TSP/HA to form a supramolecular aggregate strongly interacting with BSM, thereby displacing DS from it. As observed in Fig. 3, concerning the investigation with DS, such an ability is enhanced when the ratio TSP/HA is between 2/3 and 3/2.

In order to gain deeper insight into the origin of TSP–HA interaction processes, we measured relaxation parameters of selected resonances of TSP and HA. The ^{1}H NMR (600 MHz, D_2O, 25 °C) spectral region of TSP corresponding to anomeric proton resonances showed three well resolved signals (Fig. 4) at 5.06 ppm, 4.85 ppm and 4.45 ppm, which were respectively assigned to 2-*O*-galactosylxylose (Xyl), terminal xylose (Xyl_t) and all of the glucose and galactose (GluGal) residues (Gidley et al., 1991). Firstly we measured the transverse relaxation rate ($R_2 = 1/T_2$ (s^{-1})) of TSP anomeric protons in mixtures TSP/HA having the constant total concentration of 5 mg mL^{-1}. As can be seen in Table 4, the values for Xyl protons of TSP measured in the presence of HA were remarkably lower than those for TSP alone either at the same concentrations as in the mixtures or at the total concentration of the mixture. The decrease in the NMR parameter of TSP protons following the addition of HA cannot be attributed to the resulting viscosity increase, which should in fact produce the opposite effect. Rather,

Table 3
Selective relaxation rates (R_1^{ms}, s^{-1}; 600 MHz; 25 °C; D_2O) of H_2, H_3, H_5, H_6 protons of DS (see Fig. 1) alone (2 mM), or in the presence of BSM (11 mg mL^{-1}); or TSP (8 mg mL^{-1}); or HA (8 mg mL^{-1}); or BSM (11 mg mL^{-1}) and TSP (8 mg mL^{-1}); or BSM (11 mg mL^{-1}) and HA (8 mg mL^{-1}); or BSM (11 mg mL^{-1}) and different TSP/HA mixtures (each at the total concentration of 8 mg mL^{-1}).

	H_2	H_3	H_5	H_6
DS	0.16	0.24	0.34	0.38
DS + BSM	7.89	10.52	10.60	9.03
DS + TSP	0.22	0.28	0.38	0.43
DS + HA	0.20	0.24	0.33	0.38
DS + BSM + TSP	9.31	11.79	11.59	10.35
DS + BSM + HA	11.23	14.01	12.80	10.76
DS + BSM + TSP/HA (4/1)	6.22	5.66	6.54	6.52
DS + BSM + TSP/HA (3/2)	1.87	1.96	2.26	2.15
DS + BSM + TSP/HA (2/3)	1.91	1.94	2.21	2.21
DS + BSM + TSP/HA (1/4)	6.26	7.61	8.03	6.99

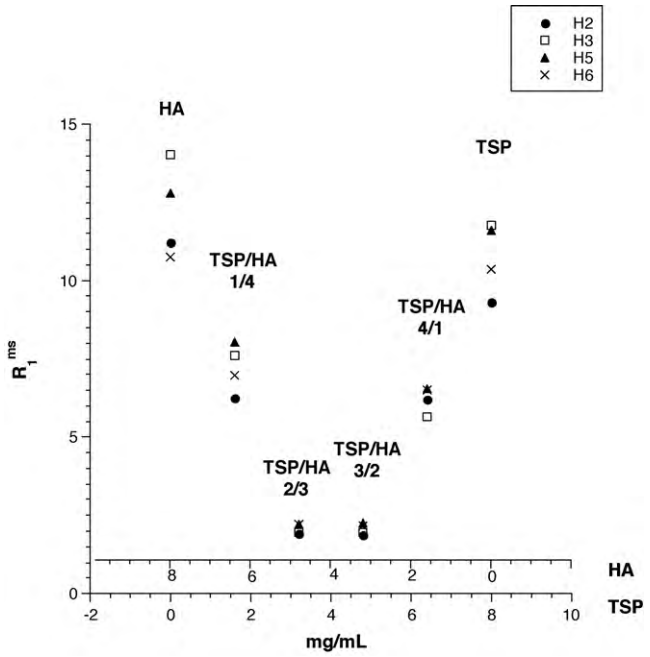

Fig. 3. Dependence of selective relaxation rates of DS (2 mM) protons on TSP and HA concentration, in the presence of BSM (11 mg mL^{-1}).

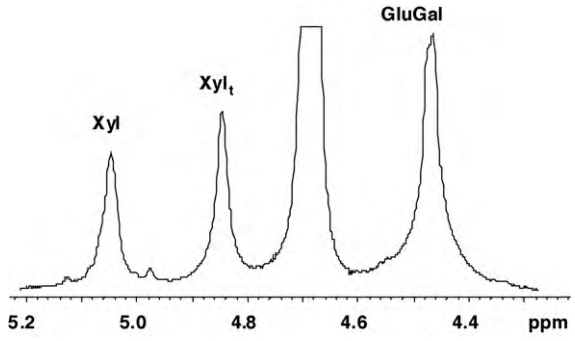

Fig. 4. ^{1}H NMR (600 MHz, D_2O, 25 °C) spectral region of TSP (5 mg mL^{-1}) corresponding to anomeric proton resonances.

it is ascribed to a variation of the environment of TSP anomeric protons consequent upon their interaction with HA. As the TSP/HA ratio was increased, a progressive increase in relaxation rate of Xyl anomeric protons was detected till the TSP/HA (3/2) ratio, passed which only negligible variations were detected. The same trend is observed for the anomeric protons of Xyl$_t$. An opposite trend appears for anomeric protons of glucose backbone and galactopyranosyl side chains (GluGal), the relaxation rate of which remarkably decreased with increasing TSP/HA ratio till the limit ratio of 3/2. It is noteworthy that the relaxation parameter for GluGal anomeric protons of unmixed TSP was nearly insensitive to concentration

Table 4
Transverse relaxation rates (R_2, s^{-1}; 600 MHz; 25 °C; D_2O) for Xyl, Xyl$_t$ or GluGal anomeric protons of TSP either alone or mixed with HA in different ratios.

C (mg mL^{-1})		R_2 (s^{-1}), Xyl		R_2 (s^{-1}), Xyl$_t$		R_2 (s^{-1}), GluGal	
TSP	HA	Alone	Mixed	Alone	Mixed	Alone	Mixed
1	4	23.9	6.7	22.6	3.2	68.5	174.3
2	3	31.0	19.8	28.5	12.3	65.1	106.3
3	2	32.0	23.3	29.2	19.3	68.8	77.8
4	1	37.3	23.9	28.7	20.7	66.2	78.2
5	0	36.3	–	28.5	–	65.3	–

Table 5
Longitudinal relaxation rates (R_1^{ms}, s^{-1}; 600 MHz; 25 °C; D_2O) and transverse relaxation rates (R_2, s^{-1}; 600 MHz; 25 °C; D_2O) of acetyl protons of HA either alone or mixed with TSP in different ratios.

C (mg mL^{-1})		R_1^{ms} (s^{-1})		R_2 (s^{-1})	
HA	TSP	Alone	Mixed	Alone	Mixed
1	4	8.46	4.75	84.3	38.4
2	3	6.20	4.42	88.5	36.5
3	2	5.14	4.13	69.6	30.6
4	1	4.79	3.67	34.5	28.4
5	0	4.38	–	32.1	–

changes, up to 5 mg mL^{-1}. Probably, beyond the TSP/HA ratio of 3/2 the excess of TSP levelled the effects produced by HA, and values more ad more similar to those measured for unmixed TSP were obtained.

The only proton resonances of HA not superimposed on TSP signals are those of acetyl groups at 1.91 ppm. Spin–spin and spin–lattice relaxation parameters were measured in the absence and in the presence of TSP. The R_1^{ms} values for unmixed HA, listed in Table 5, decrease with increasing HA concentration. As shown in the table, also in the presence of TSP the acetyl longitudinal relaxation rates progressively decrease with increasing HA concentration. However, the magnitude of the decrease is lower than in the absence of TSP. This indicates that TSP interacted with HA thus contributing to minimize repulsive electrostatic interactions among the chains of the latter. All the measured values were lower than those for the unmixed HA at corresponding concentrations. The transverse relaxation rate of acetyl protons showed a similar trend. Thus these data confirm the occurrence of TSP–HA interactions giving rise to the formation of a supramolecular aggregate.

As already stated in Section 1, the transverse relaxation rate of solvent molecules is a very sensitive probe for changes in polysaccharide dynamics. These can be the consequence of either conformational changes or aggregation processes, besides the expected effects of concentration-dependent viscosity changes. According to Hills et al. (1991) in the cases of aqueous polysaccharide systems preponderant relaxation mechanisms of water protons originate from exchange processes. Thus the water transverse relaxation rate was measured in solutions containing varying concentrations of the single TSP or HA, or their mixtures at the constant total concentration of 5 mg mL^{-1}. For the single TSP the R_2 value increased by 1.1 s^{-1} as the polysaccharide concentration increased from 1 to 5 mg mL^{-1}. The R_2 values for HA, which were significantly lower than the corresponding ones for TSP, were less sensitive to the same concentration increase as they only changed by 0.3 s^{-1}. In the solutions containing both polymers the R_2 values were higher than those for the single polymers at the respective concentrations, and changed by 1.5 s^{-1} on increasing TSP/HA ratio. The R_2 values for the TSP/HA (3/2) ratio were remarkably higher than the sum of the values for the single polymers. This indicates that the formation of the TSP–HA supramolecular aggregate strongly affects the nature of polysaccharide interaction with water molecules. This result is quite relevant to the effectiveness of the TSP/HA mixtures in binding water molecules on the ocular surface.

3.2. Comparative assessment of polysaccharide mucoadhesivity

In an attempt to assess the rank order of polysaccharide adhesivity to the eye surface, the mucin–polymer interactivity, determined *in vitro* by viscosity measurements, was compared with the mean residence time of polymer(s) in the tear fluid of rabbits. All ophthalmic drops when instilled in rabbit eyes caused no apparent irritation signs, such as conjunctival/corneal edema and/or hyperhemia. In none of the rabbits did the I_{irr} score from the mod-

Table 6
Comparison between the polysaccharide effect on the viscosity of a dispersion of mucin (15%, w/w) and polysaccharide(s) (total concentration, 5 mg mL^{-1}) and the mean residence time of polysaccharide(s) in tear fluid of rabbits. η_p, polysaccharide viscosity; η_{mp}, viscosity component of the mucin–polysaccharide dispersion due to mucin–polymer interaction; η_{mp}/η, contribution of the interactive component to the viscosity of the mucin–polysaccharide system; MRT, mean residence time of polysaccharide(s) in tear fluid of rabbits ($n = 6$).

Polymer(s)	η_p[a] (mPa s)	η_{mp}[a] (mPa s)	η_{mp}/η[a] (%)	MRT ± ES (min)
TSP	4.5[b]	63.5[b]	22.1[b]	10.42 ± 1.70[b]
HA	15.0[b]	54.0[b]	18.6[b]	14.48 ± 2.56[b]
TSP/HA (4/1)	4.6	59.7	64.3	17.85 ± 3.53
TSP/HA (3/2)	6.4	75.8	70.8	26.69 ± 3.11
TSP/HA (2/3)	9.6	50.7	57.5	16.73 ± 3.17
TSP/HA (1/4)	8.5	52.7	58.7	18.59 ± 3.41

[a] Viscosity measurements carried out at 35 °C. Values refer to a pseudoplastic behaviour (shear rate, 200 s^{-1}).
[b] Data from Di Colo et al. (2009).

ified Draize test (Lallemand et al., 2005) exceed 3, which means that the eye drops were well tolerated. In Table 6 the viscosity component due to mucin–polymer interaction, η_{mp}, and the percent contribution of this interactive component to the viscosity of the mucin–polysaccharide system, η_{mp}/η, are listed for each mucin–polysaccharide dispersion and compared with the mean residence time of polysaccharide(s) in the tear fluid of rabbits, MRT. As can be observed, for all polymer mixtures the interactive component gives an important contribution to the overall viscosity of the dispersion. For the mixtures such a contribution is greater than for the unmixed polysaccharides, in particular, the importance of the interactive component is uppermost for the TSP/HA (3/2) mixture. A similar trend is shown by the MRT values in Table 6. All elimination curves from which the corresponding MRT values were calculated (not reported) showed an exponential decay**,** in line with the elimination profile of xenobiotics from precorneal area. The mean MRT values for the polysaccharide mixtures being higher than those for the unmixed polymers cannot be ascribed to higher viscosity coefficients for the former. Indeed the viscosity coefficient of unmixed HA is seen in Table 6 to be substantially higher than the respective values for the mixtures, while the viscosity coefficient of unmixed TSP is equal to that of TSP/HA (4/1) in spite of neatly lower η_{mp}/η and MRT values. Then the *in vitro* η_{mp}/η and *in vivo* MRT values in Table 6 agree in indicating a mucoadhesivity of the mixed polysaccharides stronger than that of the unmixed ones, and hence, a synergistically enhanced mucoadhesivity of these polymers. A precise rank order of mucoadhesivity of the polysaccharide mixtures in Table 6 cannot be stated because of the variability of the *in vivo* MRT data. Nevertheless the uppermost *in vitro* mucin–polymer interactivity and *in vivo* MRT value correspond in the table to the TSP/HA (3/2) mixture, which has been shown by NMR to form a stable supramolecular aggregate. Then it appears that the strongest interpolymer TSP–HA interaction corresponds to the strongest mucoadhesivity of the polymer mixture.

3.3. Polysaccharide effect on drug residence in tear fluid of rabbits

3.3.1. Case of KT

The KT concentration of 0.7 mg mL^{-1} in the ophthalmic drops was equal to that contained in the commercial product Ketoftil (Farmigea, Italy). The polysaccharide mixtures TSP/HA (1/4), TSP/HA (2/3), TSP/HA (3/2), TSP/HA (4/1) at the total polymer concentration of 5 mg mL^{-1} were tested. The unmixed TSP and HA were not put to test because their mucoadhesivity has been shown in the preceding section to be weaker than that of the above mixtures. The resulting residence times in tear fluid are found in Table 7, where the drug fraction bound to polymer, determined by dynamic dialysis, is also reported. It appears from a comprehensive consideration

Table 7
Vehicle effect on the residence time of KT in tear fluid of rabbits. MRT, mean residence time ($n = 6$); RT_{max}, maximum residence time at measurable concentrations (≥1.1 μg mL^{-1}); f_B, drug fraction bound to polymer mixture.

Vehicle	MRT ± SE (min)	RT_{max} (min)	f_B (%)
Control	5.07 ± 0.46	12	–
TSP/HA (4/1)	4.17 ± 0.10	12	11.47
TSP/HA (3/2)	8.12 ± 0.71*	20	6.47
TSP/HA (2/3)	5.24 ± 0.86	12	10.35
TSP/HA (1/4)	5.65 ± 0.44	15	9.66

* Significantly different from control ($P < 0.05$).

Table 8
Vehicle effect on the residence time of DS in tear fluid of rabbits. MRT, mean residence time ($n = 6$); RT_{max}, maximum residence time at measurable concentrations (1.5 μg mL^{-1}); f_B, drug fraction bound to polymer mixture.

Vehicle	MRT ± SE (min)	RT_{max} (min)	f_B (%)
Control	5.55 ± 0.21	20	–
TSP/HA (4/1)	7.38 ± 0.75*	30	7.8
TSP/HA (3/2)	9.72 ± 1.78*	35	10.02
TSP/HA (2/3)	7.30 ± 0.95*	25	6.68
TSP/HA (1/4)	6.35 ± 1.62	25	8.60

* Significantly different from control ($P < 0.05$).

of data in the table that the TSP/HA (3/2) mixture has more ability than the others to retain KT on the ocular surface, in fact, the MRT value for this mixture is the only one significantly higher than the control. The relevant f_B value, i.e., 6.47%, is so low as to rule out the possibility of drug binding to polymers being involved in the prolonged drug retention in the precorneal area. On the other hand the remarkable mucoadhesivity of the TSP/HA (3/2) mixture, pointed out in the preceding section and also resulting from the NMR study, must reasonably be involved in this effect. It appears from a comparison of MRT values in Tables 6 and 7 for corresponding polymer mixtures that the residence of each of these mixtures in the tear fluid of rabbits is longer than that of the drug. This can be explained after Di Colo et al. (2009). The TSP/HA mixtures are so adhesive to the eye surface that their removal from the precorneal area is slowed down. These polymers, being hydrophilic, would retain/stabilize tear fluid on the eye surface, thereby slowing down drainage and ultimately prolonging drug precorneal residence. Nevertheless the residence time of the drug is not expected to be so long as that of the mucoadhesive polymers, considering that the f_B values in Table 7 in no case point to any strong drug–polymer binding. The free, unbound drug molecules could diffuse in the tear film even in the presence of a mucoadhesive polymer and be removed from the precorneal area. Nonetheless, this diffusion flux is likely to be significantly slower than the convective drainage that would occur in the absence of polymer.

3.3.2. Case of DS

The DS concentration of 1 mg mL^{-1} in the ophthalmic drops was equal to that contained in the commercial Voltaren Ophthalmic®. Diclofenac is chemically quite different from ketotifen, e.g., the former is an acid, the latter a base. Yet, the data on DS residence in precorneal area, reported in Table 8, show some similarities to the corresponding data for KT, discussed in the preceding section. Thus, as already observed with KT, also with DS the RT_{max} relative to TSP/HA (3/2) is greater than the values for the control and all of the other polysaccharide mixtures. Moreover, this mixture exerted the strongest enhancing effect on the MRT of this drug. Even the MRT values for KT and DS in the presence of TSP/HA (3/2) were similar, as results from a comparison between relevant data in Tables 7 and 8. In analogy with the findings by Di Colo et al. (2009) the present data altogether suggest that the vehicle, if mucoadhesive, exerts a significant influence on the drug residence time in the precorneal

Table 9
Data on KT or DS permeation across excised rabbit cornea from GBR containing 0.7 mg mL^{-1} KT or 1 mg mL^{-1} DS and 5 mg mL^{-1} TSP/HA (3/2) mixture. HL, corneal hydration level; f_F, drug fraction free from binding to polymers; P_{app}, apparent permeability. Means ± SD (n = 6).

Donor	HL (%)	Flux (μg cm^{-2} h^{-1})	f_F	P_{app} 10^5 (cm s^{-1})
KT, control	78.1 ± 1.59	62.06 ± 4.19	–	2.46 ± 0.17
KT + TSP/HA (3/2)	76.5 ± 1.15	42.80 ± 2.36*	0.94	1.81 ± 0.10*
DS, control	75.1 ± 0.66	102.45 ± 8.12	–	2.85 ± 0.23
DS + TSP/HA (3/2)	74.5 ± 0.81	82.63 ± 8.33*	0.90	2.55 ± 0.26

* Significantly different from the relevant control (P < 0.05).

area, irrespective of the chemical nature of the drug. The f_B values in Table 8 are too low to consider the molecular DS binding to polymers determinant to the polymer effect on drug MRT. Rather it was the stabilization of tear film by the mucoadhesive polymer mixture that mostly delayed drug elimination from the eye surface compared to the polymer-free control, as illustrated in the preceding section with KT.

3.4. Transcorneal permeation studies

It was reported that TSP, added to eye drops for the topical ocular administration of such antimicrobial agents as ofloxacin, gentamicin, rufloxacin, enhanced transcorneal disposition and intra-ocular penetration of these drugs in rabbits (Ghelardi et al., 2000, 2004). This effect of the polysaccharide was hypothetically ascribed to its mucoadhesivity prolonging drug residence on the corneal tissue (Ghelardi et al., 2004). Although sound, this hypothesis nevertheless disregards the possibility of the polysaccharide acting to permeabilize the corneal epithelium. In fact the permeation experiments across the excised rabbit cornea described in the present report had the purpose of investigating this possibility in the case of the interactive polysaccharide mixture TSP/HA (3/2).

The experimental model used to determine the corneal permeability has been demonstrated by previous reports to be valid (Camber, 1985; Monti et al., 2002). The HL of the corneal tissue has been considered a sensitive indicator of tissue integrity. According to the literature, the normal water content of the rabbit cornea ranges between 75% and 78% (see, e.g., Maurice, 1984) a corneal damage being indicated by hydration levels increased to 83% or more (Monti et al., 2002). In the present work the corneal hydration was determined, at the end of each permeation run, by the usual gravimetric method (Midelfart, 1987; Saettone et al., 1996; Doughty, 1999; Monti et al., 2002). The resulting HL values are listed in Table 9. None of them is higher than the value of 80.1 ± 0.69% (n = 6), reported by Monti et al. (2002) for freshly excised rabbit corneas, which indicates that neither the permeants nor the polysaccharide mixture produced any substantial damage to the tissue in the perfusion apparatus. The effects of polysaccharides on the corneal epithelium permeability may stem from polymer interactions not only with the epithelium structures, but also with the permeant in the donor solution. Indeed, the latter interaction type might depress the permeability in that the permeant fraction bound to the polymers can be considered impermeable. The concentration of the permeant fraction free from binding to polymers, represented by the product $C_0 f_F$, was introduced into Eq. (3), so that the calculated permeability values are independent of binding and only reflect possible modifications of epithelium structures produced by the polymers. The P_{app} values listed in Table 9 show the absence of any statistically significant modification of DS permeability across the cornea by TSP/HA (3/2) with respect to the relevant control. This observation suggests that the interaction of this polysaccharide mixture with the mucins of the corneal epithelium failed to promote any significant polysaccharide interaction with epithelial cells that could result in an enhancement of epithelial permeability to DS. With KT the TSP/HA (3/2) effect on P_{app} was statistically significant, only it resulted in a 26% decrease rather than an increase. To explain this finding the hypothesis is here advanced that the adhesion of polysaccharides to the membrane-associated mucins, rather than facilitating a permeabilizing action of polymers on the epithelium, might somewhat increase the epithelium barrier properties. This effect had a different strength with DS or KT because of the different chemical nature of these molecules. Altogether the *ex vivo* permeation experiments have ruled out any enhancement of corneal permeability by the TSP/HA (3/2) mixture. The enhancing effects of this mixture on either extra- or intra-ocular bioavailability should be ascribed to the polysaccharide mucoadhesivity, which prolonged drug contact with the action/absorption site.

4. Conclusions

The NMR studies have proved that relaxation parameters are a powerful tool for detecting interactions between polysaccharides that strongly affect their conformation and dynamics in comparison with the unmixed polymers. Different probes were selected, spanning from polysaccharide protons or nuclei of small molecules interacting with them, such as solvent or drug molecules. An enhanced ability of TSP/HA mixtures to displace small molecules from mucin binding sites was demonstrated by NMR and correlated to superior mucoadhesive properties of these mixtures compared to the unmixed TSP or HA. The maximum affinity with mucin was found to correspond with TSP/HA ratios ranging from 3/2 to 2/3, which demonstrates that polysaccharide mixtures originated supramolecular aggregates. Interpolymer interactions were also evidenced by the analysis of TSP or HA relaxation parameters. Among TSP anomeric protons, those of glucose backbone and galactopyranosyl side chains were particularly affected by the presence of HA, then it is concluded that these protons were deeply involved in the TSP–HA interaction. A stabilizing effect of TSP on HA is deduced from the smoothing of the remarkable concentration-dependence of the relaxation rate of acetyl protons of pure HA in the presence of TSP. The magnitude of the effect depends on the TSP/HA ratio. In particular the TSP/HA ratio of about 3/2 seems critical for the formation of stable supramolecular aggregates, the ability of which to bind water molecules is strongly differentiated from that of the single polymeric materials. In particular, the water relaxation rate increased as a result of co-operative attractive hydrogen bonding interactions, which acted upon water exchange processes with carbohydrate hydroxyl groups and increased the amount of the so-called bound water. A complementary approach involving the detection of relaxation properties of KT was also exploited. The ability of KT to bind to TSP was strongly affected by HA, as a further evidence of a co-operative behaviour of the TSP/HA mixtures.

The TSP/HA (3/2) mixture, stabilized by non-covalent co-operative interactions, fulfils the requirements of a liquid ophthalmic vehicle, such as ocular tolerability and mucoadhesivity accompanied by comparatively low viscosity. The mucoadhesivity of this mixture, in particular, is stronger than that of each of the unmixed polymers or the less interactive TSP/HA mixtures of different composition. In virtue of its mucoadhesivity the TSP/HA (3/2) mixture has shown the uppermost ability to resist elimina-

tion from tear fluid and stabilize the tear film, thereby prolonging the residence of KT and DS in the precorneal area of rabbit eyes. This enhanced retention points to a potential of KT or DS eye drops, containing the TSP/HA (3/2) mixture as excipient, to allow reduction of the frequency of instillations. The permeation experiments have shown that the above polysaccharide mixture is unable to permeabilize the corneal epithelium. Then it is concluded that mucoadhesivity is the property of TSP/HA (3/2) mainly responsible for the effect of this potential excipient to enhance either the extra- or intra-ocular bioavailability by prolonging drug contact with its action/absorption site.

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