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Mucoadhesive properties of tamarind-seed polysaccharide/hyaluronic acid mixtures: A nuclear magnetic resonance spectroscopy investigation

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ABSTRACT

Mixtures of tamarind-seed polysaccharide (TSP) and hyaluronic acid (HA), which are employed as artificial tears for ophthalmic applications in the eye dry syndrome, were investigated by NMR spectroscopy by analyzing the effect of TSP/HA ratio and total concentration on their capability to form stable aggregates with enhanced mucoadhesive properties over those of the separate polysaccharides. The effect of TSP, HA or TSP/HA mixtures on the affinity of diclofenac sodium salt (DS) to mucin (BSM) was ascertained by means of proton selective relaxation rate measurements and assumed as the basis to compare polysaccharides mucoadhesive properties. The NMR relaxation parameters of pure DS (2 mM), binary DS/BSM (5 mg/mL or 10 mg/mL) and ternary DS/BSM/polysaccharide systems (polysaccharide = TSP, HA or variable ratios TSP/HA mixtures) were compared in aqueous medium. The experimental data demonstrate that the minimum concentration of 1.5 mg/mL of each polysaccharide is needed to have formation of a stable TSP/HA aggregate endowed with NMR detectable mucoadhesive properties and inside which reciprocal synergistic interaction occurs.

1. Introduction

Polysaccharides showing mucoadhesive and/or mucomimetic activities are employed in ophthalmic applications to increase the residence time of the drug on the ocular surface and as artificial tears for the treatment of the dry eye syndrome. Among such a kind of polysaccharides, hyaluronic acid (HA) (Fig. 1) is a polyanion polysaccharide the use of which for formulations of hydrogels in the biomedical field has been strongly promoted (Cabral & Moratti, 2011; Delair, 2012; Teh, Shen, Friedland, Atlas, & Marano, 2012). The major disadvantages of the commercially available HA formulations are their low stability and fast degradability. Even though the preparation of chemically cross-linked hydrogels has been proposed in order to overcome this problem (Barbucci et al., 2006), the development of alternative ways for the stabilization of HA, which do not involve chemical derivatization, should be strongly encouraged. Tamarind-seed polysaccharide (TSP) (Fig. 1) has a cellulose-like backbone with a high degree of glycosyl substitution which is responsible for the formation of stiff extended chain structures in solution (Gidley et al., 1991; Mishra & Malhotra, 2009; Rasala et al., 2011). It possesses mucoadhesive, mucomimetic properties and also, a protective activity of the corneal surface. Its ability to generate inter-chain interactions with hyaluronic acid in TSP/HA non-covalent aggregates has been recently demonstrated and exploited in order to improve HA stability and to develop new formulations for ophthalmic applications with enhanced mucoadhesive properties.

The analysis of the mucoadhesive behavior of polymeric materials relies on the determination of their affinity toward mucin, which could be performed by exploiting several techniques, among which spectroscopic methods (Guglieri et al., 2008; Monti, Manet, & Marconi, 2011; Zartler, Yan, Mo, Kline, & Shapiro, 2003) could open interesting alternatives in terms of reliability and efficiency (Hägerström, Stromme, & Edsaman, 2005). Nuclear Magnetic Resonance (NMR), in particular, seemed to be particularly advantageous as no sample derivatization or pre-treatment is needed, thus making the analysis very simple and quick.

Polysaccharide–mucin interaction can be investigated by NMR employing a drug having high affinity to the mucin as an interaction probe: any polysaccharide to mucin interaction is expected to perturb the drug–mucin affinity and, hence, the NMR parameters of the drug. Among NMR parameters, proton selective relaxation rates were strongly responsive to the drug–mucin affinities (Neuhaus & Williamson, 1989; Valensin, Sabatini, & Tiezzi, 1986; Uccello-Barretta, Bertucci, Domenici, & Salvadori, 1991; Uccello-Barretta et al., 2008; Uccello-Barretta, Nazzi, Balzano, & Sansò, 2011) and allowed us to demonstrate the enhanced mucoadhesive and stability properties of TSP/HA mixtures quite at high total concentration (8 mg/mL) in comparison to the single polysaccharides (Uccello-Barretta et al., 2010).

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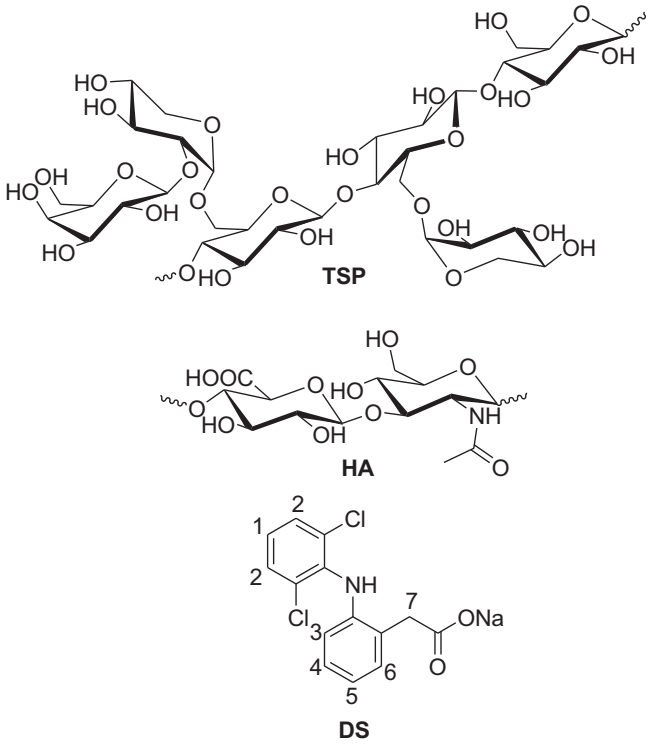

Fig. 1. Structures of TSP, HA and DS.

In consideration of the expected better patients compliance of diluted artificial tears with respect to concentrated ones, we are now prompted to extend the same kind of spectroscopic approach to the analysis of the mucoadhesive properties of TSP/HA mixtures in order to define the minimum concentration of the polysaccharide mixture which is compatible with an NMR detectable affinity for mucin. Therefore we selected diclofenac sodium (DS) salt as a probe for the polysaccharide to mucin affinity as it has been already proved to have high affinity for the mucin and low affinity for the polysaccharides (Uccello-Barretta et al., 2010) and we detected by NMR the effect of the presence of TSP, HA or TSP/HA mixtures on the DS–mucin interaction by measuring DS protons selective relaxation rates. The investigation was carried out at the total concentrations of polysaccharides of 3 mg/mL or 1 mg/mL and variable polysaccharides ratios.

2. Materials and methods

2.1. Material

Diclofenac sodium (DS) salt (Corden Pharmachem NV, Landen, Belgium), tamarind-seed polysaccharide, MW 700 kDa (TSP) (Opocrin SpA, Modena, Italy), hyaluronic acid, MW 950 kDa (HA) (Contipro, Dolní Dobrouč, Czech Republic) all were kindly gifted by Farmigea SpA (Pisa, Italy). Bovine submaxillary mucin (BSM) type I-S was purchased from Sigma.

2.2. Methods

^{1}H NMR measurements were performed by a spectrometer operating at 600 MHz. The temperature was controlled to 25 ± 0.1 °C. The longitudinal selective relaxation rates were measured in the initial rate approximation (Freeman & Wittekoek, 1969) using the inversion recovery sequence with a selective 180-pulse at the selected frequency.

2.3. Samples preparation

The stock solutions of each polysaccharide were stirred in a vortex (1000 rpm) for 2 h at 23 or 37 °C; after further 2 h these solutions were employed in the preparation of the binary, ternary and quaternary mixtures for the NMR studies. Such mixtures were stirred in a vortex (1000 rpm) at 23 or 37 °C for 14 h and, then, transferred into NMR tubes. The drug concentration was 2 mM in all samples.

3. Results and discussion

Concerning the NMR study of the drug–macromolecule interaction, the selection of methods and parameters essentially depends on the great difference in their molecular weights which imposes the need to have a very high drug-to-macromolecule ratio for obtaining a detectable NMR signal of the drug. On the fast-exchange condition, the observed parameter (P_{obs}) represents the weighted average of its value in the bound (P_b) and free (P_f) states (Eq. (1))

$$P_{obs} = P_f x_f + P_b x_b \quad (1)$$

where x_f and x_b are the molar fractions in the bound and free state, respectively.

Thus, only NMR parameters which are strongly responsive to the drug–macromolecule interaction can be usefully exploited. In particular longitudinal proton selective relaxation rates R^{ms} undergo a sharp increase in the slow motion region where fasting moving small molecules are driven as the consequence of the interaction with macromolecules (Neuhaus & Williamson, 1989; Valensin et al., 1986; Uccello-Barretta et al., 1991, 2008, 2011). Therefore, changes of selective relaxation parameters of the small molecule can be detected also in mixtures which contain a great molar excess of the small molecule with respect to the macromolecule. Proton selective relaxation rates are measured by using a selective 180° pulse centered at the selected frequency of the spin *i* and by following its magnetization recovery by leaving unperturbed the other spins.

Cross-relaxation rates σ_{ij} describe the magnetization transfer between spins pairs *ij* and depend on the reorientational correlation time τ_c of the vector connecting the two spins *ij* and on their distance r_{ij}. Above dependence can be very simply expressed by means of Eqs. (2) and (3), which holds in the fast and slow motion regions, respectively.

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

$$\sigma_{ij} = -0.1\gamma^4\hbar^2 r_{ij}^{-6}\tau_c \quad (3)$$

where γ is the gyromagnetic ratio and $\hbar$ is the reduced Planck's constant.

In particular, the interaction of small molecules with macromolecules brings about a slowing down of their molecular motion which causes a sign change of cross-relaxation parameters with the amount of the decrease of cross-relaxation rates depending on the bound molar fraction. Moreover, if the proton pair is at a fixed and known distance, σ_{ij} becomes a function of the reorientational correlation time of the vector connecting the spins *ij* and, in the hypothesis of isotropic motion, it can be assumed as the correlation time of the whole molecule.

Cross-relaxation parameters can be very simply obtained as the differences between the biselective relaxation rates R_{ij}^{bs} and monoselective relaxation rates R_i^{ms} (Eq. (4)).

$$\sigma_{ij} = R_{ij}^{bs} - R_i^{ms} \quad (4)$$

Biselective relaxation rates are obtained by applying the inversion pulse at the frequencies of the spins *i* and *j* leaving unperturbed the other spins and by following the recovery of the magnetization of the spin *i*.

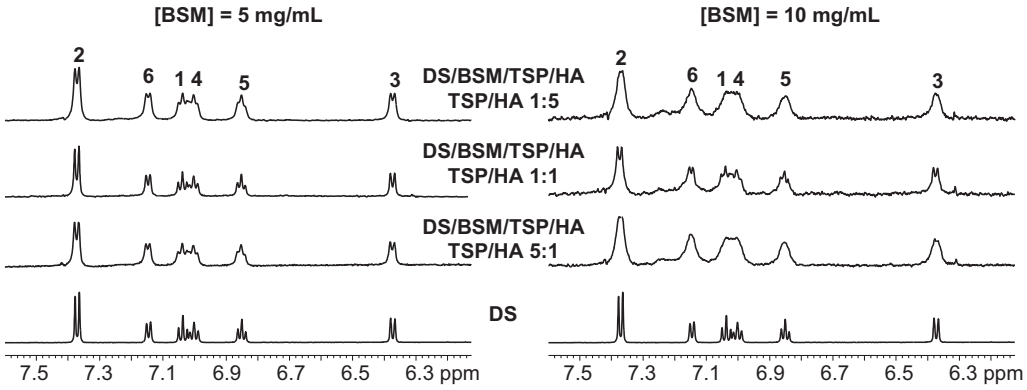

Fig. 2. ^{1}H NMR (600 MHz, D_2O, 25 °C) spectra of DS alone (2 mM) and of DS in the presence of BSM (5 or 10 mg/mL) and different TSP/HA mixtures (each at the total concentration of 3 mg/mL).

On the basis of the above said premises, we faced our problem by comparing the NMR parameters of pure DS (2 mM) and of DS in the binary mixture with BSM (5 mg/mL or 10 mg/mL), in ternary mixtures DS/BSM/polysaccharide containing the polysaccharide at 3 mg/mL or 1 mg/mL concentration and in quaternary mixtures DS/BSM/TSP/HA, where the total concentration of the two polysaccharides was kept constant at 3 mg/mL or 1 mg/mL with variable TSP/HA ratios.

Already the simple inspection of the ^{1}H NMR (600 MHz, D_2O, 25 °C) spectra gave preliminary indications regarding the effect of TSP/HA ratios on the DS to mucin affinity. All in the quaternary mixtures containing BSM 5 mg/mL (Fig. 2) and variable TSP/HA ratios remarkable broadenings of the resonances of DS were detected, as in the binary mixture DS/mucin, which reflected the immobilization of DS due to its interaction with mucin. The sole exception was the quaternary mixture containing TSP/HA at 1 to 1 ratio, where the line widths of DS resonances were reduced and partially recovered the values detected for the pure drug. Thus, in this last mixture the molar fraction of unbound free DS was greater with respect to the other mixtures. Above effect can be attributed to the ability of the TSP/HA mixture at 1 to 1 ratio to displace DS from mucin as the consequence of the enhanced mucin affinity of the polysaccharides mixture. The just discussed effect was even more pronounced in the presence of a double concentration of BSM (10 mg/mL) as shown in Fig. 2.

Due to the fact that the total concentration of the two polysaccharides was held constant and that the molecular weights of the two polysaccharides were comparable, above result supports the existence of a synergistic interaction between the two polysaccharides at the 1 to 1 ratio. It is noteworthy that similar synergistic interactions were demonstrated in a previous investigation (Uccello-Barretta et al., 2010) at remarkably higher TSP/HA concentrations (8 mg/mL total concentration) in the range of molar ratios between 2 to 3 and 3 to 2.

The above observations were strengthened by comparing the proton selective relaxation rates of DS in the different mixtures. In particular we focused on the relaxation parameters of DS aromatic protons H-2, H-3, H-5 and H-6 (Fig. 1), which gave rise to well resolved resonances and which were not superimposed to polysaccharides or mucin signals.

At first we analyzed mixtures containing 5 mg/mL of BSM, in order to have a polysaccharide to BSM ratio comparable with the one employed in the previous investigation (TSP/HA total concentration of 8 mg/mL and BSM 11 mg/mL) (Uccello-Barretta et al., 2010).

In the absence of any macromolecule, relaxation rates of DS protons were quite low (Table 1), as expected for a small molecule which is fast moving. We measured 0.16 s^{-1} for H-2, 0.24 s^{-1} for H-3, 0.34 s^{-1} for H-5 and 0.38 s^{-1} for H-6.

Similarly low values were measured in the presence of TSP or HA (as an example 0.22 and 0.20 s^{-1} for H-2 in the presence of TSP or HA, respectively), to indicate the very low drug to polysaccharides affinities (Uccello-Barretta et al., 2010). By contrast, about ten-fold increases of the relaxation rates were detected in the presence of mucin (2.33 s^{-1}, 2.78 s^{-1}, 3.03 s^{-1} and 3.13 s^{-1} for H-2, H-3, H-5 and H-6 respectively), which probed the relevant affinity of DS for mucin (Table 1). The co-presence of sole TSP did not affect significantly the DS/BSM affinity, whereas further relaxation rate increases were caused by the presence of HA to indicate that some kind of HA/BSM cooperation should be involved in the ternary mixture DS/BSM/HA, accounting for the reinforcement of DS binding (Table 1) as well as a conformational change of mucin due to the presence of HA could be involved which made easier the interaction DS/BSM. As an example, the relaxation rate of H-2 protons increased from 2.22 s^{-1} to 4.17 s^{-1} due to the co-presence of HA. To this regard it must be stressed that it has been already demonstrated that HA does not interact with DS (Uccello-Barretta et al., 2010).

In the quaternary mixtures which contained both polysaccharides, the monoselective relaxation rates remained nearly unchanged with respect to the ternary mixture DS/BSM/HA in spite of the co-presence of TSP and irrespective of the TSP to HA ratios (Table 1). Only in the case of the DS/BSM/TSP/HA mixture containing equal amounts of the two polysaccharides (1.5 mg/mL each) sharp decreases of DS relaxation rates were detected (Fig. 3A), which recovered the values measured in the DS/BSM binary mixture (Table 1).

Table 1
Proton selective relaxation rates (R^{ns}, s^{-1}) (600 MHz, 25 °C, D_2O) of pure DS (2 mM) and of DS in its binary, ternary and quaternary mixtures (BSM 5 mg/mL, total TSP/HA concentration of 3 mg/mL).

	H-2	H-3	H-5	H-6
DS	0.16	0.24	0.34	0.38
DS + BSM	2.33	2.78	3.03	3.13
DS + BSM + TSP	2.22	2.56	3.02	3.03
DS + BSM + HA	4.17	4.55	5.00	4.36
DS + BSM + TSP/HA (5:1)	3.85	4.38	5.00	4.55
DS + BSM + TSP/HA (4:1)	3.86	4.37	4.76	4.35
DS + BSM + TSP/HA (3:1)	3.70	4.17	4.76	4.55
DS + BSM + TSP/HA (2:1)	3.69	4.76	4.76	4.78
DS + BSM + TSP/HA (1:1)	2.13	2.50	2.78	2.78
DS + BSM + TSP/HA (1:2)	3.70	4.57	5.00	4.78
DS + BSM + TSP/HA (1:3)	4.01	4.76	5.25	4.76
DS + BSM + TSP/HA (1:4)	4.18	4.56	5.26	5.02
DS + BSM + TSP/HA (1:5)	4.00	4.78	5.00	4.77

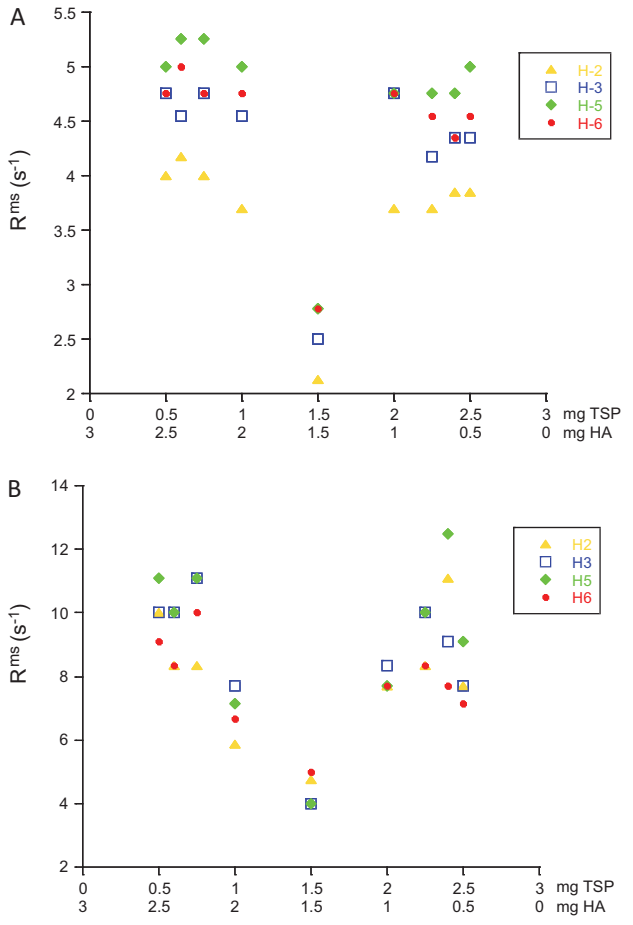

Fig. 3. Graph of proton selective relaxation rates (R^{ms}, s^{-1}) (600 MHz, 25 °C, D_2O) of pure DS (2 mM) in its quaternary mixtures with TSP/HA (total concentration of 3 mg/mL) and with: (A) BSM (5 mg/mL) and (B) BSM (10 mg/mL).

Such a kind of behavior could suggest that the synergistic interaction between the two polysaccharides is sensitive more to the concentration of each polysaccharide rather than to their molar ratio and the formation of a stable supramolecular aggregate is not possible when the concentration of each polysaccharide is lower than 1.5 mg/mL. That conclusion is fully supported by the *a posteriori* comparison with data which have been recently published (Uccello-Barretta et al., 2010) for quaternary mixtures DS/BSM/TSP/HA, where the total concentration of the two polysaccharides was much higher and equal to 8 mg/mL. In those mixtures significant decreases of the relaxation parameters of DS were observed starting from TSP/HA ratios equal to 4:1 or 1:4, in which, interestingly, the concentration of each polysaccharide was at least 1.5 mg/mL. Thus the concentration of 1.5 mg/mL of each polysaccharide constitutes the minimum value needed to have formation of a stable TSP/HA aggregate, which is further stabilized on increasing the total concentration. It is noticeably that the relaxation rates measured in the quaternary mixture with a 1 to 1 ratio between the two polysaccharides were almost the same of those measured in the absence of the polysaccharides.

Relaxation parameters are affected by local effects due to the through space dipole–dipole interaction between proton pairs, therefore they could change not only as the consequence of the slowing down of DS motion due to its interaction with the macromolecular systems, but also they could feel effects due to conformational changes both of DS or macromolecules they interact with. On the other hand, cross-relaxation parameters of proton pairs at fixed distance are a direct probe of dynamics changes

Table 2
Proton mono and selective relaxation rates (R_5^{ms}, R_{56}^{bs}, s^{-1}) (600 MHz, 25 °C, D_2O) of H-5 and H-6 protons of pure DS (2 mM) and of DS in its binary mixture with BSM and in its quaternary mixtures (BSM 5 mg/mL, total TSP/HA concentration of 3 mg/mL). Cross-relaxation parameters (σ_{56}, s^{-1}) and correlation times (τ_c, ns).

	R_5^{ms}	R_{56}^{bs}	σ_{56}	τ_c
DS	0.34	0.38	0.04	0.03
DS + BSM	3.03	2.56	−0.47	1.70
DS + BSM + TSP/HA (5:1)	5.00	4.17	−0.83	3.00
DS + BSM + TSP/HA (4:1)	4.76	3.85	−0.91	3.29
DS + BSM + TSP/HA (3:1)	4.76	3.85	−0.91	3.29
DS + BSM + TSP/HA (2:1)	4.76	3.85	−0.91	3.29
DS + BSM + TSP/HA (1:1)	2.78	2.27	−0.51	1.84
DS + BSM + TSP/HA (1:5)	5.00	4.17	−0.83	3.00

and could definitively ascertain the origin of the relaxation rates changes. Therefore we selected the proton pair H-5/H-6, which is at fixed and known distance (2.43 Å) (Uccello-Barretta et al., 1991), and calculated the cross-relaxation rate σ_{56} as the difference between the biselective relaxation rates R_{56}^{bs} of the proton H-5 and its monoselective relaxation rate R_5^{ms}. The values are reported in Table 2.

For pure DS, σ_{56} was positive, as expected for a small molecule which does not interact in solution with other molecules. On the basis of Eq. (2) we calculated the correlation time of the vector connecting the two spins H-5 and H-6, that was equal to 0.03 ns. In the presence of mucin, a sign change of the cross-relaxation parameter (−0.47 s^{-1}) was found, which demonstrated the remarkable slowing down of DS molecular motion due to the drug to mucin binding. On the basis of Eq. (3) the correlation time of 1.70 ns was calculated. In the quaternary mixtures, the cross-relaxation parameters all were negative and greater in the absolute value than they were in the presence of mucin only (Table 2). A correlation time of about 3.00 ns was calculated which was quite insensitive to the TSP/HA ratios with the sole exception of the quaternary mixture which contained equal amounts of the two polysaccharides, where the reorientational correlation time was very similar to the one measured in the mixture DS/BSM.

Therefore, also cross-relaxation rates demonstrated the formation of a supramolecular aggregate TSP/HA which was stabilized when at least 1.5 mg/mL of each polysaccharide was present in the mixture. At different polysaccharides ratios no such stabilization is possible.

However, any indication about the TSP/HA to mucin affinity was obtained at least in these experimental conditions. Therefore we carried out the analogous investigation in mixtures where the ratio between DS and mucin was more similar to the one selected in the previous investigation focused on much more concentrated TSP/HA solutions and we kept the mucin constant at 10 mg/mL in each binary or quaternary mixture.

What we could expect was a much higher DS to mucin binding, which was confirmed by the measurements of the proton selective relaxation rates (Table 3). About fifty-fold increases of the relaxation rates (Table 3) were detected. As an example the relaxation rate of the proton H-2 increased to 9.83 s^{-1} in the presence of mucin. As in the previous case (BSM 5 mg/mL), the relaxation rates seemed to be scarcely sensitive to the presence of the polysaccharides mixtures, with the exception of the mixture 1 to 1 TSP/HA, which produced sharp decreases of the relaxation rates of all the protons of DS (Fig. 3B). Interestingly, in the presence of BSM 10 mg/mL, the minimum values of the NMR parameters were lower with respect to the ones measured in the DS/BSM mixture, which demonstrated that the TSP/HA mixture containing equal amounts of the two polysaccharide was able to displace DS from mucin due to its enhanced affinity for mucin.

Table 3
Proton selective relaxation rates (R^{ms}, s^{-1}) (600 MHz, 25 °C, D_2O) of pure DS (2 mM) and of DS in its binary, ternary and quaternary mixtures (BSM 10 mg/mL, total TSP/HA concentration of 3 mg/mL).

	H-2	H-3	H-5	H-6
DS	0.16	0.24	0.34	0.38
DS + BSM	9.83	10.92	10.51	9.09
DS + BSM + TSP	8.74	9.93	10.90	8.25
DS + BSM + HA	8.33	9.09	11.32	8.19
DS + BSM + TSP/HA (5:1)	7.52	8.48	9.52	8.44
DS + BSM + TSP/HA (4:1)	10.54	9.78	12.02	7.93
DS + BSM + TSP/HA (3:1)	8.62	10.31	9.77	8.60
DS + BSM + TSP/HA (2:1)	7.56	7.89	8.03	7.53
DS + BSM + TSP/HA (1:1)	4.89	4.28	4.25	4.23
DS + BSM + TSP/HA (1:2)	6.23	7.65	7.24	7.00
DS + BSM + TSP/HA (1:3)	8.88	10.79	10.72	11.07
DS + BSM + TSP/HA (1:4)	8.60	10.82	10.43	8.02
DS + BSM + TSP/HA (1:5)	9.78	11.41	12.23	9.08

The final confirmation of our conclusions was given by the analysis of the relaxation parameters of the mixtures containing the same amounts of DS (2 mM) and BSM (10 mg/mL), but even lower polysaccharides concentration (1 mg/mL total concentration). Any synergistic TSP to HA interaction was detected as well as any mixture was able to displace DS from mucin. In fact, in this last case the concentration of each polysaccharide in the mixture TSP/HA did not reach the minimum value needed to stabilize the TSP/HA supramolecular aggregates.

4. Conclusions

NMR relaxation rate measurements were confirmed to be powerful tools for detecting synergistic interactions between polysaccharides, which can be exploited for the rational design of new efficient medical formulations. To this regard, different probes can be selected spanning from polysaccharides protons (Uccello-Barretta et al., 2010) or nuclei of small molecules interacting with them, such as solvents or drug molecules. Here, we selected DS, which is not able to bind polysaccharides, as an internal probe of the affinities of the polysaccharides for mucin.

The ability of TSP to generate non-covalent stabilizing interactions with HA was demonstrated. However, the minimum concentration of 1.5 mg/mL of each polysaccharide is needed to guarantee the formation of a stable supramolecular aggregate TSP/HA. This is probably due to the fact that hyaluronic acid is an ionic polymer the aggregation properties of which are severely affected by electrostatic factors. Above said threshold concentration is also needed in order to hold an NMR detectable affinity for mucin and, hence, mucoadhesive properties which could be exploited for the development of new diluted formulations.

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References

Barbucci, R., Leone, G., Chiumiento, A., Di Cocco, M. E., D'Orazio, G., Gianferri, R., et al. (2006). Low- and high-resolution nuclear magnetic resonance (NMR) characterisation of hyaluronan-based native and sulfated hydrogels. *Carbohydrate Research*, *341*, 1848–1858.

Cabral, J., & Moratti, S. C. (2011). Hydrogels for biomedical applications. *Future Medicinal Chemistry*, *3*, 1877–1888.

Delair, T. (2012). *In situ* forming polysaccharide-based 3D-hydrogels for cell delivery in regenerative medicine. *Carbohydrate Polymers*, *87*, 1013–1019.

Freeman, R., & Wittekoek, S. (1969). Selective determination of relaxation times in high resolution NMR. *Journal of Magnetic Resonance*, *1*, 238–276.

Gidley, M. J., Lillford, P. J., Rowlands, D. W., Lang, P., Dentini, M., Crescenzi, V., et al. (1991). Structure and solution properties of tamarind-seed polysaccharide. *Carbohydrate Research*, *214*, 299–314.

Guglieri, S., Hricovini, M., Raman, R., Polito, L., Torri, G., Casu, B., et al. (2008). Minimum FGF2 binding structural requirements of heparin and heparan sulfate oligosaccharides as determined by NMR spectroscopy. *Biochemistry*, *47*, 13862–13869.

Hägerström, H., Stromme, M., & Edsaman, K. (2005). Drug molecules as probes for studying the compatibility between gels and mucous tissue with dielectric spectroscopy. *Journal of Pharmaceutical Sciences*, *94*, 1090–1100.

Mishra, A., & Malhotra, A. V. (2009). Tamarind xyloglucan: A polysaccharide with versatile application potential. *Journal of Materials Chemistry*, *19*, 8528–8536.

Monti, S., Manet, I., & Marconi, G. (2011). Combination of spectroscopic and computational methods to get an understanding of supramolecular chemistry of drugs: From simple host systems to biomolecules. *Physical Chemistry Chemical Physics*, *13*, 20893–20905.

Neuhaus, D., & Williamson, M. (1989). *The nuclear Overhauser effect in structural and conformational analysis*. New York: VCH Publisher.

Rasala, T. M., Kale, V. V., Lohiya, G. K., Moharir, K. S., Ittadwar, A. M., & Awari, J. G. (2011). Chemistry and pharmaceutical applications of excipients derived from tamarind. *Asian Journal of Chemistry*, *23*, 1421–1423.

Teh, B. M., Shen, Y., Friedland, P. L., Atlas, M. D., & Marano, R. J. (2012). A review on the use of hyaluronic acid in tympanic membrane wound healing. *Expert Opinion on Biological Therapy*, *12*, 23–36.

Uccello-Barretta, G., Bertucci, C., Domenici, E., & Salvadori, P. (1991). Conformational and dynamic changes of D- and L-tryptophan due to stereoselective interaction with human serum albumin, as revealed by proton-selective relaxation rate measurements. *Journal of the American Chemical Society*, *113*, 7017–7019.

Uccello-Barretta, G., Nazzi, S., Balzano, F., Di Colo, G., Zambito, Y., Zaino, C., et al. (2008). Enhanced affinity of ketotifen toward tamarind seed polysaccharide in comparison with hydroxyethylcellulose and hyaluronic acid: A nuclear magnetic resonance investigation. *Bioorganic & Medicinal Chemistry*, *16*, 7371–7376.

Uccello-Barretta, G., Nazzi, S., Zambito, Y., Di Colo, G., Balzano, F., & Sansò, M. (2010). Synergistic interaction between TS-polysaccharide and hyaluronic acid: Implications in the formulation of eye drops. *International Journal of Pharmaceutics*, *395*, 122–131.

Uccello-Barretta, G., Nazzi, S., Balzano, F., & Sansò, M. (2011). A nuclear magnetic resonance approach to the comparison of mucoadhesive properties of polysaccharides for ophthalmic uses. *International Journal of Pharmaceutics*, *406*, 78–83.

Valensin, G., Sabatini, G., & Tiezzi, E. (1986). Determination of zero- and double-quantum relaxation transition probabilities by multiple-selective irradiation methods. In N. Niccolai, & G. Valensin (Eds.), *Advanced magnetic resonance techniques in systems of high molecular complexity* (pp. 69–76). Boston: Birkhauser.

Zartler, E. R., Yan, J., Mo, H., Kline, A. D., & Shapiro, M. J. (2003). 1D NMR methods in ligand-receptor interactions. *Current Topics in Medicinal Chemistry*, *3*, 25–37.