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 rates 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.

Keywords: TSP; Hyaluronic acid; mucoadhesivity; NMR; proton selective relaxation rate

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 et al., 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.
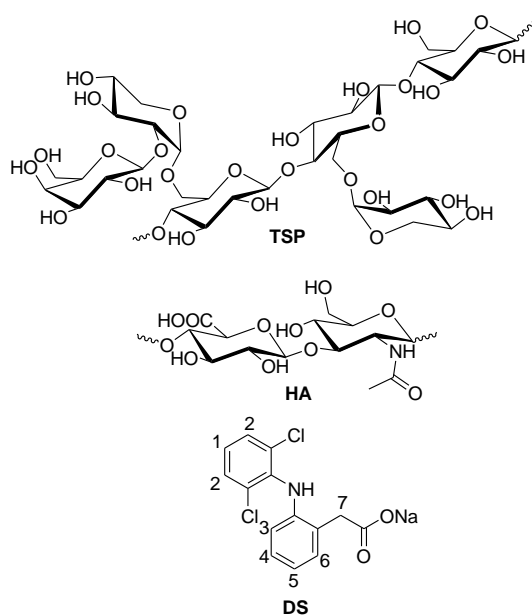
Fig. 1 - Structures of TSP, HA and DS

The analysis of the mucoadhesive behaviour of polymeric materials relies on the determination of their affinity towards mucin, which could be performed by exploiting several techniques, among which spectroscopic methods (Guglieri et al. 2008; Monti et al. 2011; Zartler et al. 2003) could open interesting alternatives in terms of reliability and efficiency (Hägerström et al., 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 et al., 1986; Uccello-Barretta et al., 1991, 2008, 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).

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 salt (DS) 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. Material and methods

2.1 Material

Diclofenac sodium salt (DS) (Corden Pharmachem NV, Landen, Belgium), tamarind-seed polysaccharide, MW 700 kDa (TSP) (Opocrin S.p.A., Modena, Italy), hyaluronic

92   acid, MW 950 kDa (HA) (Contipro, Dolní Dobrouč, Czech Republic) all were kindly

93   gifted by Farmigea S.p.A. (Pisa, Italy). Bovine submaxillary mucin (BSM) type I-S

94   was purchased from Sigma.

95

96   *2.2 Methods*

97   ^{1}H NMR measurements were performed by a spectrometer operating at 600 MHz.

98   The temperature was controlled to 25±0.1 °C. The longitudinal selective relaxation

99   rates were measured in the initial rate approximation (Freeman and Wittekoek, 1969)

100 using the inversion recovery sequence with a selective 180-pulse at the selected

101 frequency.

102

103 *2.3 Samples preparation*

104 The stock solutions of each polysaccharide were stirred in a vortex (1000 rpm) for 2 h

105 at 23 or 37 °C; after further 2 h these solutions were employed in the preparation of

106 the binary, ternary and quaternary mixtures for the NMR studies. Such mixtures were

107 stirred in a vortex (1000 rpm) at 23 or 37 °C for 14 h and, then, transferred into NMR

108 tubes. The drug concentration was 2 mM in all samples.

109

110 **3. Results and discussion**

111 Concerning the NMR study of the drug-macromolecule interaction, the selection of

112 methods and parameters essentially depends on the great difference in their molecular

113 weights which imposes the need to have a very high drug-to-macromolecule ratio for

114 obtaining a detectable NMR signal of the drug. On the fast-exchange condition, the

115 observed parameter (P_{obs}) represents the weighted average of its value in the bound

116 (P_b) and free (P_f) states (Eq. (1))

117 $P_{obs} = P_f\, x_f + P_b\, x_b$ (1)

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

119 Thus, only NMR parameters which are strongly responsive to the drug-

120 macromolecule interaction can be usefully exploited. In particular longitudinal proton

121 selective relaxation rates R^{ms} undergo a sharp increase in the slow motion region

122 where fasting moving small molecules are driven as the consequence of the

123 interaction with macromolecules (Neuhaus & Williamson, 1989; Valensin et al.,

124 1986; Uccello-Barretta et al., 1991, 2008, 2011). Therefore, changes of selective

125 relaxation parameters of the small molecule can be detected also in mixtures which

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126   contain a great molar excess of the small molecule with respect to the macromolecule.

127   Proton selective relaxation rates are measured by using a selective 180° pulse

128   centered at the selected frequency of the spin i and by following its magnetization

129   recovery by leaving unperturbed the other spins.

130   Cross-relaxation rates σ_{ij} describe the magnetization transfer between spins pairs ij

131   and depend on the reorientational correlation time τ_c of the vector connecting the two

132   spins ij and on their distance r_{ij}. Above dependence can be very simply expressed by

133   means of Eqs. (2) and (3), which holds in the fast and slow motion regions,

134   respectively.

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

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

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

138   In particular, the interaction of small molecules with macromolecules brings about a

139   slowing down of its molecular motion which causes a sign change of cross-relaxation

140   parameters with the amount of the decrease of cross-relaxation rates depending on the

141   bound molar fraction. Moreover, if the proton pair is at a fixed and known distance,

142   σ_{ij} becomes a function of the reorientational correlation time of the vector connecting

143   the spins ij and, in the hypothesis of isotropic motion, it can be assumed as the

144   correlation time of the whole molecule.

145   Cross-relaxation parameters can be very simply obtained as the differences between

146   the biselective relaxation rates R_{ij}^{bs} and monoselective relaxation rates R_i^{ms} Eq. (4).

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

148   Biselective relaxation rates are obtained by applying the inversion pulse at the

149   frequencies of the spins i and j leaving unperturbed the other spins and by following

150   the recovery of the magnetization of the spin i.

151   On the basis of the above said premises, we faced our problem by comparing the

152   NMR parameters of pure DS (2 mM) and of DS in the binary mixture with BSM (5

153   mg/mL or 10 mg/mL), in ternary mixtures DS/BSM/polysaccharide containing the

154   polysaccharide at 3 mg/mL or 1 mg/mL concentration and in quaternary mixtures

155   DS/BSM/TSP/HA, where the total concentration of the two polysaccharides was kept

156   constant at 3 mg/mL or 1 mg/mL with variable TSP/HA ratios.

157   Already the simple inspection of the ^{1}H NMR (600 MHz, D$_2$O, 25 °C) spectra

158   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.
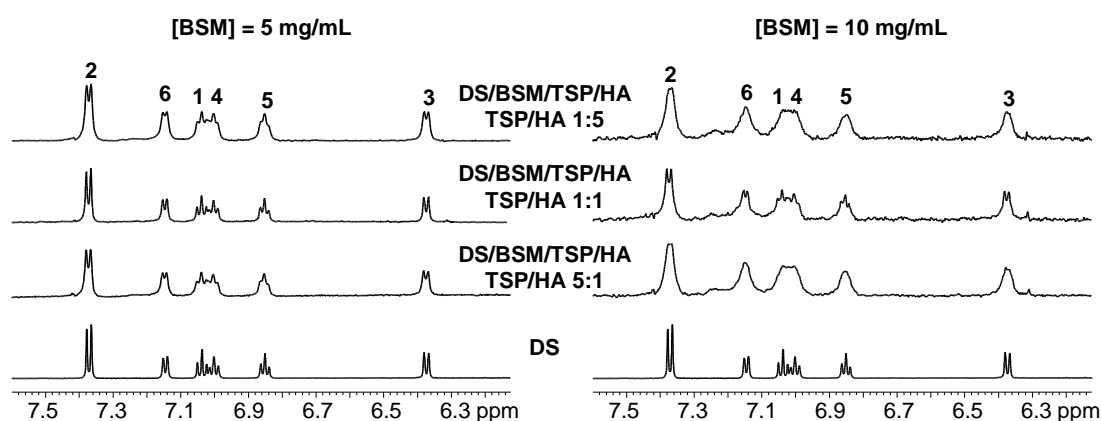
Fig. 2. ^{1}H NMR (600 MHz, D_2O, 25 °C) spectra of DS alone (2 mM) and of DS in the presence of BSM (5 or 10 mg/mL) and different TSP/HA mixtures (each at the total concentration of 3 mg/mL)

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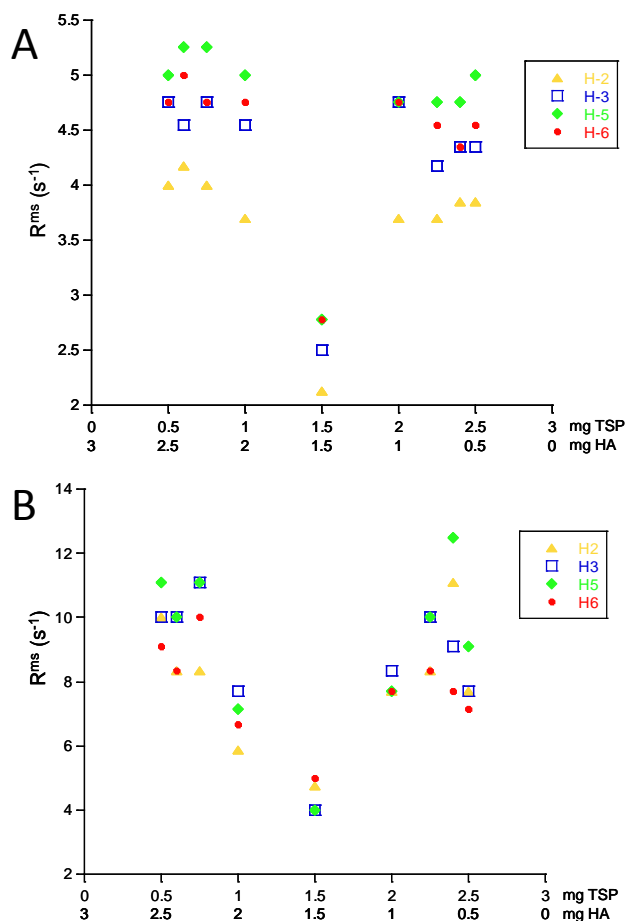
Table 1. Proton selective relaxation rates (R^{ms}, s^{-1}) (600 MHz, 25 °C, D_2O) of pure DS (2 mM) and of DS in its binary, ternary and quaternary mixtures (BSM 5 mg/mL, total TSP/HA concentration of 3 mg/mL)

	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

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 rates 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

212 made easier the interaction DS/BSM. As an example, the relaxation rate of H-2

213 protons increased from 2.22 s^{-1} to 4.17 s^{-1} due to the co-presence of HA. To this

214 regard it must be stressed that it has been already demonstrated that HA does not

215 interact with DS (Uccello-Barretta et al., 2010).

216   In the quaternary mixtures which contained both polysaccharides, the

217 monoselective relaxation rates remained nearly unchanged with respect to the ternary

218 mixture DS/BSM/HA in spite of the co-presence of TSP and irrespective of the TSP

219 to HA ratios (Table 1). Only in the case of the DS/BSM/TSP/HA mixture containing

220 equal amounts of the two polysaccharides (1.5 mg/mL each) sharp decreases of DS

221 relaxation rates were detected (Fig. 3A), which recovered the values measured in the

222 DS/BSM binary mixture (Table 1).

223 **Fig. 3**. Graph of proton selective relaxation rates (R^{ms}, s^{-1}) (600 MHz, 25 °C, D$_2$O) of
224 pure DS (2 mM) in its quaternary mixtures with TSP/HA (total concentration of 3
225 mg/mL) and with: A) BSM (5 mg/mL), B) BSM (10 mg/mL).
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227   Such a kind of behavior could suggest that the synergistic interaction between the

228 two polysaccharides is sensitive more to the concentration of each polysaccharide

229   rather than to their molar ratio and the formation of a stable supramolecular aggregate
230   is not possible when the concentration of each polysaccharide is lower than 1.5
231   mg/mL.

232   That conclusion is fully supported by the *a posteriori* comparison with data
233   which have been recently published (Uccello-Barretta et al., 2010) for quaternary
234   mixtures DS/BSM/TSP/HA, where the total concentration of the two polysaccharides
235   was much higher and equal to 8 mg/mL. In those mixtures significant decreases of the
236   relaxation parameters of DS were observed starting from TSP/HA ratios equal to 4:1
237   or 1:4, in which, interestingly, the concentration of each polysaccharide was at least
238   1.5 mg/mL. Thus the concentration of 1.5 mg/mL of each polysaccharide constitutes
239   the minimum value needed to have formation of a stable TSP/HA aggregate, which is
240   further stabilized on increasing the total concentration. It is noticeably that the
241   relaxation rates measured in the quaternary mixture with a 1 to 1 ratio between the
242   two polysaccharides were almost the same of those measured in the absence of the
243   polysaccharides.

244   Relaxation parameters are affected by local effects due to the through space
245   dipole-dipole interaction between proton pairs, therefore they could change not only
246   as the consequence of the slowing down of DS motion due to its interaction with the
247   macromolecular systems, but also they could feel effects due to conformational
248   changes both of DS or macromolecules they interact with. On the other hand, cross-
249   relaxation parameters of proton pairs at fixed distance are a direct probe of dynamics
250   changes and could definitively ascertain the origin of the relaxation rates changes.
251   Therefore we selected the proton pair H-5/H-6, which is at fixed and known distance
252   (2.43 Å) (Uccello-Barretta et al., 1991), and calculated the cross-relaxation rate σ_{56} as
253   the difference between the biselective relaxation rates R_{56}^{bs} of the proton H-5 and its
254   monoselective relaxation rate R_5^{ms}. The values are reported in Table 2.

255   For pure DS, σ_{56} was positive, as expected for a small molecule which does not
256   interact in solution with other molecules. On the basis of Eq. (2) we calculated the
257   correlation time of the vector connecting the two spins H-5 and H-6, that was equal to
258   0.03 ns. In the presence of mucin, a sign change of the cross-relaxation parameter
259   (-0.47 s^{-1}) was found, which demonstrated the remarkable slowing down of DS
260   molecular motion due to the drug to mucin binding. On the basis of Eq. (3) the
261   correlation time of 1.70 ns was calculated. In the quaternary mixtures, the cross-

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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 about of 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.

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

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 all of 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_1^{ms}, s^{-1}) (600 MHz, 25 °C, D$_2$O) 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 rates 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

315   molecules. Here, we selected DS, which is not able to bind polysaccharides, as an
316   internal probe of the affinities of the polysaccharides for mucin.

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

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