

## Synergistic Effects in Semidilute Mixed Solutions of Alginate and Lactose-Modified Chitosan (Chitlac)

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The present study specifically aimed at preparing and characterizing semidilute binary polymer mixtures of alginate and chitlac which might find an application in the field of cell encapsulation. A polyanion, alginate, and a polycation, a lactose-modified chitosan, were mixed under physiological conditions (pH 7.4 and NaCl 0.15) and at a semidilute concentration avoiding associative phase separation. The mutual solubility was found to be dependent on the charge screening effect of the added NaCl salt, being prevented below 0.05 M NaCl. A comparison with the behavior of the polyanion (alginate) under the same experimental conditions revealed that both the viscosity and the relaxation times of the binary polymer solutions are strongly affected by the presence of the polycation. In particular, the occurrence of electrostatic interactions between the two oppositely charged polysaccharides led to a synergistic effect on the zero-shear viscosity of the solution, which showed a 4.2-fold increase with respect to that of the main component of the solution, i.e., alginate. Moreover, the relaxation time, calculated as the reciprocal of the critical shear rate, markedly increased upon reducing the alginate fraction in the binary polysaccharide solution. However, the formation of the soluble complexes and the synergistic effect are reduced upon increasing the concentration of the 1:1 electrolyte. By containing a gel-forming polyanion (alginate, e.g., with  $\text{Ca}^{2+}$  ions) and a bioactive polycation (chitlac, bearing a  $\beta$ -linked D-galactose), the present system can be regarded as a first step toward the development of biologically active scaffold from polysaccharide mixtures.

### Introduction

Polysaccharides have been extensively used for a wide variety of applications encompassing food science (as thickeners, stabilizers, fat substitute, and taste release systems) and bio-(medical)technology (articular viscosupplementation<sup>1–4</sup> and cell therapy<sup>5–7</sup> above all). In particular, the latter has gained an increasing attention in the scientific community, and numerous applications of alginate, hyaluronan, and chitosan for tissue engineering and cell encapsulation have been proposed.<sup>8,9</sup>

Besides the interest elicited by natural polyelectrolytes when considered singularly, it is generally agreed that their mixing could lead to complex biomimetic systems resembling the biological milieu of entangled macromolecules, commonly termed as Extracellular Matrix (ECM), that embeds cells providing for adhesion and proper biomechanical signaling. Moreover, the simultaneous presence of different polysaccharides is expected to induce notable variation of the overall physicochemical properties of the system. In fact, it has been established that the solution properties of biopolymer mixtures can be quite different from those of the pure components,<sup>10,11</sup> due to their synergistic interactions.<sup>12–17</sup>

Alginate and chitosan are a polyanion and a polycation, respectively, easily isolated from natural sources. The former polysaccharide is produced by marine brown algae and bacteria and consists of 1→4 linked  $\beta$ -D-mannuronic acid and  $\alpha$ -L-guluronic acid arranged in different types of block-wise patterns.

At variance, chitosan is obtained from chemical deacetylation of chitin, the main component of the exoskeleton of crustaceans, and is composed of 1→4 linked  $\beta$ -D-glucosamine units with some residual 2-acetamido-2-deoxy- $\beta$ -D-glucosamine groups randomly distributed along the polymer chain. When considering the possibility of preparing solutions containing both such oppositely charged polysaccharides, it should be kept in mind that the use of pH values above  $\sim 6$  is prevented by the precipitation of highly deacetylated chitosans.<sup>18</sup> For a similar reason (gelation/precipitation of alginate), the use of pH values lower than  $\sim 3$  has to be avoided. This implies that when alginate and chitosan solutions are mixed, the contemporaneous presence of negative charges on the polyanion (alginate) and positive charges on the polycation (chitosan) leads to associative phase separation which cannot be suppressed by adding 1:1 electrolytes, as usual for mixtures of oppositely charged polysaccharides.<sup>19</sup> In fact, the intrinsic lack of mutual solubility between these two polysaccharides has been exploited to reinforce and to control the permeability of calcium alginate capsules in the field of cell microencapsulation.<sup>20,21</sup> The latter has emerged as a feasible approach for the production of insulin-producing bioreactors for the treatment of type I-diabetes.<sup>22</sup>

However, the analysis of the main features of the two polysaccharides as such might sprout the idea that a soluble combination of the two systems could be beneficial for many applications. In fact, alginate has been extensively studied for its ability to form stable gels, under cell-friendly conditions, when in contact with solutions containing the proper divalent cation (such as calcium).<sup>7,23</sup> On the other hand, chitosan is a biodegradable biopolymer with interesting antimicrobial and hemostatic properties.<sup>24</sup> Moreover, the presence of amino

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groups on the polymer backbone renders highly deacetylated chitosans particularly appealing candidates for the development of a bioactive biomaterial through the insertion of cell specific ligands such as oligosaccharide sequences. As an example, the introduction of  $\beta$ -linked D-galactose moieties (as the non-reducing end in lactitol) on the chitosan backbone led to an engineered polysaccharide, named chitlac, granted of both biological significance<sup>25,26</sup> and complete solubility at neutral pH.

Prompted by these considerations, the possibility of preparing binary polymer solutions by using the two oppositely charged polysaccharides, namely alginate and chitlac, was explored. In the present paper, the flow properties of the binary polymer solutions were analyzed by means of a rheological approach and the results compared with those displayed by the single polymeric components considered separately. In particular, the presence of a synergistic effect on the viscosity and on the relaxation time of the binary polymer solution, as a consequence of the inter-polysaccharide electrostatic interactions, was assessed.

## Materials and Methods

Sodium alginate isolated from *Laminaria hyperborea* stipe, LF 10/60 ( $F_G = 0.69$ ;  $F_{GG} = 0.56$ ;  $M_w = 1.3 \times 10^5$ ), was provided by FMC Biopolymers (Norway). Chitlac (1-Deoxylactit-1-ylchitosan (CAS registry number 85941-43-1),  $M_w$  approximately  $1.5 \times 10^6$ ) sample was prepared according to the procedure reported elsewhere.<sup>25</sup> *N*-(2-Hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) sodium salt (HEPES) and poly-L-lysine (PLL) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium chloride was from Aldrich Chemical Co. (Milwaukee, WI).

**Binary Polymer Solution.** The preparation of the binary polymer solution was accomplished by mixing different amounts of a 1% (w/V) solution of chitlac with a 3% (w/V) solution of alginate under vigorous stirring. Binary polysaccharide mixtures containing different alginate weight fractions ( $\psi_{Alg}$  refers to the binary polymer composition) were therefore obtained. The polymer solutions were prepared in the presence of 0.15 M sodium chloride maintaining a constant HEPES concentration (10 mM, pH 7.4). In addition, the mixing of the alginate and chitlac solution was also attempted at pH 5.5 in the presence of NaCl (0.15 M) and at pH 7.4 without additional 1:1 electrolyte.

**Photon Transmission Intensity (Transmittance) Measurements.** The transmittance of the binary polymer mixtures containing alginate and chitlac was measured at different wavelength values (namely 480 nm, 520 nm, and 600 nm, respectively) with a Cary4E UV-visible spectrophotometer. For comparison, the transmittance of each of the two oppositely charged polysaccharides was determined.

**<sup>1</sup>H NMR Spectroscopy.** The <sup>1</sup>H NMR spectra were recorded at 90 °C with a Bruker Advance DPX 400 spectrometer with the chemical shifts expressed in ppm downfield from the signal of 3-(trimethylsilyl)-propanesulfonate. The binary polymer solution, at a total concentration of 0.2 g/L, was treated following the procedure reported by Grasdalen,<sup>27</sup> and the pH was adjusted to approximately 7 prior to analysis.

**Viscosity Measurements.** Reduced capillary viscosity was measured at 25 °C by means of a Schott-Geräte AVS/G automatic apparatus and an Ubbelohde type viscometer. For the polysaccharides used in the present study, the intrinsic viscosity ( $[\eta]$ ) values were determined by analyzing the polymer concentration dependence of the reduced specific viscosity ( $\eta_{sp}/c$ ) and of the reduced logarithm of the relative viscosity ( $\ln \eta_{rel}/c$ ) by use of the Huggins (eq 1) and Kraemer (eq 2) equations, respectively:

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c \quad (1)$$

$$\frac{\ln(\eta_{rel})}{c} = [\eta] - k''[\eta]^2 c \quad (2)$$

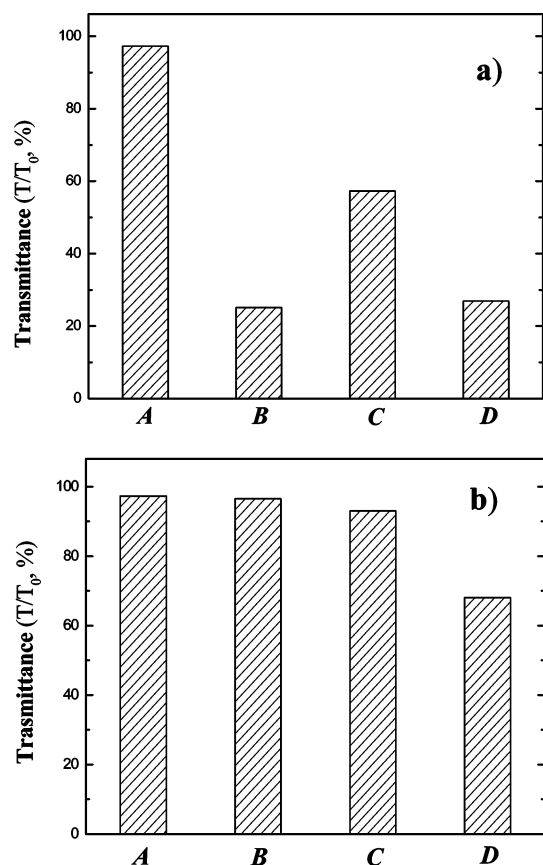
where  $k'$  and  $k''$  are the Huggins and Kraemer constants, respectively.

**Rheological Measurements.** Rheological measurements were performed on a Stress-Tech general-purpose rheometer (Reologica instruments AB, 22363 Lund, Sweden) using a cone-plate geometry (50 mm radius, 1°). The solutions of alginate, chitlac, and the different binary polymer mixtures were loaded on the plate of the rheometer, and the viscosities at different shear rates were obtained by means of steady-state measurements. Two replicate measurements were performed for each sample. The complex viscosity ( $\eta^*$ ) and the storage ( $G'$ ) and loss ( $G''$ ) moduli of the binary polymer solutions were recorded in the frequency range 0.01–50 Hz (maximum strain <3%). All the measurements were performed at 25 °C.

## Results and Discussion

**(a) Binary Polymer Solutions from Oppositely Charged Polysaccharides.** In order to study the behavior of a semidilute binary polymer mixture of alginate and chitlac, a 2% (w/V) solution with an alginate weight fraction ( $\psi_{Alg}$ ) of 0.75 was considered.<sup>28</sup> First, the possibility of preparing such a mixture containing the two oppositely charged polysaccharides under semidilute conditions avoiding extended coacervation/precipitation was assessed. As reported in Figure 1a (column A), a complete miscibility between alginate and chitlac was achieved under physiological conditions, i.e., in the presence of NaCl 0.15 M and at pH 7.4. Moreover, it was noted that both neutral pH and the presence of a sufficient amount of added salt (NaCl) are required to guarantee the mutual solubility of the oppositely charged polysaccharides. In fact, the transmittance of the binary mixture dropped when the pH of the solution was lowered to 5.5 (Figure 1a column B): it can be reasonably correlated with the simultaneous increase of charge density on the chitlac component of the mixture and the decrease on the alginate one. Similarly a decrease of the concentration of the added salt in the binary polymer mixture induced an associative phase separation (Figure 1a column C) due to (i) a net entropic gain by the counterions of the polyelectrolytes upon phase separation and (ii) a reduction of the charge screening effect between the polyelectrolyte chains (i.e., increasing the Debye length,  $\kappa^{-1}$ , defined as the distance over which the electrostatic field of an ion extends with appreciable strength, being  $\kappa^2 \propto I$ ). The use of pH = 7.4 and the presence of a 1:1 electrolyte at 0.15 M concentration did not, *per se*, imply the mutual miscibility of oppositely charged polyelectrolytes. In fact, Figure 1a (column D) reports the decrease in transmittance detected upon replacing chitlac with poly-L-lysine (PLL) in the binary polymer solution. This result was not surprising, since the polycation PLL has been extensively reported to stabilize calcium-alginate gel beads through the formation of coacervates on the surface of the negatively charged bead in the so-called “coating” process.<sup>29,30</sup> Therefore, an active role of the bulky *N*-lactit-1-yl groups of chitlac in hampering polyanion–polycation extended associations at neutral pH can be preliminarily proposed.

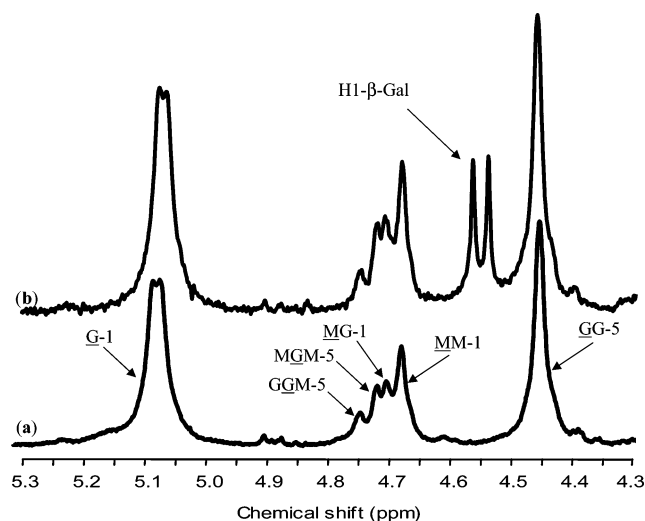
The effect of 1:1 electrolytes on the solubility of the binary mixture was further assessed by reducing the overall concentration of NaCl in the solution. In all the cases reported, the reduction of NaCl was compensated by the addition of a nonionic solute (mannitol) to attain isoosmolar conditions. As seen from Figure 1b (column C), a minimum concentration of 0.05 M of NaCl is required to allow for the mutual solubility of the two polysaccharides.



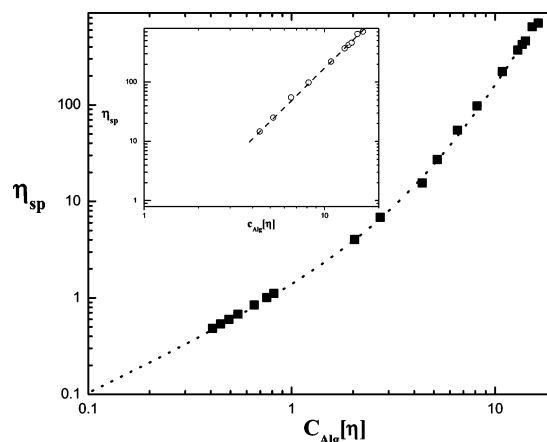
**Figure 1.** (a) Relative transmittance (%; 600 nm) of a binary polymer solution containing alginate (1.5%) and chitlac (0.5%) in (A) NaCl 0.15 M, HEPES 10 mM, pH 7.4; (B) NaCl 0.15 M, pH 5.5; (C) HEPES 10 mM pH 7.4; (D) relative transmittance (%; 600 nm) of a binary polymer mixture containing alginate (1.5%) and poly-L-lysine (0.5%) in NaCl 0.15 M, HEPES 10 mM, pH 7.4. (b) Relative transmittance (%) of a binary polymer solution containing alginate (1.5%) and chitlac (0.5%) in (A) NaCl 0.15 M, HEPES 10 mM, pH 7.4; (B) NaCl 0.1 M, mannitol 0.1 M, HEPES 10 mM, pH 7.4; (C) NaCl 0.05 M, Mannitol 0.2 M, HEPES 10 mM, pH 7.4; (D) NaCl 0.025 M, Mannitol 0.25 M, HEPES 10 mM, pH 7.4. ( $T_0$  = transmittance of the alginate solution considered separately).

The binary solution containing both alginate and chitlac was characterized by means of  $^1\text{H}$  NMR analysis (Figure 2b) which revealed, in addition to the signals assigned to the polyanion (Figure 2a), the presence of a doublet centered at around 4.55 ppm, arising from the side-chain galactose moieties on chitlac. It is noteworthy that the NMR signals of the anomeric protons of alginate in the binary polymer mixture sample have not been altered, under the experimental conditions used for the analysis, by the presence of the polycation. In fact, the diadic composition calculated for the alginate in the binary polymer mixture ( $F_G = 0.67$ ;  $F_{GG} = 0.55$ ) correlates very well with pure alginate sample ( $F_G = 0.69$ ;  $F_{GG} = 0.56$ ). Since the anomeric protons of the in-chain sugar residues of chitlac are shifted upfield at pH 7.4 (spectrum not reported), no interference of such signals in the anomeric region of the spectrum was detected.

**(b) Alginate Solutions in the Semidilute Regime.** An analysis of the main rheological properties of semidilute alginate solutions has been considered as preliminary to the investigation of any synergistic interaction of alginate with chitlac in the polymer mixture. To this end, the specific viscosity of alginate solutions was measured under the same conditions used for the preparation of the binary polymer mixture (i.e., NaCl 0.15M; HEPES 10 mM; pH 7.4). Figure 3 reports the dependence of  $\eta_{sp}$  on the coil overlap factor,  $C_{Alg}[\eta]$ , for alginate in both dilute



**Figure 2.**  $^1\text{H}$  NMR spectra of (a) alginate and of (b) the binary polymer mixture of alginate (1.5%) and chitlac (0.5%). The additional peak at around 4.55 in part b can be assigned to the anomeric proton of the  $\beta$ -galactose moieties in chitlac.

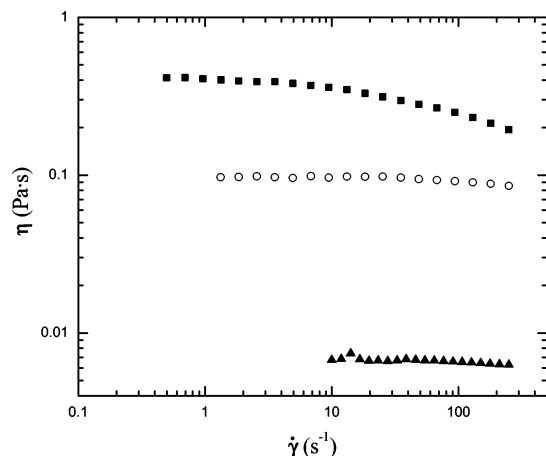


**Figure 3.** (■) Dependence of the specific viscosity on the coil overlap factor for alginate in NaCl 0.15 M, HEPES 10 mM, pH 7.4. (.....) Theoretical dependence calculated on the basis of eq 4 ( $[\eta]_{Alg} = 5.44\text{dL/g}$ ;  $k' = 0.353$  as determined from eq 1 in dilute conditions;  $c = C_{Alg}$ ). Inset: magnification of the main figure in the semidilute regime. The linear regression (---) of the experimental data in the latter concentration regime gave  $R^2 = 0.987$  and a slope of 3.1.

and semidilute regimes.  $C_{Alg}$  is the specific concentration of alginate, expressed in the units of  $([\eta])^{-1}$ . The measurements of  $\eta_{sp}$  have been performed with a capillary viscometer for  $C_{Alg}[\eta]$  values below 4, while a rotational rheometer (steady-state conditions) was used for solutions with a higher polymer concentration. In the latter case, the zero-shear viscosity,  $\eta_{\dot{\gamma}=0}$ , was extrapolated from the  $\eta - \dot{\gamma}$  dependence by means of the so-called Cross equation.<sup>31,32</sup>

In the semidilute condition, i.e., for a coil overlap factor higher than 4,<sup>33</sup> the specific viscosity of the polysaccharide solutions was found to scale with the polymer concentration according to  $\eta_{sp} \propto C_{Alg}^{3.1}$  (Figure 3 inset). The latter value is somewhat lower than expected for a semidilute entangled system, i.e.  $\eta_{sp} \propto c^{15/4}$ , but it is in good agreement with the value already reported in literature, i.e.,  $\sim 3.3$ ,<sup>33</sup> thus supporting the validity of the experimental data. In the same concentration regime, the relaxation time,  $\tau_{relax}$  ( $\tau_{relax} = 1/\dot{\gamma}_{cr}$  where  $\dot{\gamma}_{cr} = 0.8\dot{\gamma}_0$ <sup>34</sup>) scaled as  $\tau_{relax} \propto C_{Alg}^{1.8}$ , thus substantially in good agreement with the value expected for a semidilute entangled





**Figure 4.** Dependence of the viscosity ( $\eta$ ) on the shear rate ( $\dot{\gamma}$ ) for (O) alginate (1.5%), (▲) chitlac (0.5%) and (■) their binary mixture (final concentration: alginate 1.5% and chitlac 0.5%;  $\psi_{\text{Alg}} = 0.75$ ) in NaCl 0.15 M, HEPES 10 mM, pH 7.4.

system, i.e.  $\tau \approx c^{3/2}$ . For sake of comparison, a scaling law of  $\tau_{\text{relax}} \propto c^{2.2}$  was found in the case of hyaluronan.<sup>35</sup>

It has been reported that in the case of hyaluronan<sup>36</sup> the experimental trend of the specific viscosity can be reproduced by means of the four-term equation resulting from the truncated form of the exponential expression derived by Martin:

$$\eta_{\text{sp}} = ([\eta]c)e^{k'[\eta]c} \quad (3)$$

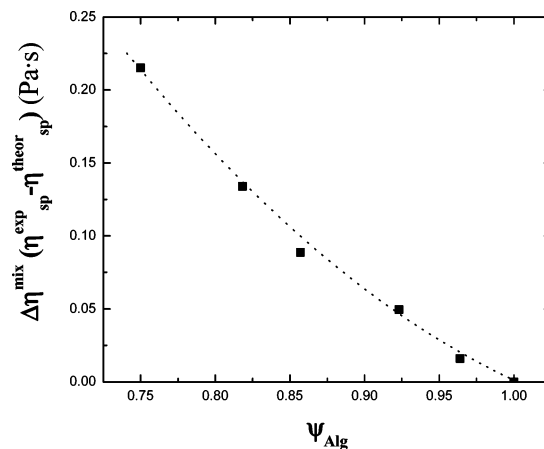
which reads

$$\eta_{\text{sp}} = c[\eta] + k'(c[\eta])^2 + \frac{(k')^2}{2!}(c[\eta])^3 + \frac{(k')^3}{3!}(c[\eta])^4 \quad (4)$$

where  $[\eta]$  and  $k'$  are the intrinsic viscosity and the Huggins constant, respectively, of the polysaccharide, and  $c$  is the polymer specific concentration. Along this line, eq 4 was used to calculate the  $\eta_{\text{sp}} - (C_{\text{Alg}}[\eta])$  relationship for alginate under the above-reported experimental conditions. A good agreement between the experimental data and the theoretical curve (Figure 3) was obtained, hence demonstrating the feasibility of this approach.

#### (c) Addition of Chitlac: Synergistic Effect on Viscosity.

The possibility of preparing binary polymer solutions containing a polyanion (alginate) and a polycation (chitlac) does not *per se* imply the complete lack of interaction between the two oppositely charged macromolecular species. On the contrary, the viscosity of a binary polymer solution (alginate weight fraction,  $\psi_{\text{Alg}} = 0.75$ : alginate concentration = 1.5% (w/V), chitlac concentration 0.5% (w/V)) (Figure 4) is about 4.2-fold higher than that exhibited by the main component of the mixture when considered separately, i.e., the 1.5% alginate solution. Taking into consideration the rather low viscosity displayed by a 0.5% solution of chitlac, it seems safe to allocate this synergistic effect on the viscosity of the binary system to the presence of interactions of electrostatic origin between the two polysaccharides. The latter statement is confirmed by considering the zero-shear viscosity of the same binary polymer solution in the presence of a higher concentration of added salt, namely NaCl 0.75 M, where the ratio between the viscosity of the binary system and that of the main component of the mixture, i.e., alginate, decreases from 4.2 to 3.1 (not reported). Once more, the increase of sodium chloride screened the electrostatic interactions between alginate and chitlac and reduced the entropy gain driving the formation of inter-polyelectrolyte complexes.



**Figure 5.** Dependence on the alginate weight fraction ( $\psi_{\text{Alg}}$ ) of the difference between the experimentally measured ( $\eta_{\text{sp}}^{\text{exp}}$ ) and theoretically calculated ( $\eta_{\text{sp}}^{\text{theor}}$ ) specific viscosities of the binary polymer solution of alginate and chitlac. The latter was calculated on the basis of eq 5 assuming no interaction between the two polymeric components of the mixture ( $[\eta]_{\text{chitlac}} = 3.49 \text{ dL/g}$ ;  $k' = 0.488$  as determined from eq 1 in dilute conditions). Solvent: NaCl 0.15 M, HEPES 10 mM, pH 7.4. The line has been drawn to guide the eye.

In view of the very good agreement between the experimental data and the theoretical predictions (as calculated from eq 4) achieved for the viscosity of alginate solutions in semidilute conditions (see Figure 3), a similar approach was used to predict the specific viscosity of the binary mixture of alginate and chitlac,  $\eta_{\text{sp}}^{\text{theor}}$ , on the basis of the relative concentration of the two polysaccharides:

$$\eta_{\text{sp}}^{\text{theor}} = [\eta]^{\text{theor}}(C_{\text{Alg}} + C_{\text{chit}})e^{k^{\text{theor}}[\eta]^{\text{theor}}(C_{\text{Alg}} + C_{\text{chit}})} \quad (5)$$

where

$$[\eta]^{\text{theor}} = \sum_i \Phi_i [\eta]_i \quad (6)$$

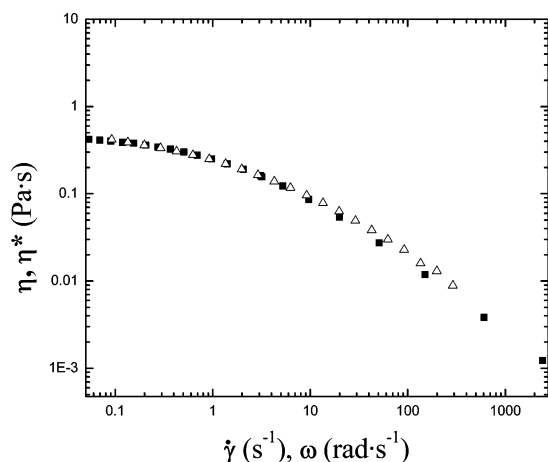
$\Phi_i$  being the weight fraction of the  $i$ -th components of the mixture (alginate or chitlac) and  $[\eta]_i$  its intrinsic viscosity.

Under the assumption that no interaction is occurring between the two oppositely charged polysaccharides, the Huggins cross-coefficient reads:

$$k^{\text{theor}} = \sum_i \Phi_i k_i \quad (7)$$

with  $k_i$  the Huggins coefficient of the  $i$ -th component of the mixture considered separately in the same solvent and at the same temperature.

Figure 5 reports the dependence of the difference between the experimental,  $\eta_{\text{sp}}^{\text{exp}}$ , and the theoretical zero-shear viscosity,  $\eta_{\text{sp}}^{\text{theor}}$ , on the alginate weight fraction in the case of binary polymer mixtures of alginate and chitlac. It should be noted that, in the  $\psi_{\text{Alg}}$ -range explored, the higher is the fraction of chitlac in the binary polymer solution (and consequently the lower the fraction of alginate present), the higher is the deviation in the zero-shear viscosity from the value expected assuming no interactions between polymers (according to eq 5). This result provided additional confidence on the interpretation of the synergistic effect on the viscosity of these binary polymer solutions as due to electrostatic interactions between the positive charges on the polycation and the negative charges on the polyanion. Moreover, the monotonic increasing curve reported in Figure 5 suggests that in the soluble complexes formed

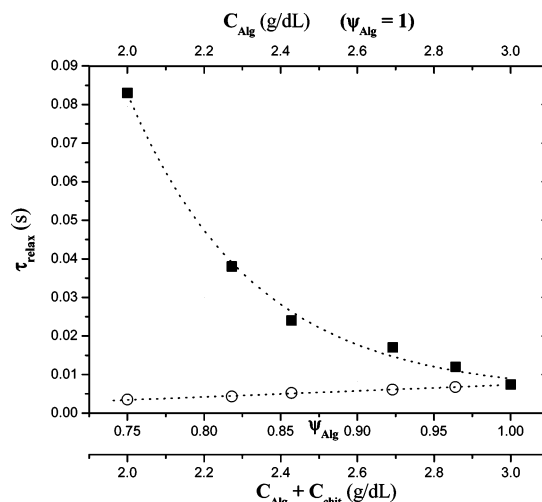


**Figure 6.** Verification of the Cox–Merz rule for the binary polymer solution containing alginate (1.5%) and chitlac (0.5%) in NaCl 0.15 M, HEPES 10 mM, pH 7.4: (■) dependence of the viscosity ( $\eta$ ) on the shear rate ( $\dot{\gamma}$ ) and ( $\Delta$ ) dependence of the complex viscosity ( $\eta^*$ ) on the angular velocity ( $\omega$ ).

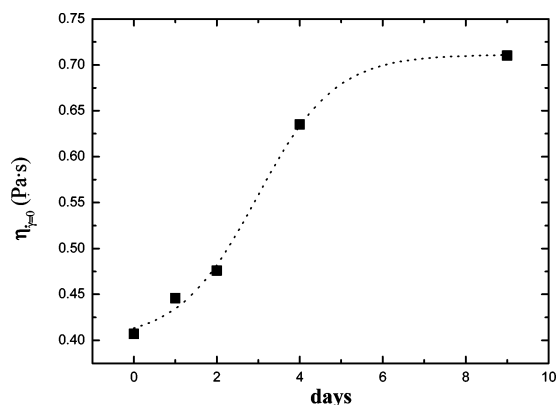
between alginate and chitlac, the polycation is likely to induce the aggregation of several alginate chains, thus forming a highly dynamic micronetwork in solution, with an overall negative charge.<sup>37</sup> It is important to note, however, that the formation of the electrostatic interactions between the macromolecules and the relevant increase in viscosity did not lead to the formation of aggregates or microgels on a large scale. In fact, it was found that the Cox–Merz rule<sup>38</sup> held in the case of the binary polymer solution, as reported in Figure 6.

The synergistic effect arising from the interaction between the polycation and the polyanion was quantified in the case of a binary polymer solution containing 1.5% (w/V) alginate and 0.5% (w/V) chitlac in NaCl 0.15 M. Under the assumption that no interaction between the two polymeric components of the mixture occurs, the zero-shear viscosity predicted by eq 5 is approximately 198 mPa·s (equal to that of a 1.95% (w/V) alginate solution). Conversely, the zero-shear viscosity extrapolated for the actual binary mixture ( $\psi_{\text{Alg}} = 0.75$ ) was found to be approximately 407 mPa·s, corresponding to the zero-shear viscosity of a ~2.45% alginate solution (Figure 3 inset). It therefore seems likely to conclude that the electrostatic contacts between the oppositely charged polyelectrolytes formed (large) soluble complexes which span over a relevant portion of the volume of the solution. The ensuing increase of viscosity corresponds to a "virtual" increase of the total polymer concentration of approximately ~0.5% (w/V).

A further insight into the main features of the binary polymer mixture of alginate and chitlac was achieved from the analysis of the viscosity ( $\eta$ )–shear rate  $\dot{\gamma}$  dependence (Figure 4). In particular, in the case of the binary polymer mixture, a more pronounced shear-thinning behavior was detected, with respect to the pure alginate solutions, directly implying a notable effect on its relaxation time. In fact, Figure 7 shows that by reducing the fraction of alginate (and its concentration) in the binary polymer solution (and consequently increasing the fraction of chitlac), a nonlinear increase of the relaxation time of the system was measured. Conversely, a linear decrease of  $\tau_{\text{relax}}$  was found for pure alginate solutions with (decreasing) concentration equaling the total polymer concentration present in the binary polymer mixtures (and then with constant  $\psi_{\text{Alg}} = 1$ ). Numerically, the relaxation time of the binary polymer solution ( $\tau_{\text{relax}} \sim 83$  ms) containing the two oppositely charged polysaccharides ( $\psi_{\text{Alg}} = 0.75$ , alginate concentration 1.5% (w/V), chitlac concentration 0.5% (w/V)) resulted to be approximately 1 order



**Figure 7.** (■, bottom x-scale) Dependence of the relaxation time  $\tau_{\text{relax}}$  ( $\tau_{\text{relax}} = 1/\dot{\gamma}_{\text{cr}}$  where  $\dot{\gamma}_{\text{cr}} = 0.8\dot{\gamma}_0$ <sup>34</sup>) on the alginate weight fraction  $\psi_{\text{Alg}}$  in the case of binary polymer solutions of alginate and chitlac (having the total polymer concentration reported as  $C_{\text{Alg}} + C_{\text{chit}}$ ). (○, top x-scale) Dependence of the relaxation time,  $\tau_{\text{relax}}$ , of alginate solutions on the concentration, with alginate concentrations equaling the total polymer concentrations of the binary systems. The lines have been drawn to guide the eyes.



**Figure 8.** (●) Zero-shear viscosity ( $\eta_{\dot{\gamma}=0}$ )–storage time relationship for the binary polymer mixture (final concentration: alginate 1.5% and chitlac 0.5%;  $\psi_{\text{Alg}} = 0.75$ ;  $T = 25^\circ\text{C}$ ). Solvent: NaCl 0.15 M, HEPES 10 mM, pH 7.4. The line has been drawn to guide the eye.

of magnitude higher than that of an alginate solution (~3.6 ms) of equal total polymer concentration (i.e., 2%). Once more, this behavior is explained by considering the presence, in the semidilute binary polymer solution, of soluble polyanion–polycation complexes which, by fastening polymer chains through electrostatic interactions, likely hamper (to some extent) their molecular motions hence accounting for the higher time required by the system to relax after the application of the stress.

The characterization so far reported has been performed on freshly prepared solutions. However, a preliminary screening of the effect of time on the properties of the mixtures has also been carried out. In particular, it was noted that the zero-shear viscosity increased upon storing the binary polymer mixture ( $\psi_{\text{Alg}} = 0.75$ ) at room temperature (Figure 8). Far from being conclusive, this observation seemed to point at the possibility of rearrangements on the soluble complexes which occur on long time-scales and induce further aggregation of polysaccharide chains. In all the cases, however, the formation of networks on a large scale was not detected, since the Cox–Merz rule still applied to the 9-day-stored binary mixture (data not

reported). However, further analyses are needed to confirm the latter observation.

### Conclusions

The use of polysaccharide mixtures to develop Extracellular Matrix (ECM)-like systems enabling cell embedding and tissue engineering is a current challenge for biomaterial engineering. In this sense, key aspects such as the polymer miscibility and the presence of synergistic effects on the overall physical properties of the mixture have to be properly addressed and explored for a successful final outcome. Two main contributions deserve to be stressed based on the results of this study:

(i) The miscibility between the polyanion alginate and the polycation chitosan is achieved, under the proper experimental conditions, when a bulky and highly hydrophilic side-chain moiety, namely lactitol, is introduced onto the latter.

(ii) The presence of negative charges on one polysaccharide and of positive charges on the other one leads to interpolymer electrostatic interactions which induce the formation of soluble complexes and account for a notable increase of both viscosity and relaxation time of the binary polymer solution (i.e., synergistic effect). However, the (likely) transient and dynamic nature of the electrostatically based network did not induce large scale aggregations nor microgel formation.

It is worth mentioning that in the present case the choice of polymeric materials for the binary mixture overcomes stringent physicochemical considerations (i.e., solubility and net charge). In fact, by containing at the same time a bioactive (chitlac) and a ionotropic (alginate) polysaccharide, the binary polymer solution presented here might be a very appealing candidate for the development of an engineered semi-IPN hydrogel obtained under cell-friendly conditions. Along this line, the semidilute mixture of alginate and chitlac described in this paper is currently under study for the development of a bioactive scaffold for articular chondrocytes encapsulation with potential applications in cartilage reconstructive surgery.<sup>28</sup> In addition, from the standpoint of a polymeric solution, a synergistic effect on the viscosity could be of interest in all those applications in which one single polysaccharidic component does not provide for adequate viscoelastic properties.

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