

Tuning Supramolecular Structuring at the Nanoscale Level: Nonstoichiometric Soluble Complexes in Dilute Mixed Solutions of Alginate and Lactose-Modified Chitosan (Chitlac)

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Two oppositely charged polysaccharides, alginate and a lactose-modified chitosan (chitlac), have been used to prepare dilute binary polymer mixtures at physiological pH (7.4). Because of the negative charge on the former polysaccharide and the positive charge on the latter, polyanion–polycation complex formation occurred. A complete miscibility between the two polysaccharides was attained in the presence of both high (0.15 M) and low (0.015 M) concentrations of simple 1:1 supporting salt (NaCl), as confirmed by turbidity measurements; phase separation occurred for intermediate values of the ionic strength (I). The binary solutions were further characterized by means of light scattering, specific viscosity, and fluorescence quenching measurements. All of these techniques pointed out the fundamental role of the electrostatic interactions between the two oppositely charged polysaccharides in the formation of nonstoichiometric polyelectrolyte soluble complexes in dilute solution. Fluorescence depolarization (P) experiments showed that the alginate chain rotational mobility was impaired by the presence of the cationic polysaccharide when 0.015 M NaCl was used. Moreover, upon addition of calcium, the P values of the binary polymer mixture in 0.015 M NaCl increased more rapidly than that of an alginate solution without chitlac, suggesting an efficient crowding of the negatively charged alginate chains caused by the polycation.

Introduction

The study of the physicochemical parameters influencing the mixing of oppositely charged polyelectrolytes has been extensively tackled from both an experimental and a theoretical point of view. The first experiments performed in the field led to describe the liquid–liquid phase separation arising from the mixture of oppositely charged polyelectrolytes as coacervation.¹ Later, the term “associative phase separation” was suggested as more appropriate to describe such a phenomenon. The formation of aggregates involving biopolymers,² micelles,³ and dendrimers⁴ was reported and has prompted relevant research in the field encompassing the study of the influence of pH, ionic strength, charge density,^{5,6} and the “critical” stoichiometry for coacervate formation in protein–polyelectrolyte and micelle–polyelectrolyte systems.^{4,7} This extensive work has, on one hand, triggered the development of several interesting applications of the coacervation process (protein separation⁸ and enzyme immobilization,⁹ to name a few) and, on the other, contributed to establish the “phase boundaries” governing the liquid–liquid-phase separation and the precipitation of oppositely charged polyelectrolytes. Moreover, this extended analysis led one to propose different interpretations of the “electrostatically driven complexation” process (very recently reviewed by Dubin and co-workers in ref 10) and to recognize the entropic significance of the counterions released when associative phase separation occurs.¹¹

From a theoretical point of view, the first model developed to describe the formation of a concentrated complex coacervate phase was proposed by Voorn and Overbeek.¹² According to them, the process of coacervation of oppositely charged poly-

electrolytes should be regarded as a “spontaneous phenomenon” resulting from the competition between the electrostatic attractive forces and the entropic dispersing effects acting toward the separation of the polymeric chains. Within the assumptions of this model (electrostatic interactions described using the Debye–Hückel theory, entropic term provided by the Flory–Huggins theory, unperturbed random coil configurations for the polyions, negligible solvent–solute interactions), a proper description of the albumin/acacia gum complex coacervation was achieved.¹² A different approach was developed by Veis and Aranyi¹³ in their “dilute phase aggregate model”, where, besides taking into account the solvent–solute interactions, a notable contribution to the overall coacervation process is allocated to the conformational entropy gain arising from the rearrangements of the polymeric chains following the formation of the very first complexes. In fact, the main innovative contribution of this model, at variance with the Voorn–Overbeek one, is the description of complex coacervation of oppositely charged polyelectrolytes as a two-step process. First, a close approach driven by the electrostatic interaction occurs between oppositely charged polyelectrolytes to form coacervate solutions of low configurational entropy. Second, an entropically driven rearrangement of the latter leads to the formation of complex coacervates. Thus, the Veis–Aranyi theory replaces the “spontaneous and instantaneous” phenomenon depicted by Voorn and Overbeek with a process that can take much longer to occur.^{10,14} The “coacervate solutions” have been later described as “soluble complexes”.^{15–17} The latter require particular conditions to be formed, such as the presence of one component with weak ionic groups. In fact, the use of strong polyelectrolytes in aqueous solution leads to the formation of non-soluble polyelectrolyte complexes. As an example, even in highly dilute solution, sodium poly(styrenesulfonate) and poly(diallyldimethylammonium chloride-*co*-acryl-amide) form quasi-soluble particles.^{18,19}

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The peculiar feature of the “soluble complex” rests on their ability to undergo structural rearrangements via polyelectrolyte exchange reaction with the polymer chains present in solution.²⁰ If the latter step is prevented or suppressed, the soluble complexes will evolve into the formation of large-scale aggregates.²¹

Izumrudov and co-workers^{22–24} extensively investigated the dependence of the stability of DNA-containing polyelectrolyte complexes on the concentration of added simple salt, the degree of polymerization of the polyelectrolytes, and the polyanion/polycation ratio. These analyses revealed that a fundamental feature for the existence of soluble aggregates is represented by the unbalanced distribution of the charges on the two polymer backbones. In fact, the presence of a net charge, whether positive or negative, on the polyelectrolyte aggregate prevents their extended association and, hence, phase separation. In this sense, either the polyanion or the polycation can be used as a solubilizing component of the polyelectrolyte complex. Recently, additional considerations regarding the electrostatically driven interactions between polyanions and polycations led one to propose a theoretical treatment of the nonstoichiometric water-soluble polyelectrolyte complexes based on Madelung's theory for ionic crystals and on Manning's counterion theory.²⁵

The behavior of semidilute binary polymer solutions containing two oppositely charged polyelectrolytes (alginate and a chitosan derivative named chitlac) was previously reported.²⁶ A synergistic effect on the overall viscosity of the system was ascribed to an electrostatically driven interpolymer interaction with the formation of soluble aggregates. It was further evidenced that the highly dynamic nature of the interpolyelectrolyte contacts did not induce the formation of microgels as inferred from the applicability of the Cox–Merz rule to the binary polymer solution. The preparation^{27,28} and characterization²⁷ of the lactose-modified chitosan, chitlac, has already been reported, and its biological functions have been explored.^{27,29} Beyond its biological significance, chitlac is a polycation characterized by a very good solubility at physiological pH (7.4) due to the presence of the lactitol side-chains on the polysaccharide backbone. At variance, highly deacetylated chitosan is soluble exclusively in acidic media. It is worth mentioning that chitlac induces a galectin-mediated²⁹ extensive aggregation of pig articular chondrocytes into nodules of considerable dimension,²⁷ stimulating, at the same time, the production of type-II collagen and GAGs.

In the present paper, the investigation on binary polymer mixtures of alginate and chitlac is aimed at assessing the electrostatically driven interpolymer interactions in dilute solution. In particular, spectroscopic and viscometric techniques were employed to study the effect of the ionic strength on the aggregation of the two oppositely charged polyelectrolytes. From these analyses, the existence of nonstoichiometric soluble complexes between alginate and chitlac, likely bearing a net negative charge, has been proposed. The present work is aimed at a preliminary characterization of this binary polyelectrolytes mixture. It is not devoted to full assessment of the range of stability of the soluble complexes under different experimental conditions. Nevertheless, to the best of our knowledge, no soluble complex formation between oppositely charged polysaccharides with molecular weight ranging from medium to high has been reported yet. In our opinion, the preliminary screening of the main features of these soluble complexes in solution might provide the basis for the design and development of bioactive material of biotechnological significance.

Materials and Methods

Materials. Sodium alginate isolated from *Laminaria hyperborea* stipe, LF 10/60 ($F_G = 0.69$; $F_{GG} = 0.56$; $M_w = 1.3 \times 10^5$, $d = 1.8$), was provided by FMC Biopolymers (Norway). Chitlac (lactose-modified chitosan, CAS registry number 85941-43-1) sample was prepared according to the procedure reported elsewhere²⁷ starting from a highly deacetylated chitosan (residual acetylation degree = 11.3%). The molecular weight of chitlac was estimated to be approximately 1.5×10^6 . *N*-(2-Hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) sodium salt (HEPES), *N*-hydroxysuccinimide (NHS), 2-[*N*-morpholino]ethanesulfonic acid (MES), fluorescein isothiocyanate (FITC), and Rhodamine 123 were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), calcium perchlorate ($\text{Ca}(\text{ClO}_4)_2$), sodium perchlorate (NaClO_4), and sodium chloride were from Aldrich Chemical Co. (Milwaukee, WI).

Binary Polymer Mixture Preparation (dAC-Solution). The preparation of the dilute binary mixtures was achieved by mixing different amounts of a 1.5 g/L alginate solution and a 1.5 g/L chitlac solution, respectively, hence obtaining different alginate weight fractions (r_{Alg}) under the condition of keeping a total polymer concentration of 1.5 g/L. In all cases, a constant supporting salt/buffer (HEPES) ratio of 15 was maintained, and the pH was adjusted to 7.4. The solutions prepared in the presence of various concentrations (x) of simple supporting salt have been termed dAC_{*x*}-solutions; in the definition, “d” stands for “dilute”, “A” and “C” stand for “alginate” and “chitlac”, respectively, and “*x*” stands for the molar concentration of NaCl.

Turbidity Measurements. The transmittance, T , of the binary polymer mixtures at various alginate weight fractions (r_{Alg}) was measured at 520 nm with a Cary4E UV–visible spectrophotometer at different values of the ionic strength (NaCl). A constant supporting salt/buffer (HEPES) ratio equal to 15 (pH 7.4) was kept throughout the measurements.

Light Scattering. A Perkin-Elmer LS50B spectrofluorimeter was used to record the intensity of the light scattered (90°) by the dAC_{0.15}- and dAC_{0.015}-solutions, respectively, upon irradiation with a 520 nm incident light ($T = 25^\circ\text{C}$). Various alginate weight fractions in the binary mixture (r_{Alg}) were used while keeping a total polymer concentration of 1.5 g/L. For comparison, the scattered intensity for each of the two components of the binary polymer solutions was measured separately.

Viscosity Measurements. Viscosity was measured at 25 °C by means of a Schott-Geräte AVS/G automatic measuring apparatus and an Ubbelohde-type capillary 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 (η_{sp}/c) and of the reduced logarithm of the relative viscosity ($\ln(\eta_{\text{rel}})/c$) by use of the Huggins (eq 1) and Kraemer (eq 2) equations, respectively:

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

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

where k' and k'' are the Huggins and Kraemer constants, respectively. The specific viscosity of dAC_{*x*}-solutions was measured at different alginate weight fractions (r_{Alg}) by means of the same apparatus. A constant total polymer concentration (1.5 g/L) was used in all of the experiments with the binary polymer mixtures.

Fluorescent Labeling of the Polymers. Chitlac was labeled with fluorescein isothiocyanate (FITC) following a procedure previously reported.²⁷ The total amount of FITC used corresponded to label about 1/2000 available amino groups. Rhodamine 123 was used to label carboxylic groups of alginate. Briefly, to a 0.3% (w/v) alginate solution in MES buffer (0.1 M, pH 4.5) containing 10% ethanol were added

NHS and EDC ([EDC]/[COOH] = 1.5; [NHS]/[EDC] = 1) followed by an amount of Rhodamine 123 sufficient to label about 1/1500 carboxylic groups. The solution was stirred overnight in dark at room temperature, dialyzed against NaHCO₃ (2 shifts), and then extensively against water (until the conductivity at 4 °C was below 4 μ S). The pH was adjusted to 7.4, and the solution was filtered through 0.45 μ m Millipore filters and then freeze-dried. Comparison of both the mild reaction conditions used and the very low degree of substitution achieved with similar cases reported in the literature leads one to safely assume that the present polymer derivatization did not affect the polymer backbone integrity or its (very low) hydrophobic features.

Fluorescence Measurements. Fluorescence quenching was measured for the dAC_{0.15}- and dAC_{0.015}-solutions, prepared from fluorescein-labeled chitlac and rhodamine-labeled alginate (total polymer concentration 1.5 g/L, $r_{\text{Alg}} = 0.5$), with a Perkin-Elmer LS50B spectrofluorimeter ($T = 25$ °C). A 450 nm excitation wavelength was used, and the emission spectrum of the binary polymer solution was recorded in the range 520–620 nm ($T = 25$ °C). Both the excitation and the emission bandwidth were set to 4.5 nm. For comparison, the emission spectra of the polymeric components of the polymer mixture were recorded separately.

The fluorescence depolarization (P) of the dAC_{0.015}-solution (total polymer concentration 1.5 g/L, $r_{\text{Alg}} = 0.5$) was evaluated by means of

$$P = \frac{I_{\perp} - G \cdot I_{\parallel}}{I_{\perp} + G \cdot I_{\parallel}} \quad (3)$$

A vertically polarized light at 535 nm was used, and the vertically (I_{\perp}) and horizontally (I_{\parallel}) polarized components of the emitted intensity, respectively, were measured at 590 nm.³⁰ Fluorescence polarization measurements were performed at 25 °C on a binary polymer solution containing rhodamine-labeled alginate and non-labeled chitlac. For comparison, the fluorescence depolarization of solutions (i) containing rhodamine-labeled alginate alone (0.75 g/L) and (ii) containing rhodamine-labeled alginate (0.75 g/L) and unlabeled alginate (0.75 g/L), respectively, were recorded. For the binary polymer mixture containing rhodamine-labeled alginate (dAC_{0.015}) and for the 0.75 g/L rhodamine-labeled alginate solution, measurements were performed before and after the addition of Ca(ClO₄)₂ to produce different R_j ratios, defined as $R_j = [\text{Ca}^{2+}]/[\text{alginate}]_0$ (the concentration of the polymer is expressed per polymer repeating unit, ru).

Zeta-Potential and Z-Average Size Measurements. The zeta-potential and the Z-average size for alginate (0.75 g/L), chitlac (0.75 g/L), and their binary mixture dAC_{0.015}, respectively, was measured in NaCl 0.015 M and pH 7.4 (supporting salt ratio/(HEPES) = 15) by using a Zetasizer Nano ZS system equipped with a 4 mW He–Ne laser (633 nm) (Malvern Instruments, Inc., Southborough, MA).

Results

(a) Effect of the Ionic Strength on the Alginate–Chitlac Mixtures. The solutions of alginate and chitlac were mixed at pH 7.4 ($r_{\text{Alg}} = 0.5$, ■) in the presence of different amounts of supporting salt (NaCl), and the transmittance of the binary systems was recorded at 520 nm (Figure 1). It is noteworthy that the binary polymer mixture displayed a window of highly reduced miscibility for I (ionic strength) spanning from 0.03 to approximately 1.3. Nevertheless, the two oppositely charged polyelectrolytes were completely miscible in both 0.15 and 0.015 M NaCl. The effect of the supporting salt on the solubility of the binary mixture of the two oppositely charged polyelectrolytes was found to be dependent on the polyanion/polycation ratio, as r (Figure 1), in accordance with the results reported by Izumrudov and co-workers.²² In particular, when the amount of chitlac overcomes that of alginate ($r_{\text{Alg}} = 0.15$), the miscibility at low concentration of supporting salt is notably hampered.

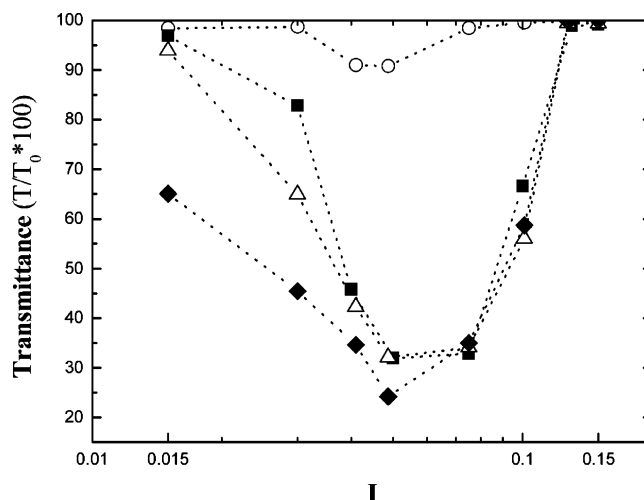


Figure 1. Dependence of the transmittance from the ionic strength (I) for binary polymer mixtures of alginate and chitlac at different values of the weight fraction, r : (○) $r_{\text{Alg}} = 0.75$, (■) $r_{\text{Alg}} = 0.5$, (△) $r_{\text{Alg}} = 0.25$, and (◆) $r_{\text{Alg}} = 0.15$ (total polymer concentration 1.5 g/L). Curves are drawn to guide the eye (T_0 = transmittance of the corresponding alginate solution).

Besides disclosing the experimental conditions for the miscibility of the two polysaccharides to be attained, it is in our opinion important to stress the peculiarity of the minimum of the solution transmittance depending on the ionic strength (I). In the presence of a (relatively) high concentration of 1:1 simple salt (i.e., NaCl = 0.15 M), the polyanion–polycation system is composed of compact macromolecules, which are only minimally affected by interchain electrostatic interactions. The overall net result is a salt-induced solubility for the binary polymer mixture. However, the long-range (and short-range) charge screening effects can be suppressed by significantly reducing the amount of supporting simple salt (i.e., for 0.015 M NaCl). In addition, the inter-polyelectrolyte interactions are obviously favored by the entropic gain arising from the release of counterions in a low-salt containing medium. The number of negative charges on the polyanion (alginate, zeta-potential -63.1 ± 1.2 mV) is much larger than the number of positive ones on the chitlac sample (zeta-potential, $+6.8 \pm 0.8$ mV) (those numbers are essentially independent of the concentration of the supporting electrolyte). Therefore, at $r_{\text{Alg}} = 0.5$ and low I , the complexes obtained from the two oppositely charged polyelectrolytes are expected to be nonstoichiometric (as confirmed by their negative zeta-potential -39.4 ± 2.9 mV), with the excess of negative charges granting a complete solubility to the binary mixture.

(b) Soluble Complex Formation in Dilute Alginate–Chitlac Solutions. *(b1) Optical Properties.* Figure 2 reports the dependence of the percentage of light transmittance for the dAC_{0.15}- and the dAC_{0.015}-solutions on the concentration of alginate (expressed as the weight fraction, r_{Alg}) present in the binary polymer system. The effect of the presence of the two oppositely charged polysaccharides in solution was negligible on T . It can be concluded that, in the r_{Alg} range explored and at pH 7.4, the opposite charges on the two different polymers do not induce the formation of large-scale coacervates or precipitates. Turbidity measurements, however, are not sensitive enough to association processes that may lead to soluble complexes. In fact, the analysis of the light scattered at 90° (which is by far a more sensitive and informative technique) by the dAC_{0.15}- and dAC_{0.015}-solutions revealed a much more

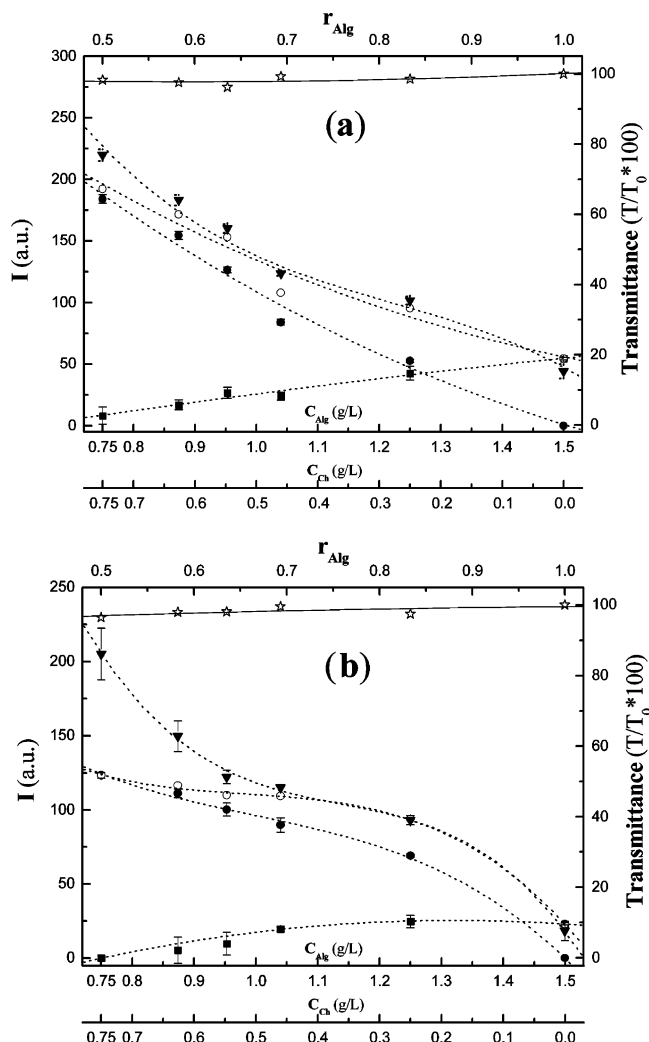


Figure 2. Transmittance (%; 520 nm) (☆, right axis) and 90° scattered light (▼, left axis) for (a) dAC_{0.15}- and (b) dAC_{0.015}-solutions at different values of the alginate weight fraction (r_{Alg} , top axis) in the binary mixture (T_0 = transmittance of the alginate solution). The total polymer concentration was 1.5 g/L in all of the cases analyzed. For comparison, the light scattered at 90° by alginate (■, left axis) and chitosan (●, left axis) solutions considered separately is reported together with their sum (○, left axis). The concentrations are reported on the bottom axes. Values are reported as mean \pm s.d. ($n = 10$). Curves are drawn to guide the eye.

detailed picture of such inter-polyelectrolyte interactions. Under the assumption of a complete lack of interaction between the polymers, the intensity of the light scattered by the mixture ($I_{\text{C}_{\text{tot}}}^{\text{theor}}$) is expected to be simply additive and hence equal to the sum of the intensity of its polymeric components considered separately:

$$I_{\text{C}_{\text{tot}}}^{\text{theor}} = I_{\text{C}_{\text{Alg}}}^{\text{Alg}} + I_{\text{C}_{\text{Ch}}}^{\text{Ch}} \quad (4)$$

where $I_{\text{C}_{\text{Alg}}}^{\text{Alg}}$ and $I_{\text{C}_{\text{Ch}}}^{\text{Ch}}$ are the intensities of the alginate and chitosan solutions, respectively, and C_{Alg} and C_{Ch} are their concentrations in the binary mixtures. C_{tot} represents the total polymer concentration in the mixture (1.5 g/L). Figure 2 reports the comparison between the theoretical “additive” curve (eq 4, ○) and the experimental curve (▼) of the scattered light in the case of dAC_{0.15}- (Figure 2a) and dAC_{0.015}-solutions (Figure 2b). While in the former case the experimental curve shows a very limited deviation from the predicted behavior (about +15%), when the amount of supporting salt is reduced to 0.015 M, that

is, in the case of dAC_{0.015}-solution, notable discrepancies between the two curves are found (about +65%). This is consistent with the schematic picture reported by Dubin and co-workers³¹ and by de Kruif and co-workers,³² and it unambiguously points to the presence of soluble aggregates of the two oppositely charged polyelectrolytes in the binary polymer solution dAC_{0.015}.

An encouraging support to this interpretation comes from the results obtained with the Zetasizer Nano system, which allowed one to achieve a preliminary evaluation of the dimension of the soluble complexes for $r_{\text{Alg}} = 0.5$ in 0.015 M NaCl: the value of the (z -average) apparent hydrodynamic size for the aggregates (172.6 ± 18.3 nm) was much larger than those separately recorded for alginate (46.4 ± 2.7 nm) and chitosan (70.9 ± 0.8 nm) under the same experimental conditions. Far from being conclusive, this analysis seems to confirm the formation of inter-polyelectrolyte complexes under the above-reported experimental conditions.

(b2) *Viscometric Properties.* Different experimental techniques were exploited to further investigate the existence and the main characteristics of the soluble alginate–chitosan aggregates. In particular, the dependence of the specific viscosity (η_{sp}) on the polymer composition of the binary solutions was measured in aqueous NaCl solutions, 0.15 and 0.015 M, respectively (Figure 3a and b). For comparison, in both cases, the η_{sp} for the two polymeric components separately considered is reported together with the theoretical value of the specific viscosity of the binary mixtures $\eta_{\text{sp}}^{\text{theor}}$ calculated, under the assumption of the simple additive contribution, by means of:^{26,33}

$$\eta_{\text{sp}}^{\text{theor}} = ([\eta]_{\text{Alg}} \cdot C_{\text{Alg}} + [\eta]_{\text{Ch}} \cdot C_{\text{Ch}} + k_{\text{Alg}} \cdot [\eta]_{\text{Alg}}^2 \cdot C_{\text{Alg}}^2 + k_{\text{Ch}} \cdot [\eta]_{\text{Ch}}^2 \cdot C_{\text{Ch}}^2 + k_{\text{mix}} \cdot [\eta]_{\text{Alg}} \cdot [\eta]_{\text{Ch}} \cdot C_{\text{Alg}} \cdot C_{\text{Ch}}) \quad (5)$$

where $[\eta]_{\text{Alg}}$ and $[\eta]_{\text{Ch}}$ are the intrinsic viscosities of alginate and chitosan, respectively, and k_{Alg} and k_{Ch} are the corresponding Huggins constants. Under the assumption that no interaction is occurring between the two oppositely charged polysaccharides, the Huggins cross-coefficient reads:

$$k_{\text{mix}} = \frac{C_{\text{Alg}} \cdot k_{\text{Alg}} + C_{\text{Ch}} \cdot k_{\text{Ch}}}{C_{\text{tot}}}, \quad C_{\text{tot}} = C_{\text{Alg}} + C_{\text{Ch}} \quad (6)$$

where C_{Alg} and C_{Ch} are the concentrations of alginate and chitosan in the binary polymer mixtures, respectively.

It can be noted that when a low amount of supporting salt (0.015 M NaCl) is added to the mixture, that is, in the case of dAC_{0.015}-solution, relevant discrepancies between the experimental and the theoretical behavior of the specific viscosity of the system are detected (Figure 3b). In particular, the dAC_{0.015}-solution with equivalent amounts of the two polymeric components ($r_{\text{Alg}} = 0.5$) displays a 3.1-fold increase in the specific viscosity with respect to the value measured for an alginate solution of equal total polymer concentration (i.e., $r_{\text{Alg}} = 1$). Conversely, a slight decrease of η_{sp} is theoretically predicted assuming the lack of interactions between the two polysaccharides, that is, by using eq 5. Moreover, by diluting the alginate solution with chitosan, that is, by reducing the alginate weight fraction r_{Alg} in the binary mixture, a monotonic increase in the specific viscosity is detected, in accordance with previous observations.²⁶ This marked increase in specific viscosity can be safely traced back to the formation of soluble complexes led by the presence of electrostatic interaction between the negative charges on one of the polysaccharides (alginate) and

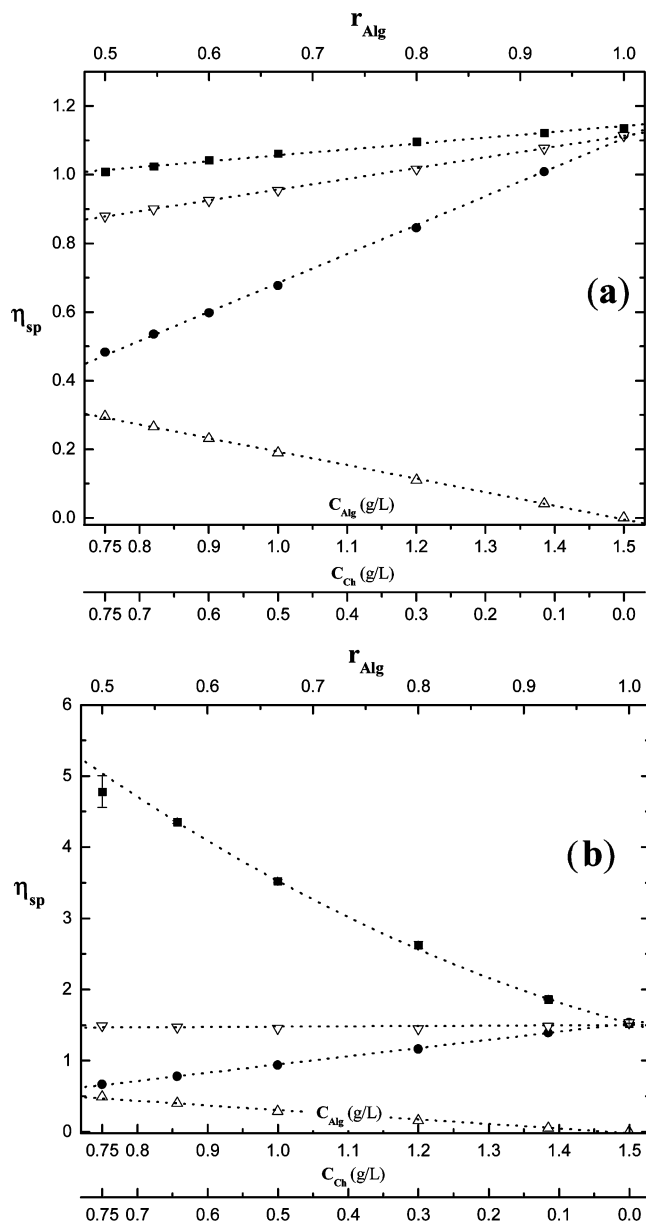


Figure 3. (■) Specific viscosity of (a) dAC_{0.15}- and (b) dAC_{0.015}-solutions as a function of alginate weight fraction (r_{Alg} , top axis) in the binary mixture. A total polymer concentration of 1.5 g/L was used in both cases. For comparison, the specific viscosity of alginate (●) and chitlac (△) solutions considered separately is reported together with their sum ($\eta_{\text{sp}}^{\text{theor}}$) (▽) calculated according to eq 5. Concentrations are reported in the bottom axes. Values are reported as mean \pm s.d. ($n = 6$). Curves are drawn to guide the eye.

the positive charges on the other one (chitlac). A 10-fold increase of the supporting salt concentration strongly limits (but not completely impedes) the latter interactions. In fact, in the case of dAC_{0.15}-solution (Figure 3a), the discrepancy between the experimental and theoretical values of η_{sp} (about +19%) is markedly suppressed, hence accounting for a salt-induced hindering of the interpolymer electrostatic associations.

To gain more information on the main features of the binary polymer mixtures between alginate and chitlac, the limiting behavior of η_{sp} for the binary dAC_{0.15}- and dAC_{0.015}-solutions was considered (Figure 4a and b). In particular, the variation of the reduced specific viscosity of the two binary solutions containing an equal amount of the two polymeric components, thus with alginate weight fraction $r_{\text{Alg}} = 0.5$, was measured upon diluting the system with the solvent. The η_{sp}/c versus c

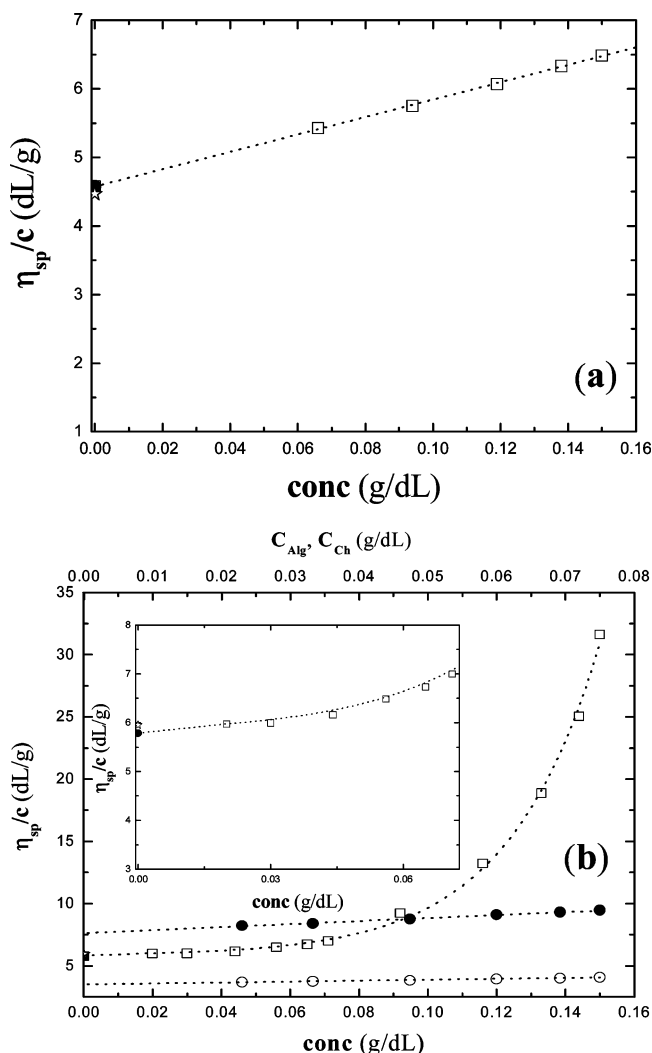


Figure 4. (□) Dependence of the reduced specific viscosity from the total polymer concentration for (a) dAC_{0.15}- and (b) dAC_{0.015}-solutions. In both cases, $r_{\text{Alg}} = 0.5$. The intrinsic viscosities extrapolated at infinite dilution (■) and calculated according to eq 7 (☆) are reported. Inset: Magnification of Figure 2b in the lower concentration range. In (b), the dependence of reduced specific viscosity on polymer concentration of alginate (●) and chitlac (○) considered separately is reported in the case on 0.015 M NaCl. Lines represent the best fit of the experimental data (linear regression for dAC_{0.15}-solution and for alginate and chitlac in 0.015 M NaCl; exponential growth $y = y_0 + a^x e^{(x/b)}$ for dAC_{0.015}-solution).

dependence in the case of the dAC_{0.15}-solution (Figure 4a) perfectly correlates with the linear relationship normally found in the case of dilute polymeric solutions. Moreover, the intrinsic viscosity extrapolated at $C \rightarrow 0$, that is, 4.57 dL/g, compares very well with the theoretical one (4.47 dL/g) calculated on the basis of eq 7, which assumes no interaction between the two polysaccharides:

$$[\eta]_{\text{mix}}^{\text{theor}} = \frac{[\eta]_{\text{Alg}} \cdot C_{\text{Alg}} + [\eta]_{\text{Ch}} \cdot C_{\text{Ch}}}{C_{\text{tot}}} \quad (7)$$

where $[\eta]_{\text{Alg}}$ and $[\eta]_{\text{Ch}}$ are the intrinsic viscosities of alginate and chitlac, respectively, separately considered (more specifically, $[\eta]_{\text{Alg},0.15} = 5.44$ dL/g; $[\eta]_{\text{Ch},0.15} = 3.49$ dL/g; $[\eta]_{\text{Alg},0.015} = 7.61$ dL/g; $[\eta]_{\text{Ch},0.015} = 4.39$ dL/g).

A different scenario is found upon diluting the dAC_{0.015}-solution (alginate weight fraction $r_{\text{Alg}} = 0.5$) (Figure 4b). In fact, a sharp increase of the reduced specific viscosity is detected

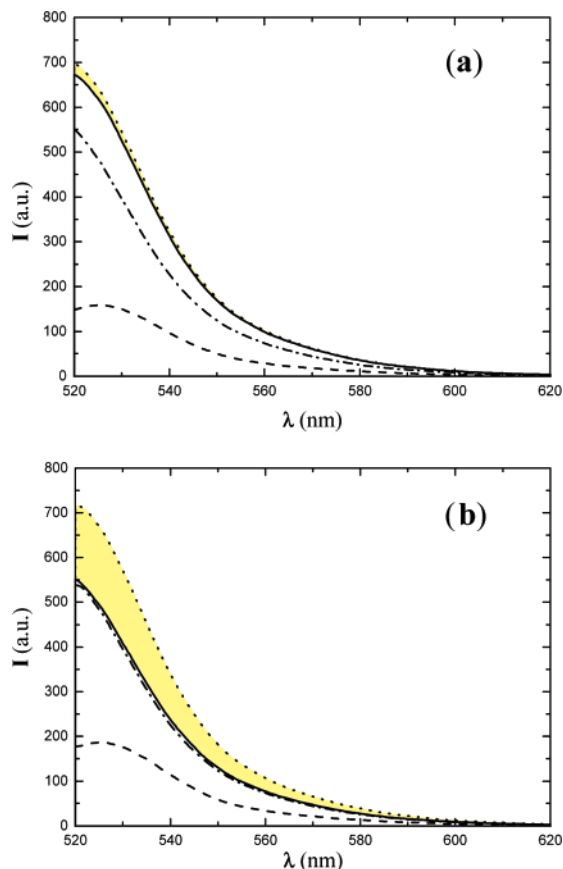


Figure 5. (—) Fluorescence quenching for (a) dAC_{0.15}- and (b) dAC_{0.015}-solutions ($r_{\text{Alg}} = 0.5$; total polymer concentration 1.5 g/L). Fluorescence spectra for rhodamine-labeled alginate ($C_{\text{Alg}} = 0.75$ g/L) (—), for fluorescein-labeled chitlac ($C_{\text{Ch}} = 0.75$ g/L) (---), and for their sum (\cdots) are also reported.

on increasing polymer concentration in the high concentration range considered. This behavior is diagnostic of overlapping of polymer coils.³⁴ However, a linear η_{sp}/c versus c dependence is regained when the total polymer concentration is lowered below 0.03 g/dL. By comparison, alginate and chitlac considered separately do show the well-known linear dependence of η_{sp}/c versus c . It is very important to note that also for the dAC_{0.015}-solution the extrapolated value of the intrinsic viscosity (5.78 dL/g) again correlates very well with the one calculated by means of eq 5 (5.95 dL/g), clearly pointing to the reversibility of the soluble complex formation upon dilution with the salt-containing solvent.

(b3) Fluorescence Quenching and Depolarization. An alginate weight ratio (r_{Alg}) of 0.5 was considered to further characterize the binary polysaccharide mixtures. The high versatility of the two polysaccharides used in the present study toward chemical modifications on the polymer backbone allowed labeling them with fluorescent dyes. In particular, alginate and chitlac were decorated with rhodamine and fluorescein, respectively, to a very low degree of substitution to minimize the perturbation of the overall physicochemical properties of the polymers. The preparation of dAC-solutions with the labeled polysaccharides allowed one to perform a fluorescence quenching investigation on the binary mixtures (Figure 5).^{35,36} In particular, the emission spectrum for the fluorescently labeled dAC_{0.15}- and dAC_{0.015}-solutions ($r_{\text{Alg}} = 0.5$) was recorded and compared to the theoretical one calculated (see eq 4) as the sum of the spectra of the two polymeric components considered separately. If a close approach between the two oppositely charged polysac-

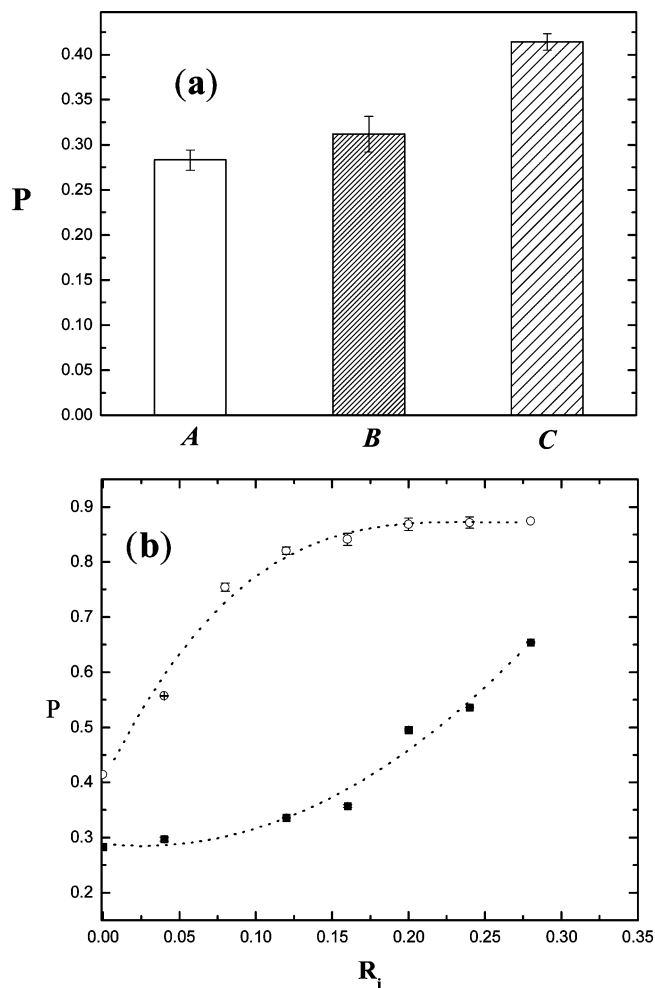


Figure 6. (a) Fluorescence depolarization (P) for rhodamine-labeled alginate ($C_{\text{Alg}} = 0.75$ g/L (column A) and $C_{\text{Alg}} = 1.5$ g/L (column B)) and for dAC_{0.015}-solution ($r_{\text{Alg}} = 0.5$; total polymer concentration 1.5 g/L) (column C), at $I = 0.015$ M. (b) Increase of the fluorescence depolarization (P) upon addition of calcium ions ($R_j = [\text{Ca}^{2+}]/[\text{Alg}]_0$) for the dAC_{0.015}-solution ($r_{\text{Alg}} = 0.5$; total polymer concentration 1.5 g/L) (○) and for the (chitlac-free) rhodamine-labeled alginate ($C_{\text{Alg}} = 0.75$ g/L) (■), at $I = 0.015$ M. Values are reported as mean \pm s.d. ($n = 10$). Curves are drawn to guide the eye.

charides is attained, a quenching of the fluorescence of the sample can be detected. In the case of the doubly labeled dAC_{0.15}-solution (Figure 5a), the theoretical and experimental emission spectra almost coincide, hence accounting for a negligible quenching effect. This means that the two fluorophores (and also the corresponding labeled polymers) on the average stay at comparatively large distances. On the contrary, the 10-fold decrease of simple salt added in the binary solution (i.e., for the dAC_{0.015}-solution) (Figure 5b) brings about a quenching of the fluorescence of the solution, as proved by the notable discrepancy between the experimental and the theoretical curves. Clearly, it must stem from a very close contact between the two polysaccharides in soluble complexes, the formation of which is largely due to the reduced of inter-polyelectrolyte charge screening.

Because the formation of soluble complexes at low I is expected to influence the local diffusional properties of the polymer chains, the fluorescence depolarization, P , of the dAC_{0.015}-solution ($r_{\text{Alg}} = 0.5$), prepared by using rhodamine-labeled alginate (and non-labeled chitlac), was evaluated (Figure 6a). It is interesting to note that the P value of the binary polymer system ($P = 0.414$) is higher than that of a solution

containing the same amount of alginate (i.e., 0.75 g/L) ($P = 0.283$). Moreover, the formal replacement of the chitlac sample in the mixture with an equal amount of alginate, that is, using a 1.5 g/L rhodamine-labeled alginate, brought about a comparatively small increase of P ($P = 0.312$), which certainly could not account for the relevant increase of P detected for the binary mixture.³⁷ This behavior can be traced back to the reduction of the mobility of the fluorescent tag in the dAC_{0.015}-solution and suggests the presence of (soluble) molecular assemblies affecting the rotational freedom of the alginate component of the mixture.

It was desired to exploit the possibility of measuring the local mobility (or microviscosity)^{38,39} of the alginate chains and the effect of the addition of calcium ions for comparing the behavior of the dAC_{0.015}-solution with that of a pure alginate solution. The strong bonding interactions occurring between the Ca²⁺ ion and the G residues present in alginate lead to junction formation (and eventually to a wall-to-wall hydrogel): two comparatively long ordered sequences containing G residues from two opposing alginate chains stiffen and face each other, “glued” by a sequence of bonded calcium ions. As a result, the fluorescence depolarization (P) of the alginate solution is expected to increase with increasing $[Ca^{2+}]/[Pol]_m$ (R_j) ratio (Figure 6b). In the case of calcium-treated 0.75 g/L (chitlac-free) alginate solution, a smooth increase of P was detected at low calcium concentrations followed by a sharp growth when R_j exceeds 0.16. On the contrary, in the case of calcium-treated dAC_{0.015}-solution (Figure 6b), the qualitative dependence of P on R_j somehow seems to mirror the former one. The very first additions of calcium ions induced a marked increase of the fluorescence depolarization, thus accounting for a very rapid decrease of the mobility of the system even when just a few Ca-induced contacts between the alginate chains are formed. They are followed by a leveling off at (already) very high levels of microviscosity, higher than the highest attained in chitlac-free case at the same R_j .

Discussion

This Article deals with the preparation of soluble complexes between two oppositely charged polysaccharides, alginate and chitlac, in dilute condition. The formation of such aggregates takes place mainly as a consequence of the presence of the small residual positive charge on chitlac^{27,40} and of the excess negative charges on alginate.

The analysis of Figure 1 is a good starting point to elucidate the characteristic features of the soluble complexes formed between alginate and chitlac. The inverted bell-shaped dependence of transmittance on ionic strength shows that full solubility can be attained on both extremes of the investigated interval of I , but for a peculiar, albeit not unusual, minimum of solubility at intermediate I values. In fact, it neatly parallels the theoretically and experimentally described case of nonstoichiometric soluble complexes (NSC).^{22,25,41}

The observed behavior can be interpreted assuming that it stems from the concurrent interplay of two different, and somehow opposite, mechanisms. The first one can be generically addressed as stemming from the long-range attractive interactions between individual polyion chains of opposite charge. This attractive term is expected to depend strongly on the ionic strength of the system, in particular rapidly decreasing on increasing I up to the level of practically swamping out all attractive interactions at, say, $I = 0.15$ M. On the other hand, the absolute value of the Z potential of alginate is 10 times larger than that of chitlac under the experimental conditions of

pH 7.4. This is consistent with the structural features of the two polysaccharides used in the present study and brings to the conclusion that a net negative charge occurs in the 1:1 w/w (i.e., $r_{Alg} = 0.5$) alginate and chitlac solution, with the negative groups on alginate by far overcoming the total number of the protonated amine groups on chitlac. A more quantitative account of the actual charge density of macroions would imply the knowledge of chain expansion and respective hydrodynamic volume, the latter being smaller for alginate than for chitlac. Therefore, albeit within such a qualitative approach, it can be concluded that the soluble complexes formed in the presence of a limited amount of sodium chloride (0.015 M NaCl) are characterized by a nonstoichiometric interaction between the two polymeric components.

While the aggregation of the soluble complexes (i.e., phase separation) is prevented by the negative net charge bore by the complexes at low ionic strengths, upon increasing the ionic strength by the addition of 1:1 simple salt, a “salting out” effect takes place and associative phase separation occurs, as previously described by Izumdurov and co-workers.²² Under these circumstances (intermediate ionic strength), the increase in the supporting salt concentration induces the nonstoichiometric soluble complexes to rearrange into stoichiometric aggregates that phase separate.

Eventually, further additions (above $I = 0.13$ in the present case) of the supporting salt start to efficiently screen the electrostatic interactions between positive (chitlac) and negative (alginate) charges on the two polysaccharides and reduce the entropic gain upon inter-polyelectrolyte interaction, thus inducing a “salting-in” effect on the ternary system.

On an overall basis, upon increasing ionic strength the long-range attractive forces between oppositely charged polyions are screened and the stoichiometry of the alginate–chitlac complexes varies. The net effect results in the minimum solubility of the system at intermediate ionic strength.

In the case of the formation of soluble nonstoichiometric complexes (i.e., with 0.015 M NaCl), the interchain electrostatic “cross-links” lead to a very close approach of the polymer chains bearing opposite charges, thus explaining both the fluorescence quenching detected with the labeled polysaccharides (Figure 5b) and the higher microviscosity of the system (measured by P) found in the case of rhodamine-labeled dAC_{0.015}-solution (Figure 6a). This effect is even more striking when calcium ions are added to binary polymer solution (Figure 6b); in this case, the electrostatic cross-links⁴² apparently add to the calcium-induced ones, inducing a more pronounced “molecular freezing” of the binary polymer solution. Actually, this effect can be undoubtedly attributed to the very high local concentration of alginate chains in the domain of the inter-polyelectrolyte complex. By definition, the junction formation implies the coming together of two polymer chains, and high polymer concentrations are known to produce stronger gels than are dilute solutions. As a consequence, synergistic effects of the interpolymer electrostatic interactions on the overall mechanical properties of the hydrogels obtained from the binary mixture can be expected.⁴³

The remarkable effect observed on the viscosity of the system (see Figure 3b for the dAC_{0.015}-solution) is highly indicative of the existence of nanoscale multiple complexes formed by contacts between the bio-macromolecules in the case of the binary polymer solution with a low amount of added simple salt. An additional proof supporting this interpretation can be found in Figure 4b. In fact, the deviation of the dAC_{0.015}-solution from the behavior expected for dilute polymer systems, that is, a linear dependence of the (reduced) specific viscosity of the

solution from the concentration, is highly suggestive of the presence of a interpolymer domains, which are characterized by a very high hydrodynamic volume even at a low polymer concentration. However, it is interesting to note that such soluble complexes fade out upon dilution, determining a limiting behavior for the system (i.e., the intrinsic viscosity extrapolated to zero total polymer concentration) equal to that of a non-interacting mixture of the two polysaccharides. Hence, the formation of electrostatic contacts between alginate and chitlac chains in the nonstoichiometric soluble complexes is not incompatible with their exchange,¹¹ because the interpolyelectrolyte contacts can be constantly broken and reformed.⁴⁴

Literature reports on the conformational effects induced by the close interactions between polycation and polyanion. In particular, poly(lysine) is able to induce pectate (i.e., poly(galacturonate): the configurational analogue of poly(gulonate)) to assume an helical conformation. Preliminary results seem to indicate that alginate (or at least its guluronate component) shows, to some extent, the same behavior in the presence of chitlac. If confirmed, this would add a flavor of conformational ordering to a process otherwise apparently ruled by nonspecific long-range interactions only. This point, however, falls well beyond the scope of this paper, which is devoted to the detailed phenomenological description and identification of the physical process. Work is in progress to shed light on that aspect.

Conclusions

The preparation of aqueous binary systems containing a polyanion and a polycation of polysaccharidic nature has been explored. In particular, it was noted that, depending on the amount of simple 1:1 supporting salt added to the solvent, alginate and chitlac, a lactose-modified chitosan, can undergo an aggregative phase separation. However, a complete miscibility is attained in the presence of either a high (0.15 M) or a low (0.015 M) amount of supporting salt (NaCl). The notable electrostatic interactions present in this latter case lead to the formation of nonstoichiometric soluble complexes (NSC), characterized by an excess of negative charges arising from the uronic moieties present in alginate. The analyses performed in dilute and in semidilute conditions allowed one to suggest the formation of nanoscale multiple complexes for the NSC that, by leading to structures with very high hydrodynamic volumes, strongly affects the viscosity of the binary polymer solution.

As a whole, the nonstoichiometric soluble complexes reported here can be envisaged as a supramolecular system with nanoscale association induced by interchain electrostatic contacts. In this sense, this Article strengthens the results proposed in our previous study regarding the synergistic effects on semidilute binary solutions containing alginate and chitlac. These two works together address the specific topic of the effects of molecular crowding on the overall properties of the system.

As it is known that cells embedded within tissue can strongly be affected by the physicochemical and mechanical properties of their milieu, it can be easily foreseen that the proper assessment of the mutual behavior of the polymers in the mixtures is of paramount importance in designing applications in the field of tissue engineering, thus allowing the gap between wet chemistry and biomaterial engineering to be covered.

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