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ARTICLE *in* BIOMACROMOLECULES · NOVEMBER 2011

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# Polysaccharide-Based Polyanion–Polycation–Polyanion Ternary Systems. A Preliminary Analysis of Interpolyelectrolyte Interactions in Dilute Solutions

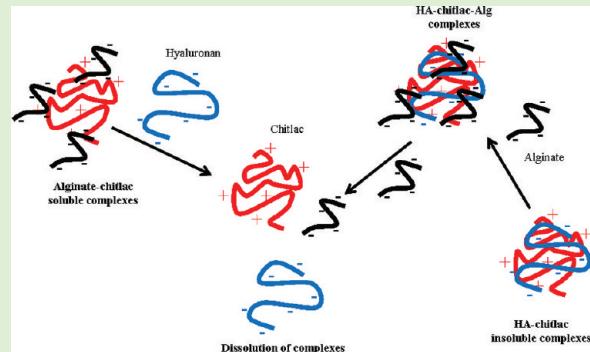
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## Supporting Information

**ABSTRACT:** The present contribution deals with the preparation and characterization of ternary mixtures of polysaccharides with potential applications in the field of tissue engineering. Two natural polyanions, i.e., alginate and hyaluronic acid, and a polycation, a lactose-modified chitosan (chitlac), were mixed in dilute conditions. The miscibility between the three components was explored in the presence of different amounts of supporting simple salt. These analyses allowed to identify the experimental conditions avoiding polymer phase separation and leading to either solution of independent polymers or soluble nonstoichiometric interpolyelectrolyte complexes. The characterization of the interpolyelectrolyte complexes was tackled by means of viscometry, light scattering, fluorescence quenching, and energy transfer. The electrostatic interactions taking place among the different polyelectrolytes led to synergistic effects on the viscosity of the polymer mixtures which strongly depend on the ionic strength. It has been found that, starting from binary soluble complexes of alginate and chitlac, the addition of hyaluronan led to the dissolution of the complexes. At variance, the addition of alginate to a phase-separated binary mixture of hyaluronan and chitlac led to the formation of soluble complexes composed of all three polysaccharides and, eventually, to their dissolution. In addition, the results showed that at low ionic strength the overall properties of the ternary mixtures depend on their order of mixing.



## INTRODUCTION

The compatibility of oppositely charged polyelectrolytes is a challenging field of research from both the experimental and theoretical points of view. Parameters such as ionic strength, charge density, degree of polymerization, polymer stiffness, and polycation/polyanion weight ratio are of paramount importance in determining the final outcome from the mixing of polyanion–polycation solutions. Very frequently, a liquid–liquid phase separation, accompanied by a large desolvation,<sup>1</sup> occurs as a consequence of the electrostatic attraction<sup>2</sup> between the fixed charges on the polymer arrays. Associative aggregates are described as true equilibrium systems which can be stable over months<sup>3,4</sup> and reach the maximum when the two oppositely charged species are at stoichiometry.<sup>5</sup> Electrostatic driven interactions between oppositely charged polyelectrolytes could lead to hierarchical structures which can be modulated by variation of physical chemical parameters such as ionic strength and/or dilution.<sup>6</sup>

The definition of the physical–chemical conditions leading to the formation of aggregates of oppositely charged polyelectrolytes is of particular importance when considering their possible applications. As an example, complexes of

oligonucleotides with polycations such as chitosan, called polyplexes, have attracted increasing interest as vehicles for cells gene delivery.<sup>7,8</sup> The complex interplay of polyplexes with other polyelectrolytes naturally surrounding the cells was shown to have a notable impact on their final fate and transfection efficiency.<sup>9</sup> In addition, polyanion–polycation complexes have been proposed for applications in the biomaterials field.<sup>10–13</sup>

When mixing solutions of oppositely charged polyelectrolytes, the transition from a clear solution to an associative phase separation is clearly detected as an abrupt change in the turbidity of the system. The interzone between the soluble system and the phase separation is represented by the soluble complexes. A precise separation between the noninteracting polyelectrolyte regime and the soluble complexes is subtle, and the boundary among these two conditions is characterized by variation of small entity in the transmittance.<sup>1</sup> Aspects such as the stability of the nonstoichiometric complexes as well as their molecular organization and dynamics have been tackled by

Received: July 28, 2011

Revised: September 28, 2011

Published: October 13, 2011

several authors. Izumrudov investigated in detail soluble complexes containing polycations and nucleic acids pointing out the effect of ionic strength and of polycation/polyanion ratio on their stability.<sup>14</sup> Moreover, the presence of bulky groups in the side chain of the polycation was reported to have a notable effect on the complexation with the highly charged polyanion DNA.<sup>8,14</sup> The complex role of the electrostatic interactions among oppositely charged polyelectrolytes in nonstoichiometric conditions has been extensively analyzed and the existence of charged complexes of different morphology experimentally confirmed.<sup>6,15</sup> Moreover, the effect of different formulation pathways and mixing protocols on the morphology of the complexes has been proved, eventually leading to different bulk and surface properties.<sup>16</sup>

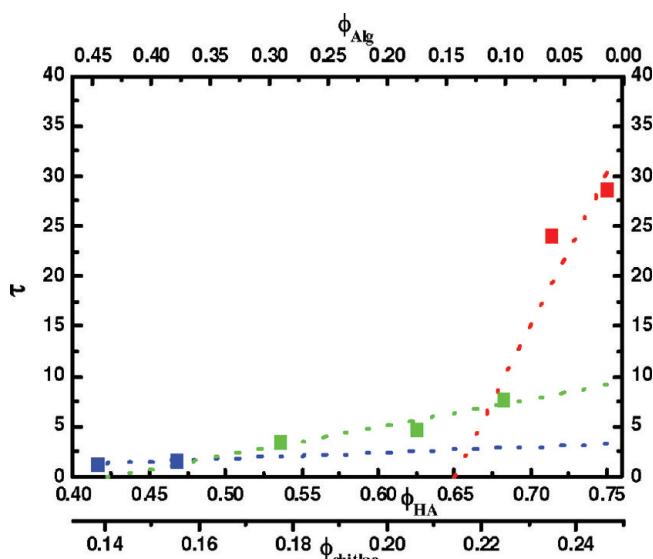
In the present work, ternary mixtures of polysaccharides, namely alginate, hyaluronan (polyanions), and a lactose-modified chitosan named chitlac (a polycation), have been analyzed. The selection of the polysaccharides was based on their large applicability in the field of biomaterials. In fact, alginate<sup>17</sup> has been largely used for cell encapsulation processes and, more in general, for the preparation of lyotropic hydrogels,<sup>18,19</sup> while hyaluronan has been proposed for tissue engineering purposes.<sup>20</sup> While hyaluronan plays both structural and rheological roles, the biological significance of this polysaccharide is of no lesser importance, being recognized by the CD44 receptor with a direct involvement in cell adhesion and proliferation.<sup>21–23</sup> As to the polycation, chitlac was selected for its bioactive features being recognized by galectins<sup>24</sup> and able stimulate the production of type II collagen and glycosaminoglycans (GAGs) by primary chondrocytes.<sup>25</sup> Binary mixtures of alginate and chitlac revealed a synergistic behavior in semidilute condition<sup>26</sup> and showed to have interesting biological properties when used for the encapsulation of chondrocytes for cartilage regeneration.<sup>27</sup> In this work the complexity of the system is increased by adding a third component represented by hyaluronan. The ternary systems tackled in the present work represent a nontrivial extracellular matrix mimic which, once used for biomaterials development, could lead to a biopolymer-based matrix with bioactive signals targeted to different adhesive proteins, i.e., a bioactive biomaterial of second generation.<sup>28,29</sup>

## MATERIALS AND METHODS

Alginate, isolated from *Laminaria hyperborea* (*L. hyp.*), and hyaluronan were purchased from Novamatrix. Their main characteristics are reported in Tables 1 and 2 of the Supporting Information. Low molecular weight hyaluronan (LowHA, MW  $\sim 1.2 \times 10^5$ ) was a kind gift of Fidia SpA (Abano Terme, Italy). Chitlac (lactose-modified chitosan, CAS registry number 85941-43-1) was prepared according to the procedure reported elsewhere starting from a highly deacetylated chitosan (residual acetylation degree  $\sim 18\%$ ).<sup>25</sup> The main characteristics of chitlac are reported in Table 3 of the Supporting Information. High molecular weight mannuronan (polyM, fraction of galuronic residues, F<sub>G</sub>, <0.001, M<sub>w</sub>  $\sim 1.4 \times 10^6$ ) was kindly provided by Prof. Gudmund Skjåk-Bræk, Institute of Biotechnology, University of Trondheim (Norway).<sup>30</sup> S,6-Diamino-N,N,N',N'-tetraethylrhodamine B (DAR), fluoresceinamine, fluorescein isothiocyanate (FITC), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, (Hepes), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and sodium chloride were purchased from Sigma.

**Miscibility Studies.** Ternary mixtures were prepared at different polysaccharide weight fractions ( $\phi$ ), and their transmittance was measured at 550 nm by means of a UV-vis spectroscopy (Cary 4E). Supporting salt/buffer (Hepes) ratio equal to 15 (pH 7.4), total

polymer concentration, and ionic strength were kept constant throughout the measurements. For the turbidity measurements, the instrument was calibrated against the solvent setting %T = 100. The qualitative distinction between solution, soluble complexes, and phase separation (aggregation) was done on the basis of the transmittance following the procedure proposed by Dubin and co-workers.<sup>1</sup> In particular, the onset of phase separation for the polymer mixtures was identified from an abrupt change of the percent transmittance identified with a value of  $\tau$ , defined as  $100 - \%T$  (i.e., a dimensionless number), exceeding 10. All the single polymer solutions tested showed, with respect to the solvent, a  $\tau < 3$ . Soluble ternary systems displayed a  $\tau$  below 3 and a quasi-zero slope of the best fit line between data points. The region corresponding to the soluble complexes is defined by  $3 < \tau < 10$  and by a best fit line between data points with positive slope (see Figure 1).



**Figure 1.** Dependence of  $\tau$  from the composition of the ternary mixtures of alginate (Alg), hyaluronan (HA), and chitlac. The compositions reported correspond to the black dashed line in Figure 2d. Ternary compositions corresponding to soluble systems, to phase-separated aggregates, and to soluble complexes are represented in blue, red, and green squares, respectively. The lines represent the linear best fit of the experimental data. Total polymer concentration: 1.5 g/L, NaCl 0.015 M (ratio [NaCl]/[Hepes] of 15 and a pH of 7.4).  $\phi_{\text{Alg}}$ ,  $\phi_{\text{chitlac}}$ , and  $\phi_{\text{HA}}$  refer to the weight fraction of alginate, chitlac and hyaluronan, respectively.

**Viscosity Measurements.** Specific viscosity of polysaccharides and of their mixtures was measured at 25 °C by means of a Schott-Geräte AVS/G automatic measuring apparatus and an Ubbelohde-type capillary viscometer. The intrinsic viscosity ( $[\eta]$ ) values were determined at 25 °C and at different ionic strengths for the three polysaccharides considered separately by analyzing the polymer concentration dependence of the reduced specific viscosity ( $\eta_{\text{sp}}/c$ ) 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:

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

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

where  $k_{\text{H}}$  and  $k_{\text{K}}$  are the Huggins and Kraemer constants, respectively. Polysaccharide solutions were filtered through 0.45  $\mu\text{m}$  Millipore filters prior to the measurements and to their mixing. The specific viscosity of the binary and ternary mixtures was measured at different polysaccharide weight fractions ( $\phi$ ). A constant total polymer

concentration (1.5 g/L) and supporting salt/buffer (Hepes) ratio equal to 15 (pH 7.4) were used throughout the experiments.

**Polysaccharide Labeling.** Fluorescein-labeled chitlac (Fluo-chitlac): Chitlac (200 mg) was dissolved in a 0.5 M NaHCO<sub>3</sub> solution (70 mL), and FITC was added to label 1/2000 of available amino groups. The solution was stirred overnight in the dark at room temperature, dialyzed against 0.05 M NaHCO<sub>3</sub> (2 shifts), 0.05 M NaCl (2 shifts), and then extensively against deionized water (until the conductivity at 4 °C was below 4 μS). The pH of the solution was adjusted to 4.5; the solution was filtered through 0.45 μm Millipore filters and then freeze-dried.

Rhodamine-labeled alginate (DAR-Alg) and fluorescein-labeled hyaluronan (Fluo-HA): NHS and EDC ([EDC]/[PolymRU] = 1.5; [NHS]/[EDC] = 1; PolymRU refers to the polymer repeating units) were added to a polysaccharide solution (3 g/L) in 2-N-morpholinoethanesulfonic acid (MES) buffer (50 mM, pH 5.5) containing 10% ethanol. A solution of the fluorescent tag (DAR or fluoresceinamine, respectively) in ethanol was added to label 1/1000 of the carboxylic groups of the polysaccharide. The solution was stirred overnight in the dark at room temperature, dialyzed against 0.05 M NaHCO<sub>3</sub> (2 shifts), 0.05 M NaCl (2 shifts), and then extensively against deionized water (until the conductivity at 4 °C was below 4 μS). The pH was adjusted to 7.4; the solution was filtered through 0.45 μm Millipore filters and then freeze-dried.

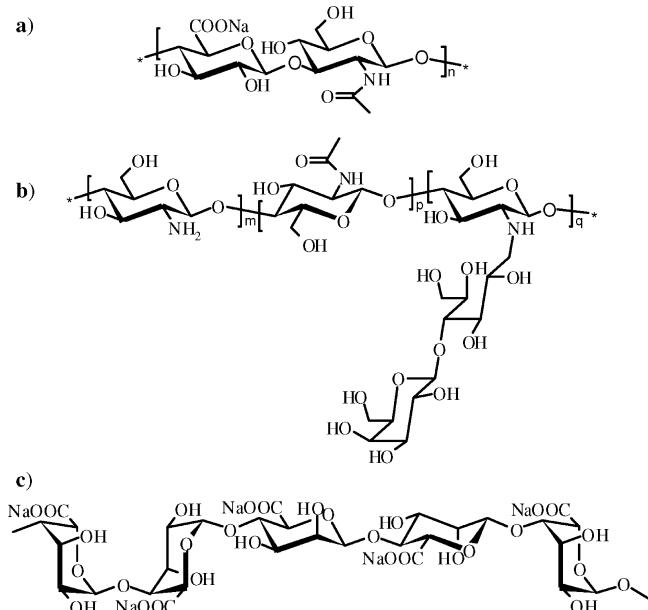
**Fluorescence Spectroscopy.** Fluorescence measurements were performed with a Perkin-Elmer LS50B spectrophotometer at  $T = 25^\circ\text{C}$ . For fluorescence resonance energy transfer (FRET) measurements, rhodamine-labeled alginate (DAR-alg) and fluorescein-labeled hyaluronan (Fluo-HA) were used in the ternary mixtures. A 480 nm excitation wavelength was used, and the intensity of the emitted light was recorded in the range 460–800 nm. The emission relative to the fluorescein tag was measured at 514 nm and the one allocated to rhodamine at 573 nm. For fluorescence quenching measurements, ternary mixtures were prepared by using fluorescein-labeled chitlac (Fluo-chitlac) and fluorescein-labeled hyaluronan (Fluo-HA). A 480 nm excitation wavelength was used, and the intensity of the emitted light was recorded in the range 460–800 nm. The emission relative to the fluorescein tag was measured at 514 nm. Supporting salt/buffer (Hepes) ratio equal to 15 (pH 7.4), total polymer concentration (1.5 g/L), and ionic strength were kept constant throughout the measurements.

**Light Scattering.** A Perkin-Elmer LS50B spectrophotometer was used to record the intensity of the light scattered at 90° by binary and ternary mixtures of alginate, hyaluronan, and chitlac at different weight fractions ( $\phi$ ) upon irradiation with a 550 nm incident light ( $T = 25^\circ\text{C}$ ). Supporting salt/buffer (HEPES) ratio equal to 15 (pH 7.4), total polymer concentration (1.5 g/L), and ionic strength were kept constant throughout the measurements.

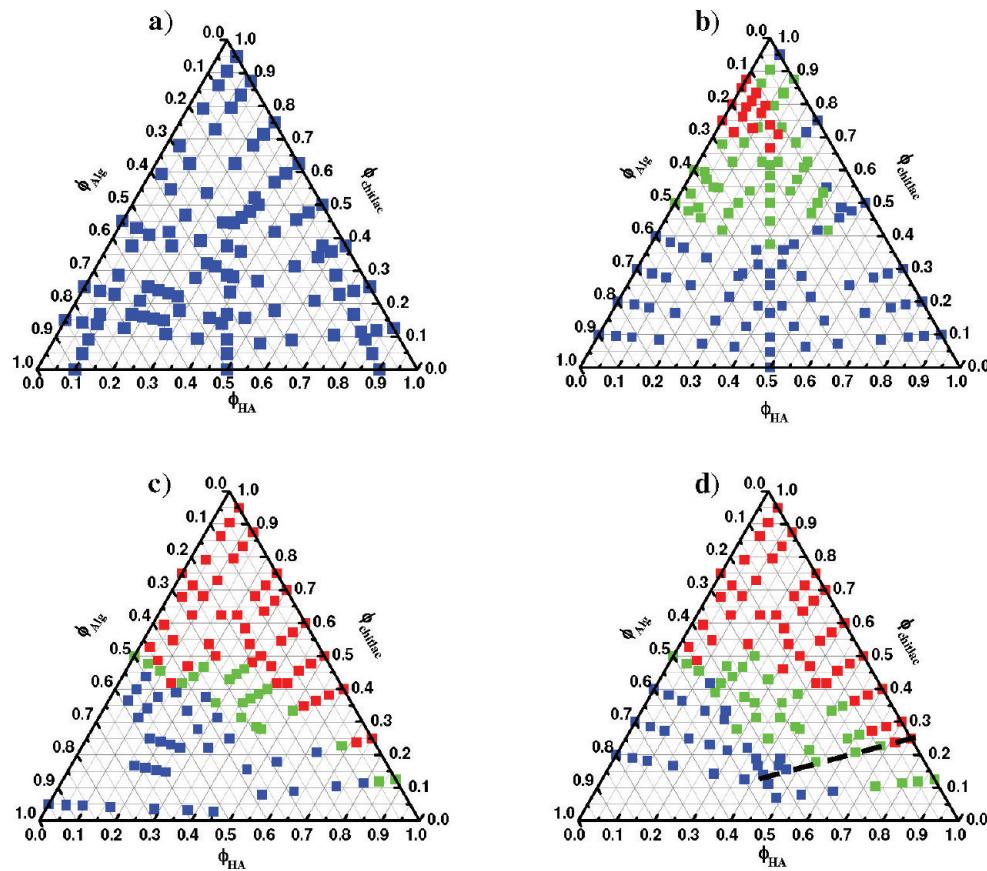
## RESULTS AND DISCUSSION

**Miscibility of Ternary Systems Alginate–Chitlac–Hyaluronan.** The miscibility of the ternary mixtures of alginate, chitlac, and hyaluronan (Scheme 1) was evaluated at constant pH of biological relevance, namely 7.4, and at different weight fractions,  $\phi$ , by means of transmittance (turbidity) measurements. The ternary diagrams obtained were qualitatively divided into three areas defined as soluble region (in blue), insoluble region (in red), and a region pertaining to soluble complexes (in green). The qualitative distinction among these regions was based on the absolute value of  $\tau$  as well as on the slope of the best fit line connecting the experimental data points of different composition, as suggested by Dubin and co-workers.<sup>1</sup> Figure 1 reports an example of qualitative definition of the different regions as soluble system, soluble complexes, and phase-separated (coacervate) system. The points showing  $\tau < 3$ , thus comparable with the one of the polymer solutions considered separately, and a negligible slope

**Scheme 1. Schematic Structure of the Polysaccharides Used in the Present Study: Hyaluronan (a), Chitlac (b,  $m = 0.20$ ;  $p = 0.18$ ;  $q = 0.62$ ), Alginate (c, GGMMG Sequence)**



of best fit line of the  $\tau$  vs  $\phi_x$  relationship were defined as soluble composition (blue points). At variance, compositions with  $3 < \tau < 10$  and related by a nonzero slope of the best fit line are defined as soluble complexes (green points). Finally, when the change of  $\tau$  is abrupt upon variation of the composition, the ternary mixtures evolve into an aggregated system. As qualitative as this distinction might be, it still provides preliminary information on the features of the mixtures, although additional experiments are needed to properly identify and characterize in particular the region of soluble complexes. Figure 2a–c reports the effect of ionic strength on the miscibility of the ternary polysaccharide system, using the above definitions. In aqueous NaCl 0.15 M (Figure 2a), all the compositions tested led to a soluble systems. On the contrary, upon reducing the concentration of the supporting salt (NaCl) to 0.075 M (Figure 2b), a zone of immiscibility appears in the area close to  $\phi_{\text{Alg}} = 0.2$  and  $\phi_{\text{chitlac}} = 0.8$ . In addition, a large area corresponding to the formation of soluble complexes is detected. This covers almost all the upper part of the ternary diagram. Upon decreasing further the amount of supporting salt, namely to NaCl 0.015 M, the zone of immiscibility is increased. In particular (Figure 2c), all the upper part of the triangular graph corresponds to compositions leading to phase separation (coacervation). It should be noticed that the nonmiscibility area is not symmetric. The binary compositions containing alginate and chitlac are nonmiscible up to a  $\phi_{\text{Alg}} = 0.5$ . On the contrary, binary hyaluronan–chitlac systems become miscible when the fraction of the polyanion in the mixture exceeds 0.8. The lack of symmetry in the ternary alginate–hyaluronan–chitlac system might arise from the difference in charge density of the two polyanions (see Tables 1–3 in the Supporting Information). This is a fundamental feature, together with the molecular weight of the components of the mixtures, in defining the conditions for the phase separation or for soluble complexes formation upon mixing polyanions and polycations. In Figure 2c, it can be noticed that the boundary between the insoluble and soluble zones is



**Figure 2.** Miscibility of ternary mixtures of alginate (Alg), hyaluronan (HA), and chitlac in aqueous (a) NaCl 0.15 M; (b) NaCl 0.075 M; (c) NaCl 0.015 M with a total polymer concentration of 1 g/L, and (d) in aqueous NaCl 0.015 M with a total polymer concentration of 1.5 g/L. In all cases, a ratio [NaCl]/[Hepes] of 15 and a pH of 7.4 were maintained.  $\phi_{\text{Alg}}$ ,  $\phi_{\text{chitlac}}$ , and  $\phi_{\text{HA}}$  refer to the weight fraction of alginate, chitlac, and hyaluronan, respectively. Color legend as in Figure 1. Black dashed line represents the compositions considered in Figure 1.

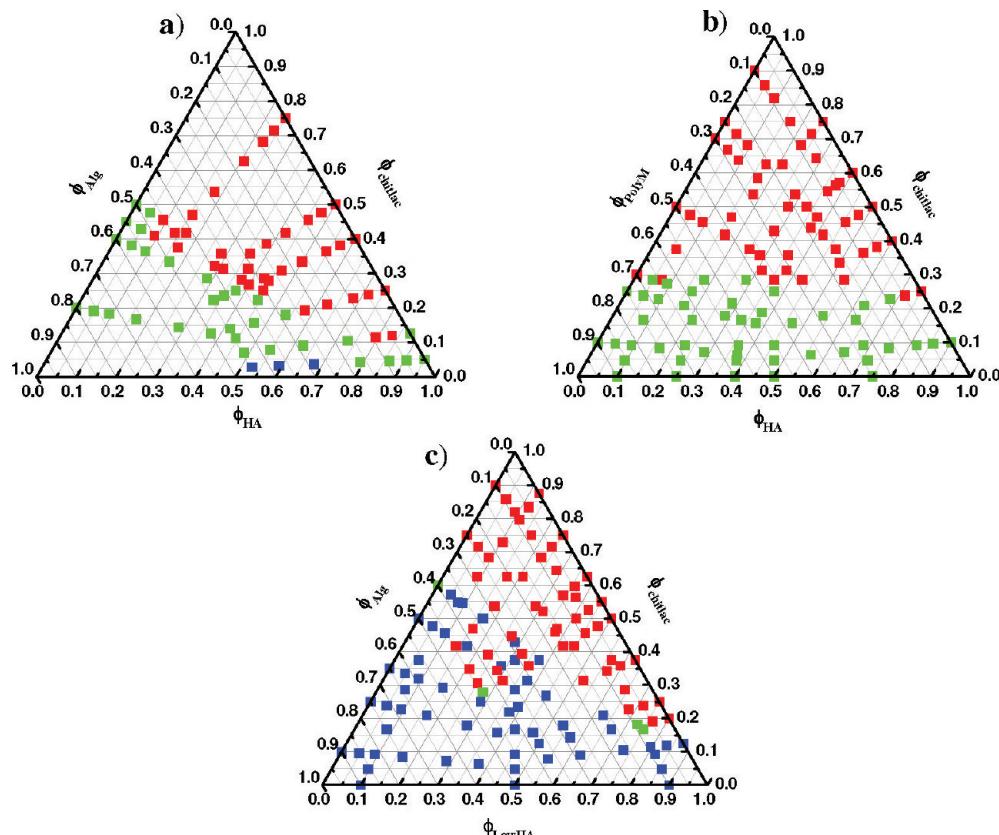
represented by an area in which soluble complexes are formed. In these cases, as it will be discussed in detail further in this work, electrostatically driven complexes are present in solution but, due to unbalanced charge stoichiometry among the components of the system, they remain in solution. Focusing on the side representing the hyaluronan–chitlac mixtures of Figure 2c, it is interesting to note that, starting from an insoluble binary mixture, i.e., with a fraction of hyaluronan of 0.75, the addition of alginate leads to the formation of soluble complexes and, eventually, to soluble ternary mixtures stressing on the solubilization effect of alginate on chitlac–hyaluronan aggregates. Figure 2d reports the solubility of the three polysaccharides in aqueous NaCl 0.015 M upon increasing the total polymer concentration by 50% (i.e., to 1.5 g/L). By comparing Figures 2c and 2d, it can be noticed that this variation in polymer concentration has little effect on the zone of immiscibility of the system. At variance, the zone corresponding to the soluble complexes is markedly increased when the polymer concentration is increased. This conclusion holds even in the case of an increase of the total polymer concentration to 3 g/L (Figure 3a), where the upper part of the ternary graph is occupied by the zone of phase separation while soluble complexes cover basically all its lower part.

The effect of the molecular mass on the miscibility of the three polysaccharide components of the system was also explored (Figure 3b,c). In particular, in Figure 3b commercial alginate from *L. hyperborea* was replaced in the ternary mixture by high molecular weight mannuronan (PolyM). This

polysaccharide sample is an homopolymer of mannuronic acid residues (i.e., homopolymeric alginate) with a molecular mass of more than  $1 \times 10^6$ . There is a minor variation (below 12%) on the linear charge density of the polyelectrolyte with respect to alginate from *L. hyperborea*.<sup>31</sup> Figure 3b shows that the presence of mannuronan has a notable effect on the areas of phase separation and soluble complexes formation: both are increased with respect to the ternary mixtures prepared in the presence of alginate with lower molecular mass (Figure 2c), in line with results reported by Dubin and co-workers.<sup>1</sup> A different situation was noticed when the high molecular weight hyaluronan was replaced by a low molecular weight sample. Although little variation on the area of the immiscibility of the ternary mixtures was detected, a major effect was seen on the area corresponding to the soluble mixtures which basically disappear from the ternary graph. This is in good agreement with the results reported by Izumrudov and co-workers,<sup>14</sup> who noticed a decrease of the content of soluble complexes upon shortening one of the oppositely charged polyelectrolytes.

#### Physical–Chemical Properties of Ternary Mixtures

**Alginic–Chitlac–Hyaluronan.** The investigation of the properties of ternary mixtures composed of alginate, chitlac, and hyaluronan was started from binary polyanion–polycation mixtures and evaluating the effect produced by the introduction of the third component to the system. Two different systems were separately considered. In the first one, indicated as  $\text{AC}_\text{H}$  ternary mixtures, hyaluronan was added to a binary mixture of alginate and chitlac. On the contrary, in the  $\text{HC}_\text{A}$  system, the



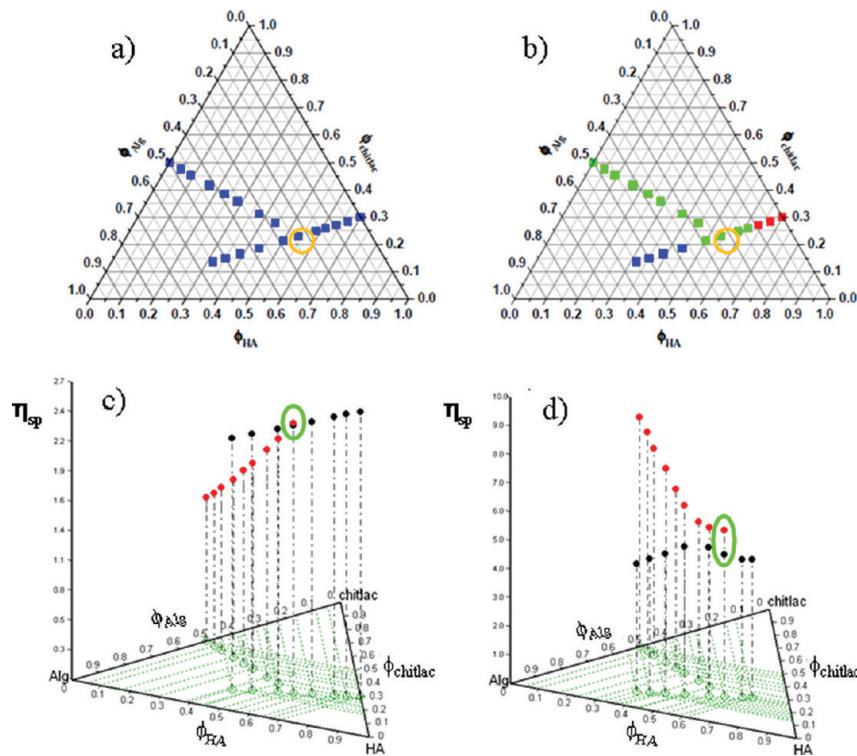
**Figure 3.** (a) Miscibility of ternary mixtures of alginate (Alg), hyaluronan (HA), and chitlac in aqueous NaCl 0.015 M with a total polymer concentration of 3 g/L. Miscibility of ternary mixtures of (b) mannuronan (PolyM), hyaluronan (HA), and chitlac and of (c) alginate, low molecular weight hyaluronan (lowHA), and chitlac. For (b) and (c), total polymer concentration was 1 g/L with NaCl 0.015 M. In all cases, a ratio [NaCl]/[Hepes] of 15 and a pH of 7.4 were maintained.  $\phi_{\text{Alg}}$ ,  $\phi_{\text{chitlac}}$ ,  $\phi_{\text{HA}}$ ,  $\phi_{\text{PolyM}}$ , and  $\phi_{\text{LowHA}}$  refer to the weight fraction of alginate, chitlac, hyaluronan, mannuronan, and low molecular weight hyaluronan, respectively. Color legend as in Figure 1.

starting point was represented by a binary hyaluronan–chitlac system to which an alginate solution was added.

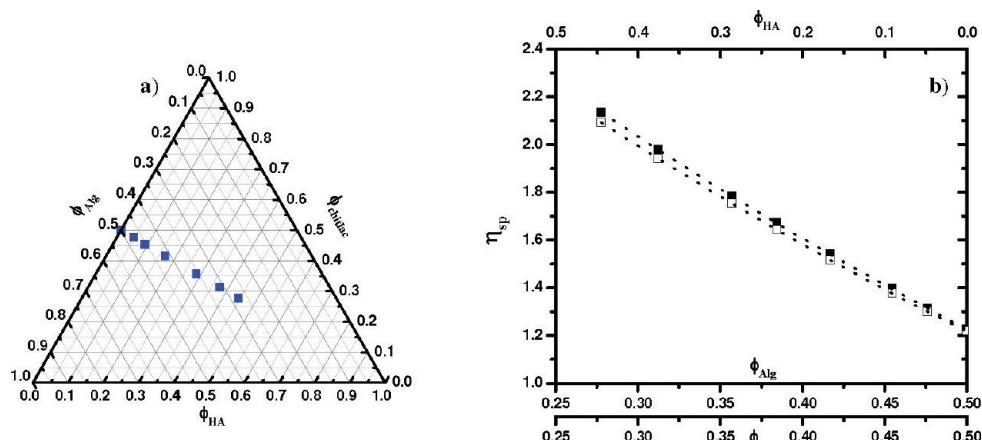
It should be stressed from the beginning that, as the preparation of ternary mixtures of polyelectrolytes may lead to complex systems which range from solutions to soluble complexes to aggregative associations, the final properties of the mixtures could be strongly influenced by factors like the order of mixing of the components.<sup>16,32</sup> This aspect was investigated further for the ternary mixtures at different ionic strength (Figure 4). In particular, hyaluronan was added to a binary alginate–chitlac mixture with a  $\phi_{\text{Alg}} = \phi_{\text{chitlac}} = 0.5$  ( $\text{AC}_\text{H}$  system), and the variation of the specific viscosity was recorded. The curve was compared with the one obtained starting for a binary mixture of hyaluronan ( $\phi_{\text{HA}} = 0.7$ ) and chitlac ( $\phi_{\text{chitlac}} = 0.3$ ) to which alginate was added ( $\text{HC}_\text{A}$  system). The two different ternary systems were devised to cross, i.e., at one point to have the same weight composition of the three polysaccharides, namely at  $\phi_{\text{Alg}} = 0.23$ ,  $\phi_{\text{chitlac}} = 0.23$ , and  $\phi_{\text{HA}} = 0.54$ . When the ternary mixtures were prepared in aqueous NaCl 0.15 M, hence when no complexes of any kind were formed in solution (Figure 4a), the specific viscosity,  $\eta_{\text{sp}}$ , of the ternary mixtures with the same polysaccharide composition but obtained through a different path is basically the same (Figure 4c). However, when the same analysis was performed in aqueous NaCl 0.015 M, a different result is found (Figure 4b). In this case, in the  $\text{AC}_\text{H}$  mixture soluble complexes are formed, as previously discussed. At variance, the  $\text{HC}_\text{A}$  mixtures span from phase-separated systems to soluble

complexes, depending on the additions of alginate. The two ternary mixtures prepared differently but with the same composition do display a neat difference as to  $\eta_{\text{sp}}$  (Figure 4d). In particular, the  $\text{AC}_\text{H}$  system has a higher  $\eta_{\text{sp}}$  than that obtained from the  $\text{HC}_\text{A}$  mixtures. This peculiar behavior stresses on the complex interplay of factors like polyelectrolyte charge density, ionic strength, and polyanion/polycation ratio in determining the true thermodynamic stability of the polyelectrolyte complexes. In particular, the impact of the mixing order could likely originate from the depletion of the hyaluronan–chitlac interactions when alginate is present from the beginning in the ternary system.<sup>32</sup> Although additional analyses are needed, it may be speculated that a different order of mixing could lead to different morphologies of the two ternary systems, as recently reported by Cousin and Chapel.<sup>16,32</sup>

**Addition of Hyaluronan to Alginate–Chitlac Mixtures ( $\text{AC}_\text{H}$  System).** The behavior of the  $\text{AC}_\text{H}$  system was explored by means of different techniques and at two values of the ionic strength, namely NaCl 0.15 M and a NaCl 0.015 M solution, respectively. High molecular weight hyaluronan was added to binary mixtures of alginate and chitlac, and the specific viscosity was measured (Figures 5 and 6). The experimental value of  $\eta_{\text{sp}}$  was compared with the theoretical one calculated for a noninteracting mixture of the three polyelectrolytes, hence under the assumption that simple additive contribution holds (see Supporting Information).



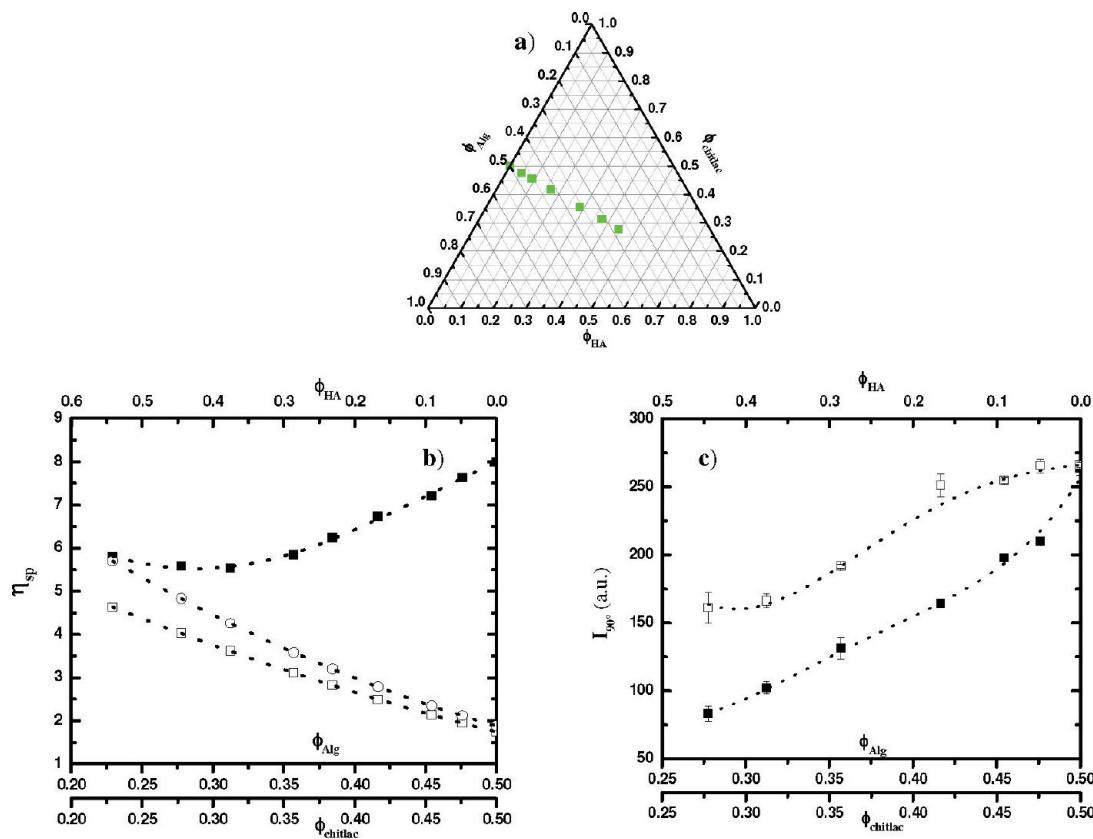
**Figure 4.** (a, b) Miscibility of ternary mixtures of alginate (Alg), hyaluronan (HA), and chitlac in aqueous NaCl: (a) NaCl 0.15 M and (b) NaCl 0.015 M. Color legend as in Figure 1. (c, d) Specific viscosity of two ternary systems of alginate, hyaluronan, and chitlac with different polysaccharide compositions in aqueous NaCl: (c) NaCl 0.15 M and (d) NaCl 0.015 M. Red circles represent the specific viscosity of the  $\text{AC}_H$  system while the black circles refer to the  $\text{HC}_A$  mixtures (for definitions, see text). Green circles indicate ternary mixtures with the same weight compositions, namely  $\phi_{\text{Alg}} = 0.23$ ,  $\phi_{\text{chitlac}} = 0.23$ , and  $\phi_{\text{HA}} = 0.54$ . In all cases, a ratio of  $[\text{NaCl}]/[\text{Hepes}] = 15$  (pH 7.4) was used and the total polymer concentration was 1.5 g/L.  $\phi_{\text{Alg}}$ ,  $\phi_{\text{chitlac}}$ , and  $\phi_{\text{HA}}$  refer to the weight fraction of alginate, chitlac, and hyaluronan, respectively.



**Figure 5.** (a) Graph relative the compositions of the  $\text{AC}_H$  ternary mixtures of alginate, hyaluronan, and chitlac analyzed in aqueous NaCl 0.15 M. Color legend as in Figure 1. (b) Experimental (■) and theoretical curve (□, calculated with the Huggins equation truncated to the second term, see Supporting Information) of the specific viscosity of the ternary polysaccharide mixtures in aqueous NaCl 0.15 M. In all cases, a ratio  $[\text{NaCl}]/[\text{Hepes}]$  of 15 and a pH of 7.4 were maintained.  $\phi_{\text{Alg}}$ ,  $\phi_{\text{chitlac}}$ , and  $\phi_{\text{HA}}$  refer to the weight fraction of alginate, chitlac, and hyaluronan, respectively. Total polymer concentration = 1.5 g/L. Lines are drawn to guide the eye.

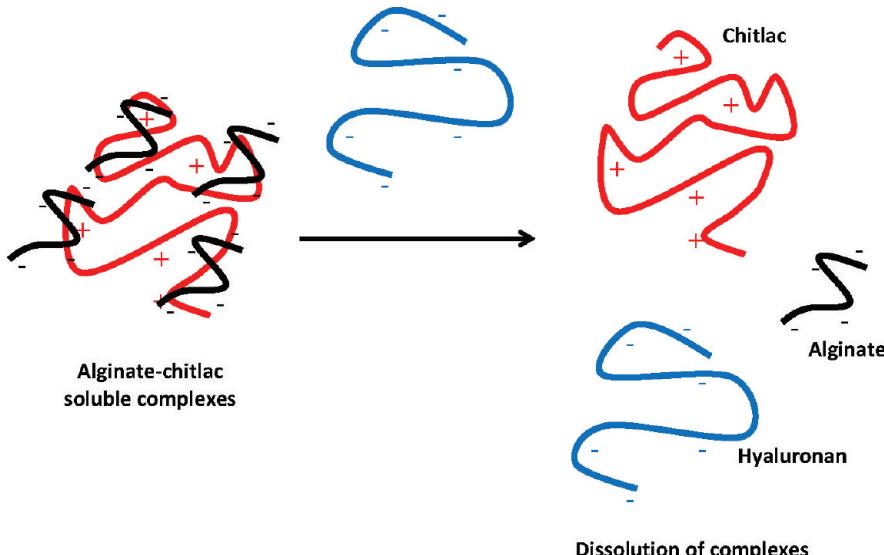
In aqueous of NaCl 0.15 M (Figure 5b), it can be noticed that the experimental values of specific viscosity for the ternary mixture are very close to the theoretical ones calculated with the Huggins equation truncated after the second term of the expansion series (see eq 1 in Supporting Information), suggesting that these conditions guarantee an effective charge screening preventing association between oppositely charged polyelectrolytes.

The same ternary mixtures were analyzed in aqueous NaCl 0.015 M, i.e., upon a 10-fold reduction of the ionic strength (Figure 6a–c). The specific viscosity values of the binary mixtures of alginate–chitlac are much higher than the theoretical values calculated either truncating the Huggins equation to the second term (as high as ~4.6-fold increase in the absence of HA, eq 1 in Supporting Information) or to the third term (as high as ~4.2-fold increase in the absence of HA, eq 5 in Supporting Information) (Figure 6b). As already



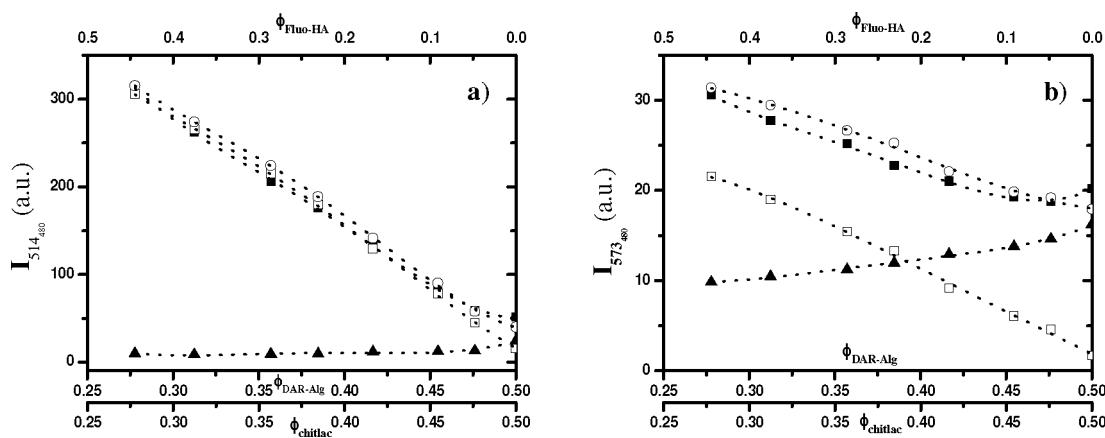
**Figure 6.** (a) Graph relative to the compositions of AC<sub>H</sub> ternary mixtures of alginic acid, hyaluronan, and chitlac mixtures analyzed in aqueous NaCl 0.015 M. (b) Experimental (■) and theoretical curve (□, ○ calculated by means of Huggins equation truncated to the second and third term, respectively, see Supporting Information) of the specific viscosity of the ternary polysaccharide mixtures in aqueous NaCl 0.015 M. (c) Scattered intensity of the ternary polysaccharide mixtures in aqueous NaCl 0.015 M (■) compared with the binary alginic acid/chitlac mixture diluted with the same solvent (□). In all cases, the total polymer concentration was 1.5 g/L and the ratio [NaCl]/[Hepes] was 15 (pH 7.4).  $\phi_{\text{Alg}}$ ,  $\phi_{\text{chitlac}}$ , and  $\phi_{\text{HA}}$  refer to the weight fraction of alginic acid, chitlac, and hyaluronan, respectively. Lines are drawn to guide the eye.

**Scheme 2. Graphical Representation of the Effect of Addition of Hyaluronan (Blue) to Soluble Complexes of Chitlac (Red) and Alginic Acid (Black)**

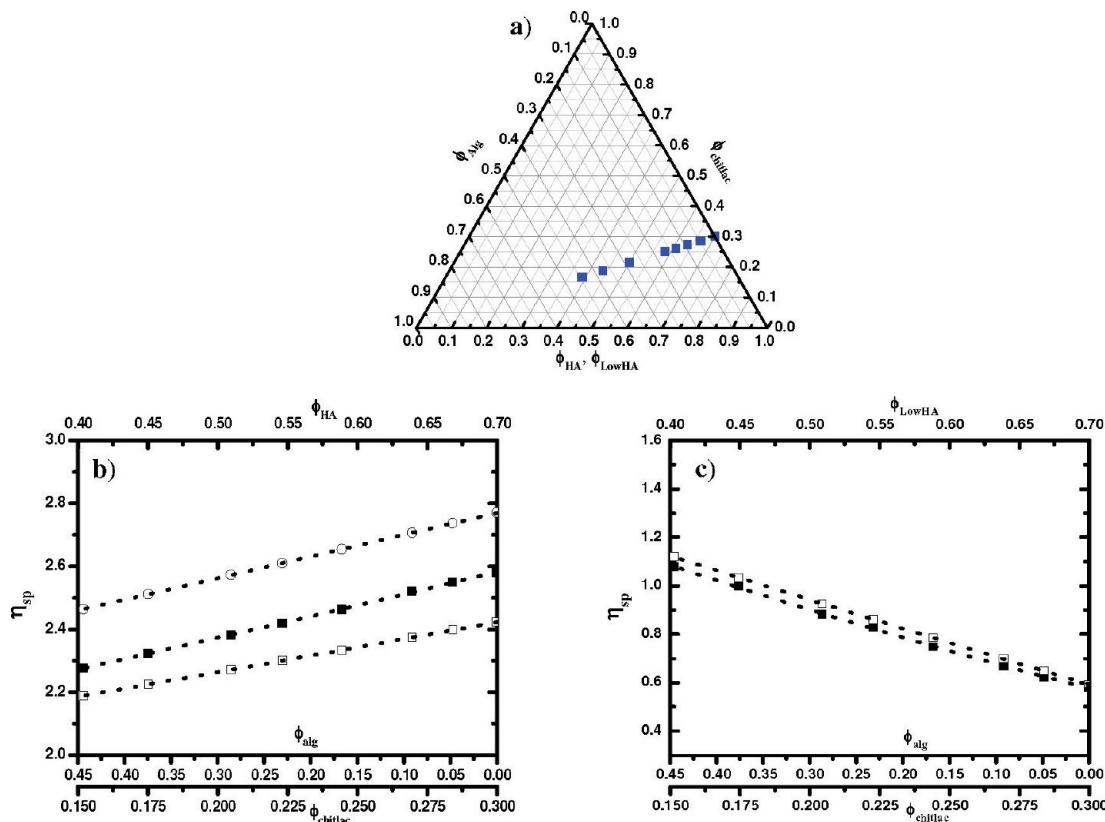


pointed out in a previous work on alginic acid/chitlac binary system, this stems from the formation of nonstoichiometric complexes of the two oppositely charged polyelectrolytes.<sup>33</sup> The addition of the third component, i.e., the polyanion hyaluronan, to the binary system leads to a nonmonotonic

dependence of the experimental  $\eta_{sp}$  from the composition of the ternary mixture (Figure 6b). The discrepancy between the experimental and the theoretical curve is progressively reduced by the additions of hyaluronan to the system. Specifically, when the theoretical curve is calculated with the Huggins equation



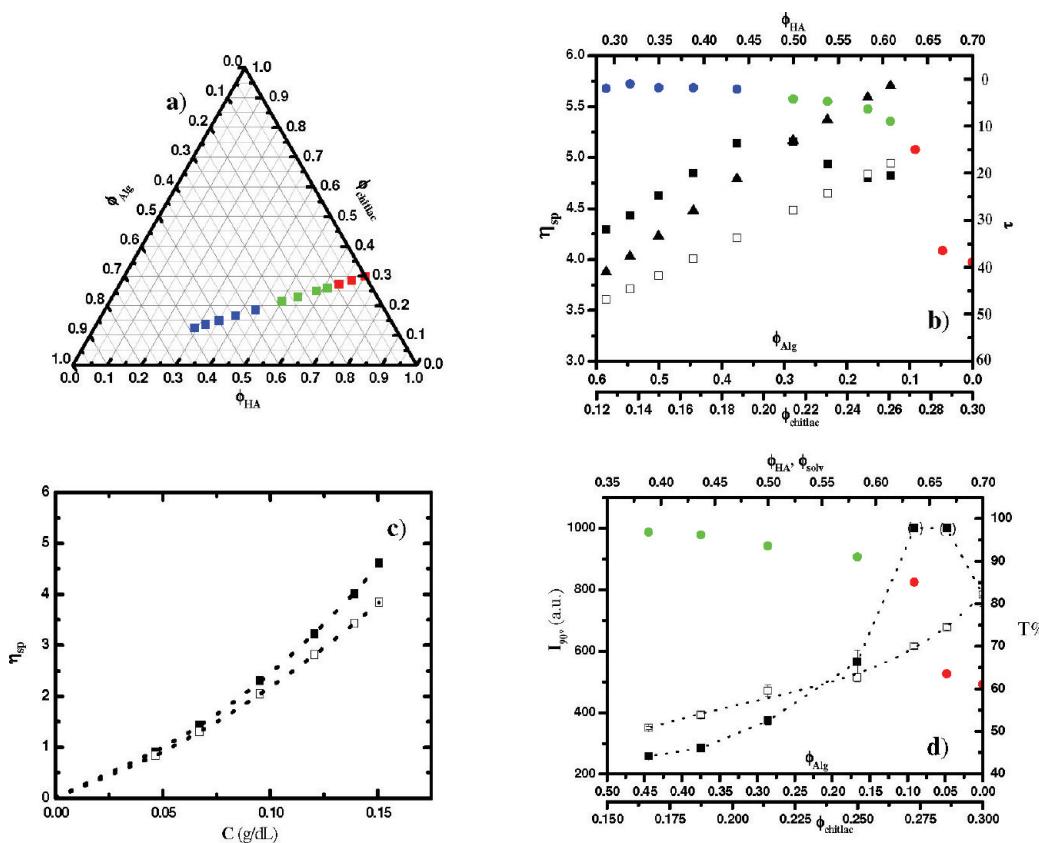
**Figure 7.** FRET of ternary mixture of rhodamine-labeled alginate (Dar-Alg) (■), chitlac, and fluorescein-labeled hyaluronan (Fluo-HA) (□). The excitation wavelength was 480 nm, and the emission wavelength were 514 nm (a) or 573 nm (b). For comparison, the fluorescence signal of DAR-Alg (▲) and Fluo-HA (□) at the same concentration, together with the sum of the two signals (○), is reported. In both cases, the concentration of aqueous NaCl was 0.015 M, and a ratio of [NaCl]/[Hepes] = 15 was used (pH 7.4). Total polymer concentration was 1.5 g/L.  $\phi_{\text{Alg}}$ ,  $\phi_{\text{chitlac}}$ , and  $\phi_{\text{HA}}$  refer to the weight fractions of alginate, chitlac, and hyaluronan, respectively. Lines are drawn to guide the eye.



**Figure 8.** (a) Graph relative to the compositions of the  $\text{HC}_A$  ternary mixtures analyzed in aqueous NaCl 0.15 M. (b) Experimental (■) and theoretical curve (□, ○) calculated by means of Huggins equation truncated to the second and third term, respectively, see Supporting Information) of the specific viscosity of the ternary polysaccharide mixtures in aqueous NaCl 0.15 M. (c) Experimental (■) and theoretical curve (□, ○) calculated by means of Huggins equation truncated to the second) of the specific viscosity of the ternary polysaccharide mixtures containing the low molecular weight hyaluronan in aqueous NaCl 0.15 M. In all cases, the total polymer concentration was 1.5 g/L and the ratio [NaCl]/[Hepes] = 15 (pH 7.4).  $\phi_{\text{Alg}}$ ,  $\phi_{\text{chitlac}}$ ,  $\phi_{\text{HA}}$ , and  $\phi_{\text{LowHA}}$  refer to the weight fraction of alginate, chitlac, hyaluronan, and low molecular weight hyaluronan, respectively. Lines are drawn to guide the eye.

truncated after the second term of the expansion series (eq 1 in Supporting Information), the difference in specific viscosity with the experimental results is reduced from 6.24 ( $\phi_{\text{HA}} = 0$ ) to 1.16 ( $\phi_{\text{HA}} = 0.54$ ). Conversely, when using the Huggins equation truncated to the third term, the difference in specific viscosity between the theoretical and experimental curves

ranges from 6.10 ( $\phi_{\text{HA}} = 0$ ) to only 0.1 ( $\phi_{\text{HA}} = 0.54$ ). The analysis of the data so far reported seems to indicate that the addition of the hyaluronan to the alginate–chitlac mixtures leads to an electrostatic destabilization of the soluble complexes among the latter oppositely charged polyelectrolytes leading to their dissolution (Scheme 2).



**Figure 9.** (a) Ternary graph relative to the compositions of the  $\text{HC}_\text{A}$  ternary mixtures of alginate, hyaluronan, and chitlac analyzed in aqueous NaCl 0.015 M. Color legend as in Figure 1. (b) Experimental (■) and theoretical curve (□, ▲ calculated by means of Huggins equation truncated to the second and third term, respectively, see Supporting Information) of the specific viscosity of the ternary polysaccharide mixtures of alginate, hyaluronan, and chitlac in aqueous NaCl 0.015 M. The percent transmittance ( $\tau$ , %) is also reported (circles). (c) Experimental (■) and theoretical (□, calculated by means of Huggins equation truncated to the second term) dependence of the specific viscosity from the total polymer concentration for a ternary mixture  $\phi_{\text{Alg}} = 0.5$ ,  $\phi_{\text{chitlac}} = 0.15$ , and  $\phi_{\text{HA}} = 0.35$ . A ratio  $[\text{NaCl}]/[\text{Hepes}] = 15$  was maintained throughout the measurements. (d) Intensity of the light scattered at  $90^\circ$  by the ternary mixtures in aqueous NaCl 0.015 M (■) compared to the binary hyaluronan/chitlac mixture diluted with the same solvent (□). Parentheses indicate that the points reached the instrument response limit. The transmittance ( $\tau$ , %) is also reported (circles). In (a, b, d), the total polymer concentration was 1.5 g/L and the ratio  $[\text{NaCl}]/[\text{Hepes}] = 15$ .  $\phi_{\text{Alg}}$ ,  $\phi_{\text{chitlac}}$ , and  $\phi_{\text{HA}}$  refer to the weight fraction of alginate, chitlac, and hyaluronan, respectively.  $\phi_{\text{solv}}$  represents the fraction of added solvent (aqueous NaCl 0.015 M). Lines are drawn to guide the eye.

To shed light on the destabilization effect of hyaluronan, the scattering of the light at  $90^\circ$  for the ternary mixtures was recorded upon its addition to the alginate–chitlac binary mixtures (Figure 6c). The high value of the scattered intensity when hyaluronan is not present in solution can be traced back to the formation of the large soluble complexes between the alginate and chitlac, as previously pointed out.<sup>33</sup> It can be noticed that the addition of the polyanion with the lowest charge density leads to a decrease of intensity of the scattered light which is much more marked than the one detected for the same alginate–chitlac mixture treated with the polymer-free solvent (aqueous NaCl 0.015 M).

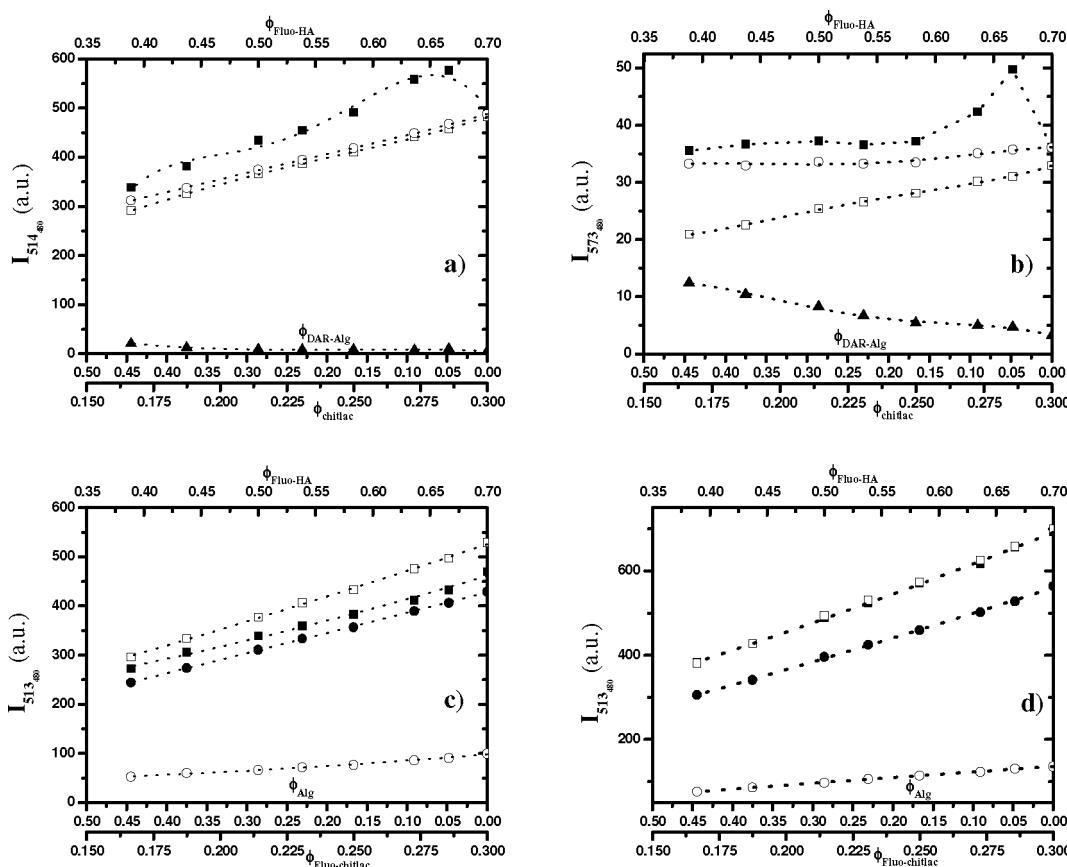
A FRET experiment was performed on ternary systems using fluorescently labeled polyelectrolytes (Figure 7a,b). In particular, alginate was labeled with rhodamine (Dar-Alg) and hyaluronan with fluorescein (Fluo-HA). The ternary polysaccharide mixtures were irradiated at a wavelength corresponding to the excitation of the fluorescein, and the intensity of the emitted light was measured at the wavelength corresponding to both the fluorescein and rhodamine tags. The experimental emission curve detected at different weight fraction of the polymeric components was compared with the theoretical one calculated from the contribution of the polysaccharides when

considered singularly,  $I_{C_{\text{tot}}}^{\text{Theor}}$ . The latter stems simply from the sum of the signal arising from labeled alginate and hyaluronan (eq 3).

$$I_{C_{\text{tot}}}^{\text{Theor}} = I_{C_{\text{Alg}}}^{\text{Dar-Alg}} + I_{C_{\text{HA}}}^{\text{Fluo-HA}} \quad (3)$$

Figure 7a,b shows that at both wavelengths (corresponding to the emission of fluorescein and of rhodamine, namely 514 and 573 nm, respectively) the intensity of the emitted light of the experimental mixture parallels very closely the theoretical value calculated according to eq 3. It follows that the two labeled polyanions do not reach distance close enough for the transfer of fluorescence energy from fluorescein to rhodamine to take place. These results confirm that during the addition of the polyanion hyaluronan no ternary no complex composed by the polycation and both polyanions, i.e., alginate and hyaluronan, is formed.

**Addition of Alginate to Hyaluronan–Chitlac Mixtures ( $\text{HC}_\text{A}$ ).** The analyses of the characteristics of these ternary mixtures were also studied starting from binary mixtures of hyaluronan and chitlac upon addition of alginate. In particular, the viscosimetric properties of the ternary mixtures were studied in aqueous NaCl 0.15 M (Figure 8) and NaCl 0.015 M



**Figure 10.** (a, b) FRET of ternary HC<sub>A</sub> mixtures of rhodamine-labeled alginate (DAR-Alg), chitlac, and fluorescein-labeled hyaluronan (Fluo-HA) in aqueous NaCl 0.015 M (■). The excitation wavelength was 480 nm, and the emission wavelengths were 514 nm (a) or 573 nm (b). For comparison, the fluorescence signal of DAR-Alg (▲) and Fluo-HA (□) at the same concentration, together with the sum of the two signals (○), is reported. (c, d) Fluorescence quenching of ternary HC<sub>A</sub> mixture of alginate, fluorescein-labeled chitlac (Fluo-chitlac) and fluorescein-labeled hyaluronan (Fluo-HA) (■) in aqueous NaCl: (c) NaCl 0.015 M and (d) 0.15 M. The excitation wavelength was 480 nm, and the emission wavelength was 513 nm. For comparison, the fluorescence signal of Fluo-chitlac (●) and Fluo-HA (○) at the same concentration, together with the sum of the two signals (□), is reported. In all cases, a ratio of [NaCl]/[Hepes] = 15 was used, and the total polymer concentration was 1.5 g/L (pH 7.4).  $\phi_{\text{Alg}}$ ,  $\phi_{\text{chitlac}}$ , and  $\phi_{\text{HA}}$  refer to the weight fraction of alginate, chitlac, and hyaluronan, respectively. Lines are drawn to guide the eye.

(Figure 9). In both cases, the experimental specific viscosity of the ternary mixtures was compared with the one theoretically calculated for noninteracting polysaccharides (see Supporting Information). When the system was studied in the presence of a high ionic strength, i.e. NaCl 0.15 M, the ternary mixtures of chitlac, hyaluronan, and alginate under analysis are completely miscible for all compositions (weight fractions) (Figure 8a). The experimental curve of the specific viscosity (Figure 8b) is located in between the theoretical curves calculated with a truncation to the second and to the third term of the Huggins expansion series, respectively. This behavior can be traced back to specific characteristics of hyaluronan sample used whose dependence of the specific viscosity from the polymer concentration is efficiently described by a Huggins expansion series with a fractional exponent  $\nu$  (eq 4), as reported by Cowman and co-workers.<sup>34</sup>

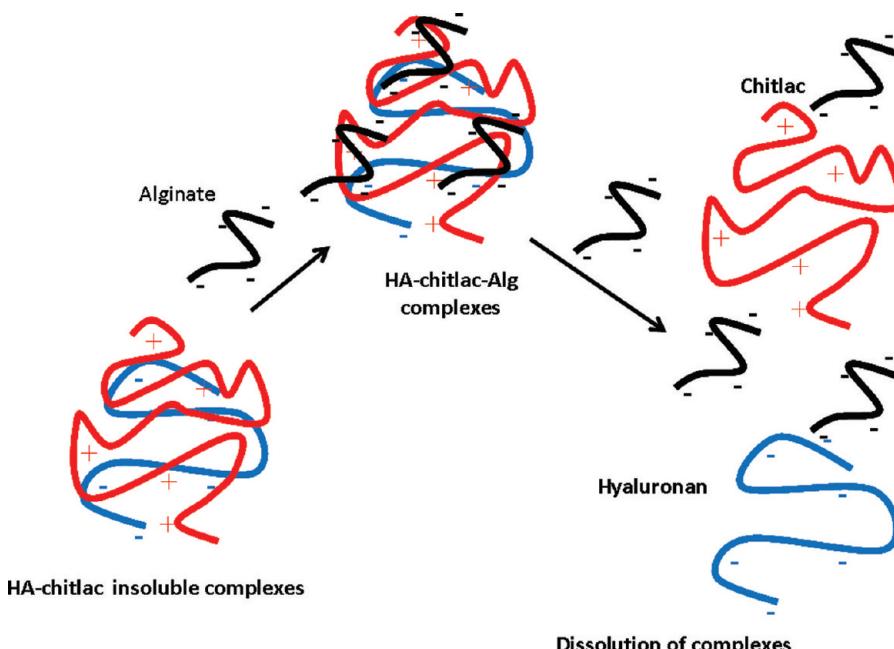
$$\eta_{sp} \propto (c[\eta])^\nu \quad (4)$$

where the exponent  $\nu$  is a real number. In the case of the present work, the deviations from the theoretical curves should be regarded as arising from the intrinsic macromolecular characteristics of the hyaluronan sample used, being the main component of the ternary HC<sub>A</sub> mixtures rather than an indication of the presence of interpolyelectrolyte complexes. To

prove this hypothesis, ternary polysaccharide mixtures were prepared starting from a low molecular weight hyaluronan (Figure 8c). In this case, the experimental  $\eta_{sp}$  of the ternary mixtures at different weight fractions of the three polysaccharides is very closely paralleled by the theoretical curve calculated for noninteracting polymers truncating to the second term the Huggins expansion series.

The analysis of the specific viscosity of the ternary mixtures at different polysaccharide (weight) composition was performed upon reducing the overall ionic strength of the solution, i.e., in aqueous NaCl 0.015 M. In this case, the solubility of the ternary mixtures is highly dependent on the polymer composition (Figure 9a). In fact, starting from a phase-separated binary mixture composed of hyaluronan ( $\phi_{\text{HA}} = 0.7$ ) and chitlac ( $\phi_{\text{chitlac}} = 0.3$ ), the addition of alginate progressively leads to the formation of soluble complexes and, eventually, to soluble mixtures of the individual polymers. The specific viscosity of the ternary mixtures corresponding to soluble complexes and soluble systems was recorded and compared with the theoretical curves calculated for noninteracting polysaccharides truncating the Huggins expansion series to the second or to the third term, respectively (Figure 9b). The experimental curve is nonmonotonic and displays a maximum in the  $\eta_{sp}$  corresponding approximately to  $\phi_{\text{HA}} = 0.5$ ,  $\phi_{\text{chitlac}} =$

**Scheme 3.** Graphical Representation of the Effect of Addition of Alginate (Black) to Phase-Separated Aggregates of Chitlac (Red) and Hyaluronan (Blue)



0.21, and  $\phi_{\text{Alg}} = 0.29$ . This is followed by a decrease of the specific viscosity for further additions of alginate. The first part of the experimental curve, i.e., the increase of the specific viscosity upon addition of alginate, can be safely traced back to the persistent presence of small amounts of phase-separated hyaluronan–chitlac complexes which are solubilized by addition of alginate. This conclusion is supported by the  $\eta_{\text{sp}}$  measurements performed starting from the phase-separated binary mixtures, which resulted to be lower than the values reported in Figure 9b (not reported). Focusing on the second part of the experimental dependence of  $\eta_{\text{sp}}$  on composition, when complete solubilization of such binary complexes is attained, the additional increase of the fraction of alginate in the system causes an overall decrease in the specific viscosity. This effect leads to conclude that the complexes present in solution are progressively disentangled eventually ending up in non-associated polysaccharides in solution. However, although polyelectrolyte–polyelectrolyte association does not occur, long-range electrostatic interactions still take place with a net effect on the overall physical properties of the system. In fact, looking at the dependence of the experimental  $\eta_{\text{sp}}$  of the ternary mixture (with  $\phi_{\text{Alg}} = 0.5$ ,  $\phi_{\text{chitlac}} = 0.15$ , and  $\phi_{\text{HA}} = 0.35$ ) on the total polymer concentration (Figure 9c), it can be noticed that, at higher polysaccharide concentration, this deviates from the theoretical curve as a consequence of the presence of long-range electrostatic contributions besides the merely hydrodynamic ones.<sup>35,36</sup>

The intensity of the light scattered at  $90^\circ$  recorded at different polysaccharide weight fractions seems to support these conclusions (Figure 9d). In fact, the first additions of alginate to the binary solution of hyaluronan and chitlac induce a sharp increase of the intensity of the scattered light stemming from the formation of larger aggregates in the system. However, upon further increasing the amount of alginate in the system, the intensity of the light scattered rapidly decreases indicating that the complexes are progressively disentangled. This decrease is much more marked than that obtained upon

addition of polymer-free solvent (i.e., NaCl 0.015 M) to the phase-separated binary hyaluronan–chitlac mixture, meaning that it is not a sole effect of dilution.

It remains to determine whether the destabilizing effect of alginate is due to an electrostatic interference/competition on the hyaluronan–chitlac complexes or to an active part played by the more highly charged alginate polyanion on the formed complexes. Once more it was resorted to FRET, using fluorescently labeled polyanions, namely Dar-Alg and Fluo-HA. Rhodamine-labeled alginate at different values of the weight composition were added to binary mixtures of chitlac and fluorescein-labeled hyaluronan (Figure 10a,b). The experimental data of emitted light were compared with the theoretical values calculated from the sum of the signals of the labeled polyanions considered separately (see eq 3). Figure 10a,b shows that, at the emission length of both fluorescein and rhodamine, the experimental data deviate from the theoretical ones, displaying a fluorescence energy transfer from the former fluorescent tag to the latter one. This is particularly evident for the first additions of alginate to the binary mixture of hyaluronan and chitlac. This result is of particular importance as it establishes the existence, when starting from binary nonsoluble systems composed of hyaluronan and chitlac, of complexes containing three components, specifically one polycation and two polyanions. Upon further additions of alginate, the experimental curve of the intensity of emitted light converges to the theoretical one, stemming from the dissolution of the complex containing both alginate and hyaluronan. A further insight into the characteristics of these ternary polysaccharide systems was obtained by means of fluorescence quenching using fluorescein as a tag on both hyaluronan (Fluo-HA) and chitlac (Fluo-chitlac). In this case, the ternary mixtures were irradiated at the excitation wavelength of the fluorescein and the intensity of the light were recorded at the emission wavelength of the same fluorescent tag. The experimental curve obtained for different weight fraction of polysaccharides in the ternary mixtures was

compared with the theoretical one calculated as the sum of the signals relative to chitlac and hyaluronan at the same concentration as in the mixture but considered separately (eq 3) (Figure 10c,d). When the ternary mixtures are prepared in aqueous NaCl 0.15 M, the experimental curve is perfectly superimposable with the theoretical one confirming that no complex formation takes place. At variance, when the added salt is reduced, i.e. with NaCl 0.015 M, the experimental curve of emitted light by fluorescent tags obtained for the ternary mixtures of the polysaccharides is markedly lower than the theoretical curve. This evidence, in combination with the results reported in Figure 10a,b, indicates that the complexes contain, in close contact to hyaluronan, both alginate and chitlac. When the weight fraction of alginate in the ternary mixture is increased above  $\phi_{\text{Alg}} = 0.3$ , the fluorescence quenching between fluorescein-labeled hyaluronan and chitlac is suppressed, indicating disruption of the interpolyelectrolyte complexes. The effect of the addition of alginate to hyaluronan–chitlac complexes is graphically represented in Scheme 3.

## CONCLUSIONS

The present article focuses on the characterization of ternary mixtures of alginate, hyaluronan, and chitlac in dilute conditions. The miscibility of the three components has been studied as a function of ionic strength, total polymer concentration, and, to some extent, molecular weight. In particular, the presence of insoluble and soluble regions, together with the presence of soluble complexes, has been identified. It is interesting to note the ionic strength strongly influences the interchain association affecting all the three possible conditions of the ternary mixtures. At variance, the polymer concentration has a notable effect on the miscible compositions and soluble complexes. The extended analysis of the behavior of the ternary mixtures has revealed that in the presence of low ionic strength their overall properties are strongly dependent on the order of mixing. This stresses the high complexity of such systems and the importance of a thorough control of all the parameters for understanding and exploiting completely the potentials of these mixtures. It is noteworthy that there is a nonequivalent effect of the addition of a polyanion to the binary mixture polyanion–polycation. In fact, when alginate–chitlac binary mixtures are considered, the addition of hyaluronan induces dissolution of the complexes. At variance, the hyaluronan–chitlac binary mixtures undergo, upon addition of alginate, the formation of ternary polyanion–polycation–polyanion complexes. For further alginate additions, a disruption of the complexes is again attained. The use of this complex system and the possibility of tuning the formation or disruption of complexes opens up additional question which shall be tackled in forthcoming work. In particular, a tracking of the variation of the net charge on the complexes as a function of the composition, the detailed analysis of the effect of the mixing protocol, and the quantitative analysis of the amount of polysaccharides not involved in the complexation process could give a more detailed comprehension of this complex system.

The study of these ternary mixtures has potential implications for the development of novel biomaterials for tissue engineering, in particular in semidilute conditions and hydrogels. The present work represents a starting point to shed some light onto the interpolyelectrolyte interchain associations providing the fundamental knowledge to move further toward

ternary systems closer to the needs of the biological applications (in progress).

## ASSOCIATED CONTENT

### Supporting Information

(i) Theoretical specific viscosity for ternary mixtures of noninteracting polymers and (ii) tables with the main characteristics of the polysaccharides used in the present study. This material is available free of charge via the Internet at <http://pubs.acs.org.org>.

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## ACKNOWLEDGMENTS

Mr. Alessandro Lunardelli is thanked for skilful technical assistance.

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