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Formation of a biocompatible polyelectrolyte complex: chitosan-hyaluronan complex stability

L. Rusu-Balaita, J. Desbrières, M. Rinaudo (✉)

Centre de Recherches sur les Macromolécules Végétales (CERMAV,CNRS), affiliated with Joseph Fourier University, BP53, 38041 Grenoble cedex 9 (France)
e-mail address:marguerite.rinaudo@cermav.cnrs.fr fax : +33476547203

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Summary

This paper concerns the formation of a biocompatible electrostatic complex; a conductimetric method is proposed to control the complex formation. The dissociation of chitosan in acidic medium is considered and the method for determination of the degree of acetylation is revisited. The degree of association of the complex is determined and the role of the auto-dissociation of the chitosan hydrochloride on the formation of the complex is discussed. The complex formed is very stable and cooperative for low degree of acetylation of the chitosan. The stability of the complexes formed is tested as a function of the pH. The role of the different parameters involved are examined (degree of acetylation of chitosan, external salt concentrations).

Introduction

Interpolymer complexes become a very important domain of research due to the versatility of the systems produced. H-bonding mechanism is involved in some water soluble polymer mixtures containing electron-donating protons (such as poly(carboxylic acids)) and electron-donating groups (such as poly(ethyleneglycol) or poly(vinylalcohol))[1]. But mainly interpolyelectrolyte complexes were developed in the field of permselective membranes [2], drug release [3], preparation of nanoparticles [4] or multilayers [5] ... for biological (immobilization of enzymes and protein purification) and industrial applications (water purification, cosmetic and paint formulations). The mechanism involving polyanion-polycation complexes was largely developed by Kabanov and his group [6-9]. The stability of such complexes depends upon the nature and the polarity of the polymers, their degrees of polymerisation, the pH of the reaction medium [10]... The preparation of polyelectrolyte complexes appears to offer an important reservoir of materials, the property of which may be tailored according to the requirements.
This paper is devoted to the preparation of polyelectrolyte complexes from two natural polymers, chitosan and hyaluronan. The stability of the complex as well as the mechanism of complexation and the role of different parameters are discussed.

Experimental

Chitosan is produced by Pronova (Norway); it is purified as exposed previously [11] and characterised using NMR to determine the degree of acetylation (DA= 0.12) [12] and GPC multidetection to determine the molar mass (Mw= 190,000 g/mol) [13]. Chitosans with different degrees of acetylation were prepared by G. Roberts; they were obtained by homogeneous reacetylation of chitosan [14] and characterized by NMR and GPC. The chitosan is dissolved in presence of stoichiometric amount of HCl; hence, it is a polycationic molecule and the concentration in $-NH_2$ is calculated from the DA and the exact dried weight of polymer dissolved. The charge parameter (λ) of this chitosan at complete protonation equals 1.21.

The exact chitosan concentration is determined by neutralisation of this polymer solution by titrated NaOH followed by potentiometry or conductimetry.

Hyaluronan (HA) is a bacterial polymer produced by ARD Cy (Pomacle, France); its molar mass is Mw= 800,000 g/mol. It is a carboxylic polysaccharide isolated under the Na salt form. The charge parameter is nearly half that of chitosan and is $\lambda = 0.7$.

The HA solutions are prepared by dissolution of the exact dried weight in distilled water. The concentration is controlled by exact neutralisation with NaOH of the acid form obtained passing through an ion exchanger (under H form). To control the ionic concentration, the polymer solutions prepared in water are diluted with a defined solution of NaCl. The experiments are performed using a CD 78 conductimeter from Tacussel (France) equipped with a platinated platinum electrode and a Minisis 6000 pH-meter from Tacussel (France) equipped with a glass electrode. The temperature is fixed at 25.0 ± 0.1°C.

Results and discussion

A-Chitosan dissociation in acid medium.

As we discussed previously [15], the chlorhydrate form of chitosan is hydrolysed and an equilibrium is established in water with a partial production of HCl and a fraction of undissociated chitosan depending on polymer concentration. This was described from pH measurement (the initial pH of the solution gives the fraction of proton release, α') and demonstrated by conductimetry combined with pH-metry during neutralisation by titrated NaOH solution. This phenomenon is important if potentiometry is used to determine DA and to follow the interaction with polyanion.

In a first series of experiments, one introduces the exact amount of HCl molecules to transform chitosan in the chlohydrate form; the pH and conductivity curves are given in Figures 1 and 2. A first step variation of pH occurs when the neutralisation starts; in conductivity, for the first additions of NaOH, the conductivity decreases due to neutralisation of free H_3O^+ and then, neutralisation of chitosan hydrochloride occurs; the stoichiometry corresponding to the total content in $-NH_2$ in the sample is indicated by an arrow (V= 7.7 mL of NaOH added in the choiced conditions).

The same curves were realized in the presence of a known excess of HCl (equivalent to 4 mL NaOH solution); these conditions correspond to that adopted to determine the degree of acetylation (named DA) when it is unknown. The results are given in Figures 3 and 4. In the pH curve, two transition points are located by arrows on the curve (and precisely determined using the derivative of the curve); between the two transitions, one gets $\Delta V = 7$ mL of the NaOH solution (i.e. lower than 7.7 mL supposed to correspond to the total $-NH_2$ groups). This means also that the potentiometric method is not convenient to determine precisely the DA; from ΔV

between the two inflexions, the DA is overestimated. By conductimetry, it is shown that this excess of HCl corresponds to the departure from linearity of the first decreasing part of the conductivity curve as indicated by the first arrow. Then, we conclude that the conductimetric method should be preferred.

Figure 1. Potentiometric titration of chitosan in presence of stoichiometric amount of HCl based on DA determination from NMR

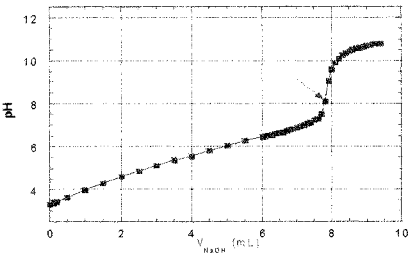


Figure 2. Conductimetric titration of chitosan in presence of stoichiometric amount ofHCl based on the DA determination from NMR

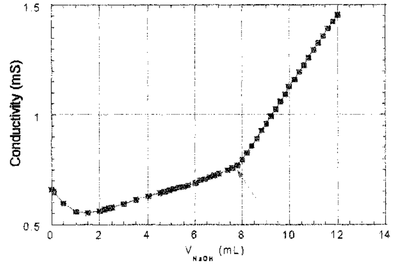


Figure 3. Potentiometric titration of chitosan in the presence of an excess of HCl based on DA determination from NMR. Two arrows indicate the inflexions.

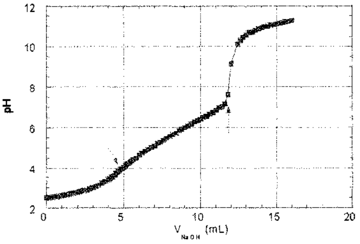
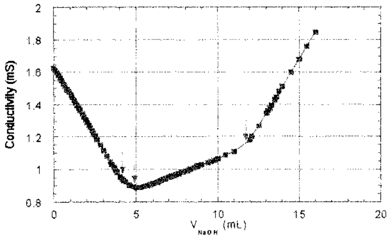


Figure 4. Conductimetric titration of chitosan in presence of an excess of HCl based on the DA determination from NMR. Two arrows indicate the inflexions from potentiometry.



B-Complex formation.

a)Theoretical approach.

When a polyanion is mixed with a polycation usually a phase separation occurs up to a stoichiometric ratio Z =1 ($Z = [-COO^-]/[-NH_3^+]$). The complex formation corresponds to charge neutralisation at least partially and then, can be followed by conductimetry as we have already discussed [16,17].

In fact, a specific problem occurs here with the chitosan solution: it is partially hydrolysed in water, then, even in stoichiometric condition, it coexists a fraction (x) of HCl and chitosan with a degree of charge lower than corresponding to DA. This was evidenced by conductimetric titration (Figure 2).

The complex formation when one equivalent of charge is considered can be expressed following the relation considering that chitosan (CH) is added to hyaluronan (HA):

$$\begin{array}{cccccccc} \text{HA-COONa} & + & \text{CH-NH}_3\text{Cl} + \text{HCl} & \leftrightarrow & \text{HA-COO...NH}_3\text{-CH} & + \text{HCl} + \text{NaCl} \\ (1-Z\theta) & & Z(1-\theta) \quad x Z & & Z\theta & xZ \quad Z\theta \end{array}$$

in which θ is the degree of complexation with charge neutralisation.

Then, the conductivity along the addition of chitosan solution is described by the relation:

$$\sigma_1 = P_1 (1 - Z\theta) + P_2 Z (1 - \theta) + xZP_4 + Z\theta P_3 \qquad (1)$$

where P_1, P_2, P_3 and P_4 are respectively the effective equivalent mobilities of HA, CH, NaCl and HCl.

We can also express the additivity of the species mixed in terms of conductivity:

$$\sigma_2 = P_1 + P_2 Z + xZP_4 \qquad (2)$$

For simplification, we can express the following relation:

$$(\sigma_1 - \sigma_2)/\sigma_2 = Z\theta\,[P_3 - P_2 - P_1]/[P_1 + Z(P_2 + x P_4)] \qquad (3)$$

allowing the determination of the degree of complexation θ. The content of HCl in chitosan solution was determined by titration of chitosan hydrochloride by NaOH in the same range of polymer concentration. The factor Pi for the polyelectrolytes can be expressed as:

$$P_i = f(\lambda p + \lambda c)$$

in which f is the transport coefficient as introduced by Manning [18]; λp and λc represent the ionic mobility of the polyion and of the counterion respectively as discussed previously [19]. Pi is given by the slopes of the curves obtained by conductimetry for addition of excess of polyelectrolytes in distilled water or electrolyte. From the literature or our own experiments, it comes $P_1 = 82$, $P_2 = 107$, $P_3 = 126.45$ and $P_4 = 426$ ($\times 10^4$ $m^2.S.mol^{-1}$)

The addition of chitosan hydrochloride in hyaluronan gives a smooth curve (not given) where the characterisation of 1/1 stoichiometry ($Z=1$) is difficult to determine. The calculated values of θ are given in Table 1a. The degree of complexation θ is around 1 whatever is Z (for $Z<1$).

For progressive addition of hyaluronan in a solution of chitosan hydrochloride, the relation (3) is modified and becomes:

$$(\sigma_1 - \sigma_2)/\sigma_2 = Z\theta\,[P_3 - P_2 - P_1]/[ZP_1 + P_2 + x P_4] \qquad (4)$$

The curves are represented on Figure 5; they are easier to be analysed and we choice this method to investigate the role of DA on the stability of the complex; especially, θ is determined at $Z=1$. The stoichiometry is indicated on the curve; after this point, the specific conductivity decreases in the presence of an excess of HA. This is interpreted as due to the acidification of the carboxylate group. For $Z>1.4$, HA in excess remains in its sodium form allowing to determine the equivalent conductivity (P_1) from the slope which is in good agreement with values obtained from the additivity of HA solution to distilled water.

In Figure 6, the pH variation was examined in the same conditions. The pH evolves slowly during complex formation up to 9.5 mL HA added; this indicates that ion pairs are formed between the two charged polymers involving the part of chitosan in the salt form.Then the pH increases rapidly in the zone where the conductivity decreases first and then slowly increases again. This is due to the consumption of H_3O^+ to acidify HA added. At end (over 14.5 mL HA added), HA causes the normal increase in conductivity and a slow increase in pH. The values obtained for θ are given in Table 1b.

Figure 5. Conductimetric titration of Chitosan, HCl/Hyaluronan; the arrow indicates Z=1. [HA]=3.92x10^{-3} equiv.L^{-1} [CH]= 3.72x10^{-3} equiv.L^{-1}.

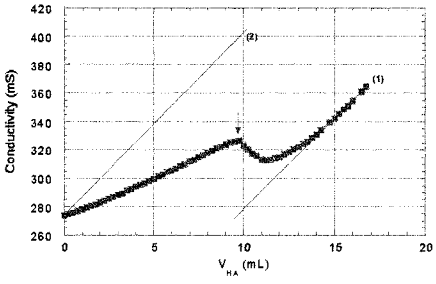
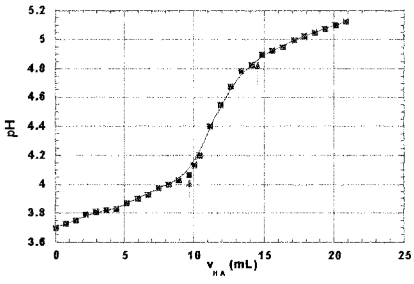


Figure 6. pH evolution during titration of Chitosan,HCl by Hyaluronan. The two arrows indicate Z=1 and the end of the equilibrated reaction of Hyaluronan and HCl.



The two ways seem not to be identical (θ is lower in the second case) and indicate that the complex formed when chitosan, with the higher charge parameter, is in presence of an excess of carboxylic groups is more stable than in the opposite way, related with the higher θ values.

Table 1. Determination of the degree of complexation for different Z ratios taken on the conductivity curves.

(a) CH added to HA

Z= [CH]/[HA]	θ
0.2	1.1
0.5	1.09
0.7	0.943
1	0.85

(b) HA added in CH

Z = [HA]/[CH]	θ
0.2	0.614
0.5	0.592
0.7	0.568
1	0.548

b) Role of the degree of acetylation

The HA solution is dropped into chitosan solutions having the same ionic concentration, taking in consideration their degree of acetylation; all the samples tested are obtained by homogeneous reacetylation. The curves obtained are given in Figure 7. The experimental data are given in Table 2.

From these results it comes first that the degree of autodissociation of the chitosan chlorhydrate decreases when DA increases. This also modifies the curve for which progressively the minimum after Z =1 disappears. The degree of complexation θ is nearly independent of DA at least up to 40%. For DA=60%, the mechanism is different and implies certainly a less cooperative mechanism. The conductivity (see Figure 7) increases progressively without appearance of a critical point for Z =1.

Table 2. Influence of the degree of acetylation on the solution characteristics during complex formation.

DA (%)	Initial pH	pH for Z=1	Degree of complexation θ
12	3.73	4	0.516
12*	3.70	4.05	0.548
25	3.89	4.2	0.561
40	4.12	4.5	0.585
60	5.03	5.6	------

*heterogeneous sample

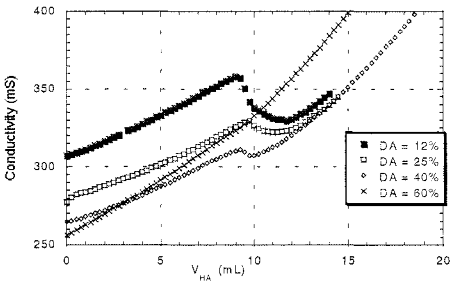


Figure 7. Conductivity titration of Chitosan,HCl with different DA by Hyaluronan. [CH]= 3.96 x 10^{-3} equiv.L^{-1}.

C-Complex stability

First, the stability based on the dissolution of the complex was tested in acidic or basic medium; whatever the conditions, the insoluble complex seems not to redissolve in excess of NaOH. It only dissociates in acidic conditions; the turbidity is followed during progressive additions of HCl solution (0.2M).

Table 3. Influence of the NaCl external salt concentration on the complex stability(*).

[Na Cl] (M)	V (mL 0.2M HCl) added	pH of complex dissolution
0	6	1.33
0.033	4.6	1.41
0.066	3.9	1.48
0.133	2.9	1.55
0.166	2.4	1.57
0.33	1.8	1.68
0.5	1.6	1.74
0.66	------	-----

*The total polymer concentration was fixed at 1.5x10^{-3} equiv./L, Z = 1 and the total volume was 30mL.

The pH value and the volume of acid added up to the complete dissolution, in a given volume of solution containing the complex, were determined .

The stability of the complex was shown to depend on the ionic concentration in Table 3. When NaCl concentration increases, the stability of the complex is decreasing; for a concentration larger than 0.5M, the complex no more forms due to electrostatic screening.

Chitosan having different DA with homogeneous distribution of the acetyl groups are examined in the same experimental conditions to test the complex stability and compared to the commercial sample (DA=12%) tested previously for which the acetyl distribution is heterogeneous along the chains. These results indicate that the stability of the complex decreases when DA increases, i.e. that the distance between charged groups increases. The complex is less cooperative for large DA (Table 4).

The higher value of HCl volume added for DA =12% compared with DA=0% may be related with the microstructure of the chain

Table 4. Role of the degree of acetylation on the complex stability for a stoichiometric ratio Z=1(*).

DA (%)	V (mL 0.2M HCl) added	pH of complex dissolution
0	6	1.25
12**	7	1.18
25	5.6	1.33
40	5	1.29
60	3.4	1.49

*Total volume 20mL ; initial concentration 3.925×10^{-3} equiv./L for the two polyelectrolytes.

** heterogeneous distribution of acetyl groups.

Conclusion

In this paper, the potentiometric and conductimetric titrations of chitosan chlohydrate are discussed; comparison of titration in stoichiometric amount or excess of HCl is analysed and allows to conclude that the value of degree of acetylation is overestimated by potentiometry in excess of acid compared with the degree of acetylation obtained by NMR. Conductimetry should be preferred.

Then conductimetry is used to analyse the complex formation between Hyaluronan and Chitosan with a low DA (i.e. a large charge density). In the conditions proposed, the conductivity is demonstrated as a good technique to estimate the stoichiometry when the polyanion is progressively added in the polycation.

The analysis of the curves based on additivity of ionic mobility allows to determine the degree of ion pair formation, θ, which is around 1 independently of the stoichiometric ratio of the two polyelectrolytes when Chitosan,HCl is added to Hyaluronan. In the opposite way, it is found lower values for θ independently of Z. This may be due to the presence of an excess of HCl giving a large contribution to the conductivity and a lack of precision.

Then, different parameters are tested to identify their role on the complex stability. It was first demonstrated that the complex looks stable whatever the pH in basic conditions; it must be recalled that chitosan turns to insoluble over pH=7 and it becomes difficult to identify the chitosan and the complex separately. The complex is dissociated in acidic medium; in acid conditions, the dissociation of carboxylic charge of Hyaluronan is completely repressed and the Chitosan completely ionised and soluble.

The complex is destabilized in excess of NaCl (and disappears in 0.66M NaCl). The role of the degree of acetylation on the complex formation is also described and especially it is shown that its stability decreases when DA increases in relation with the lower cooperativity of the electrostatic interaction.

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