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Chitosan–chondroitin sulfate and chitosan–hyaluronate polyelectrolyte complexes. Physico-chemical aspects

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When fully deacetylated chitosan is contacted in solution with chondroitin sulfates and hyaluronic acid, pure polyelectrolyte complexes are formed. pH and conductometric measurements, as well as Fourier-transform infra-red spectrometry or X-ray diffraction allow us to demonstrate that strong electrostatic interactions take place between the $-NH3^+$ functions of chitosan and the $-OSO3^-$ and/or the $-COO^-$ groups of the two other GAG's studied. These complexes are formed even in acidic media and then, are quite stable whatever the pH. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Chitosan is now well known for its numerous and interesting biological properties (Muzzarelli *et al.*, 1988) leading to consider this polysaccharide as a biocompatible (Fradet *et al.*, 1985), bioresobable (Hirano *et al.*, 1990; Arai *et al.*, 1968) and bioactive (Olsen *et al.*, 1988) polymer. The physico-chemical study of its interactions with the most important components of living matter appears as an essential point for the interpretation of the biological responses induced by the presence of chitosan in living media. Another aspect of this kind of study is to try to prepare new biomaterials from the association of chitosan and other biomolecules. We began with the study of the interactions between collagen and chitosan (Taravel & Domard, 1993) as well as between chitosan and lipids (Demarger-André & Domard, 1993, 1994).

Although they are little abundant in mammals, glycosaminoglycans (GAG's) play a major part in living media in particular as components of some connective tissues. In mammals, the two main GAG's are chondroitin sulfate and hyaluronic acid. To our knowledge there are only few results in the literature concerning the interactions between chitosan and other GAG's (Hirano *et al.*, 1978; Takayama *et al.*, 1990) but none concerns the case of fully deacetylated chitosan.

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This paper deals with the physico-chemical study of the interactions between fully deacetylated chitosan and chondroitin 4 and 6 sulfates as well as with hyaluronic acid. The interactions between polyelectrolytes of opposite charge depend on the degree of neutralisation, the strength of the alkaline or the acidic sites they bear and their charge density. Thus, very strong polyelectrolyte complexes (PEC) are generally formed between polymers including anions and cations of strong acids or bases in their structure (Kikushi & Oda, 1976; Shinoda & Nakajima, 1975). In contrast, weak PEC's are obtained when these acids and bases are weak and in the free form (Terrayama, 1952; Domard & Rinaudo, 1983; Gelman *et al.*, 1972; Gelman & Blackwell, 1974; Shinoda & Nakajima, 1975).

Chitosan is a weak base with an intrinsic pKa near 6.5 (Domard, 1987) and a low charge density, with a maximum of only one charge per residue *i.e.* every ≈5.15 Å. Chondroitin sulfates are mixed structures including both a weak and a strong acid but with a low charge density. Hyaluronic acid is a weak polyacid with a very low charge density since only one charge can be present every two residues.

It has often been demonstrated (Katchalsky, 1970; Domard & Rinaudo, 1980) that the PEC formation obeys simply the stoichiometric reaction represented by the equation: $\alpha[PA] = \beta[PC]$ where α and β are the degrees of ionization of the ionizable sites beared by the polyanionic and polycationic chains, respectively (inde-

pendently of their nature) and [PA] and [PC] represent the total concentrations of ionizable sites brought about by the polyanion and the polycation chains, respectively, at the maximum complexation.

MATERIALS AND METHODS

Materials

Chitosan was a sample kindly supplied by Aber Technologies (Plouvien, France) (lot BGL 25) with a degree of acetylation (DA) near 2.5% deduced from I.R. spectra, according to Miya *et al.*, 1980. It was fully deacetylated in our laboratory according to the method described by Domard & Rinaudo, 1983. The average molecular weight, determined by viscosimetry (Domszy & Roberts, 1982) was 9.52×10^5 g×mol^{-1}. The complete deacetylation was verified by F.T.I.R. spectrometry (Miya *et al.*, 1980).

Three different chondroitin sulfates, in the sodium salt form were used. Two of them, from Fluka and Sigma, were chondroitin 4 sulfates from bovine tracheas with 55 and 70% of sulfated residues on the 4 position, respectively. Their weight average molecular weights determined by light scattering measurements were near 45 500 g mol^{-1} (provided by the manufacturers). A third one from Sigma was a chondroitin 6 sulfate from shark cartilage with 90% of 6 sulfated residues. Its average molecular weight determined as above was near 59 000g mol^{-1}. Hyaluronic acid sodium salt was a gift from Merck-Clevenot (Nogent-sur-Marne, France). All these polymers were used as received with no further purification or characterization. For some experiments, these GAG's were acidified by regeneration of the acidic form on an Amberlite IR-120 H^+ ion exchange resin.

Methods

In order to take into account the water content in the calculation of the concentrations, all the biopolymers were initially dried overnight under *vacuum* at 80°C, then cooled in a dry atmosphere until ambient temperature. The residual water content, as well as the thermal stability of the products, were measured on a Dupont Thermogravimetric analyser, TGA 2950. Conductometric measurements were made with a Tacussel CD 810 conductometer equipped with a Tacussel electrode. pH's were measured with a Tacussel, Minisis 8000 pH-meter equipped with a double junction electrode. In both cases the studies were performed in a thermostated cell at 25±1°.

F.T.I.R. spectra were recorded by means of a Jasco F.T.I.R. 5300 spectrophotometer. The samples in the KBr pellet form were studied by transmission. In the case of polyelectrolyte complexes, the precipitates were isolated by centrifugation, then rinsed thoroughly with distilled water, lyophilised and dried overnight under vacuum at 60°C before use.

For X ray diffraction analyses, the spectra were plotted in the range $2\theta = 3°–35°$ by means of a Siemens D500 X-ray diffractometer operating with the Cu K_α radiation.

Solutions

Stock solutions (2.5×10^{-2} equiv l^{-1}) of each polysaccharide under their salt forms were prepared by weighting the exact amount of product to which a given volume of distilled water was added in order to prepare a concentrated sample of well defined concentration. In each case, the weight of polymer was taking into account and the content of water previously measured by thermogravimetry. In the case of chitosan, a stoichiometric amount of 0.1 M HCl was added to a dispersion of the polymer in the free amine form in order to prepare the hydrochloride salt (Domard, 1987). These solutions were then diluted to prepare the solutions used for the experiments.

RESULTS AND DISCUSSION

pH and conductometric study

In a first part of this work, the interactions between chitosan and GAG's were studied by conductometry and pH-metry. As supposed by the relation above, the maximum electrostatic interaction between polyelectrolytes of opposite charge is obtained from their fully ionized form. As a consequence, we used chitosan in its hydrochloride form and the other GAG's in their sodium salt form. In another kind of experiments we were interested in the study of the competition between the protonation of the carboxylic sites of GAG's and their complexation with ammonium sites of chitosan. In this case, GAG's were prepared in their acidic form, but, with no excess of acid, in order to be sure that the pH of the media was only due to the presence of the polyelectrolytes and not to an excess of acid. It would also have been interesting to test the direct interaction between chitosan in its free amino form and GAG's in their fully ionized form. But, unfortunately the insolubility of chitosan under this form did not allow this kind of study.

Interaction between chitosan and chondroitin sulfates

Figure 1 shows the variations of conductivity observed when a solution of chitosan hydrochloride is added to solutions of chondroitin sulfates (CHOS) with various proportions of sulfation on carbons 4 and 6. Whatever the CHOS structure, the curves are similar and show a change of behaviour for $\rho = 1$. ρ is the ratio between the number of glucosamine residues and the total number

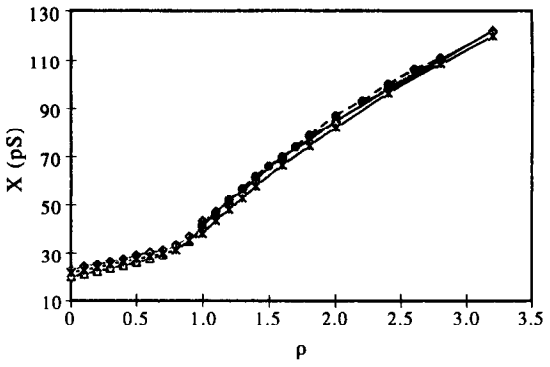

Fig. 1. Variations of conductance, as a function of ρ, when chitosan hydrochloride 2.5 × 10^{-2} equiv l^{-1} is added to 2.5 × 10^{-4} equiv l^{-1} chondroitin sulfate sodium salt with 55% (–◇–), 70% (–×–) of 4 sulfate; (–△–)10% of 6 sulfate and, to 2.5 10^{-4} equiv l^{-1} NaCl (–•–).

of sulfate and carboxylate sites of CHOS present in solution. Before $\rho = 1$, the conductivity increases linearly. This behaviour illustrates the formation of a PEC with a 1/1 stoichiometry, according to the equation:

$$\left|\begin{matrix}-OSO_3^- & Na^+\\ -COO^- & Na^+\end{matrix}\right| + \left|\begin{matrix}-NH_3^+ & Cl^-\\ -NH_3^+ & Cl^-\end{matrix}\right| \rightarrow \left|\begin{matrix}-OSO_3 & H_3N-\\ -COO & H_3N-\end{matrix}\right| + \begin{matrix}2Na^+\\ 2Cl^-\end{matrix} \qquad (I)$$

Therefore, the introduction of chitosan hydrochloride in solution induces the elimination of the $-OSO_3^-$ and $-COO^-$ anions which are stoechiometrycally replaced by Cl^-. As the limit equivalent conductivitys of $-OSO_3^-$ and $-COO^-$ are 66,5 and 35 (m^2.s.mol^{-1}), respectively and that of Cl^- is 75.5 10^{-4} (m^2. s. mol^{-1}), the conductivity of the media increases with ρ. Assuming that both kinds of polyelectrolytes used in this work have a low charge density, the osmotic coefficient of their counter-ions should be close to one (Domard & Rinaudo, 1980). As a consequence, the linearity of the curves before $\rho = 1$ demonstrates the completion of the reaction after each addition of polycations as well as the cooperativity of the mechanism. Therefore, if each addition of chitosan involves the reaction of all its -NH_3^+ functions with, for one half, the -OSO_3^- groups and, for the other half, the -COO^- functions of CHOS, the conductivity observed for $\rho = 1$ must be exactly the conductivity of a solution of NaCl with a concentration corresponding to the total number of $-NH_3^+$ functions introduced in the media. This prediction is quite well verified experimentally by the comparison with the conductivity of a solution of NaCl 2.5×10^{-4} M. During the addition of chitosan, a precipitate appears and a maximum turbidity is obtained for $\rho = 1$. Beyond this value, no new precipitation is observed and the supernatants obtained after centrifugation never contain CHOS, but, only the excess of added chitosan. The independence of the results of the position of the sulfate substituents on carbons 4 or 6, agrees with a sufficiently strong interaction which is not influenced by structural problems. It signifies that this kind of PEC could be of ladder type (Michaels, 1965) but, in this situation, the conformations of the two polymers would be highly constrained, in relation to the differences of glycosidic linkages of the two kinds of chains but also to the position of the ionizable functions on their backbones. The second possibility corresponding to a scrambled mixture of coils seems more acceptable.

If we consider the variations of pH obtained in the same conditions (Fig. 2), the curves resemble those of a classical pH titration with an equivalent point for $\rho = 1$. These results confirm the formation of a pure PEC in which all the ionic sites of the polymers are used at $\rho = 1$. Thus the pH of the solution on this point is simply the pH of a solution of NaCl 2.5×10^{-4} M. Moreover, as for conductometric measurements, the curves obtained for $\rho > 1$ are similar to the one corresponding to the addition of a solution of chitosan hydrochloride in a media which contains only NaCl 2.5×10^{-4} M.

The results corresponding to the reverse situation where CHOS are added to a solution of chitosan (not shown) also confirm those discussed above.

This kind of 1/1 complex usually observed for $\alpha = \beta = 1$ was also obtained during the study of the interactions between glycol-chitosan and CHOS (Shinoda & Nakajima, 1975) or, between partially acylated chitosans and CHOS (Hirano *et al.*, 1978). These authors also observed the independence of the position of the sulfate groups on the results.

CHOS bear a carboxylic site every two residues, alternately with a sulfate group. In previous works, in other circumstances, it has been shown (Hirano *et al.*, 1978; Domard & Rinaudo, 1980; Fukuda & Kikushi, 1977) that at low pH there exists a competition between the protonation of polyanionic sites and their complexation with polycations. This situation should not exist with sulfate sites which are always dissociated and always give a 1/1 PEC with polycations, even in

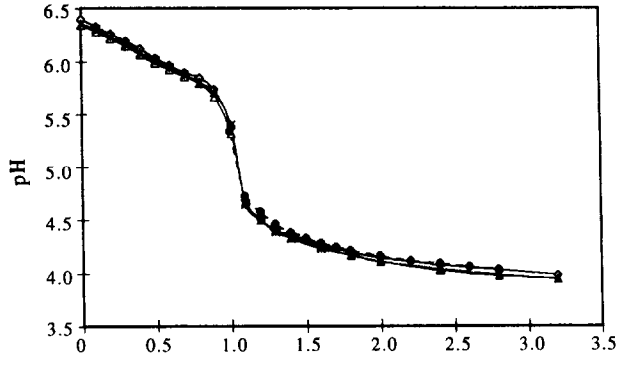

Fig. 2. Variations of pH, as a function of ρ, when chitosan hydrochloride 2.5 × 10^{-2} equiv l^{-1} is added to 2.5 × 10^{-4} equiv l^{-1} chondroitin sulfate sodium salt with 55% (–◇–), 70% (–×–) of 4 sulfate; (–△–)10% of 6 sulfate and, to 2.5 10^{-4} equiv l^{-1} NaCl (–•–).

acidic media (Kikushi & Fukuda, 1974; Shinoda & Nakajima, 1975). The situation is different with weak polyacids such as polycarboxylic polymers. As a consequence it was important to study the role of the apparent charge density of CHOS by changing the protonation state of their carboxylic functions. For this purpose, we prepared solutions of CHOS for which all the counter-ions of the anionic sites were exchanged by protons after elution on a proton exchange resin and thus, as we mentioned it above with no excess of external acid.

On Fig. 3, we show the variations of conductivity observed when a solution of chitosan is added to a solution of CHOS (with 70% of 4 sulfates) in the acidified form. There again, the results (not shown) are independent of the substitution on the 4 or 6 carbons. We observe an equivalent point corresponding to a maximum precipitation for $\rho = 0.75$. If we neglect the polyelectrolyte behaviour due to the relatively low charge density of the CHOS, whatever the pH, the sulfate groups can be considered as fully dissociated and they always allow the formation of a PEC with the $-NH_3^+$ functions of chitosan. In contrast, due to the intrinsic pK_a (pK_0) of the carboxylic funtions of glucuronic residues which is similar to the one for weak acids (3.83) (Park & Chakrabarti, 1978), initially, the carboxylic sites are partially in the free undissociated acidic form and must be deprotonated to give rise to the same interaction. As a consequence, we can consider that $\rho = 0.75$ corresponds to a situation for which all the $-NH_3^+$ are used to form a PEC with all the $-OSO_3^-$ and only half the $-COO^-$ of CHOS. In the latter case two reactions are necessary, the deprotonation;

$$|\text{–COOH} \rightleftharpoons |\text{–COO}^- + \text{H}^+ \qquad \text{(II)}$$

this reaction is obviously dependent on the presence of an excess of acid and then an excess of external acid should disfavor the formation of a complex. The second reaction corresponds simply to the following equation.

$$|\text{–COO}^- + |\text{–NH}_3^+ \rightarrow |\text{–COO H}_3\text{N–}| \qquad \text{(III)}$$

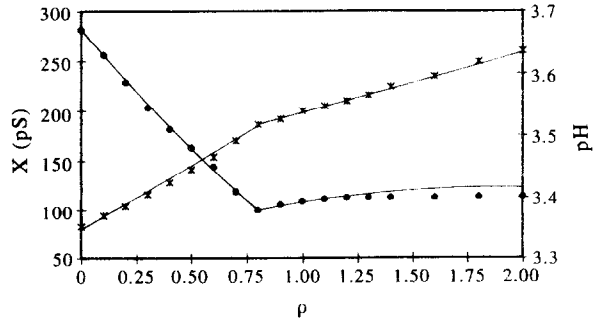

Fig. 3. Variation of conductance (–×–) and pH(–•–), as a function of ρ, when chitosan hydrochloride 2.5×10^{-2} equiv l^{-1} is added to 2.5×10^{-4} equiv l^{-1} protonated chondroitin sulfate with 70% of 4 sulfate.

The mechanism of deprotonation and complexation is verified by an increase of conductivity after each addition of chitosan which is much higher than in the previous case (Fig. 1) and agrees with the liberation of high conductivity H^+ ions. Nevertheless, the conductivity varies linearly which necessitates the same reaction after each addition of chitosan. As a consequence, the reaction does not seem to yield to equilibrium laws and should be considered as total after each addition of polymer and completely achieved for $\rho = 0.75$. As revealed in our experiments (not shown), this value does not seem to depend on the concentration of the polymer initially present in the media. This behaviour reinforces the demonstration of the absence of equilibrium in this kind of reaction. This mechanism is confirmed by the variation of pH (Fig. 3) in the case of the addition of chitosan to acidified CHOS. Indeed, in this case, although the pH of the solution of added chitosan is over that of the solution of CHOS, the pH decreases up to $\rho = 0.75$ and then does not change significantly beyond this value, except for a small increase due to the higher pH of chitosan solutions (this increase depends on the initial concentration of CHOS and increases with it). As a consequence, the reaction with the carboxylic groups of CHOS can be simply schematised as follows:

$$\begin{vmatrix} \text{–COOH} \\ \text{–COOH} \end{vmatrix} + {}^+\text{H}_3\text{N–}\Big| \rightarrow \begin{vmatrix} \text{–COOH} & \\ \text{–COO} & \text{H}_3\text{N–} \end{vmatrix} + \text{H}^+ \qquad \text{(IV)}$$

The decrease of pH observed after each addition of chitosan does not seem to disfavor the complexation. Nevertheless, we must mention that as the remaining concentration of the caboxylic sites decreases, their dissociation increases. This kind of complex disagrees with the simple mechanism proposed by Katchalsky for which the possible deprotonation of the carboxylic sites is not considered. It has not been observed or studied by Shinoda & Nakajima (1975) or Hirano *et al.* (1978) in the case of the interaction of glycol chitosan or *N*-acylated chitosans with CHOS. This complex is particularly interesting since, in contrast to the one described on Fig. 2, it can be charged during an increase of pH, leading to a dissociation of the free carboxylic acid functions which are not involved in the PEC. There also, the results are in agreement with a model of scrambled coils.

Interaction between chitosan and hyaluronic acid

Figure 4 shows the variations of conductivity when a solution of chitosan in the hydrochloride form is added to a solution of hyaluronic acid (HA) in the sodium salt form (1.3×10^{-4} M). The behaviour is quite similar to the one observed with CHOS, in particular a maximun precipitation for $\rho = 1$ and a curve beyond this value which is the same as when a solution of chitosan is added to a solution of NaCl 1.3×10^{-4} M. The curve

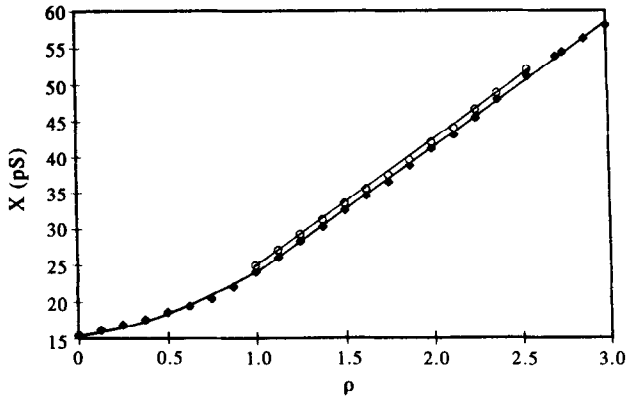

Fig. 4. Variation of the conductance, as a function of ρ, when chitosan hydrochloride 1.3×10^{-2} equiv l^{-1} is added to 1.3×10^{-4} equiv l^{-1} sodium hyaluronate (–◆–) and to 1.3×10^{-4} equiv l^{-1} NaCl (–○–).

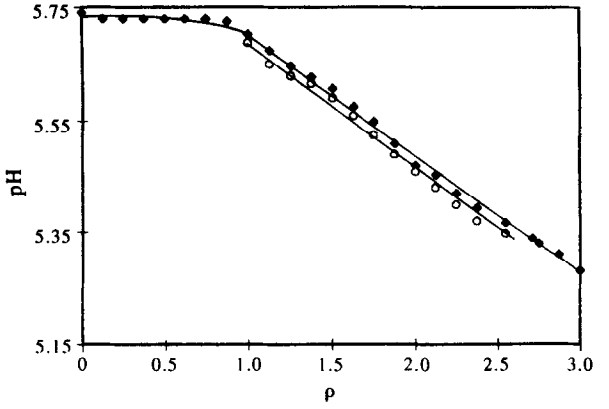

Fig. 5. Variation of the pH, as a function of ρ, when chitosan hydrochloride 1.3×10^{-2} equiv l^{-1} is added to 1.3×10^{-4} equiv l^{-1} sodium hyaluronate (–◆–) and to 1.3×10^{-4} equiv l^{-1} NaCl (–○–).

below $\rho = 1$ is also linear and should correspond to a complete reaction after each addition of chitosan. Then, at $\rho = 1$, all the anionic sites of HA are involved in a PEC of 1/1 type with all the $-NH_3^+$ functions of chitosan. As for CHOS, the experiments (not shown) corresponding to the reverse situation in which HA is added to a chitosan solution give symmetrical results and confirm the PEC mechanism. pH measurements (Fig. 5), also agree with this behaviour, in particular a pH for $\rho = 1$ which corresponds to the pH of a NaCl solution with the same concentration as the initial solution of chitosan. Thus, at $\rho = 1$, the media contains only NaCl according to the simple equation:

$$|{-}COO^- Na^+ + Cl^- H_3^+N{-}| \rightarrow |COO\,H_3N{-}| + NaCl \qquad (V)$$

As shown on Fig. 6, we also studied the interactions between chitosan in the pure ammonium form and HA in the free acidic form obtained after elution on a proton-ion exchange resin. The variations of conductivity reveal an important increase below $\rho = 1$ and a curve, beyond $\rho = 1$ which resembles the one obtained when chitosan is added to a solution of HCl 1.3×10^{-4} M. These results agree with the formation of a pure 1/1 PEC according to the equation:

$$|-COOH + Cl^- H_3^+N-| \rightarrow |-COO\,H_3N-| + HCl \qquad (VI)$$

in which all the protons of the free carboxylic acid borne by HA are liberated to allow the formation of the 1/1 PEC. Thus, whatever the pH, the same PEC is formed, a behaviour relatively different from the one of CHOS, for which depending on pH, two kinds of complexes can be formed. In this case, there is a total deprotonation of the carboxyl groups and these results are in agreement with those obtained in the case of the interactions between glycol-chitosan and hyaluronic acid (Shinoda & Nakajima, 1975). The difference of behaviour with CHOS can be attributed to differences

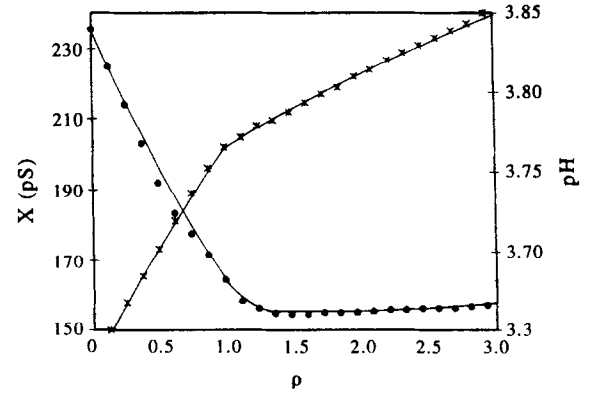

Fig. 6. Variation of conductance (–×–) and pH (–·–), as a function of ρ, when chitosan hydrochloride 1.3×10^{-2} equiv l^{-1} is added to 1.3×10^{-4} equiv l^{-1} Hyaluronic acid.

in conformation between these two polysaccharides but also to the lower pk_0 of hyaluronic acid (2.9; Cleland *et al.*, 1982) allowing an easier deprotonation in the case of HA. There also, the formation of a 1/1 PEC with HA should also be of scrambled coils form.

Characterisation of the complexes in the solid state

X-ray diffraction

The GAG's used in this work, due to the inhomogeneity of composition for CHOS but also due to the fact they are studied in their salt forms are relatively amorphous in the solid state. Chitosan in the hydrocHloric form is also slightly crystalline in the solid state but its diffractogram exhibits well defined peaks at $2\theta = 12°$ and 20° (Demarger-André & Domard, 1994). The complexes give diffractograms showing only the amorphous part and, the two peaks mentioned above, characteristic of chitosan salt crystallites, are completely absent. Thus, we can conclude that in the solid state, the complexes are not in an organized form, in agreement with the model of scrambled coils.

Infrared spectroscopy

If we consider the complex formed between chitosan hydrochloride and CHOS in the sodium salt form, the spectra of this complex are quite similar to those obtained from a KBr pellet prepared from a physical mixture of an equivalent amount of the two polymers in their salt forms (not shown). No new band appears and, the frequencies characteristic of Na–CHOS (Cael *et al.*, 1976) are unchanged. As for the results described above, there is no significant influence of the position of the sulfate groups. The I.R. spectra obtained with the complex formed between chitosan hydrochloride and sodium hyaluronate lead to similar results. We can conclude that the interaction between GAG's and chitosan in their salt form are purely electrostatic. They simply correspond to the interaction between $-NH3^+$ and $-OSO3^-$ and/or $-COO^-$ as described above.

The most interesting results are certainly those obtained with the complexes prepared with the acidic form of GAG's. The I.R. spectra of the complex prepared with chitosan hydrochloride and CHOS in the free acidic form confirms the typical behaviour described above. Indeed the I.R. spectra of the free acidic form of CHOS show a strong absorption band at 1738 cm^{-1} corresponding to –COOH. On the spectra of the complex obtained between this form and chitosan hydrochloride, this band is much less important and, we notice the appearance of a shoulder at 1640 cm^{-1} which reveals the presence of $-COO^-$ groups. Moreover, the spectra of the precipitates corresponding to the complexes formed in these conditions, for $\rho = 0.25$ or $\rho = 0.375$, are quite similar to the one recorded at $\rho = 0.75$. This result confirms the completion of the reaction after each addition of chitosan and the fact that it does not obey an equillibrium law. In the case of the complex between HA in the acidic form and chitosan hydrochloride, the –COOH band at 1738 cm^{-1} disappears completely and the $-COO^-$ band only is present although it is normally absent on the spectra of HA in the acidic form.

All these results are in good agreement with those obtained above. They confirm, quite well, the fact that the interaction between GAG's in their acidic form and chitosan hydrochloride is strong enough to induce the deprotonation of the carboxylic sites which is partial in the case of CHOS and complete with HA.

Stability of the complexes

On Fig. 7 are represented the thermograms of the complexes obtained with the salted forms of the polymers. We can notice that the thermal degradation of the two kinds of complexes formed between chitosan and CHOS or HA arises at a lower temperature compared to the case where the polysaccharides are studied alone.This difference is somewhat important in the case of the complex chitosan/CHOS (50°). This destabilisation can be related to the X-ray diffraction results showing the absence of organization of the complexes in the solid state. Thus, the very strong interactions occuring between these polyelectrolytes lead to scrambled coil systems where the chains are certainly highly constrained. It is also interesting to notice the very strong stability of these complexes in water which contrary to numerous other PEC's cannot be dissolved whatever the pH and thus can have interesting applications.

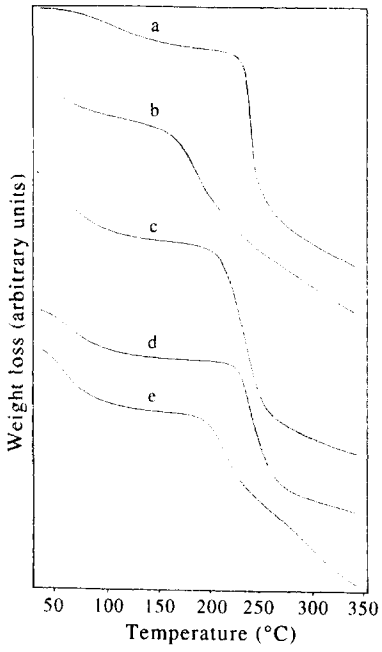

Fig. 7. Thermograms of (a) chondroitin sulfate sodium salt, (b) complex: chondroitin sulfate/chitosan, (c) chitosan hydrochloride, (d) sodium hyaluronate and (e) complex hyaluronate/chitosan.

CONCLUSION

This work demonstrates that very strong PEC's can be formed between fully deacetylated chitosan and GAG's whether they are in their acidic or salt forms. In the acidic form, the interaction induces a deprotonation of the carboxylic sites of GAG's which depends on their pK_0. This behaviour must be related to the acidity of these functions which is higher than in the case of usual polycarboxylic acids for which the pk_0 is often within 4.3–4.6 and for which PEC's cannot be formed with the free acidic forms of their carboxylic functions.

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