Polyelectrolyte Complexes of Chitosan with Sodium Carboxymethylcellulose

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(Received February 27, 1979)

A new water-insoluble precipitate was obtained by mixing chitosan with sodium carboxymethylcellulose, an anti-thrombogenic agent. The mole ratio of N/COO^- in the polyelectrolyte complexes was estimated to be 0.3935—0.9071. It was found that pH, mole ratio of N/Na in the reaction mixture and the order of mixing play an important role in determining the composition ratio of N/COO^-. In order to clarify the relationship between the molecular conformation of the polyelectrolyte complexes and their properties, IR spectroscopic studies, elementary analyses, color reaction with Toluidine Blue, studies on solubility and blood clotting test *in vitro* were performed. The polyelectrolyte complex, prepared at higher pH, differed from that prepared at lower pH, particularly in the density of –COOH group site. These results were interpreted in terms of the variation in the degree of electrolytic dissociation of the weak polyelectrolytes, caused by pH change. All the polyelectrolyte complexes revealed clot-inhibition *in vitro*. The clot-inhibiting behavior seems to be due to the clot-inhibiting surface of the polyelectrolyte complex, not to the elution of the activity from the surface.

A solution of charged polyelectrolyte reacts with one of oppositely charged polyelectrolyte to form a water-insoluble hydrous complex whose property is determined by various factor such as the degree of electrolytic dissociation of polyelectrolyte, composition of reaction mixture, order of mixing, pH and the polyion concentration. The polyion interaction or intermolecular reaction is remarkable not only as a model reaction *in vivo*, such as polyion interaction between charged polysaccharide and protein, but also as functional polymers or biomedical materials. Studies have been made on this subject.[1-3] However, the mechanism of polyion interaction and the probable structure of the polyelectrolyte complex (PEC) remains unclarified. Only a few papers[4,5] deal with the polyion interaction between polysaccharides or polysaccharide and synthetic macromolecule in spite of its importance as regards model reaction *in vivo* or biomedical applications.[6,7] The property of PEC can be made to satisfy the requirement.

Reports have appeared on novel reactions of sodium dextran sulfate with chitosan [poly(N-deacetylated chitin)],[8-11] sodium dextran sulfate with [2-(diethylamino)ethyl] dextran,[12] and sodium carboxymethyldextran with chitosan.[13] This paper describes a novel chemical interaction of sodium carboxymethylcellulose (CMC) with chitosan and studies on the PEC.

Experimental

Materials. CMC (Wako Pure Chemical Industries Co. Ltd., sodium content 7.79%, degree of substitution 0.753/A.G.U) and chitosan (Tokyo Kasei Co. Ltd., nitrogen content 7.95%) were used, the latter being purified by reprecipitation from its acidic solution with sodium hydroxide solution, but not CMC because of its high purity.

Preparation of PEC. When the pH of solution is below 2.0, no precipitates are formed by mixing chitosan with CMC, and when the pH of the chitosan solution is higher than 6.5, chitosan remain insoluble. When the pH of the chitosan solution is higher than 5.5, gelation of precipitate takes place. The reactions were thus carried out at pH 2.5 and 5.0, respectively. CMC was dissolved in water, and the pH of solution was adjusted to 2.5 and 5.0 with hydrochloric acid solution. Purified chitosan was dissolved in a 0.1 M hydrochloric acid solution, the pH of solution being adjusted to 2.5 and 5.0 with sodium hydroxide solution. These chitosan solutions were added dropwise to the corresponding CMC solutions, with stirring (rate of addition about 50 ml/30 min) under the conditions given in Table 1. Water-insoluble precipitates, the polyelectrolyte complexes PEC, were obtained. After standing for a half hour, the precipitates were washed with 0.01 M hydrochloric acid, water and methanol, and then separated by centrifugation, dried *in vacuo* for a week, until a constant weight was attained.

The elementary analyses of the PEC were performed at the Institute of Physical and Chemical Research, Wako-shi, Saitama, and the infrared spectra were taken by the KBr method. Nitrogen analysis was carried out by the Kjeldahl method.

Color Reaction of PEC with Toluidine Blue. The color change of PEC was examined in two ways: (a) addition of Toluidine Blue solution to PEC, (b) soaking of PEC in water for 1 day, and addition of Toluidine Blue solution to the filtrate. (a) was to confirm whether the surface of PEC is clot-promoting or clot-inhibiting, and (b) to see whether the clot-promoting or clot-inhibiting activities are transferred from the surface of PEC, since the reaction of anti-thrombogenic CMC with Toluidine Blue gives purple coloration.

Blood Clotting Test in Vitro. The blood clotting test was carried out according to the procedure of Imai and Nose[14] by gravimetrically measuring the amount of the clot formed at appropriate time intervals, after adding a calcium chloride solution (0.1 M, 0.02 ml) to ACD blood (0.2 ml), which had been in contact with a sample tablet: 200 mg PEC was pressed (8 t/4.5 cm^2) with poly(vinyl chloride) powder in a vacuum for 10 min. The ACD blood was prepared by adding blood (type A supplied by the Red Cross Hospital Blood Center) to an anti-coagulant citrate dextrose solution consisting of sodium citrate, citric acid and dextrose. The clotting tests were carried out concurrently under comparable conditions using the same method and the same ACD blood, after the blood had been stored for 3 days in a thermostat at 4—6 °C.

Solubility of PEC. Polyelectrolyte complexes are hardly soluble in most organic solvents. However, in order to clarify the property of PEC, it is necessary to find an appropriate solvent. Solubility tests were made on formic acid, dimethyl sulfoxide, *N,N*-dimethylformamide and two different ternary solvent mixtures (acetone/potassium bromide/water), (dioxane/water/hydrochloric acid).

Results and Discussion

Experimental conditions and yields in the preparation of PEC are given in Table 1, and, elementary analyses and compositions of PEC and pH of solutions after reaction in Table 2. Primarily, CMC is a salt of weak acid and chitosan is a salt of weak base, so that in a solution of lower pH the number of binding site $-OCH_2COO^-$ is smaller and that of binding site $-NH_3^+$ larger, as compared with a solution of higher pH.

$$-OCH_2COO^-Na^+ + H^+ \rightleftarrows -OCH_2COOH + Na^+$$

$$-NH_3OH + H^+ \rightleftarrows -NH_3^+ + H_2O$$

Thus, provided that $-COO^-$ groups in CMC participate just sufficiently to bind with $-NH_3^+$ in chitosan, in the lower pH condition (series A) the number of binding site $-NH_3^+$ is significantly larger than that of binding site $-OCH_2COO^-$. Consequently, the smaller the mole ratio of N/Na in the reaction mixture, the larger the yield for PEC. On the other hand, at pH 5.0 (series B) the number of binding site $-OCH_2COO^-$ in CMC increases and that of binding site $-NH_3^+$ decreases as compared with those at pH 2.5 (series A), so that the yield of PEC is not much affected by the mole ratio of N/Na. In general, the yield of PEC of series A is larger than that of series B. The tendency is similar to that in the PEC of chitosan with carboxymethyldextran[13] or chitosan with dextran sulfate.[10] The yield of PEC is influenced by the mixing order. When a polyelectrolyte of a large mole fraction in the reaction mixture is mixed with a polyelectrolyte solution, of a small mole fraction, the yield of PEC is larger than that obtained by reversing the order of mixing. In the case of polyelectrolyte, it seems that changes in the reaction mechanism, which affects the yield or molecular conformation of the PEC, are caused by the change in the order of mixing. The pH of reaction mixture tends to drop slightly after completion of PEC in both series. This indicates that the proton is liberated from the PEC on its formation.[6] In the pH range 2.5—5.0, the degree of electrolytic dissociation in polycation is significantly larger than

Table 1. Experimental conditions, yields in the preparation of polyelectrolyte complexes[a]

Sample code	Volume of chitosan solution / ml	Volume of CMC solution / ml	Mixing order	Mole ratio of N/Na in the reaction mixture	Yield / g/100 ml chitosan solution
A-1[b]	200	110	CMC to chitosan	3/1	0.0531
A-2[b]	200	110	Chitosan to CMC	3/1	0.1006
A-3[b]	100	167.5	CMC to chitosan	1/1	0.2696
A-4[b]	100	167.5	Chitosan to CMC	1/1	0.3856
A-5[b]	50	251	CMC to chitosan	1/3	1.0434
A-6[b]	50	251	Chitosan to CMC	1/3	0.7712
B-1[c]	200	110	CMC to chitosan	3/1	0.0925
B-2[c]	200	110	Chitosan to CMC	3/1	0.1003
B-3[c]	100	167.5	CMC to chitosan	1/1	0.2131
B-4[c]	100	167.5	Chitosan to CMC	1/1	0.3830
B-5[c]	50	251	CMC to chitosan	1/3	0.2861
B-6[c]	50	251	Chitosan to CMC	1/3	0.2351

a) Concentrations of chitosan and sodium carboxymethylcellulose (CMC): 3.0 g/l. b) Both solutions adjusted to pH 2.5. c) Both solutions adjusted to pH 5.0.

Table 2. Elementary analyses, compositions of PEC and pH of solutions after reaction

Sample code	Nitrogen content[a] / %	Sodium content[b] / %	Chlorine content[b] / %	Mole ratio of N/COO^- in the PEC	pH of mother liquor after completion of PEC
A-1	1.71	—	—	0.4595	2.40
A-2	1.77	—	—	0.4804	2.41—2.50
A-3	1.55	0.15	0.13	0.4062	2.40
A-4	1.51	0.00	0.11	0.3935	2.40
A-5	1.74	—	—	0.4701	2.40
A-6	1.79	—	—	0.4878	2.40
B-1	2.54	—	—	0.7877	4.78—5.00
B-2	2.57	—	—	0.8017	4.72
B-3	2.53	0.73	0.63	0.7833	4.80
B-4	2.69	0.35	0.32	0.8580	4.80
B-5	2.68	—	—	0.8579	4.90
B-6	2.79	—	—	0.9071	4.88—4.90

a) Nitrogen analysis carried out by the Kjeldahl method. b) Analyses performed at the Institute of Physical and Chemical Research.

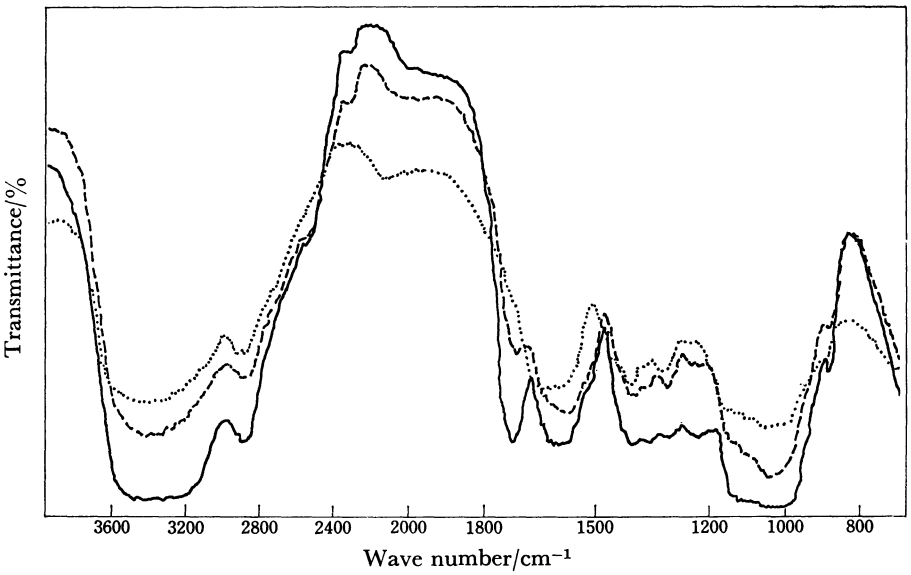

Fig. 1. IR spectra of polyelectrolyte complexes (PEC), series A and B, and of a blend polymer chitosan with sodium carboxymethyl cellulose (CMC).
——·: PEC of series A in Table 1, ——: PEC of series B in Table 1, ------: blend polymer chitosan with CMC, mole ratio of N/Na=1/3.

that in polyanion, all protons favoring release from the undissociated carboxyl groups. The nitrogen content in the PEC is not much influenced by the difference in the order of mixing or the mole ratio of N/Na. These results differ from those in the PEC, chitosan with carboxymethyldextran.[13] However, the nitrogen, sodium and chlorine contents in the PEC of B series are larger than those of series A, the results being similar to those in the PEC, chitosan with carboxymethyldextran[13] and chitosan with dextran sulfate.[10] However, the deviation of nitrogen content in the PEC, chitosan with carboxymethyldextran, with pH is larger than that in the PEC, chitosan with CMC. The results might be related to the fact that the difference in their viscous behavior,[15] or in effective molecular volume of polyelectrolyte in reaction solution, is caused by the pH change in carboxymethyldextran and CMC. The mole ratio of N/COO^- in the PEC, consequently, the nitrogen content in the PEC of series A should be smaller than that of series B. The results given in Table 2 (the mole ratios of N/COO^- in the PEC are calculated from the nitrogen contents in chitosan and PEC, and from the degree of substituent $-OCH_2COOH$ in CMC) are in line with the above consideration. This is also supported by IR spectra.

IR spectra of the PEC in series A are roughly similar to those of the PEC in series B and to those of blend polymer (blended chitosan with CMC in the same ratio as the complex composition), differing from each other in detail (Fig. 1). The absorption band around 1740 cm^{-1}, assigned to –COOH group, arises from the PEC, but not from the blend polymer. The absorption band around 1740 cm^{-1} in the PEC of series A is stronger than that of series B. This is in line with the electrolytic dissociation of $-OCH_2COO^-Na^+$ groups, the stronger the absorption band around 1740 cm^{-1}, the smaller the nitrogen content. The absorption band around 3500 cm^{-1}, attributable to –OH group in the blend polymer, seems to shift slightly towards lower wave number in the PEC. The absorption bands in the PEC of series A are assumed to become broader than those in the PEC of series B and in the blend polymer. The –OH groups in the PEC interact sparsely with other PEC molecules, probably through its –COOH groups. No such interaction was observed in the PEC of chitosan with carboxymethyldextran.[13] From the difference in absorption intensity of –COOH groups between the PEC of series A and series B, it seems that the intermolecular interactions in the PEC of series A through its –OH groups with other –COOH groups, are stronger than those of series B. This is supported by the results of solubility measurements. A weak absorption band around 1520 cm^{-1} assigned to $-NH_3^+$ groups is observed in the PEC, but none in the blend polymer. The sodium and chlorine contents in the PEC are distinctly smaller than those in CMC and chitosan, suggesting that the $-NH_3^+$ groups in chitosan participate in the binding with CMC

Table 3. Color reaction of PEC with Toluidine Blue

Sample code	Color of PEC itself	Color of solution in contact with PEC
A-1	blue	light blue
A-2	blue	light blue
A-3	blue	light blue
A-4	blue	light blue
A-5	blue	light blue
A-6	blue	light blue
B-1	light bluish purple	light blue
B-2	light bluish purple	light blue
B-3	light bluish purple	light blue
B-4	light bluish purple	light blue
B-5	bluish purple	light blue
B-6	bluish purple	light blue

probably through its $-COO^-$ groups, although the number of the ionic binding sites is small. The IR spectra of PEC are similar to those of CMC, being strongly affected by CMC rather than by chitosan. However, this might not be related to the fact that the mole ratio of N/COO^- in the PEC is small (Table 2).

Table 3 gives the results of color reactions of PEC with Toluidine Blue. No elution of CMC is observed in the solutions in contact with each PEC. The color reaction takes place not only on the surface but throughout the bulk of the complex, indicating a homogeneous dispersion of CMC into the PEC. The tint of the PEC differs with the conditions for the preparation of PEC. This seems to be closely related to the difference in the conformation in PEC.

TABLE 4. SOLUBILITIES OF PEC

Sample code	Formic acid			Dimethyl sulfoxide		
	R.T.	B	D.H.	R.T.	B	D.H.
A-1	×	△	▲	×	×	×
A-2	×	△	▲	×	×	×
A-3	×	△	▲	×	×	×
A-4	×	△	▲	×	×	×
A-5	×	△	▲	×	×	×
A-6	×	△	▲	×	×	×
B-1	△	▲	○	×	×	×
B-2	△	▲	○	×	×	×
B-3	△	▲	○	×	×	×
B-4	△	▲	○	×	×	×
B-5	△	▲	○	×	×	×
B-6	△	▲	○	×	×	×

All PEC are insoluble, even on direct heating, in N,N-dimethyl formamide and two sorts of ternary solvent mixture [acetone/potassium bromide/water (20:20:60 wt %)] [(dioxane/water/hydrochloric acid (50:5:45 wt %)], other than dimethyl sulfoxide. ○: almost soluble, ▲: considerably soluble, △: partially soluble, ×: insoluble, R.T.: room temperature, B:heating on a water bath, D.H.: direct heating.

All PEC are insoluble in most organic solvents, even on heating, such as dimethyl sulfoxide, N,N-dimethylformamide and two different ternary solvent mixtures {[acetone/potassium bromide/water (20:20:60 wt%)] [dioxane/water/hydrochloric acid (50:5:45 wt%)]} (Table 4), but become partially soluble in formic acid on heating. There is an appreciable difference in solubility between the PEC of series A and series B. It seems that the number of intermolecular binding sites through –OH groups with –COOH groups in the PEC of series B is smaller than that of series A. This is in line with the IR spectra.

The PEC of series A and B show clot-inhibition *in vitro*; they suppress the coagulation of blood (Fig. 2). The amount of thrombus on the PEC is 10—20% of that on glass and 30—50% of that on poly(vinyl chloride) as a reinforcing agent. The difference in anti-thrombogenic behavior between the PEC of series A and that of series B can hardly be observed. The anti-thrombogenic property may be due to the clot-inhibiting surface of the PEC and not to the eluted

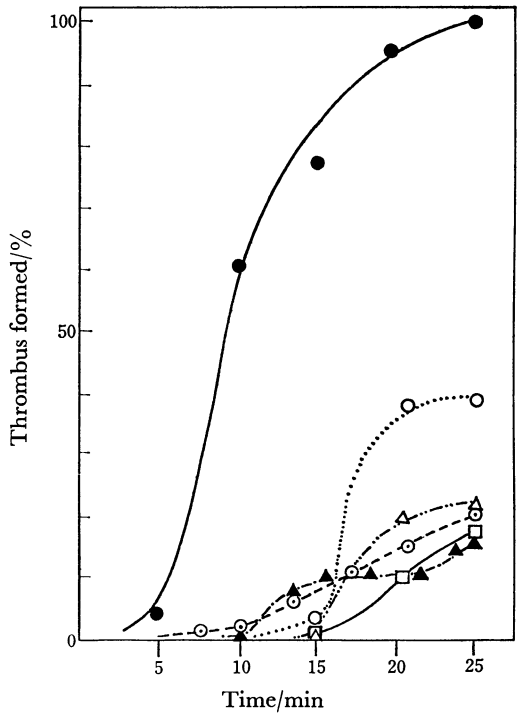

Fig. 2. Quantity of thrombus formed on polyelectrolyte complexes (PEC) compared with that on glass after a lapse of 25 min as a criterion.
●: Glass, □: A-3, △: A-4, ⊙: B-3, ▲: B-4, ○: poly(vinyl chloride) correspond to the PEC in Table 1.

activity from the surface as considered from the color reaction with Toluidine Blue. The clot-inhibiting factors of the PEC might be related to its molecular conformation or configuration.

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