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Colloidal Titration of Chitosan and Critical Unit of Chitosan to the Potentiometric Colloidal Titration with Poly(vinyl sulfate) Using Toluidine Blue as Indicator

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Three sorts of characterized chitosan were measured by visual titration and potentiometric titration with poly(vinyl sulfate) using Toluidine Blue as the indicator, to evaluate the stoichiometry and the accuracy. The measurement values for two dialyzed chitosans by both colloidal titration methods agreed with the values obtained by the acid-base titration. The relative differences between the acid-base titration and the colloidal titration were $< 2\%$, and the relative standard deviation of the potentiometric colloidal titration was $< 1\%$. For a oligo-chitosan (ChO) which consisted of several D-glucosamine oligosaccharides, the value obtained by the potentiometric colloidal titration was much smaller than the value from the acid-base titration. The inadequate result was due to the low stability constant of ChO–PVS. The observed values in the colloidal titration of ChO were evaluated for the content of each D-glucosamine oligosaccharide, and a critical unit that indicated the least unit of D-glucosamine oligosaccharide that contributed to the measurement value was estimated. The critical unit was four, tetra(D-glucosamine saccharide).

Chitosan, a polysaccharide, is a natural polyelectrolyte cation in origin. Since it was an antimicrobial activity and biocompatibility, its applications have been broadened into the biomedical and food chemical industry as well as chitin.[1—5] With these increases in the practical use of chitosan, a conventional method of measurement is required. Since chitosan is produced from chitin by deacetylation, it necessarily contains *N*-acetyl groups. The content of the amino groups strongly affects the chemical properties of chitosan. Therefore several methods for the measurement of the amino groups or the degree of *N*-acetylation have been reported: elemental analysis, acid-base titration,[6—8] colloidal titration,[9] ultraviolet-visual spectrophotometry,[10—14] near infrared spectrophotometry,[15] infrared spectrophotometry,[7,16—20] nuclear magnetic resonance spectroscopy,[8,21—24] high-pressure liquid chromatography,[25,26] pyrolysis-gas chromatography.[27—30] Among these methods, colloidal titration is one of the most conventional. The colloidal titration of chitosan, however, is suspected of inadequate accuracy.[31]

A general method for colloidal titration has been established.[32—38] The directions for use of this were reported to maintain the stoichiometry of the titration reaction. The concentration and the ionic strength of the sample solution must be low, heavy metal ions must be removed or ion-exchanged with alkali metal ions, and all of the reactive groups must be ionized. When a weak base such chitosan is measured, the pH of the sample solution must be made low. However, even if the optimum conditions of the measurement of chitosan are selected, it is hard to measure an oligo-chitosan that has a low molecular weight because the oligo-chitosan do not react quantitatively with poly(vinyl sulfate). Therefore some samples of low molecular weight oligo-chitosans cannot be measured accurately.

Tsuchida and Osada studied the polyelectrolyte complex between poly(methacrylic acid) and quaternized alkylamines and polyethylenimines with various chain lengths, and reported that the stability constants of the polyelectrolyte complexes increased with the chain length of the polyelectrolyte cation.[39] Then, since the magnitude of their stability constants was drastically changed in the range of the oligomers, they proposed the existence of a critical chain length for the polyelectrolyte complex reaction.[40] Such chemical reactivity may also appear in the complex reaction between oligo-chitosans and poly(vinyl sulfate). It is assumed that there is a threshold unit contributed to the measurement value (critical unit) for the colloidal titration of chitosan. When the critical unit of the colloidal titration becomes clear, the measurement value will be significant for a characterization of chitosan. For example, if the critical unit of chitosan is four, the measurement value of the colloidal titration can be regarded as the concentration of the amino groups of more than the tetramer in that sample of chitosan.

In this paper three sorts of characterized chitosan were titrated. The stoichiometry was evaluated by the visual method and the potentiometric method with a surfactant ion

sensor. Moreover, the critical unit of chitosan on the colloidal titration using Toluidine Blue was discussed and estimated.

Experimental

Materials. Three samples of chitosan with different molecular weights were prepared. One was a high molecular weight chitosan (ChH), which was manufactured by Katakura Chikkarin Co., Ltd. (Chitosan Highly Purified, degree of deacetylation; 79%). An adequate amount of ChH was dissolved in dilute aqueous HCl solution, and insoluble matter was removed with a membrane filter (pore size 1 μm). The ChH solution was dialyzed in deionized water before use. Another was a chitosan lactic acid with low molecular weight (ChL), which was manufactured by Piasu Co., Ltd. (Low Molecular Chitosan MH, degree of deacetylation; 85%). An adequate amount of ChL was dissolved in deionized water, and dialyzed in deionized water several times until the lactate was removed. Then the ChL solution was dialyzed in dilute aqueous HCl solution. The third was D-glucosamine oligosaccharide (ChO), which was prepared by an enzymatic hydrolysis of fully deacetylated chitosan.[41] The normality (equiv mol dm^{-3}) of chitosan was measured by an acid-base titration using a pH glass electrode. Two endpoints were obtained in a titration curve.[6] Since the concentration to the first end-point is the concentration of hydrochloric acid, the concentration of chitosan was measured from the difference between the first end-point and the second end-point. The viscosity-average molecular weights of ChH and ChL were 1.09×10^6 and 6.4×10^3, respectively.[42] The molecular weight distribution of ChO was measured by HPLC.[41] Potassium poly(vinyl sulfate) (PVS) (Wako Pure Chemical Ind., Ltd., for colloidal titration use, esterification degree 92.2%) was dissolved in deionized water, and dialyzed in deionized water. The concentration of 2.5×10^{-3} equiv mol dm^{-3} PVS solution as titrant was measured by an acid base titration as previously described.[43] 3-Amino-7-dimethylamino-2-methylphenothiazin-5-ium chloride (Toluidine Blue) from Chroma Gesellshaft Shumid GmbH & Co. was used without further purification.

Apparatus. An automatic potentiometric titration apparatus was used as previously described.[43] The plasticized poly(vinyl chloride) membrane was prepared by the method reported by Masadome and Imato.[44—46] The electrochemical cell was Ag/AgCl|satd. KCl|NPOE–PVC membrane | sample solution⁝satd. KCl|Ag/AgCl.

An HPLC system was constructed from a dual pump (CCPD, Tosoh, Tokyo, Japan) joined with a sample injector (Model 7125, 50 μl, Rheodyne Inc.), an analytical column (TSK gel NH_2-60, ϕ4.6×250 mm, Tosoh, Tokyo, Japan), and a refractive index detector (RI-71, Syowadenko, Tokyo, Japan). The column temperature was ambient (25±3 °C). An aqueous 58% (v/v) acetonitrile solution was used as an eluent at a flow rate of 0.8 ml min^{-1}. Each measurement value for the oligomers was calculated from the peak area in the chromatogram.

Procedure. Visual Titration. Sample solution consisting of $1—10\times10^{-5}$ equiv mol chitosan was accurately pipetted into a 200-ml conical beaker, 5 ml of 3 mol dm^{-3} acetic acid was added, and the solution was diluted to 100 ml with deionized water. The pH range of the sample solution was from 2.5 to 2.8. After adding one drop of 1% w/v Toluidine Blue solution, the sample solution was titrated with 2.5×10^{-3} equiv mol dm^{-3} PVS solution.

Potentiometric Titration. Sample solution consisted of $1—10\times10^{-6}$ equiv mol chitosan was accurately pipetted into a 100-ml beaker, 5 ml of 3 mol dm^{-3} acetic acid was added, and the solution was diluted to 50 ml with deionized water. To the sample solution, 0.1 ml of 1 mmol dm^{-3} Toluidine Blue was added, and two electrodes were immersed in the sample solution. After the observed potentials became constant, the sample solution was automatically titrated with 2.5×10^{-3} equiv mol dm^{-3} PVS solution.

Results and Discussion

Visual Titration. Three sorts of chitosan were titrated with PVS using Toluidine Blue as the indicator. In the titration of ChH a clear end-point was detected by finding the metachromasy, in which the color changed from blue to red purple. Although in the titration of ChL the metachromasy cannot be observed, the end-point was detected by noticing a coagulation point of the precipitation. For the titration of ChO, however, there was no detectable end-point. The titration results of two chitosans are listed in Table 1. Each obtained value agrees with the values calculated by the acid-base titration within 2% of relative differences. The stoichiometry of both chitosans is maintained in the range of $1—10\times10^{-5}$ equiv mol of the sample solution.

Potentiometric Titration. In a previous paper we used a plasticized PVC membrane doped with an ion pair between Toluidine Blue and dodecylbenzenesulfonate (DBS), and tritolyl phosphate (TTP) as a plasticizer.[43] In this study, however, a NPOE–PVC membrane without such an ion pair was used. The potentiometric titration curves obtained are shown in Fig. 1. The potentiometric curves in the vicinity of the end-point are apparently asymmetrical. The potential difference changed after the end-point. This surfactant ion sensor responded to the concentration of Toluidine Blue, although no ion exchangers were added to the NPOE-PVC membrane. The change of the potential difference in the vicinity of the end-point was smaller than that of the TTP–PVC membrane doped with Toluidine Blue-DBS.[43] On the former method by the (Toluidine Blue-DBS)–TTP–PVC

Table 1. Determination of Two Chitosans by Visual Titration

ChH			ChL		
Taken	Found[a]	R.D.[b]	Taken	Found[a]	R.D.[b]
10^{-5} equiv mol	10^{-5} equiv mol	%	10^{-5} equiv mol	10^{-5} equiv mol	%
1.658	1.674±0.013	101.0	2.064	2.077±0.010	100.6
3.315	3.378±0.010	101.9	4.128	4.164±0.010	100.9
5.525	5.589±0.010	101.2	6.880	6.947±0.010	101.0
7.735	7.884±0.016	101.9	9.632	9.706±0.032	100.8

a) Mean value of three titrations. b) Relative difference between taken value and found value.

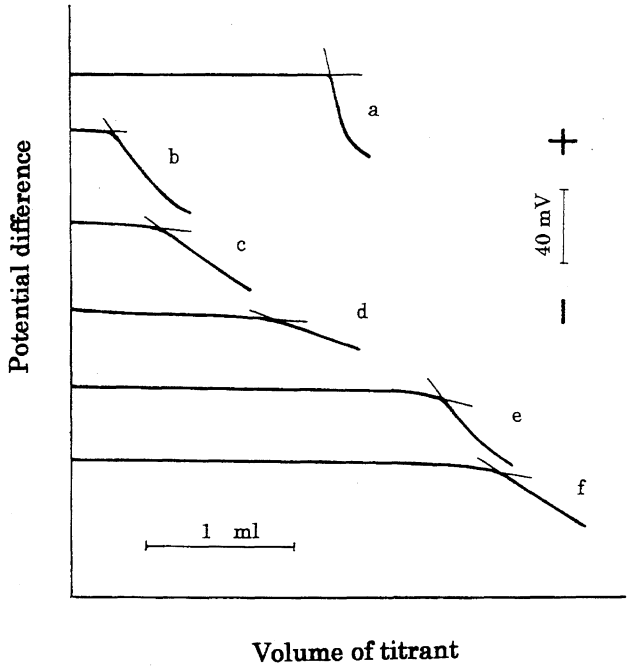

Fig. 1. Colloidal titration curves of chitosans with the NPOE–PVC membrane surfactant ion sensor using Toluidine Blue as the indicator. Sample solution (50 ml), a; 4.42×10^{-6} equiv mol of ChH, b; 1.46×10^{-6} equiv mol of ChO, c; 2.92×10^{-6} equiv mol of ChO, d; 5.84×10^{-6} equiv mol of ChO, e; mixture of 5.53×10^{-6} equiv mol of ChH and 1.46×10^{-6} equiv mol of ChO, f; mixture of 5.53×10^{-6} equiv mol of ChH and 2.92×10^{-6} equiv mol of ChO. Titrant, $2.5\times10\times10^{-3}$ equiv mol dm^{-3} of PVS.

membrane electrode, the deviation of the end-point from the equivalence point needed correction. This present method with the NPOE–PVC membrane, however, did not require correction of the blank value.

The titration reaction between chitosan and PVS competes with the indicator reaction between Toluidine Blue and PVS. The shape of the potentiometric curves is affected by the stability constants of the polyelectrolyte complex between chitosan and PVS. The titration of ChH and ChL gave a clear end-point, as shown in Fig. 1a. The sharpness of the end-point was independent of the sample concentration. The sharp end-point indicates that the stability constants of ChH–PVS and ChL–PVS are quite large. In the titration curves for ChO, however, the change of potential difference is small in the vicinity of the end-point, as shown in Fig. 1b, c, and d. The increment of the concentration of ChO dulled the sharpness of the end-point. The obscure end-points indicate that the stability constant of ChO–PVS is small. The end-point was hardly detected at more than 6×10^{-6} equiv mol of the concentration of ChO.

The end-points were detected by extrapolation as shown in Fig. 1. The titration results of ChH, ChL, and ChO are listed in Table 2. The values for ChH and ChL agree with the values calculated by the acid-base titration within 2% of relative difference. The RSD shows good reproducibility. The stoichiometry of ChH and ChL is maintained in the range of $1—10\times10^{-6}$ equiv mol of the sample solution. The measurement values for ChO, however, disagree with the values calculated by the acid-base titration. The values are much smaller than the values from the acid-base titration. The relative difference to the acid-base value decreases with the concentration of ChO. The inadequate results for ChO are due to the low stability constant of ion association with PVS.

Critical Unit of Chitosan. The measurements results of ChO by HPLC are listed in Table 3. The sample of ChO consists of five D-glucosamine oligosaccharides. The stability constants of the polyelectrolyte complexes increased with the numbers of D-glucosamine in the molecule. Then, the value obtained from the colloidal titration of ChO depended on the portion of the oligomers with the large numbers of D-glucosamine. Since the values of ChO by potentiometric colloidal titration were from 49.3 to 58.9% of the acid-base titration, it is assumed that the obtained value relates to the concentration of hexamer, pentamer, and tetramer, as listed in Table 3. It is clear that the tetramer is the critical unit of chitosan for the potentiometric colloidal titration with Toluidine Blue.

In the colloidal titration of chitosan, the measurement of oligo-chitosan only is seldom done. Mixed solutions of ChH and ChO were examined. Their titration curves are shown in Fig. 1e and f. The shapes of the titration curves of Fig. 1e and f are similar to those of Fig. 1b and c, respectively, in their vicinity of end-points. It is clear that ChH preferentially reacts with PVS, and the concentration of ChO strongly affects the shape of the end-point. Then, the

Table 2. Determination of Three Chitosans by Potentiometric Titration

ChH				ChL				ChO			
Taken	Found[a]	RSD[b]	R.D.[c]	Taken	Found[a]	RSD[b]	R.D.[c]	Taken	Found[a]	RSD[b]	R.D.[c]
10^{-6} equiv mol	10^{-6} equiv mol	%	%	10^{-6} equiv mol	10^{-6} equiv mol	%	%	10^{-6} equiv mol	10^{-6} equiv mol	%	%
1.11	1.13	0.82	100.9	1.38	1.37	0.73	99.3	1.46	0.72	1.46	49.3
2.21	2.23	0.31	100.9	2.75	2.72	0.33	98.9	2.92	1.56	1.38	53.4
3.32	3.33	0.29	100.3	4.13	4.08	0.32	98.8	4.83	2.67	1.08	55.3
4.42	4.45	0.27	100.7	5.50	5.47	0.22	99.5	5.84	3.44	0.86	58.9
5.53	5.57	0.18	100.7	6.88	6.81	0.16	99.0				
11.05	11.20	0.09	101.4	11.01	10.86	0.12	98.6				

a) Mean value of five titrations. b) Relative standard deviation. c) Relative difference between taken value and found value.

Table 3. Components of D-Glucosamine Oligosaccharide (ChO)

Component	wt %[a)]	Sum of wt%[b)]
Hexamer (6)	7.8	7.8 (6)
Pentamer (5)	22.1	29.9 (6)+(5)
Tetramer (4)	30.4	60.3 (6)+(5)+(4)
Trimer (3)	28.6	88.9 (6)+(5)+(4)+(3)
Dimer (2)	11.1	100.0 (6)+(5)+(4)+(3)+(2)

a) The values obtained from the chromatogram by HPLC.[41)] b) The sum of each component of the number put in parenthesis.

Table 4. Determination of the Mixtures of ChH and ChO by Potentiometric Titration

Taken	Found[a)]	R.D.[b)]	Sum of Col. Titr.[c)]
10^{-6} equiv mol	10^{-6} equiv mol	%	10^{-6} equiv mol
3.67 ($2.21_{ChH}+1.46_{ChO}$)	2.95±0.02	80.4	2.95
6.13 ($2.21_{ChH}+2.92_{ChO}$)	3.79±0.01	61.8	3.79
6.99 ($5.53_{ChH}+1.46_{ChO}$)	6.22±0.04	89.0	6.29
8.45 ($5.53_{ChH}+2.92_{ChO}$)	7.20±0.03	85.2	7.13

a) Mean value of three titrations. b) Relative difference between taken value and found value. c) The sum of each obtained value of ChH and ChO by the colloidal titration (Table 2).

shape of the end-point is a clue to find the existence of the oligomers. The values for the mixtures are listed in Table 4. The values agree with the sum of each obtained value of ChH and ChO by the colloidal titration. So, the critical unit can be also applied to mixed samples.

With using the surfactant ion sensor of the NPOE–PVC membrane, it is possible that many amphiphilic ions may become an indicator of the colloidal titration.[45)] The sort of amphiphilic ions will affect the change of potential difference in the vicinity of the end-point. The critical unit may be variable with the sort of amphiphilic ions. Therefore, the critical unit obtained in these results should be limited to the colloidal titration using Toluidine Blue as the indicator.

In conclusion, it was confirmed that the colloidal titration reaction of chitosan with PVS was adequately accurate, essentially. From the examination of the characterized oligochitosan, a critical unit of chitosan on the colloidal titration using Toluidine Blue was estimated. Therefore, it is pointed out that the measurement value of the colloidal titration becomes significant for the characterization. This is valuable in the measurement of chitosan because many analytical methods cannot distinguish the monomer and the oligomer, and the polymer. These results and manner will be a helpful guideline and a direction for use of colloidal titration, as well as the measurement of the stability constants for the reaction of colloidal titration.[47,48)]

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