Effect of Degree of Deacetylation of Chitin on the Properties of Chitin Crystallites

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ABSTRACT: In the present article, chitin from crab shell was systematically deacetylated using a NaOH treatment with control of the reaction time. The degree of deacetylation, monitored using solid-state NMR, revealed that the reaction was pseudo-first order. Based on this, swollen and NaOH-saturated particles are proposed as the reaction system. The weight loss of the partially saponified and neutralized samples after HCl hydrolysis increased linearly with the degree of deacetylation. The crystallinity of the samples was found to increase after acid hydrolysis. According to conductimetric titration, the surface charge density of the crystallites, after acid hydrolysis, was found to increase with base treatment time. The effect of surface charge on the formation of a chiral nematic phase, due to the rodlike nature of the crystallites, was explored. These results show that because the contribution of charged particles to the ionic strength was significant the double layer compression was affected, especially since the surface charge density was close to the Manning limit. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **65:** 373–380, 1997

Key words: chitin crystallites; deacetylation; chiral nematic; pseudo-first order; surface charge; acid hydrolysis; chitosan

INTRODUCTION

The seafood processing industry produces large quantities of shell waste which is approximately 30% by weight in chitin,[1] a linear polysaccharide composed of β-(1-4)-linked 2-deoxy-2-acetamido-D-glucose units (Glu–$NHCOCH_3$).[2] On a worldwide basis, about 10^5 metric tons of chitin waste is available annually for industrial use,[3] with crab and shrimp waste as the principal sources.[4] Chitin is biosynthesized by chitin synthetase[5] and it is reported that deacetylation is due to a chitin deacetylase.[6,7] Partial removal of acetyl groups to leave amine groups on the backbone has a profound effect on chitin properties such as solubility. Chitin with a degree of deacetylation of 75% or above[4] is usually called chitosan and will be referred to as Glu–NH_2.

Typical applications for chitin and chitosan include water purification, paper wet web strength enhancer, cosmetics additive, and pharmaceutical adjuvant.[8] Medical gauzes, ointments, and wound dressings have been made from chitin or its derivatives[9] because of its antibacterial, moisture retaining, and healing characteristics.

Nascent chitin is a crystalline microfibrillar material usually having the α-polymorphic form such as occurs in crab and shrimp shell waste. It usually exhibits a degree of deacetylation (DD) of approximately 5% "as received."[6] Both the degree of crystallinity and the DD of chitin depend on the source and the method of purification. The unit cell parameters and three-dimensional crystalline structure were reported by Carlström[10] for α-chitin and the same information for β-chitin from Pogonophora was derived by Blackwell.[11]

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When purified chitin isolated from crab shell is heterogeneously acid hydrolyzed, the product, fragmented microfibrils or chitin crystallites, can be called hydrochitin by analogy with hydrocellulose. These crystallites are rodlike colloids from chain scission occurring at random locations along microfibrils. The colloidal stabilization is due to the protonation of amino groups on the crystallites which provides a positive surface charge, hence, repulsive forces between particles.[12–14] Above a certain concentration, chitin crystallite suspensions spontaneously separate into an isotropic and an anisotropic phase,[13,14] with the surface charge influencing the relative amount of the two phases. The anisotropic phase was shown to be chiral nematic and to dry down to a film whose texture mimics the naturally occurring organization of chitin microfibrils in arthropods.[13] The effect of electrolyte on the boundary concentration for phase separation—isotropic to anisotropic—has been reported.[13,14] The electrostatic effect due to the surface charge of rodlike particles on phase separation was discussed by Onsager[15] and Odijk et al.[16] The latter[16] predicted that the boundary concentration for phase separation decreased with increasing surface charge density.

In the present study, chitin samples with a range of DD were prepared under conditions used to prepare chitosan. Changes in crystallinity of the samples were monitored using X-ray diffraction. Solid-state NMR (CP-MAS ^{13}C-NMR) and conductimetric titration were used to measure the DD and the surface amino groups (SAG). The weight loss of the samples having different DD due to acid hydrolysis under standard conditions[14] was recorded. The surface charge density was calculated from the titration data. The actual surface potentials for the suspensions of chitin crystallites having various DD at pH 4 were evaluated. The phase-separation behavior of crystallite suspensions were compared using phase diagrams. Finally, the optical anisotropy of concentrated suspensions with a high degree of deacetylation was explored.

EXPERIMENTAL

Technical-grade crab shell was ground to 20 mesh in a Wiley mill. Then, the shell was deproteinized in 1% NaOH at 60°C and demineralized using 1% HCl at room temperature overnight. In both cases, the ratio of the shell material to the 1% NaOH or 1% HCl was 1 g per 10 mL. Treatment of samples with 50% NaOH provided material corresponding to various levels of DD by treating crab chitin using 50% NaOH with a ratio of liquor to solid (L/S) of 4/1, for various lengths of time at 87–99°C. The overall reaction time ranged from 1 to 7 h. One of the samples was treated with 50% NaOH (L/S, 4/1) at 87–99°C in a capped jar in an incubator for 7 h in the presence of 3% $NaBH_4$ (by weight based on the chitin) to prevent alkali-provoked depolymerization and weight loss. All the alkali-treated samples were washed to neutrality and air-dried.

Acid Hydrolysis of Chitin and Weight Loss

Crystallites from microfibril fragments which form suspensions of rodlike particles were prepared by hydrolyzing washed and air-dried material (blank and alkali-treated) in 3N HCl at the boil for 1.5 h. The ratio of chitin to 3N HCl was 1 g per 10 mL. After hydrolysis, a dilution–centrifugation–decantation process was used for removing the excess acid. This procedure was repeated until the pH of the suspension was around 1.6, at which point a spontaneous colloidal state developed.[12] The suspension of approximately 2% solids was transferred to a dialysis bag with a cutoff molecular weight of 12,000–14,000 and was dialyzed against deionized water until its pH reached 4. This was followed by immersing the dialysis bag in a solution of concentrated poly(ethylene glycol) (M_w 20,000) at pH 4 to concentrate the suspension. For a more uniform dispersion of the chitin crystallites, the suspensions were then ultrasonicated at room temperature in a Branson sonifier (Model 350) for about 1 min for each 10 mL of the aliquot. For the weight loss measurement, the weight of the solids after hydrolysis was determined from the volume and concentration of the colloidal suspension.

X-ray Diffraction Observations

Powder diffractograms for alkali-treated chitin after purification were recorded using a Rigaku D/MAX 2400 automated powder diffractometer with a rotating anode and equipped with single-crystal monochromator and scintillator detector. CuKα radiation with a voltage of 40 kV and a current of 120 mA was obtained using a graphite monochromator. The wavelength λ was 1.541 Å. Approximately 0.25 g of the sample was used in the measurement. The measurement was controlled by a Pentium PC using the Rigmeas program. A

typical $\theta/2\theta$ scan range was from 2° to 69.9°. The degree of crystallinity of the sample based on the $\theta/2\theta$ scan from 5° to 50° was calculated according to the method suggested by Challa et al.[17] which requires separation of the crystalline peaks from the diffuse background.

Determination of DD of Chitin Samples

Two methods were used: solid-state NMR and conductimetric titration.[18] The former provides the average amino group content in the sample. The latter provides only the surface amino content.[14] As described in our previous publication,[19] a Fisher Scientific pH meter (Accumet, pH meter 50) was used to monitor the pH change during titration. The conductance of the suspension was plotted as a function of the volume of 0.01*N* NaOH used in the titration. The volume of alkali, needed to neutralize the protonated surface amino groups in the sample, was calculated from the points corresponding to the sharp change in the slope of the titration curve. The calculation of the surface charge density was discussed in our previous article.[14]

Zeta Potential Measurements

Zeta potentials for the suspensions of chitin crystallites from blank and alkali-treated material (treated with NaOH for 1 and 3 h prior to acid hydrolysis) at pH 4 were obtained using a Lazer Zee Meter as described in our previous article.[14]

Phase Separation and Microscopy

Phase-separation observations were made using NMR tubes of 5 mm internal diameter. Several milliliters of the concentrated suspensions at pH 4 were first transferred to 10 mL vials and then diluted with a pH 4 solution of HCl. The vials were shaken vigorously followed by transfer of some suspension into NMR tubes which were stoppered. The height of the isotropic phase was recorded by viewing between cross-polars after 1 week standing at room temperature. The volume fraction of the anisotropic phase was determined by dividing the height of that phase by the total height. All the crystallite suspension samples were examined in a Nikon Microphoto-FAX optical microscope.

Transmission electron microscopy of chitin crystallites was performed as previously described[14] using a 400T Philips instrument. Diffraction contrast imaging was used.

RESULTS AND DISCUSSION

The objective of this work was to control the surface charge density of chitin crystallites by varying the time of NaOH deacetylation. Ideally, the axial ratio of the crystallites in the suspensions should remain unchanged, thereby allowing a study of the effect of surface charge on the phase-separation behavior of chitin crystallite suspensions.

The resistance of solid chitin to deacetylation can be overcome by using temperature, L/S, and aqueous NaOH concentration as variables and assuming the following reaction:

$$\text{Glu}-\text{NHCOCH}_3 + \text{NaOH} \rightarrow \text{Glu}-\text{NH}_2 + \text{NaOCOCH}_3 \quad (1)$$

This treatment was performed in a pilot reactor from which samples were periodically removed. It was observed that viscosity of the reaction mixture increased with time. This is attributed to the development of microfibril fragments dispersed in a partially deacetylated chitin or chitosan matrix. However, this treatment changes the properties of chitin including crystallinity, surface charge, swelling, and interfacial properties. Most notable among these changes is the degree of crystalline perfection as measured using X-ray diffraction.

X-ray powder diffractograms of chitin and alkali-treated chitin samples are shown in Figure 1. Compared with the d-spacings reported by Carlström,[10] the X-ray pattern in Figure 1 can be identified as α-chitin. The same peaks occur in each spectrum including the sample hydrolyzed in 50% NaOH for 7 h in the presence of $NaBH_4$. It was found that the line broadening and width at half-maximum intensity (WHMI) of the composite peak at 20° (2θ) increased with increase of the reaction time. For the sample hydrolyzed in 50% NaOH for 7 h in the presence of $NaBH_4$, the WHMI is larger than in the others.

Chitin Deacetylation

In concentrated solutions of NaOH (above 43%), chitin crystallites swell followed by formation of alkali-chitin; thus, sodium hydroxide penetrates into the crystalline structure of chitin and more

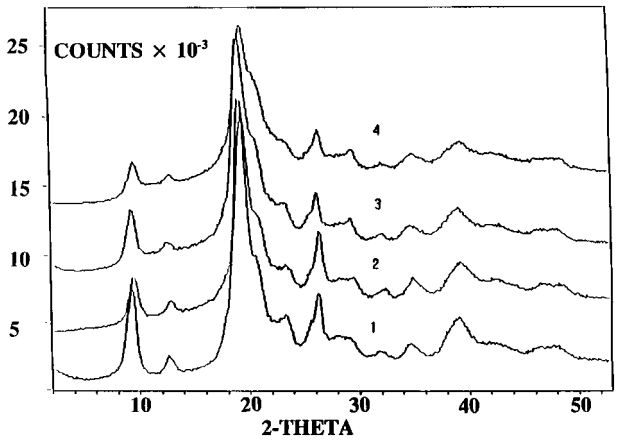

Figure 1 X-ray diffractometer scans of α-chitin and alkali-treated chitin samples from crab shell: (1) starting chitin; (2) chitin sample hydrolyzed in 50% NaOH for 3 h; (3) chitin sample hydrolyzed in 50% NaOH for 7 h; (4) chitin sample hydrolyzed in 50% NaOH for 7 h in the presence of $NaBH_4$. The reaction temperature was 87–99°C.

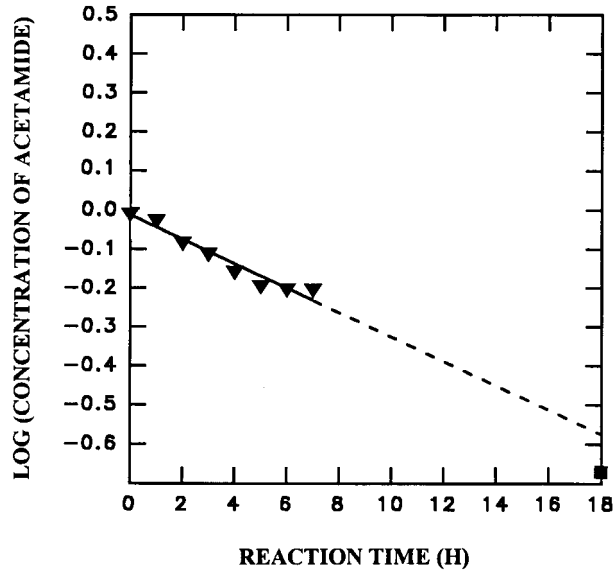

Figure 2 Plot of Log c vs. deacetylation time at 87–99°C. The value for 18 h treatment (■) as a reference was measured by Vanson Inc. using a UV method.

than 0.75 equivalents of sodium can combine with each *N*-acetylglucosamine.[6] Alkali-chitin is partially crystalline,[20] disperses rapidly in ice water,[21] and regains its original crystalline structure after washing to neutrality. When temperature increases, deacetylation takes place. For each swollen crystallite, the reaction can be considered as pseudohomogeneous since the lattice of alkali-chitin is nematiclike when swollen and the sodium hydroxide accesses the acetamide group as in a reaction vessel. The deacetylation reaction is second order with respect to acetamide and OH^-. The reaction is pseudo-first order when sodium hydroxide is in excess as in this case. The kinetic equation[22] is

$$kt = 2.303 \text{ Log}(c_0/c) \qquad (2)$$

where t is reaction time; k, a constant; c, the fractional concentration of acetamide groups at time t; and c_0, the original concentration of the acetamide group.

In Figure 2, Log c is plotted as a function of reaction time t. A straight line is obtained, showing that the reaction is pseudo-first order. The acetamide concentration extrapolated to 18 h, which is when the reaction was terminated, is 73% deacetylation. This is comparable to the observed DD, using the UV method, which was found to be 80%.[23]

Figure 3 shows the crystallinity of the alkali-treated samples as a function of alkali hydrolysis time in 50% NaOH. It was found that the alkali treatment reduced the crystallinity of the samples until a plateau was reached. The crystallinity change as a function of hydrolysis time is due to deacetylation taking place in the swollen crystallites in the samples. Alternatively, one can think in terms of a distribution of crystallite perfection and the less perfect ones are converted in part to

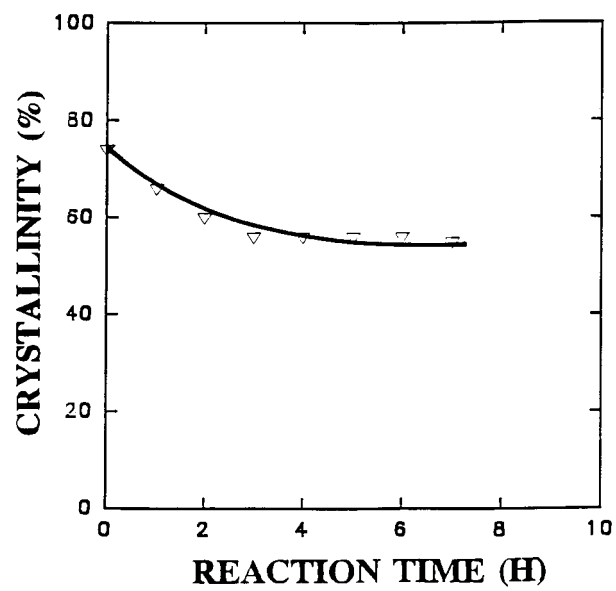

Figure 3 Percent crystallinity of the alkali-treated chitin samples after washing and air drying.

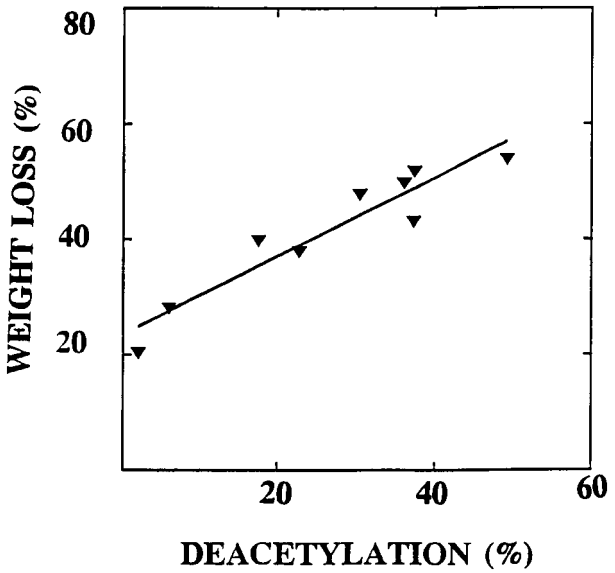

Figure 4 Weight loss of the purified and dried alkali-treated chitin samples after acid hydrolysis at the boil vs. DD.

chitosan which is ultimately dissolved. The loss of the crystalline structure of chitin depends on deacetylation which prevents the intermolecular hydrogen-bonding characteristic of α-chitin and breaks the regularity of lateral packing between chains.

For the sample hydrolyzed using 50% NaOH in the presence of $NaBH_4$, the crystallinity of the sample was 47%, which was significantly lower than that of the other samples, and the DD was 49%, which was much higher than that of the other samples. This is attributed to the effect of $NaBH_4$ as a reducing reagent which accelerates the deacetylation process, although $NaBH_4$ itself does not reduce amide groups.[24]

Effect of HCl Hydrolysis on Partially Deacetylated Chitin

Acid hydrolysis of chitin should follow first-order kinetics if all glycosidic bonds were equally accessible and reactive.[25,26] In fact, the kinetic behavior is affected by the physical state of the chitin chains, which pack antiparallel and are linked through interchain hydrogen bonding between the acetyl groups. In Figure 4, the weight loss of the chitin and alkali-treated chitin samples from acid hydrolysis is plotted as a function of the DD. It is found that the weight loss increases linearly with increasing DD, an expected result since the accessibility of glycosidic linkages to H^+ is influenced by disruption of chain packing. However, after acid hydrolysis, the remaining chitin crystallites have a higher crystallinity (see Table I) due to removal of the noncrystalline fraction which is mostly the partially deacetylated materials. In fact, some recrystallization may take place as a result of acid hydrolysis.

Table I Crystallinity of the Alkali-treated Chitin Samples Before and After Acid Hydrolysis

Reaction Time (h)	Crystallinity Before HCl Hydrolysis (%)	Crystallinity After HCl Hydrolysis (%)
0	74	—
2	60	78
4	56	76
5	56	64
7	55	61
7 + $NaBH_4$	47	—

The DD of the samples after acid hydrolysis was measured using solid-state NMR (see Table II). It was found that the DD of the alkali-treated samples after acid hydrolysis is significantly lower than that before the acid hydrolysis. This supports the conclusion that acid hydrolysis preferentially removes chitosan blocks of repeating units especially if they were at the crystallite surface. The recovered hydrochitin is almost like the original chitin in composition and degree of crystallinity.

After acid hydrolysis, the surface amino groups (SAG) of the chitin samples, which determined the surface charge property of hydrochitin in suspension, were measured using conductimetric titration.[18] Figure 5 plots the SAG of chitin samples as a function of deacetylation time. It was found

Table II DD of the Alkali-treated Chitin Samples Before and After Acid Hydrolysis

Reaction Time (h)	DD Before HCl Hydrolysis from CP-MAS (%)	DD After HCl Hydrolysis from CP-MAS (%)
2	17.6	2.3
4	30.5	6.1
5	36.1	5.7
7	37.4	8.4
7 + $NaBH_4$	49	—

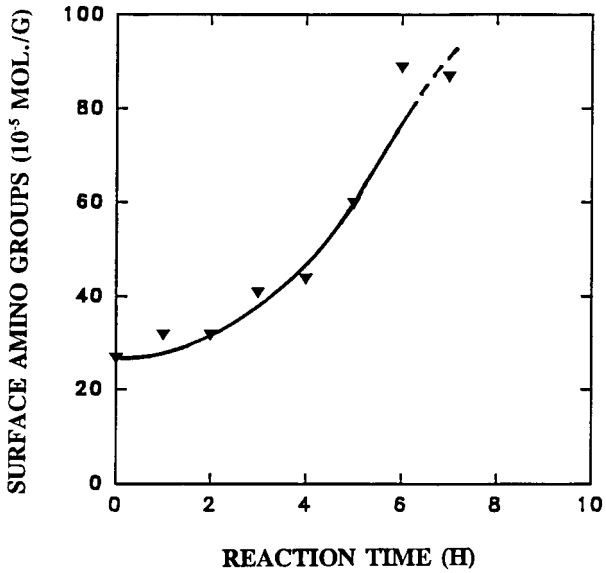

Figure 5 SAG of the alkali-treated chitin samples vs. reaction time, by conductimetric titration.

that SAG of alkali-treated and acid-hydrolyzed chitin samples increased with reaction time.

Effect of Surface Charge on Phase Separation

In acidic media, the protonation of the SAG of chitin crystallites provides positive surface charges which affect the phase behavior of suspensions of chitin crystallites. The surface charge density of crystallites therefore increases with increasing SAG (see Table III), assuming that the crystallite texture persists and the surface area does not change significantly, as confirmed by a TEM study (see Fig. 6). Figure 6 shows that the rod-like character of the particles was retained and there is no significant change of the crystallite size after NaOH treatment. The TEM study also indicates that the crystallites from the $NaBH_4$-treated sample have the same appearance as those without $NaBH_4$. Table IV shows that for the suspensions at pH 4 the zeta potential increases with an increase of surface charge density resulting from deacetylation. This change in the zeta potential ultimately changes the boundary concentrations for the phase separation of suspensions if other factors remain unchanged (cf. below).

Table III Surface Charge Density of Chitin and Alkali-chitin Samples Calculated from Conductimetric Titration Data

Reaction Time (h)	Surface Charge Density (e/nm^2)	SAG (10^{-5} mol/g)
0	0.45	27
1	0.53	32
3	0.69	41
6	1.58	89
7 + $NaBH_4$	1.58	89

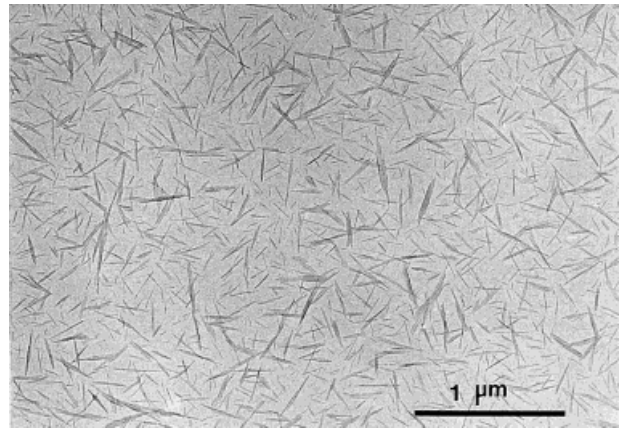

Figure 6 A TEM micrograph of chitin crystallite sample after 7 h of alkali treatment in the presence of $NaBH_4$.

As shown in our previous articles,[13,14] the chitin crystallites in the suspensions self-assemble into a chiral nematic phase above a critical concentration. As mentioned in the Introduction, the boundary concentration for phase separation depends on both the surface charge of the crystallites, i.e., surface charge density and zeta potential, and the electrolyte concentration of the suspensions. Figure 7 is a phase-separation diagram for the suspensions at pH 4 for chitin and alkali-treated chitin samples, demonstrating that the boundary concentration increases with increasing treatment time in 50% NaOH; the boundary concentration for phase separation increases with increasing surface charge. This is opposite to theoretical predictions.[17] In the present work, the alkali-treated chitin crystallites are highly charged and their contribution to the ionic strength of the suspensions is significant, thereby causing a double-layer compression. In addition, since we are working close to the Manning limit, i.e., the apparent linear charge density where condensation of counterions starts to occur, the charge density remains constant.[27] Table IV shows that the boundary concentration for phase separation increases with increasing zeta potential. This is opposite to what is observed for the suspensions prepared from untreated chitin.[14] The reason is that the zeta poten-

Table IV Zeta Potentials of Chitin Suspensions at pH 4 and Boundary Concentration for Phase Separation

Reaction Time (h)	Surface Charge Density (e/nm^2)	Zeta Potential at pH 4 (mV)	Boundary Concentration for Phase Separation (%)
0	0.45	32	2.18
1	0.53	41	3.84
3	0.69	44	4.43

tials were measured in diluted suspensions (less than 0.1%) where the contribution of the crystallites to the ionic strength of the suspensions was negligible. On the other hand, when phase separation occurs, the concentration is well above 1% and the contribution of the crystallites to the ionic strength should be considered.[28]

For the suspensions prepared from the samples hydrolyzed in 50% NaOH for 5 h or above, the suspensions are too viscous to phase separate. For the suspensions prepared from alkali-treated chitin samples for less than 3 h, chiral nematic patterns similar to those of the suspensions prepared from untreated chitin samples were observed in polarized microscopy. For the suspensions prepared from the chitin samples hydrolyzed in 50% NaOH for more than 4 h, the higher viscosity prevents formation of the chiral nematic texture. Interestingly, the suspension hydrolyzed in 50% NaOH for 6 h has less birefringence than has the one hydrolyzed in 50% NaOH for 7 h in the presence of $NaBH_4$, although the SAG from conductimetric titration show that both have the same degree of surface deacetylation. We propose that the presence of $NaBH_4$ speeds up the deacetylation reaction and minimizes the degradation of chitin chains, thereby leading to a higher effective axial ratio.

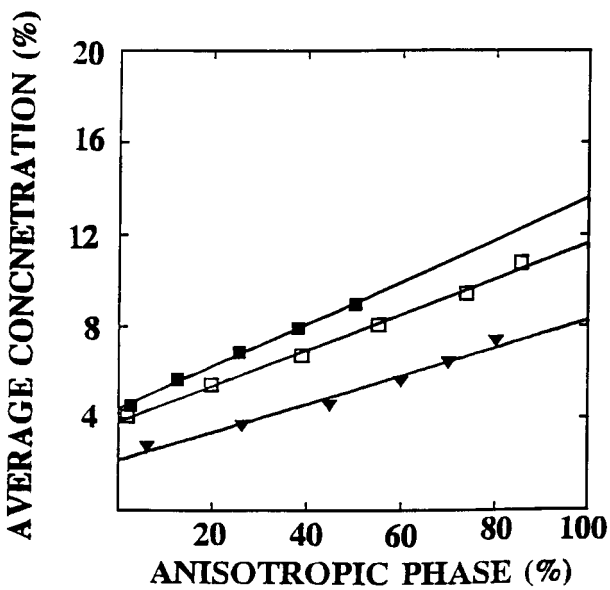

Figure 7 Phase-separation diagram for biphasic region of chitin crystallite suspensions prepared from chitin and alkali-treated chitin samples with different surface charge density: (▲) starting chitin; (□) alkali-treated chitin (1 h); (■) alkali-treated chitin (3 h). The boundary concentration is obtained by extrapolating to 0% anisotropic phase.

CONCLUSIONS

In this article, chitin samples with different DD were prepared by base hydrolysis through control of the reaction time. It was found that the treatment using a constant strength of alkali reduced the crystallinity of the sample, but the crystalline structure of α-chitin was retained after washing to neutrality and drying. Considering that NaOH is in excess within each alkali-chitin crystallite, the reaction is pseudo-first order. Both DD and SAG, from solid-state NMR and conductimetric titration, respectively, increase with hydrolysis time. The increase of surface charge density resulting from the increased SAG was found to affect the boundary concentration for the formation of the anisotropic phase. However, the observed change of boundary concentration with surface charge is opposite to that predicted by theories.[17] This is attributed to the contribution of the charged crystallites to the ionic strength. The suspensions of crystallites prepared from the samples treated with alkali for 6 and 7 h do not phase separate due to high viscosity, chiral nematic structures were not observed for the suspensions of crystallites beyond 4 h treatments.

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