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SEC-MALS study on aggregates of chitosan molecules in aqueous solvents: Influence of residual *N*-acetyl groups

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Abstract

The size-exclusion chromatography equipped with a multi-angle light scattering detector (SEC-MALS) method was applied to chitosan samples with different average molecular mass (MM) and degree of N-acetylation (DNAc) values. The influence of these parameters on dispersion state of chitosan molecules in aqueous media were preliminarily examined for conformation analysis. It was shown that even a slight amount of residual *N*-acetyl groups (as low as 2% DNAc) caused the presence of aggregates or insufficiently dispersed residues of chitosan molecules in aqueous solutions, which gave critical effects on the data for the conformation analysis. Such aggregates could not be removed or prevented by common techniques such as preparative centrifugation (7740*g*, 10 min), microfiltration (0.1 μm) or lowering ionic strength of the solution. On the other hand, completely deacetylated samples had no such structures, and gave consistent data regardless of their MM values. Therefore, complete deacetylation is highly recommended in advance of the conformation analysis of chitosan, although some depolymerization is unavoidable during the repeated deacetylation treatments.

Keywords: SEC-MALS; Chitosan; Degree of N-acetylation; Aggregate

1. Introduction

Chitosan has been studied for long years as a functional polysaccharide, and applied to various industrial fields such as pharmaceuticals, cosmetics, food additives, environmental restoration reagents, cationic retention aids and others. Since in some cases molecular characteristics of chitosan themselves play important roles in their functionalities, a lot of basic researches have been performed using light scattering (LS), osmometry, sedimentation equilibrium, viscometry, size-exclusion chromatography and other related techniques (Anthonsen, Vårum, & Smidsrød, 1993; Beri, Walker, Reese, & Rollings, 1993; Berth, Dautzenberg, & Peter, 1998; Berth & Dautzenberg, 2002; Domard & Rinaudo, 1983; Duin & Hermans, 1956; Errington, Harding, Vårum, & Illum, 1993; Fee et al., 2003; Hasegawa, Isogai, & Onabe, 1994; Miya, Iwamoto, Yoshikawa, & Mima, 1986; Rinaudo, Milas, & Dung, 1993). Particularly, molecular conformation of chitosan in aqueous solutions and its dependence on molecular mass (MM), degree of N-acetylation (DNAc) and ionic strength of the solution have been the most attractive subjects (Beri et al., 1993).

On the other hand, however, it is still difficult to evaluate conformation of chitosan molecules, because in most cases aqueous chitosan solutions contain aggregates or insufficiently dispersed residues to some extent by nature (Anthonsen, Vårum, Hermansson, Smidsrød, & Brant, 1994; Domard & Rinaudo, 1986; Matsumoto & Zenkoh, 1989; Matsumoto, Kawai, & Masuda, 1991a, 1991b), which critically disturbs the data for the conformation analysis. This may be the reason why some discrepancies were found between the results in the literatures cited above. Strictly speaking, "aggregates" and "insufficiently dispersed residues" in polymer solutions are formed

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through different routes. In the former case molecularly dispersed states once formed in solutions turn to aggregates by some intermolecular interactions, while in the latter case insufficiently dispersed residues are formed during the course of dissolution of solid polymers in solutions. In this paper, however, we use the term ''aggregates'' in both cases hereafter for convenience.

In this paper, therefore, the existence of persistent aggregates in aqueous solutions of commercial chitosans is revealed by the size-exclusion chromatography equipped with a multi angle light scattering detector (SEC-MALS) method. SEC-MALS has already been utilized for the conformation analysis of chitosan in aqueous solutions by some researchers (Beri et al., 1993; Fee et al., 2003; Rinaudo et al., 1993), although their results were somewhat inconsistent with each other and/or those theoretically predicted. Then, the reason for the presence of the aggregates is studied in relation to the amount of residual *N*-acetyl groups in chitosan samples. Detailed discussion in molecular conformation of chitosan as one of the β-1,4-linked polysaccharides (Yanagisawa & Isogai, 2005) and its dependence on MM and ionic strength of the solutions will be carried out in the following paper.

2. Materials and methods

2.1. Chitosan samples

Two commercial chitosan samples were used as starting materials in this study. Chitosan-100 and Chitosan-100D-VL (denoted as chitosans A and B, respectively) were purchased from Wako Pure Chemicals Co., Japan and Dainichiseika Color & Chemicals Mfg. Co., Japan, respectively. These samples were further deacetylated by treatment with 50% (w/V) aqueous NaOH at 105 °C for 3 h. These deacetylated samples were named chitosans A+, A++ and B+, respectively, where the number of + after each capital means that of the alkali treatment applied. Chitosan B was *N*-acetylated with acetic anhydride (0.5 mol per each hexosaminide residue) under homogeneous conditions according to the method reported by Hirano, Ohe, and Ono (1976)which was named chitosan B-*N*Ac. Number and weight average MM (M_n and M_w, respectively), polydispersity indices (M_w/M_n) and DNAc values of these six chitosan samples were summarized in Table 1.

Table 1
Number and weight average molecular mass values, polydispersity and degree of N-acetylation of six chitosan samples

Sample	$M_n/10^3$	$M_w/10^3$	M_w/M_n	DNAc (%)
A	164	416	2.54	12.6
A+	71.9	149	2.07	1.5
A++	69.1	133	1.92	0.9
B	18.6	36.3	1.95	2.0
B+	14.5	24.4	1.68	0.9
B-*N*Ac	17.1	38.5	2.25	47.0

2.2. Other materials

Distilled water of HPLC grade, acetic acid, acetic anhydride, sodium hydroxide and sodium nitrate of reagent grades were purchased from Wako Pure Chemicals. A pullulan standard (M_w 22,800; Shodex, Japan) was exclusively used to normalize the MALS photo-detectors (ASTRA for Windows user's guide version 4.90).

2.3. Preparation of sample solutions

Each chitosan sample was dissolved in 0.5 M aq. AcOH, and the stock solution thus obtained was left standing in dark at room temperature for 2–14 days. Then, a certain amount of 2 M aq. $NaNO_3$ was added to the solution to adjust the salt concentration to 0.1 M just before the SEC-MALS measurement. The d*n*/d*c* value of chitosan in aqueous solutions (0.203 mL/g) was obtained from the literature of Berth and Dautzenberg (2002) and used consistently in this study. Differences in d*n*/d*c* values for chitosans having different degree of N-acetylation were reported in some literatures (Terbojevich, Cosani, Conio, Marsano, & Bianchi, 1991; Terbojevich, Cosani, Focher, Naggi, & Torri, 1992; Wang, Bo, Li, & Qin, 1991). However, they are not taken into account in this paper because accurate determination of MM of chitosan is not our present concern.

2.4. Degree of N-acetylation (DNAc) measurement

The DNAc values of chitosan samples were determined by ^{1}H NMR according to a reported method (Hasegawa, 1992). ^{1}H NMR spectra of the chitosan samples in 0.4% (w/V) acetic acid-d_4/D_2O solutions were obtained with a JEOL ALPHA-500 spectrometer.

2.5. SEC apparatus and conditions

The SEC system used consisted of an on-line degasser (DGU-12A; Shimadzu, Japan), a high-pressure pump (LC-10ADVP; Shimadzu, Japan), a stainless steel in-line filter with a 0.1 μm hydrophilic polytetrafluoroethylene (PTFE) membrane (Millipore, USA), a manual injector (Model 7725; Rheodyne, USA), a column oven (CTO-10ACVP; Shimadzu), a pre-column filter with a 0.5 μm stainless steel frit (A-310; Upchurch Scientific, USA), a guard column (SB-G; Shodex, Japan), a SEC column packed with polyhydroxymethacrylate gel (SB-806M; Shodex), a MALS detector (DAWN EOS, λ 690 nm; Wyatt technologies, USA) and a refractive index detector (RID-10A; Shimadzu). Data acquisition and processing were carried out using the ASTRA IV software (Wyatt technologies). The SEC columns were exclusively used for cationic polymers.

SEC conditions were as follows: the sample concentration of 0.05–0.2% (w/V), injection volume of 100 μL, flow rate of 0.5 mL/min and the column temperature of 40 °C.

The detector cells of MALS and RI were kept at ambient temperature. Before injection, the sample solutions were centrifuged at 7740*g* for 10 min, and the supernatants were filtered through a 0.1 μm inorganic disposable membrane (Anotop 25; Whatman, UK) or a 0.2 μm hydrophilic PTFE disposable membrane (Millex-LG; Millipore, USA). The aqueous eluent (0.5 M AcOH + 0.1 M $NaNO_3$) was filtered through a 0.1 μm hydrophilic PTFE membrane (Omnipore; Millipore, USA) before use.

3. Results and discussion

3.1. Light scattering (LS) and refractive index (RI) signals

In Fig. 1, the light scattering (LS; 90°) and the refractive index (RI) signals were shown for all chitosan samples used in this study. This type of dual patterns provides some qualitative information about the solution state of the system examined. For example, the LS pattern of chitosan A has a large shoulder peak around 7 mL, while the correspondent RI peak is rather small. This kind of disproportion or discontinuity of correlation between LS and RI signals gives a strong indication of the presence of aggregates (or insufficiently dispersed residues), because it implies heterogeneity of the solution system. Thus, chitosan A is suspected to have some aggregates in the solution. The same is true for chitosan B-*N*Ac. Because such shoulder peak is apparently absent for its original sample (chitosan B), the introduction of *N*-Ac groups is exactly the cause for the shoulder peak observed. Thus, the amount of residual *N*-Ac groups (or the DNAc value) is clearly related to the presence of aggregates. Moreover, it should be noted that the LS patterns of chitosans A and B-*N*Ac had no concentration dependence. This indicates that these aggregates

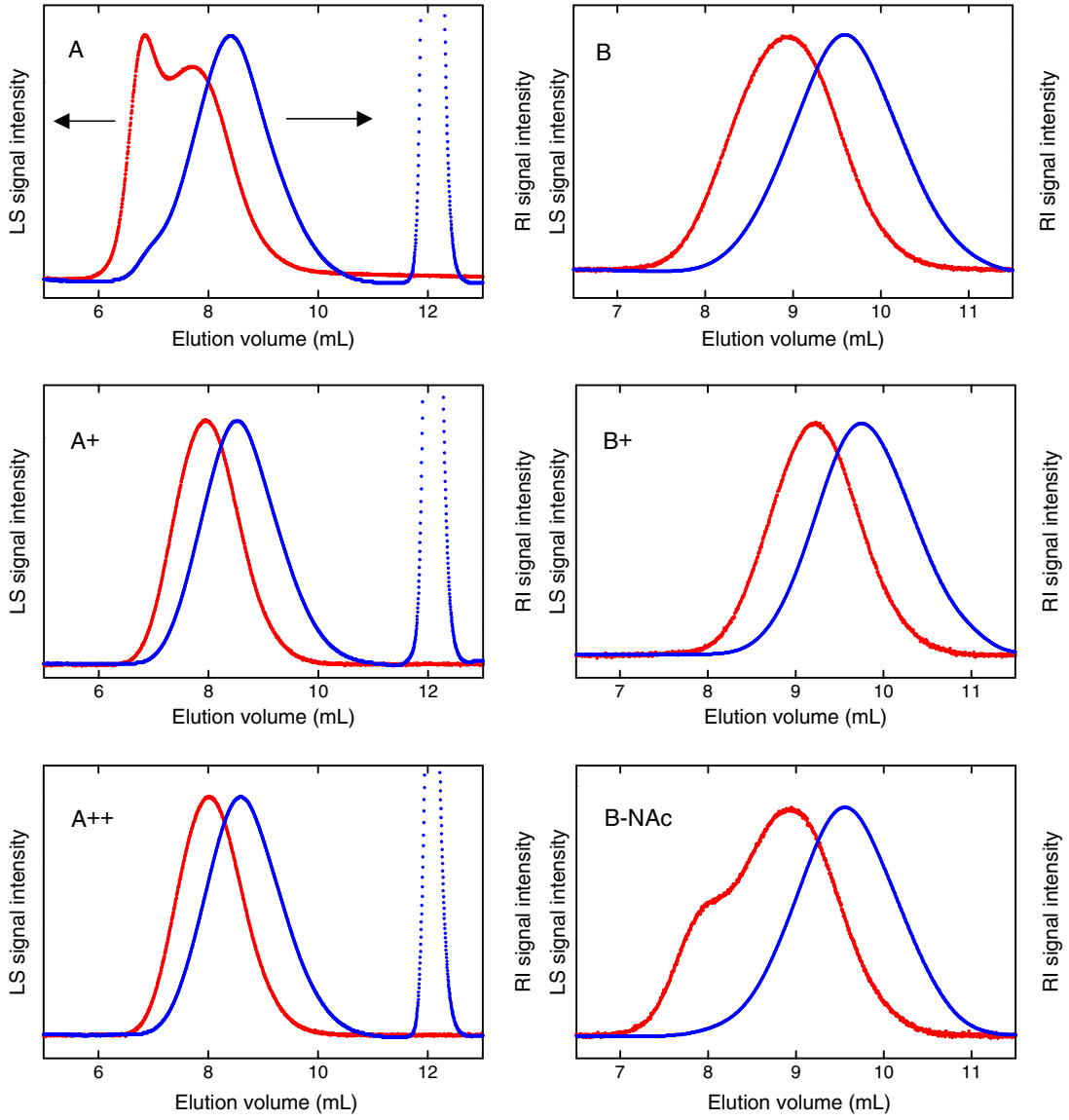

Fig. 1. Signals from the 90° light scattering (LS: red) and the refractive index (RI: blue) detectors for chitosan samples having various DNAc values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

are stably present in the solutions without in reversible equilibrium. On the other hand, no such aggregates were present in the solutions of other samples (chitosans A+, A++ and B+) with low DNAc.

In addition, detailed observation of the LS pattern of chitosan A reveals that the LS signal intensity does not recover to the initial level even after the solvent region (around 12 mL). This kind of tailing phenomenon is explained by the following hypothesis; the aggregates consisting of *N*-Ac rich chitosan molecules in chitosan A have relatively high hydrophobic affinity to the column packing material because of their hydrophobic *N*-Ac groups, and thus they elute slowly irrespective of their hydrodynamic sizes. This non-ideal elution means that such structures elute and affect the entire SEC sampling range. It is highly doubtful whether the data from such systems can be applied to conformation analysis for individual chitosan molecules.

3.2. Elution patterns and molecular mass (MM) plots

Fig. 2 shows the elution patterns and the M_w plots of each elution slice for chitosans A and A+. The elution pattern of chitosan A+ shifted to higher elution volume, showing the occurrence of some degradation of chitosan molecules during the alkali treatment. However, it is noticeable that the alkali treatment had qualitative influences on the elution patterns and the M_w plots; the shoulder peak of chitosan A around 6–7 mL disappeared, and the M_w plots became linear and monotonically decreased for chitosan A+, instead of curved and re-ascending plots in the high elution volume for chitosan A. These normalization phenomena as well as the drastic decrease in the average MM values (>60% loss; see Table 1) are explained by extinction or removal of aggregates in chitosan samples through the alkali treatment. Here, it should be noted that the alkali treatment significantly decreased the DNAc value.

On the other hand, the impact of the second alkali treatment seems rather small in terms of the SEC elution patterns and the M_w plots (see Fig. 3), as well as the average MM and DNAc values (Table 1). Additionally, the M_w plots for chitosans A+ and A++ were consistent well with each other in the entire M_w range measured. Generally, SEC elution behavior primarily depends on the hydrodynamic dimension of the polymer and secondarily on some hydrophilic/hydrophobic/ionic interactions between the polymer and the packing materials in the column. Thus, the obtained result shows that chitosan molecules in these two samples in Fig. 3 are essentially the same in terms of these factors. Probably, the first alkali treatment led chitosan molecules to a plateau state in terms of some molecular properties, while the second treatment gave no essential impact on chitosan molecules other than the slight decrease in MM and DNAc. Of course, it is possible that some minor functional groups are introduced to chitosan molecules by oxidation through the alkali treatment, although it seems to have no significant influence on molecular properties concerning SEC elution behavior.

Similar discussion can be applied to chitosan B series, although their average MM values are rather small and apparent shoulder peak is originally absent in the elution pattern of chitosan B in Fig. 4. However, the M_w plots have some slight but unignorable gap between chitosans B and B+ in their high M_w regions, indicating some difference between chitosans B and B+ as to their molecular properties. This type of gap was highlighted by N-acetylation, as shown in Fig. 5. Thus, formation of some aggregates may have brought about this type of gap in the M_w plots, which is clearly related to the amount of residual *N*-Ac groups or DNAc values of chitosan. On the other hand, the elution patterns of chitosans B and B-*N*Ac were consistent well with each other in the entire elution range. This may support the hypothesis that the DNAc values of chitosans have little influence on their hydrodynamic size or molecular conformation (Berth & Dautzenberg, 2002;

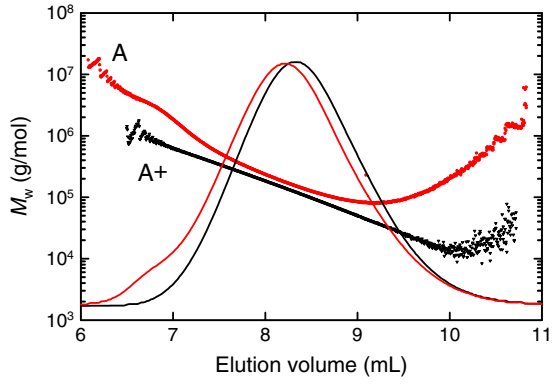

Fig. 2. SEC elution patterns and weight-averaged molecular mass (M_w) plots of chitosans A (red) and A+ (black) in 0.5 M AcOH + 0.1 M $NaNO_3$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

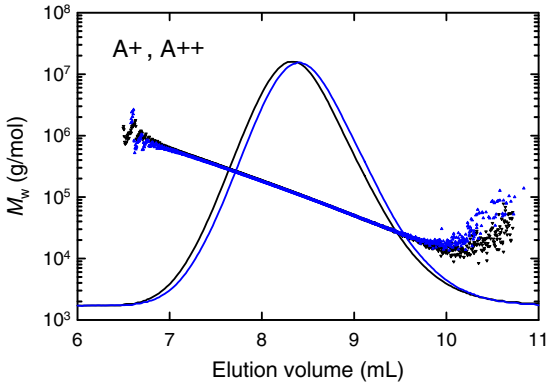

Fig. 3. SEC elution patterns and weight-averaged molecular mass (M_w) plots of chitosans A+ (black) and A++ (blue) in 0.5 M AcOH + 0.1 M $NaNO_3$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

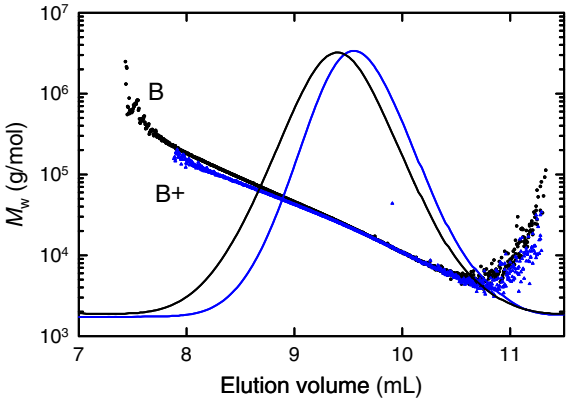

Fig. 4. SEC elution patterns and weight-averaged molecular mass (M_w) plots of chitosans B (black) and B+ (blue) in 0.5 M AcOH + 0.1 M $NaNO_3$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

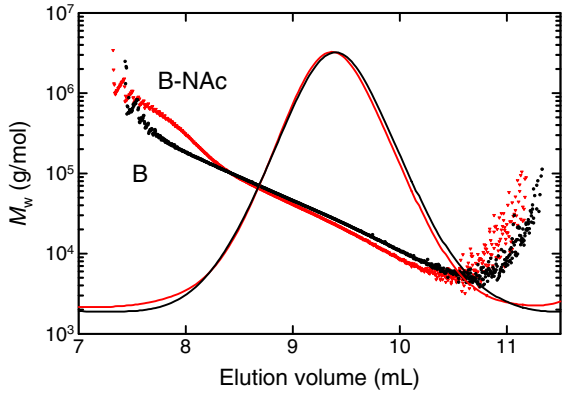

Fig. 5. SEC elution patterns and weight-averaged molecular mass (M_w) plots of chitosans B (black) and B-*N*Ac (red) in 0.5 M AcOH + 0.1 M $NaNO_3$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Rinaudo et al., 1993), while they have clear relation with the presence of aggregates.

3.3. Conformation plots

In the SEC-MALS experiments, M_w and $R_{g,z}$ of each elution slice can be successively measured, and thus M_w and $R_{g,z}$ distributions are consequently obtained for polydispersed polymer samples. Relationship between these two-dimensional parameters provides valuable information about the molecular conformation of the polymer with the aid of classical solution theories. In this paper, this type of plots is called 'conformation plot' for convenience. Easy and rapid generation of the conformation plot is one of the significant abilities of SEC-MALS. However, at the same time, it should be cautioned that the conformation plot might be substantially influenced by efficiency of SEC separation and existence of the aggregates, consequently giving false information.

In Fig. 6, the conformation plots of chitosans A, A+ and A++ are shown together. The latter two plots are consistent well with each other in the entire M_w range measured, and are almost on a common straight line. The slopes for chitosans A+ and A++ are theoretically acceptable, indicating that the molecular conformation of chitosan in these two samples are mostly the same in the solvent and can be regarded as expanded random coils as a whole. Because no improvement or change was observed between the plots of chitosans A+ and A++, they are evaluated to reach a plateau state concerning molecular conformation. Detailed discussion of molecular conformation of these purified chitosan samples will be carried out in the following paper, using not only SEC-MALS but also some other evaluation methods such as viscometry.

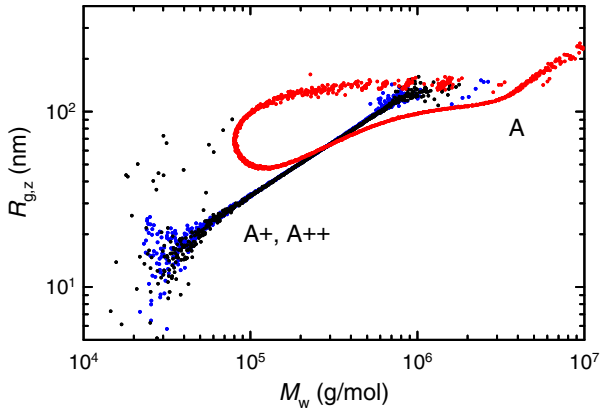

Fig. 6. Double logarithmic plots of $R_{g,z}$ vs. M_w for chitosans A (red), A+ (black) and A++ (blue) in 0.5 M AcOH + 0.1 M $NaNO_3$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

On the other hand, the conformation plots of chitosan A were rather strange and completely different from those of chitosans A+ and A++. The plots had a backward curling in the low M_w region, and exclusively distributed in the range of $M_w > 100{,}000$. However, the aggregates are rather minor in mass and the M_w of most components are in the range of 10,000–1,000,000, judging from the elution pattern in Fig. 2. Thus, the conformation plot of chitosan A does not seem to reflect the actual solution state. Probably, the contribution of the aggregates overwhelmed that of the molecularly dispersed components as to LS intensity, and distorted the entire plots. It is highly suspicious whether or not reliable information about conformation of individual chitosan molecules could be obtained from such data, except for an evidence of the presence of aggregates.

Fig. 7 shows the conformation plots of chitosans B, B+ and B-*N*Ac, along with chitosan A++ for comparison. The most remarkable point here is that only the plots of chitosan B+ are consistent with those of chitosan A++ regardless of their MM, showing that only chitosan B+ has the same molecular properties with chitosan A++ as to molecular conformation. On the other hand, the plots of chitosans B and B-*N*Ac had a clear discrepancy with those of chitosans A++ and B+ in their high MM regions, and these downward curvatures could not be resolved by varying concentration of the chitosan solution or lowering ionic

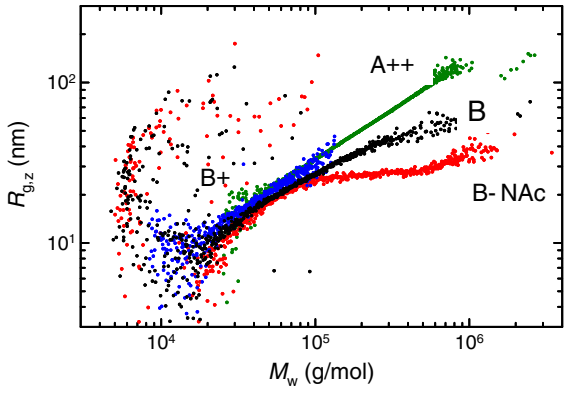

Fig. 7. Double logarithmic plots of $R_{g,z}$ vs. M_w for chitosans B (black), B+ (blue), B-*N*Ac (red) and A++ (dark green) in 0.5 M AcOH + 0.1 M $NaNO_3$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

strength of the system (down to 0.01 M, data not shown). This indicates that the aqueous solutions of chitosans B and B-*N*Ac are haunted by some stable aggregates, which seems to be quantitatively related to the DNAc values of the chitosan samples. It is noticeable that the N-acetylation increased the amount of aggregates formed in the chitosan solutions.

Of course, intra- and inter-molecular distributions of *N*-Ac groups may have significant influence on the amount of aggregates formed (Anthonsen et al., 1994). Comparison between chitosans A and B-*N*Ac may provide a typical example for this issue, where chitosan A seems to be annoyed by much more aggregates than chitosan B-*N*Ac, although the DNAc value of chitosan A is substantially lower than that of chitosan B-*N*Ac. Homogeneous introduction or distribution of *N*-acetyl groups along one chitosan molecule seems to decrease the amount of aggregates formed in chitosan solutions. Anyway, this means that the presence of aggregates is intrinsic for the chitosan samples with certain degree of N-acetylation, and thus it seems to be difficult to exclude the influence of these aggregates without changing some composition of the chitosan samples. These results lead to a somewhat strict conclusion that conformation of individual chitosan molecules in aqueous solutions can be studied only for some limited samples.

Complete removal of aggregates in chitosan solutions (Domard & Rinaudo, 1983) or complete N-deacetylation shown in this study has to be applied to chitosan samples. At least, the aggregates in chitosan solutions could not be removed in this study by common techniques such as preparative centrifugation (7740*g*, 10 min), microfiltration (0.1 μm) or lowering ionic strength of the solution. The pressure solubilization technique reported by Ross-Murphy and his co-workers (Picout, Ross-Murphy, Errington, & Harding, 2003) may be a promising alternative to work around the problems without chain degradation or N-deacetylation, although it belongs to our future work.

4. Conclusions

The SEC-MALS method was applied to chitosan samples having different average MM and DNAc values. It was shown that the amount of residual *N*-Ac groups or DNAc values of the chitosan samples have influences on their dispersion states in aqueous solvents, and even a slight amount of residual *N*-Ac groups (as low as 2% DNAc) can cause the presence of aggregates in chitosan solutions. This makes the solution system heterogeneous, and has critical influences on the data for the conformation analysis. Such aggregates could not be removed or avoided by preparative centrifugation, microfiltration or lowering ionic strength of the system. Thus, accurate evaluation of conformation of individual chitosan molecules in aqueous media may be possible only for some limited samples; complete deacetylation through alkali treatments is one of the reliable methods to circumvent these problems.

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