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Characterization of chitosan and its derivatives using asymmetrical flow field-flow-fractionation: A comparison with traditional methods

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Abstract

Chitosan and its derivatives are an important group of polymers used extensively in pharmaceutics. Thus, their physicochemical properties are of considerable interest and need to be characterized carefully. The most important feature to determine is their molecular weight and molecular weight distribution while the molecular weight of polymers plays an important role as pharmaceutical excipients. In this study, the feasibility of using asymmetrical flow field-flow-fractionation (AF4) connected online to a multi-angle light scattering (MALS) detector to measure the molecular weight of chitosans, trimethyl chitosans were studied and compared with the results from some traditional measurement methods. It was found that the influence of trimethyl chitosan synthesis process on the resulting molecular weight decrease depends on the initial molecular weight of chitosan. Significant molecular weight decrease was observed when chitosan molecular weight was larger than 100 kDa. In contrast, the influence was marginal when the molecular weight of chitosan was less than 50 kDa. The AF4-MALS was found to be a suitable method for the characterization of pure chitosans and trimethyl chitosans.

Keywords: Chitosan; Trimethyl chitosan; Asymmetrical flow field-flow-fractionation (AF4); Multi-angle laser scattering; Molecular weight

1. Introduction

Chitosan (poly[β-(1-4)-2-amino-2-deoxy-D-glucopyranose]) is a non-toxic and biocompatible cationic polysaccharide produced by partial deacetylation of chitin isolated from naturally occurring crustacean shells. It is comprised of copolymers of glucosamine and *N*-acetyl glucosamine. The term chitosan embraces a series of polymers that vary in molecular weight (from approximately 10,000 to 1 million Da) and degree of deacetylation (in the range of 50–95%). Since chitosan displays mucoadhesive properties, owns strong permeation enhancing capabilities for hydrophilic compounds and has a safe toxicity profile [1], it has received considerable attention as a novel excipient in drug delivery system and has been included in the European Pharmacopoeia since 2002. In addition, chitosans as dietary control agents have drawn much attention recently [2].

Despite its biocompatibility, the use of chitosan in biomedical fields is limited by its poor solubility in physiological media. Chitosan has an apparent pK_a value between 5.5 and 6.5 and upon dissolution in acid media the amino groups of the polymer are protonated rendering the molecule positively charged. At neutral and alkaline pH, most chitosan molecules lose their charge and precipitate from solution. To improve the poor water-solubility of chitosan at physiological pH, trimethyl chitosan (TMC) was synthesized by quaternization of the amino groups of chitosan [3]. In general, it is assumed that quaternization of chitosan will not change the molecular weight of chitosan significantly; therefore, the molecular weight of trimethyl chitosan was commonly labeled based on the initial molecular weight of chitosan used for the modification without detailed characterization. Since sodium hydroxide was used in the synthesis process, we assume that the molecular weight (MW) of the resulting TMC might decrease, indicated by the significantly decreased intrinsic viscosity of TMC 400 kDa compared with that of chitosan 400 kDa (0.208 l/g versus 1.03 l/g). However, no experimental data are available to support this assumption.

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How will the initial molecular weight of chitosan influence the extent of molecular weight decrease is also unclear and needs to be clarified. Therefore, TMC molecular weight characterization is essential.

In general, the various instrumental methods for polymer molecular weight measurements can be categorized into absolute and relative ones. Absolute methods include colligative property measurements such as osmometry, to determine number average molecular weight (M_n) and light scattering techniques to determine mass average molecular weight (M_w). Relative methods include gel permeation and solution viscosity measurements, which mostly require calibration with known molecular mass standards. The absence of standards prevents the application of relative methods on TMC characterization. Recently, polymer characterization using field-flow-fractionation (FFF), a method of separation particularly well suited for the fractionation and characterization of large macromolecules, draws much attention [4–6]. The central unit of the FFF equipment is a separation channel. The separation is done inside the channel by a field perpendicular to the flow direction of the probe. According to the nature of the force field, FFF is subdivided into various types, such as sedimentation, electrical, thermal or flow FFF. For the method flow FFF the field is achieved by a cross flow of background solvent. If the cross flow solvent is only able to penetrate through the lower side of the channel, where the upper wall of the channel consists simply of a flat float glass plate, the method is called asymmetrical. Via the glass plate, it is possible to visually detect leaking and separation asymmetry as well as optimizing sample injection and focusing of the sample. The underside is covered by a semi permeable membrane which acts as a barrier for macromolecules, but not for low molecular weight compounds like ions. The probe is separated by its particle size or molecular weight [7]. Compared to chromatographic methods there is no stationary phase and effects of adsorption are not the reason for separation. By the cross flow field especially larger particles move close to the membrane and smaller particles move back to the inner side of the channel by their Brownian motion. There they are located in a faster current and elute out of the channel first. On the other hand, connecting flow FFF to a multi-angle light scattering detector (MALS) greatly increases the applicability of the method to polymers while the MALS detector allows obtaining absolute molecular weight and distribution directly [8,9]. The flow FFF separation prior to detection increases the potential of MALS to recognize molecular weight and size distributions, especially if broad distributed samples are characterized. Furthermore, a sample prefiltering is normally not required because aggregates and dust particles elute at different times from channel [10].

In this paper, combination of asymmetrical flow field-flow-fractionation (AF4) with multi-angle light-scattering (MALS) instruments for the characterization of polydisperse polysaccharides, chitosan and its derivatives was tested. Due to its broad separation ranging from 1 Da to several GDa, the AF4 technique is a viable and desirable alternative to size-exclusion chromatography (SEC). The separation quality of AF4 is high, especially for large macromolecules and even aggregates can be detected [11]. In combination with MALS, absolute molecular weight can be calculated without resorting to calibration. The limitations of SEC-MALS in chitosan characterization such as potential interaction with the stationary phase due to its cationic structure were already described [12]. Here, three different molecular weight TMCs were synthesized and the obtained TMC molecular weights were characterized with AF4-MALS and compared with their initial material chitosan. Moreover, the reliability of flow FFF for chitosan and TMC characterization was further demonstrated by comparing the obtained data with that measured from other experimental methods such as intrinsic viscosity and GPC measurements.

2. Materials and methods

2.1. Materials

Chitosan (MW ~ 400 kDa, degree of deacetylation 84.7%) was purchased from Fluka (Steinheim, Germany) and depolymerized according to a method described previously [13] to obtain chitosans of different molecular weights. All other chemicals used were of analytical grade.

2.2. Synthesis of different molecular weight TMCs

Different molecular weight TMCs were synthesized according to a two-step method described previously using depolymerized chitosans of appropriate molecular weight as starting materials and were subsequently characterized by ^{1}H NMR [3]. We used the abbreviation TMC 100 kDa to denote the polymer prepared from chitosan 100 kDa, and the same for the other polymers. Since the degree of quaternization of TMC plays an important role in opening the tight junctions between adjacent cells and a higher degree of substitution had improved permeation enhancement [14,15], TMCs with a 40% degree of substitution were prepared.

2.3. Determination of intrinsic viscosity

Intrinsic viscosity of chitosans in 2% HAc/0.2 M NaAc was measured using an automated Ubbelohde capillary viscometer (Model Schott AVS-360, Germany) in a constant-temperature water bath at 25 ± 0.01 °C in triplicate, as described previously [13]. The capillary diameter used was 0.63 mm. Solution concentrations were adjusted based on the viscosity of the samples and the flow through time was kept in the range of 100–150 s. Six different concentrations were tested for each sample. The viscosity average molecular weights of chitosans were calculated using the classical Mark–Houwink equation:

$$[\eta] = K(M_v)^a$$

where $[\eta]$ is the intrinsic viscosity of the depolymerized chitosan, K and a are constants for given solute–solvent system and temperature. For chitosan, they are influenced by the degree of deacetylation, pH, and ionic strength of the solvent [16]. For chitosans with a DD value of 85%, the constants $K = 1.38 \times 10^{-5}$ and $a = 0.85$ were reported [17].

2.4. Fourier transformed infrared (FTIR)

FTIR spectral studies were conducted on a FT-IR 510P spectrometer in the range of 4000 and 400 cm^{-1}, with a resolution of 2 cm^{-1}. All powder samples were compressed into KBr disks for the FTIR measurement.

2.5. Gel permeation chromatography (GPC)

GPC experiments were carried out using a Supremamax 3000 column (Polymer Standard Service, Mainz, Germany) with 2% HAc/0.2 M NaAc as an eluent (1 ml/min). The system comprised a pump (Hitachi, Darmstadt, Germany), an autosampler device (Merck Hitachi model AS-2000A) and a vacuum in-line degasser. The amount of injected sample volume per run was 40 μl. The samples were analyzed with a differential refractive index (RI) detector RI-71 from Merck. Molecular weights were calculated using Astra software (Wyatt Technology Corp.).

2.6. Asymmetrical flow field-flow-fractionation

The AF4-MALS experiments were carried out with an Eclipse F suitable for both organic and aqueous solvents and a Dawn EOS detector purchased from Wyatt Technology Europe. A RI-Detector from Shodex was used (RI-101). Pump and degasser were 1100 series from Agilent Technologies. The used membrane consisted of regenerated cellulose with MWCO 10 kDa (Microdyn Nadir). The channel thickness was 350 μm. All probes were measured in 0.1 M HAc/NaAc acetate buffer at pH 4.2 containing 0.02% NaN_3 (w/v) to prevent bacterial growth. Due to the broad molecular weight distribution of chitosan, a special method using a multi-step cross flow gradient was applied. This was necessary to detect low and high molecular weight components in appropriate time within one run. Briefly, the detector flow was kept constant at 1 ml/min during all the measurements. After 2 min of cross flow adjustment and 1 min of focus flow equilibration at 2 ml/min, 100 μl of 0.2% (w/v) sample solution were injected for 2 min with a flow rate of 0.2 ml/min and focused further for 1 min. Thus, the elution mode started at 6 min in all fractograms. Subsequently, the cross flow was kept at 2 ml/min for 2 min, then decreased to 1 ml/min in 5 min and further decreased to 0 ml/min in 17 min. Each measurement was performed at least in duplicate. Molecular weights were calculated using Astra software (Wyatt Technology Corp.). The Zimm equation was used for calculation. To achieve differential molecular weight distributions, the values were fitted using polynomial model fourth order.

For pure chitosans in acetate buffer pH 4.2, a d*n*/d*c* value of 0.181 ml/g was used as reported in the literature [7]. For all the other measured samples, the refractive index increment was measured by direct injection into the RI detector. A value of 0.145 ± 0.003 ml/g was measured for TMCs. Although MALS data of TMCs was already presented in the past, a comparison to literature is difficult due to the absence of d*n*/d*c* value [18]. From the observed change of refractive index increment, it can be concluded that covalent bonds were successfully generated.

2.7. Calculation and statistics

Results are depicted as mean ± S.D. from at least two measurements. Significance between the mean values was calculated using ANOVA one-way analysis (Origin 7.0 SRO, Northampton, MA, USA). Probability values $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Characterization of chitosan

To characterize the feasibility of using AF4 as a tool for chitosan molecular weight characterization, the depolymerized chitosan of different molecular weight (100, 50 and 25 kDa, respectively) was measured with AF4-MALS, as shown in Fig. 1.

The peaks in Fig. 1(A) show the concentration of samples measured with the RI detector. The dotted lines give the corresponding molecular weight. It was noticed that there was a characteristic void peak at elution time of approximately 6 min. Such a void peak is caused by materials having low molecular weight such as buffers so that it is not retained [5]. At high elution times bigger particles elute under very low field strength and they represent unavoidable dust or eventually chitosan aggregates as described previously [19]. Therefore, the light scattering signal should not be evaluated at higher elution times and the middle range of approximately 8–24 min was used for polymer characterization in this study. As anticipated, different chitosans elute at different times. The smaller the molecule weight, the sooner it elutes. For example, the elution peak maximum is 9 min for chitosan 25 kDa compared to approximately 14 min

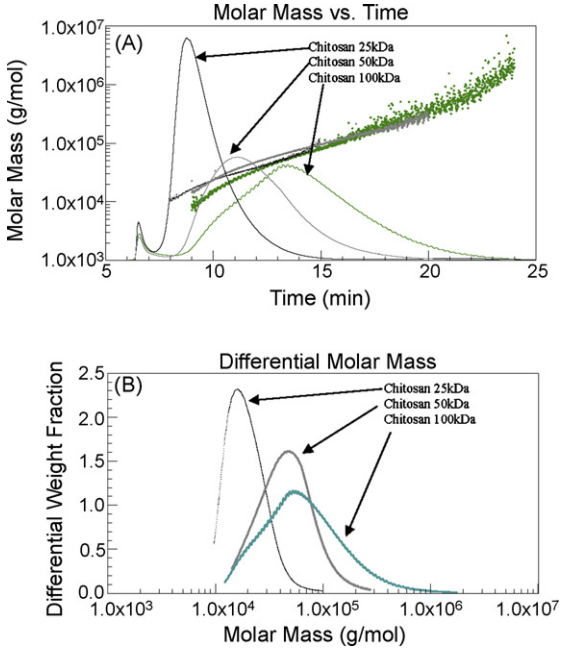

Fig. 1. (A) Chitosan fractograms where peaks represent the concentration signals and points the respective molecular weight. (B) Chitosan differential molecular weight plots.

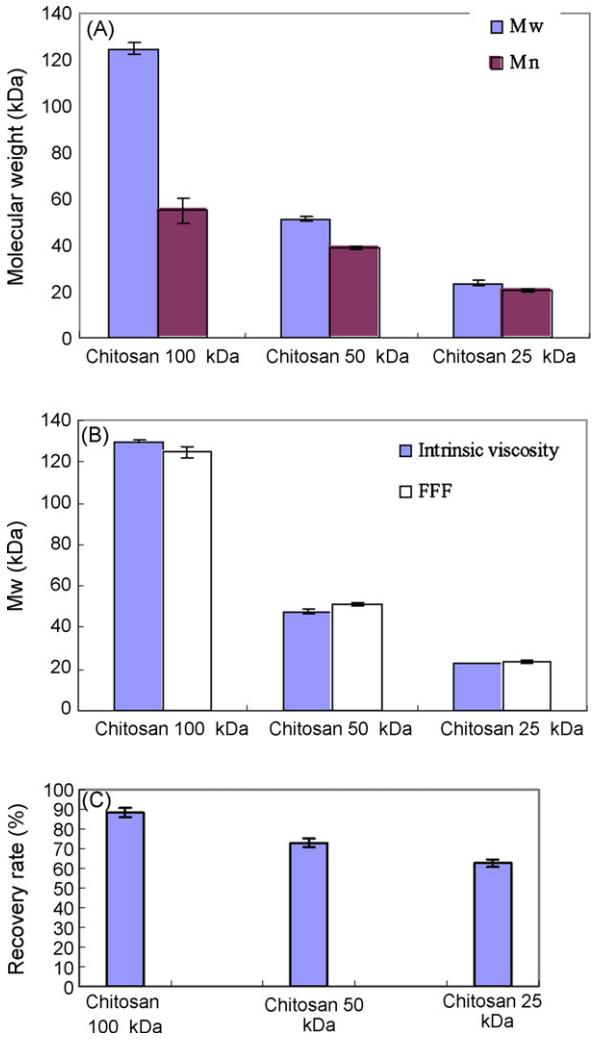

Fig. 2. (A) M_w and M_n of chitosan measured from AF4-MALS method. (B) M_w comparison of chitosan measured from intrinsic viscosity and AF4-MALS methods. (C) Proportional recovery rates of different molecular weight chitosans from AF4-MALS methods.

for chitosan 100 kDa, which is in agreement with the flow FFF theory of Giddings [20]. In addition, the weight (M_w) and number average molecular weight (M_n) values were calculated. Both values should be given with respect to the possible influence of bigger particles or aggregates [19]. As shown in Fig. 2(A), the higher the molecular weight of chitosan, the bigger is the difference between M_w and M_n, implying that chitosan molecular weight distribution is broader for higher average molecular weight samples. In contrast, similar values of M_w and M_n were found for chitosan 25 kDa, suggesting that the polydispersity is small when the molecular weight of chitosan was low. The measured polydispersity values were 2.3, 1.3 and 1.2 for chitosan 100, 50 and 25 kDa, respectively, and decreased with decreasing chitosan molecular weight. Fig. 1(B) is a detailed view of chitosan molecular weight distribution given as differential plots calculated from MALS data. The peak maxima depended on average molecular weight. Furthermore, it can be seen that the peaks were broader for higher average molecular weight samples. For example, molecular weight values of chitosan 100 kDa ranged over several magnitudes from approximately 10 kDa to values about 1 MDa.

In order to compare asymmetrical flow FFF data with results from other techniques, intrinsic viscosity was characterized. It is another method to measure the molecular weight of chitosan [17]. The weight average molecular weight (M_w) of pure chitosan was calculated from AF4-MALS measurements and compared with that measured from intrinsic viscosity, as shown in Fig. 2(B). Similar values were achieved for the molecular weight measured by the two methods ($P > 0.01$). Thus, the viscosity data supports the AF4-MALS findings although they are based on measurements of different parameters.

Prerequisite for a reliable asymmetrical flow FFF separation is the basic knowledge of adsorption of sample components on the membrane. The proportional recovery related to a regular direct injection into the RI detector is given in Fig. 2(C). This value is mainly influenced by sample adsorption on membrane or elsewhere in the system and probe loss through the membrane. The used AF4-MALS system was checked for channel leakage and RI detector calibration. The high yield of 88% for chitosan 100 kDa and the constant recoveries for fresh and several times used membranes indicated that the adsorption of chitosan on the membrane was minimal. Thus, the missing amount of chitosan 100 kDa and the molecular weight dependent decrease of proportional recovery down to only 62% for chitosan 25 kDa could be attributed to small molecules lost through the 10 kDa cut off membrane. However, a high percentage of the samples was able to be characterized, implying that the determined average values represent the main part of the sample.

3.2. Characterization of trimethyl chitosan

Trimethyl chitosan was synthesized to increase the solubility of chitosan. In order to test whether quaternization of chitosan will change the molecular weight significantly, different TMCs were synthesized using varied molecular weight chitosan as initial materials and the final products were characterized by ^{1}H NMR. The compositions of TMCs after undergoing a two-step synthesis are listed in Table 1 and no significant difference in the degree of quaternization was observed with different molecular weight TMCs ($P > 0.05$). Using the ninhydrin complexation reaction, Sabnis and Block [21] found that the reactivity of chitosan was inversely proportional to its molecular weight. However, in our study, degree of quaternization of different molecular weight TMC was similar, implying that the activity

Table 1
Degrees of substitution of different trimethyl chitosans after second-step reaction

%[a]	TMC 400 kDa	TMC 100 kDa	TMC 50 kDa	TMC 25 kDa
$N^+(CH_3)_3$	42.4	39.2	39.6	40.4
$N(CH_3)_2$	12.4	13.0	12.7	7.2
3-OCH_3	6.1	5.4	7.8	12.0
6-OCH_3	8.0	6.7	11.9	14.7
$NHCOCH_3$	10.3	10.7	7.4	8.7
NH_2 free	30.8	37.0	40.3	43.6

[a]Calculation based on ^{1}H NMR analysis.

Table 2
Characteristics of trimethyl chitosan intermediates after first-step reaction

%[a]	TMC 50 kDa	TMC 25 kDa
$N^+(CH_3)_3$	17.2	18.2
$N(CH_3)_2$	35.8	32.8
$NHCOCH_3$	12.1	16.8
NH_2 free	34.9	43.6

[a] Calculation based on ^{1}H NMR analysis.

of primary amino groups was molecular weight independent. Our result is consistent with Flory's theory, which suggests the intrinsic activity of all functional groups on a polymer remains the same [22].

Characteristics of the intermediates of TMC 50 kDa and TMC 25 kDa after undergoing a one-step reaction are described in Table 2. Comparing Tables 1 and 2, it is evident that the degree of substitution of trimethylated amino groups was only about 20% after one-step reaction. In contrast, the substitution degrees of dimethylated amino group were quite high. Therefore, a two-step synthesis was essential for the preparation of TMC with a higher $N^+(CH_3)_3$ substitution degree. Additionally, when the molecular weight of chitosan was <50 kDa, it resulted in O-methylation in the 3- and 6-position, and the degree of 3-OCH_3 and 6-OCH_3 substitutions increased considerably with TMC 50 kDa and TMC 25 kDa, which can be explained by the decreased steric hindrance of the low molecular weight polymers. It was also noticed that the degree of deacetylation of chitosan decreased slightly due to the sodium hydroxide used in the process, and chitosans with molecular weight <50 kDa were more sensitive to the existence of sodium hydroxide compared with the ones with larger molecular weight.

FT-infrared spectroscopy was used to investigate the structure of TMC and compared with that of chitosan, as depicted in Fig. 3. Pure chitosan 100 kDa shows a distinct amide II band at 1580 cm^{-1} (s, δ_{NH}) and amide linkage at 1406 cm^{-1} (ν_{C-N}) [23], the area between 1000 and 1150 cm^{-1} was saturated, maybe due

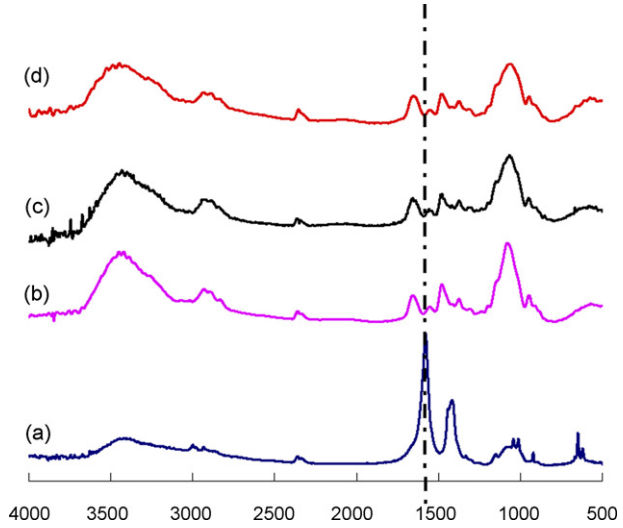

Fig. 3. IR spectra comparison of trimethyl chitosan with chitosan (a) chitosan 100 kDa, (b) TMC 100 kDa, (c) TMC 50 kDa and (d) TMC 25 kDa.

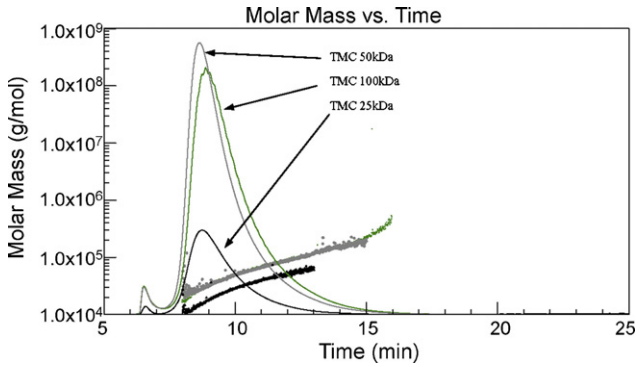

Fig. 4. TMC fractograms where peaks represent the concentration signals and points the respective molecular weight.

to the presence of three distinct modes of C–O–C, C–OH, and C–C ring vibrations. N–H stretching and O–H stretching vibrations can be characterized by the broad signals in the region of 3200–3500 cm^{-1}. After preparation of TMC, the absorption at 1580 cm^{-1} (δ_{NH}) decreased significantly. Normally there should be no absorption in this area for pure tertiary amine. However, the TMC prepared with this method is a mixture of primary amine, secondary amine and trimethyl amine; hence a small signal remains at this wavelength. Comparing the spectra of TMCs with that of chitosan, the signals at 1453 cm^{-1} ($\delta^{as}_{CH_3}$) and 1337 cm^{-1} ($\delta^{s}_{CH_3}$) became stronger due to the formation of $N^+(CH_3)_3$ group. This is in good agreement with the structure of TMC.

The obtained TMCs were characterized with AF4-MALS and GPC, respectively. The fractograms of different TMC are shown in Fig. 4. If no depolymerization occurred during the synthesis process, increased molecular weight should be observed due to the conjugation of methyl group on the back bone of chain. However, as shown in Fig. 4, compared to pure chitosan 100 kDa, TMC 100 kDa elute faster, indicating decreased molecular weight. This implies that the polymer chains of chitosan 100 kDa were degraded during the synthesis process. Moreover, their corresponding M_w values were calculated using the signals from light scattering and compared with that of the initial chitosan (Fig. 5). It was noticed that significant molecular weight decrease was observed only when chitosan M_w was high, such as in the case of 100 kDa. For chitosan 50 kDa, only approx-

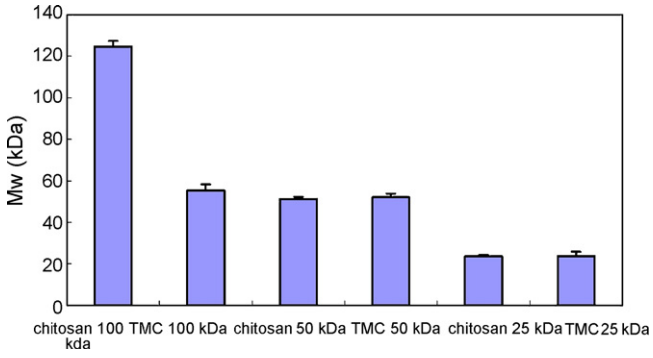

Fig. 5. Comparison of the molecular weight of TMC with the corresponding chitosan measured by AF4-MALS.

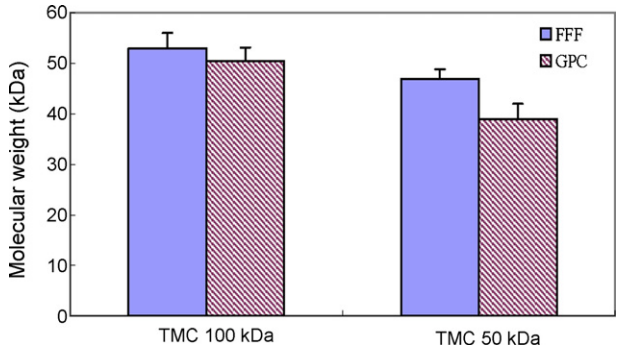

Fig. 6. Molecular weight comparison measured from two different methods.

imately 12% M_w decreased was found. In contrast, 7% M_w increase was observed for TMC 25 kDa as a result of methyl group conjugation. This study demonstrated that the influence of TMC synthesis process on the resulting molecular weight decrease depends on the initial molecular weight of chitosan. Significant molecular weight decrease is anticipated when chitosan molecular weight is more than 100 kDa. In contrast, the influence is marginal when the molecular weight of chitosan is less than 50 kDa.

TMC 100 and 50 kDa were further characterized with GPC. As shown in Fig. 6, no significant difference between the M_w measured from two different methods was found ($P > 0.05$), implying that AF4-MALS can be used to characterize TMC with simplicity compared to the traditional GPC method.

4. Conclusions

Asymmetrical flow FFF (AF4) with MALS is a fast and convenient method to characterize chitosan and trimethyl chitosans. All polymers investigated showed monomodal molecular weight and elution distributions. For chitosans, the molecular weights measured with AF4 and intrinsic viscosities were similar. When chitosans with molecular weight larger than 100 kDa were used as initial materials for synthesis, the molecular weight of the resulting TMC decreased significantly. In contrast, the influence was marginal when the M_w of chitosan was less than 50 kDa. TMC molecular weight measured from AF4-MALS and GPC has great agreement. These data indicated that AF4-MALS could be used as a powerful tool for the characterization of chitosan and TMC.

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