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Fractionation and characterization of chitosan by analytical SEC and ^{1}H NMR after semi-preparative SEC

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ABSTRACT

Heterogeneity in molecular weight and degree of deacetylation (DDA) of chitosans from different sources and preparation methods were studied by fractionating chitosans, using semi-preparative SEC, and then determining molecular weight profiles of fractions by analytical SEC with multi-angle laser light scattering (SEC–MALLS), and degree of deacetylation (DDA) by ^{1}H NMR. Fractionation of two high molecular weight chitosans from different manufacturers, produced fractions that spanned a wide range of molecular weight (number-average M_n), from 65 to 400 kDa in one case, that was not evident when unfractionated material was directly analyzed by SEC providing M_n = 188 kDa and PDI = M_w/M_n = 1.73. In a second case, fractions ranged from 20 to 600 kDa with unfractionated M_n = 145 kDa and PDI = 1.83. Fractionation of low molecular weight chitosans also showed a broad range of molecular weight in the original material, however, the fractions obtained with the TSKgel G4000W column in the M_n range of 5–100 kDa were essentially monodisperse with PDIs between 1.0 and 1.4. The DDA of one low molecular weight chitosan (10 kDa) produced by nitrous acid degradation was dependent on the M_n of the fraction. This semi-preparative fractionation procedure revealed important compositional heterogeneities of chitosans not evident in unfractionated material, and permitted the production of monodisperse low molecular weight chitosans with homogeneous properties.

1. Introduction

Polymers derived from biomass have drawn increasing attention due to favourable environmental and toxicity profiles (Sorlier, Denuzière, Viton, & Domard, 2001), and among them, chitosan is a biodegradable, biocompatible, non-toxic, and bioactive polysaccharide derived by deacetylation of the naturally abundant chitin (Borchard, 2001; Chatelet, Damour, & Domard, 2001; Koide, 1998; Köping-Höggård et al., 2001, 2004; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Mao et al., 2001). Chitosan is a linear copolymer of β-(1,4)-2-deoxy-2-amino-D-glucopyranose (D-glucosamine) and β-(1,4)-2-deoxy-2-acetamido-D-glucopyranose (*N*-acetyl-D-glucosamine) (Di Martino, Sittinger, & Risbud, 2005; Liu & Yao, 2002), where the D-glucosamine content, also called degree of deacetylation (DDA), is above 50% (Chatelet et al., 2001). Chitosan is a polyelectrolyte with a cationic character enabling electrostatic binding to polyanions such as glycosaminoglycans (Di Martino et al., 2005) and DNA, making it a suitable candidate for biomedical applications in orthopaedic tissue-engineering (Chevrier, Hoemann, Sun, & Buschmann, 2007; Hoemann, Sun, Légaré, McKee, & Buschmann, 2005b; Hoemann et al., 2005a, 2007; Khor, 2002) and non-viral gene delivery (Borchard, 2001; Di Martino et al., 2005; Lavertu, Méthot, Tran-Khanh, & Buschmann, 2006; Liu & Yao, 2002).

Due to the strong dependence of the physicochemical, rheological and biological properties of chitosan on its molecular weight and degree of deacetylation (Beri, Walker, Reese, & Rollings, 1993; Denuzière, Yagoubi, Baillet, & Ferrier, 1995; Fee et al., 2003), accurate control and characterization of these two main parameters of chitosan is essential. Unfortunately, chitosans from various manufacturers and different preparation methods can have large size polydispersity, and may form aggregates in solution (Anthonsen, Varum, Hermansson, Smidsrod, & Brant, 1994; Liu & Yao, 2002; Philippova, Volkov, Sitnikova, & Khokhlov, 2001; Schatz, Viton, Delair, Pichot, & Domard, 2003). DDA of chitosan is also usually measured as a bulk average by ^{1}H NMR (Lavertu et al., 2003), without appreciating possible compositional heterogeneity. Some previous workers have examined the heterogeneity of chitosan preparations. For example, distinct DDAs were associated with an acid-soluble versus an acid-insoluble fraction in one commercial

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chitosan (Ottoy, Varum, & Smidsrod, 1996b). In another commercial chitosan, semi-preparative SEC revealed a low molecular weight minority component (7% of the mass) that was chitin-like with DDA in the range of 0–40% while the bulk (75% of the mass) had DDA around 80% (Berth & Dautzenberg, 2002). Chitosan fractionation with semi-preparative SEC has been used by other groups as well to examine the relationships between molecular weight and chemical composition (Bahrke et al., 2002; Ottoy, Varum, Christensen, Anthonsen, & Smidsrod, 1996a; Tommeraas, Varum, Christensen, & Smidsrod, 2001), and intrinsic viscosity and radius of gyration (Berth & Dautzenberg, 2002; Berth, Dautzenberg, & Peter, 1998). For gene delivery applications, oligomers have been obtained by fractionation to identify those with high transfection efficiency in chitosan/DNA complexes (Köping-Höggård, Mel'nikova, Varum, Lindman, & Artursson, 2003). We recently reported a strong influence of molecular weight and DDA in determining the transfection efficiency of chitosan/DNA complexes (Lavertu et al., 2006). Transfection efficiency could change by up to three orders of magnitude, depending on the specific choice of M_n and DDA. Careful inspection of molecular weight profiles of these nitrous acid degraded materials revealed heterogeneity in molecular weight within each preparation that was particularly evident at low molecular weight and low DDA. These findings of a high sensitivity of gene delivery efficiency to M_n and DDA and the likely heterogeneity of these parameters within each chitosan preparation motivated the current study where chitosans are fractionated to characterize heterogeneity in M_n and DDA and to produce chitosans with more homogeneous properties and reduced polydispersity.

Characterization of chitosan molecular weight is achieved by aqueous size exclusion chromatography coupled with multi-angle laser light scattering (SEC–MALLS) to supply absolute number-average molecular weight, M_n, weight-average molecular weight, M_w, and polydispersity index, PDI = M_w/M_n. TSK-gel PW and PW_{XL} columns from Tosoh Bioscience (Barth, 1980; Tosoh Bioscience) are often used for the molecular weight characterization of chitosan by SEC (Fee et al., 2003; Knaul, Kasaai, Bui, & Creber, 1998; Kubota, Tatsumoto, Sano, & Toya, 2000; Kujawa, Moraille, Sanchez, Badia, & Winnik, 2005; Lamarque, Lucas, Viton, & Domard, 2005; Lavertu et al., 2006; Li, Du, & Xu, 2004; Liu, Du, Wang, Hu, & Kennedy, 2004; Muzzarelli, Xia, Tomasetti, & Ilari, 1995; Ottoy et al., 1996a; Tommeraas et al., 2002; Tsaih & Chen, 1999). We recently demonstrated improved reproducibility (reduced coefficient of variation of M_n) in molecular weight analysis of chitosans in SEC–MALLS by reducing the ionic strength of the mobile phase (Nguyen, Winnik, & Buschmann, 2009). We also found that the selection of the appropriate column or set of columns within the PW_{XL} series for any particular chitosan was problematic due to the large polydispersity of chitosans. For example, certain columns provide good separation for a particular range of M_n but if the chitosan molecular weight range is larger than the capacity of the column, the high molecular weight component can be partly excluded from the pore volume, creating split peaks and errors in the obtained values of M_n and M_w. One may use a linear column, such as the TSK-gel $GMPW_{XL}$, with a large molecular weight range ($500–8 \times 10^6$ g/mol for polyethylene glycols and oxides) to circumvent this problem, however, with the attendant reduction in separation efficiency that limits sensitivity and resolution. Yet another approach is to use analytical columns with narrower molecular weight ranges e.g. $G3000PW_{XL}$, $G4000PW_{XL}$, $G5000PW_{XL}$, and $G6000PW_{XL}$, from low to high molecular weights, respectively. However, as mentioned above, few chitosans actually contain M_n distributions that are within the functional range of these individual columns, and combining them in series does not eliminate the problem of artefactual peak splitting due to a high molecular weight component being excluded from one of the columns.

Faced with the above described difficulties of direct SEC–MALLS analysis of heterogeneous chitosans and our need for precise molecular weight information, we initially fractionated chitosans and then characterized molecular weight on SEC–MALLS and DDA on ^{1}H NMR. A summary of semi-preparative SEC methods used previously to fractionate chitosans is shown in Table 1. Since chitosans from different manufacturers can be dissimilar, we analyzed a high molecular weight chitosan from each of two manufacturers in addition to two low molecular weight chitosans, all with DDA at ~80%. These chitosans were fractionated and the fractions characterized by SEC–MALLS and DDA on ^{1}H NMR. We found a surprisingly wide range of molecular weight across the fractions from any one chitosan, and this was not evident prior to fractionation where PDIs were in a range (1.5–2.0) that is not usually considered highly polydisperse. We also observed after fractionation that DDA can depend on M_n in some degraded low molecular weight preparations. Fortunately, we found that our particular fractionation procedure was successful in creating homogeneous and monodisperse chitosans with molecular weights that are low (5–100 kDa) but above the oligomeric range that has been previously obtained (Ottoy et al., 1996a). This latter finding is particularly promising since well controlled and accurately characterized chitosans are required for potential biomedical applications that involve clinical and non-clinical testing subject to oversight from regulatory agencies.

2. Materials and methods

2.1. Chemicals

Acetic acid (EMD, glacial, 99.7%), sodium acetate (Sigma–Aldrich, ACS reagent, 99+%), sodium azide (Sigma–Aldrich, 99%), and double deionized water, ddH_2O, were used for mobile phase preparation. Toluene (HPLC grade, Fisher) and sodium chloride, NaCl (Sigma, ACS reagent, ⩾99.0%) were used for the calibration of DAWN EOS and Optilab DSP, respectively. Deuterium oxide, D_2O (Cat. No. 15,188-2) and deuterium chloride, DCl 35 wt-% in D_2O (Cat. No. 54,304-7) were obtained from Sigma–Aldrich.

2.2. Chitosans

The chitosans (Table 2) were provided by Bio Syntech Canada Inc. (Laval, Que., Canada), and Wako Chemicals USA (Richmond, VA, USA), and were used as received or degraded to low molecular weight samples with a previously described procedure using nitrous acid (Lavertu et al., 2006), or by autoclaving in dilute HCl solution at pH 5.5. DDA was determined by ^{1}H NMR (Lavertu et al., 2003), and nominal molecular weight approximated by analytical SEC as indicated (Table 2).

2.3. Analytical SEC

2.3.1. Analytical SEC–MALLS system

The system was composed of an Agilent 1100 Series Chromatography system, with light scattering and refractive index detectors: a multi-angle laser light scattering (MALLS) DAWN EOS, calibrated with toluene, and a differential refractometer Optilab DSP, calibrated with anhydrous sodium chloride (Wyatt Technology Corporation), which both functioned at 690 nm. The DAWN EOS was normalized with a 5.00 mg/mL solution of a nearly monodisperse pullulan standard P-5 (Shodex Standard P-82, M_w of 5900 Da and M_w/M_n of 1.07) in the acetate buffer used as SEC mobile phase.

2.3.2. Column configurations and mobile phases for analytical SEC

TSKgel PW_{XL} series columns, of 7.8 mm ID and 30 cm length, were used either individually or in combination: $G3000PW_{XL}$,

Table 1
Semi-preparative SEC methods for chitosan fractionation

DDA of chitosan (%)	Chitosan molecular weight or DP[a]		SEC column	Mobile phase	Flow rate	Fraction volume (mL)	Reference
	Before fractionation	After fractionation					
85	M_n 130,000, PDI 2.1	M_n 15,000–490,000, PDI 1.2–1.5	Sepharose CL-6B and Sepharose CL-4B packing (Pharmacia) in series, 9 cm ID × 95 cm	0.02 M sodium acetate buffer, 0.1 M NaCl, 0.02 w/v % NaN_3, pH 4.5	250 mL/h (4.2 mL/min)	182	Ottoy et al. (1996a)
48	M_n 67,000, PDI 1.7	M_n 20,000–100,000, PDI 1.3-1.6					
98		DP 1–13 and above	Biogel P4 (BioRad), 2.5 cm ID × 200 cm	0.05 M ammonium acetate buffer, pH 4.2	25 mL/h (0.42 mL/min)	6	Bahrke et al. (2002)
8	NA[b](oligomers)	DP 1–9					
81		DP 1–13					
41 and >99.9 (Tommeraas et al., 2001)	NA[b] (oligomers)	NA (Tommeraas et al., 2001)	2 Superdex 30 (Amersham Biosciences) in series, 2.5 cm ID × 100 cm	0.15 M ammonium acetate, pH 4.5	0.8 mL/min	4	Tommeraas et al., (2001)
>99.8 (Köping-Höggård et al., 2003)		1200–2800 Da, monodisperse (Köping-Höggård et al., 2003)					Köping-Höggård et al. (2003)
75	M_n 29,300, PDI 2.05	10,000–10,000,000 Da	Sepharose CL-2B packing (Pharmacia), 2.6 cm ID × 95 cm				Berth et al. (1998)
77.5	M_n 18,900, PDI 1.92						
93	M_n 71,900, PDI 2.56						
69–73	M_w 66,900	M_w 30,000–4,000,000	Sepharose CL-2B, 2.5 cm ID × 90 cm	0.02 M sodium acetate buffer, 0.1 M NaCl, pH 4.5	15 mL/h (0.25 mL/min)	16	Berth and Dautzenberg (2002)
73–78	M_w 41,700	M_w 10,000–700,000	Sepharose CL-6B, 2.5 cm ID × 90 cm				
69–75	M_w 153,700	M_w 50,000–10,000,000	Sepharose CL-2B, 2.5 cm ID × 90 cm, and Sephadex G-75, 2.5 cm ID × 45 cm				
97	M_w 178,000	M_w 20,000–400,000					
69	M_w 180,000	NA					
76	M_w 144,000	M_w 60,000–2,000,000					
55	M_w 141,000	NA					
47	M_w 95,000	M_w 20,000–4,000,000					

[a] Degree of polymerization.
[b] Not available.

Table 2
Chitosans used in this study, labeled as T-DDA%,-M_n, with T being the type of chitosan, DDA%, and M_n their nominal degree of deacetylation and number-average molecular weight.

Chitosan code	DDA (%)	M_n (kDa)
B-77-190	77.1	188[a]
W-79-145	78.8	145[a]
W-83-20	82.8	20[b]
N-86-10	85.6	10[b]
A-81-180	80.6	180[a]
A-95-100	94.5	100[b]

Type is B for products from Bio Syntech Canada Inc., W for products supplied by Wako Chemicals USA, N for nitrous acid-degraded Bio Syntech products, and A for autoclaved Bio Syntech products.
[a] Analytical column set used: Tosoh TSKgel G6000 PW_{XL}-G5000 PW_{XL}.
[b] Analytical column used: Tosoh TSKgel G4000 PW_{XL}.

G4000PW_{XL}, G5000PW_{XL}, G6000PW_{XL}, along with a PW_{XL} guard column (Tosoh Bioscience, Montgomeryville, PA, USA). The reference compound for the column exclusion limits was dextran of molecular weight of ca. 2,000,000 g/mol (Sigma, Product No. D-5376). The mobile phases were acetate buffers of different ionic strengths: regular buffer consisting of (0.3 M acetic acid, 0.2 M sodium acetate, and 0.8 mM sodium azide) in ddH_2O, and half-diluted acetate buffer (0.15 M acetic acid, 0.1 M sodium acetate, and 0.4 mM sodium azide), corresponding to total acetate concentrations of 0.501 and 0.250 M, respectively. The use of the half-diluted buffer as mobile phase was recently demonstrated to reduce variability in SEC–MALLS analyses of chitosans (Nguyen et al., in press). Mobile phases were filtered before use through a hydrophilic Durapore® membrane with 0.1 μm pore size (47 mm diameter, Millipore). The diluted buffer was obtained by mixing equal volumes of filtered regular buffer and filtered ddH_2O. The pH of the buffers was between 4.47 and 4.51 (Corning meter, model 320 with Orion electrode model 8115 BN).

2.3.3. Analytical SEC procedure and analysis

Chitosan was dissolved in the above buffers at 1.0 mg/mL (separately weighed replicates) with shaking (LabQuake shaker, Barnstead Thermolyne, model 4002110) at room temperature for 2 days. Solutions were filtered with a syringe filter containing a hydrophilic Durapore® membrane (0.45 μm pore size, 13 mm diameter, Millipore). Analyses were performed at room temperature in the buffer solution as mobile phase, at a flow rate of 0.8 mL/min, with sample injection volume of 100 μL. SEC data were recorded and analyzed with Astra V software (Wyatt Technology Corporation). Values of dn/dc of chitosan were previously determined in half-diluted buffer at 690 nm for five chitosan samples spanning high to low molecular weights (Nguyen et al., in press). The average dn/dc, 0.192 mL/g, was used in the analysis of any chitosan for which dn/dc was not specifically determined. Specific values of dn/dc for B-77-190 and N-86-10 were 0.194 and 0.191 mL/g, respectively. Recovered mass and weight fractions of chitosan were determined using the polymer concentration, obtained from the differential refractometer signal as follows (Wyatt, 1993). For each slice of elution volume, the concentration in slice i was:

$$C_i = \frac{(n_{s,i} - n_r)}{\mathrm{d}n/\mathrm{d}c} \quad (1)$$

where $n_{s,i}$ was the refractive index of the solution in slice i and n_r the refractive index of the pure solvent (reference). Therefore, the total mass of the polymer recovered was determined by Astra software, according to:

$$\text{SEC mass} = \sum_i c_i V_i \quad (2)$$

where V_i was the volume of elution slice i. Since only 0.100 mL from each 5.0 mL fraction from semi-preparative SEC described below was injected in analytical SEC–MALLS, the actual mass of chitosan

in the 5.0 mL fraction was 50 times the calculated mass from Eq. (2) and the % weight fraction in fraction *j*, W_j (%), was simply the % ratio of the calculated mass for fraction j to the sum of the mass of all fractions.

2.4. Fractionation by semi-preparative SEC

2.4.1. Semi-preparative SEC system

The SEC system included a quaternary pump (Beckman system Gold, 126 solvent module), a refractometer (ERC Inc., model ERC-7515A) as detector, and a fraction collector (Waters, fraction collector III). Manual injections were performed with a 2 mL sample loop (Rheodyne, Product No. 3055-018). Prior to use, mobile phases were filtered with a hydrophilic Durapore® membrane (0.1 μm pore size, 47 mm diameter, Millipore) and degassed by sonication for at least 30 min. The semi-preparative column was TSKgel G4000PW (I.D. 21.5 mm, length 60 cm, Tosoh Bioscience, Montgomeryville, PA, USA).

2.4.2. Sample preparation and fractionation

Chitosan solutions were in the mobile phase and filtered with a syringe filter containing a hydrophilic Durapore® membrane (0.45 μm pore size, 25 mm diameter, Millipore). Chitosan concentrations were 2.5 mg/mL for B-77-190 and W-79-145 due to their high viscosity and peak asymmetry observed on the SEC RI trace at higher concentrations. Concentrations for chitosans W-83-20 and N-86-10 were 7.0 mg/mL since they were less viscous. Duplicate solutions were prepared for B-77-190, W-79-145, W-83-20, and N-86-10 and each was injected 3 times for a total of 6 fractionations per chitosan. Runs were performed at room temperature at 5.0 mL/min, with total run time of 43.0 min. The collection time per fraction was 1.0 min, providing 5.0 mL per fraction.

2.5. 1H NMR analyses

2.5.1. Chitosan fractions from semi-preparative SEC

After sampling for SEC analyses, the remainder (~12 mL, except for the fractions individually characterized for repeatability where ~7 mL remained) of each fraction was freeze-dried for 3 days directly from acetate solution, using a Virtis Sentry freeze-dryer (Benchtop 3 L). Each fraction used to reconstruct the chitosan molecular weight profile (see below) was redissolved in a mixture (1.00 mL) of D_2O and DCl solution, with the volume ratio D_2O:DCl solution:0.99:0.01. The deuterated solutions were shaken overnight at room temperature and fractions were analyzed to obtain DDA using a specific procedure that overcame the presence of acetic acid from the acetate buffer. Analyses were performed with a Bruker BioSpin Avance 500 MHz NMR spectrometer equipped with a Bruker Biospin Inverse Triple resonance multinuclear (TBI) 5 mm probe, with ca. 750 μL of deuterated solution. Two measurements at 60 °C were performed on all sample solutions: a preliminary experiment (single 90° pulse, relaxation delay, d1 of 1 s, acquisition time, at of 3.5 s, and number of scans, ns of 8) was performed with the spectral window of 0–4735 Hz, to examine the anomeric peak region and verify magnet shimming. Then a selective excitation of the anomeric protons was applied (90° shaped pulse with d1 = 6 s, at = 3.5 s and ns = 64) to avoid excitation of water and acetic acid and specifically increase the peak sensitivity of the anomeric protons. Data were recorded and analyzed using X-WIN NMR software (version 3.5, Bruker BioSpin), including signal to noise, S/N, ratios (measured after automatic baseline correction) and signal integration (measured after manual baseline correction of the anomeric protons for the selective excitation experiments).

2.5.2. Original unfractionated chitosans

The chitosans used as starting materials (B-77-190, W-79-145, W-83-20, and D-86-10) for fractionation were analyzed using a 400 MHz Varian instrument. The polymers (dry powders without any acetic acid) were dissolved directly in D_2O and DCl solution (volume ratio D_2O:DCl solution:0.99:0.01). To be consistent, the solutions were run using the same procedures and instrumentation as their corresponding fractions.

3. Results and discussion

3.1. The broad range of molecular weights present in many chitosan preparations requires careful examination to choose the appropriate column

The difficulty in selecting the appropriate analytical SEC column or combination of columns arises from the typically large range of molecular weights present in commercial chitosans. As an illustration, Fig. 1 shows the SEC–MALLS traces obtained for chitosan A-81-180 with different analytical TSKgel PW_{XL} columns. We found monomodal traces on the linear $GMPW_{XL}$ and on the combination of $G6000PW_{XL}$ and $G5000PW_{XL}$, but with a wider peak width for the latter ~12 versus ~5 min, showing enhanced separation with the $G6000PW_{XL}$ and $G5000PW_{XL}$. M_n and PDI were 196,900 and 1.55 for $GMPW_{XL}$, and 175,700 and 1.38 on $G6000PW_{XL}$–$G5000PW_{XL}$. In contrast, when analyzed with the lower molecular weight range columns $G4000PW_{XL}$, $G3000PW_{XL}$, individually and in combination, artefactual peak splitting was observed (Fig. 1), with a peak eluting at a lower elution time, indicating a large molecular weight, at 6.8, 6.4, and 12.5 min on the LS traces for runs on $G4000PW_{XL}$, $G3000PW_{XL}$, and the combination of the two, respectively. These peaks were in the exclusion volume of these columns, since they occurred prior to the elution time of a dextran with nominal MW 2,000,000 g/mol whose maximal LS peak signal

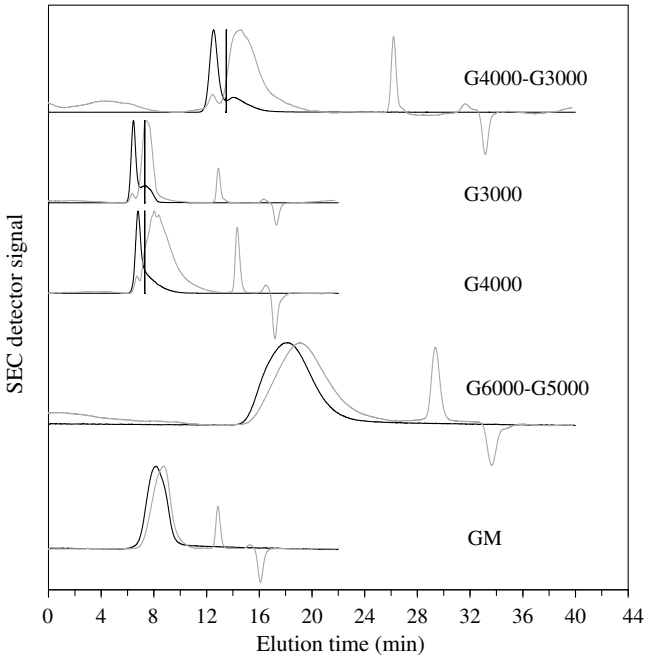

Fig. 1. SEC–MALLS traces for chitosan A-81-180 in regular buffer, with LS traces (90° angle) in black and RI traces in grey, using different columns (from top to bottom): guard PW_{XL}–$G4000PW_{XL}$–$G3000PW_{XL}$ (G4000–G3000), guard PW_{XL}–$G3000PW_{XL}$ (G3000), guard PW_{XL}–$G4000PW_{XL}$ (G4000), column set guard PW_{XL}–$G6000PW_{XL}$–$G5000PW_{XL}$ (G6000–G5000), $GMPW_{XL}$ (GM). Vertical lines on the top three traces correspond to the elution times for the excluded volume of the column or column combination.

occurred at elution times of 7.3, 7.3, and 13.5 min., for the G4000PW$_{XL}$, G3000PW$_{XL}$ individually and in combination, respectively. Another chitosan sample of lower molecular weight, A-95-100, displayed similar characteristics when run on the same columns and combinations of columns.

The above results suggest that chitosans with a molecular weight higher or similar to chitosan A-95-100, i.e. with M_n above 80,000–100,000 g/mol and PDI ratio of ~1.6, should be analyzed with the column set G6000PW$_{XL}$–G5000PW$_{XL}$, since lower molecular weight columns introduced peak splitting due to some polymer eluting in the excluded volumes. Thus one may expect that chitosans with lower molecular weight could be precisely analyzed with the columns G4000PW$_{XL}$ and/or G3000PW$_{XL}$, however, we found this to not be the case for all chitosans. For example, chitosan N-86-10 with a nominal molecular weight of 10,000 g/mol, was obtained by nitrous acid degradation and showed a large polydispersity with a bimodal distribution in the SEC–MALLS traces, even with the linear column GMPW$_{XL}$, where two distinct populations were found - a high molecular weight component of M_n = 463,000 and PDI = 2.84 containing ~4 wt% of the recovered mass, and the main polymer peak with M_n = 7500 and PDI = 1.29 containing ~96 wt% of the recovered mass (Fig. 2). However, resolution of these two peaks was incomplete. The high molecular weight component could have been the result of the aldehyde containing terminal group produced by HONO at the new reducing end which could either react with another aldehyde terminal group by aldolization, or with the amino groups of a deacetylated unit (Tommeraas et al., 2001). Yet another possibility is that this high molecular weight component was present in the original undegraded chitosan as a chitin-like component, which was less susceptible to HONO degradation, as the cleavage was shown to occur at the glycosidic bond following a deacetylated unit where the amino group had reacted with nitrous acid (Varum, Ottøy, & Smidsrod, 2001). We are currently investigating these possibilities. This same chitosan was also run on the G4000PW$_{XL}$, G3000PW$_{XL}$, and the combination of the two, resulting in peak splitting as for the previously described high molecular weight chitosans. Thus, even for this low molecular weight chitosan, some of the polymer eluted in the excluded volumes of the three low molecular weight column configurations, and two poorly resolved peaks were found in the linear and high molecular weight configurations. Thus none of these column sets could accurately measure the molecular weight profile of samples such as N-86-10, produced by HONO degradation that had high polydispersity, and was one of our most effective chitosans for gene delivery (Lavertu et al., 2006). Similar issues were observed for the chitosan W-83-20. For this reason and the observed peak splitting for the high molecular weight chitosans in the low molecular weight range column configurations, we decided to fractionate chitosans and then analyze the collected fractions on single analytical columns with a molecular weight range appropriate for each particular fraction.

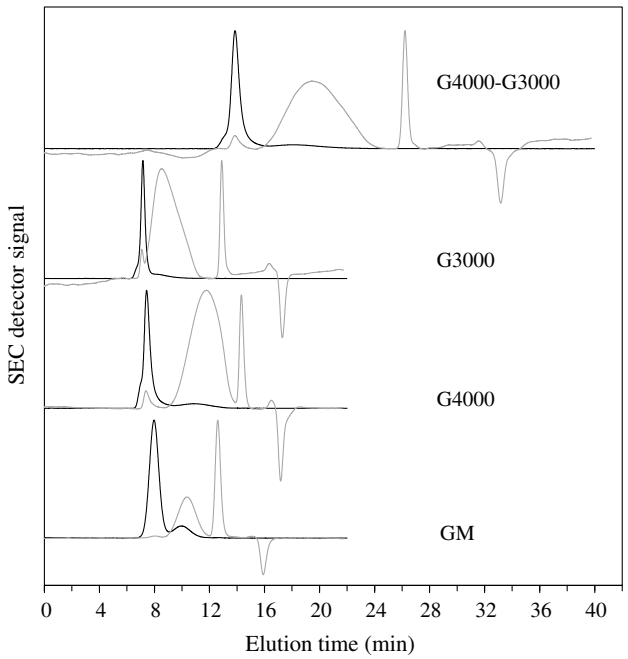

Fig. 2. SEC–MALLS traces for chitosan N-86-10 in regular buffer, with LS traces (90° angle) in black and RI traces in grey, from top to bottom: on guard PW$_{XL}$–G4000PW$_{XL}$–G3000PW$_{XL}$ (G4000–G3000), guard PW$_{XL}$–G3000PW$_{XL}$ (G3000), guard PW$_{XL}$–G4000PW$_{XL}$ (G4000), GMPW$_{XL}$ (GM).

3.2. Fractionation on semi-preparative SEC followed by analytical SEC permitted estimation of chitosan molecular weight profile

Four chitosans were fractionated using the semi-preparative SEC system with the semi-preparative column G4000PW, namely B-77-190, W-79-145, W-83-20, and N-86-10. B-77-190 and N-86-10 were obtained from Bio Syntech Canada Inc., while W-79-145 and W-83-20 were from Wako Chemicals, USA. B-77-190 and W-79-145 had high nominal molecular weight, 190 and 150 kDa, respectively, while W-83-20 and N-86-10 were low molecular weight at ~30 and ~10 kDa, respectively. We chose the DDA of all four chitosans to be ~80% in order to be comparable (see Table 2). Analytical SEC was performed in the mobile phase that was half-diluted, (0.15 M acetic acid, 0.1 M sodium acetate) since we previously showed this choice to reduce variability in M_n compared to the undiluted mobile phase (0.3 M acetic acid, 0.2 M sodium acetate) (Nguyen et al., 2009). For each chitosan, duplicate solutions were prepared and each was injected three times producing triplicates of each duplicate. Representative RI elution traces on the semi-preparative G4000PW revealed monomodal distributions for B-77-190 and W-79-145, with one peak eluting between the 17th and 28th minute for B-77-190, and between the 16th and 29th minute for W-79-145 (Fig. 3). For the low molecular weight chitosans (W-83-20, N-86-10), bimodal distributions were observed with an additional high molecular weight peak appearing between the 16th and 18th minute for W-83-20, and between the 16th and 19th minute for N-86-10. Fractions (5.0 mL) at the same collection time from the three triplicates were pooled together to provide sufficient mass for ^{1}H NMR analyses described below. In addition, we examined the reproducibility of the fractionation between the three triplicates on analytical SEC, using four fractions equally distributed along the main polymer peak.

For both pooled fractions and the selected individual fractions, approximately 1.5 mL was taken, filtered, and analyzed in SEC–MALLS as described above in half-diluted buffer, and the remainder of each pooled fraction was freeze-dried for three days for DDA determination of the fractions using ^{1}H NMR. The fractions were analyzed on SEC–MALLS from last (low molecular weight) to first (high molecular weight) starting with the low molecular weight range G3000PW$_{XL}$ column and then changing to the G4000PW$_{XL}$ for the fraction where the main polymer peak appeared partly in the excluded volume of the G3000PW$_{XL}$. The column was then similarly changed to the G5000PW$_{XL}$, and then to the G6000PW$_{XL}$ when a portion of the main polymer peak appeared in the excluded volume of the current column. M_n, PDI and the weight fraction, W_j (%), for each fraction were calculated.

3.3. Fractionation of low molecular weight chitosan N-86-10

Based on the RI elution trace of N-86-10 (Fig. 3) fractions were collected from the 16th minute to the 34th minute, i.e. F16 to

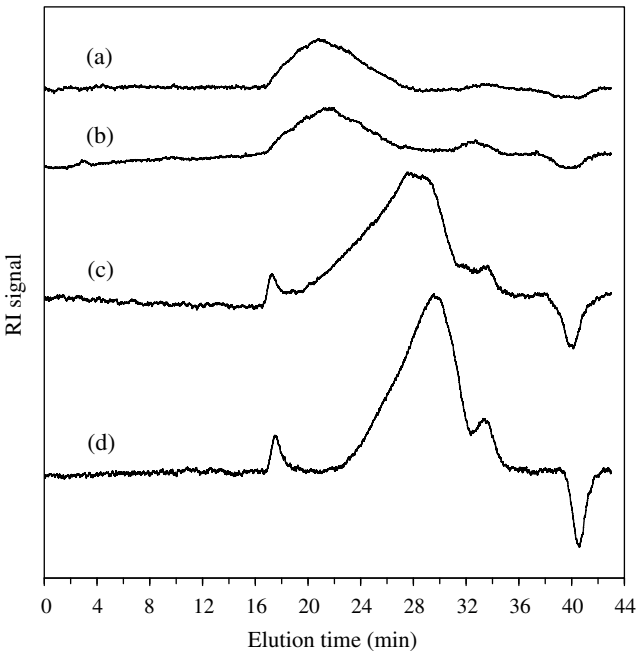

Fig. 3. Representative RI signal traces on the semi-preparative column G4000PW of chitosans (a) B-77-190, (b) W-79-145, (c) W-83-20, (d) and N-86-10.

F34. F34 to F28 were run on the G3000PW$_{XL}$, F28 to F23 on G4000PW$_{XL}$, and F23 to F16 were run on G6000PW$_{XL}$ (Fig. 4). A small but insignificant high molecular weight peak began to emerge on F30 and then grew in intensity for earlier fractions (Fig. 4). This high molecular weight peak was in the excluded volume of the G3000PW$_{XL}$ and G4000PW$_{XL}$ but not in the G6000PW$_{XL}$, where it was effectively separated and was the dominant mass in fractions F22 to F17. The other low molecular weight chitosan that was fractionated, W-83-20, had similar characteristics on analytical SEC.

3.4. Fractionation of high molecular weight chitosans

Collection of the fractions for chitosans B-77-190 and W-79-145 were performed from the 16th minute to the 31st minute, F16 to F31, in accordance with the RI trace of their preliminary runs (Fig. 3). SEC–MALLS analyses of the fractions were similar for both high molecular weight chitosans where in F28 to F31, the chitosan peak eluted in the exclusion volumes of both columns G3000PW$_{XL}$ and G4000 PW$_{XL}$, so it could not be properly analyzed. F27 was run on G5000PW$_{XL}$ while F17 to F26 were run on G6000PW$_{XL}$ and showed monomodal distribution.

3.5. Fractionation was repeatable according to low M_n variability between triplicates

We examined the repeatability of fractionation since obtaining sufficient mass in a single fraction for either analytical or experimental purposes may require pooling of fractionation runs. The coefficient of variation of M_n for the triplicate runs (CV (%) = standard deviation/mean of M_n) was calculated for fractions F18, F21, F24, and F27 for the high molecular weight chitosans (B-77-190 and W-79-145) and F18, F23, F28, and F32 for the low molecular weight chitosans (W-83-20 and N-86-10). In general, when sufficient mass was present in the fraction to perform an accurate SEC analysis, the obtained CVs were low in the range 3–12%, indicating good repeatability. In contrast, the CV of F27 for high molecular weight chitosans was 32–49% since this represented the tail of the elution profile (Fig. 3), where the concentration of chitosan in the fraction of 0.014–0.034 mg/mL was insufficient for accurate analytical SEC, whose lower limit is ~0.07 mg/mL.

3.6. Recovered mass is nearly complete

The total recovered mass was determined from the sum of the masses in the tubes of the fractions based on the RI signal as described in Materials and Methods. For the high molecular weight chitosans, a small loss occurred corresponding to 6.4% and 4.9% of the total 5 mg injected for the duplicates of B-77-190 and of 12% and 14% for the duplicates of W-79-145, possibly due to sample filtration through a 0.45 μm syringe filter prior to the fractionation. For the low molecular weight chitosan W-83-20, the total mass loss during fractionation was negligible at 0.27% and 0.47% for duplicates. In contrast, there was a calculated mass gain for N-86-10 of 8.0% and 6.3% for the duplicates, possibly due to a small error in the dn/dc value used in the calculation, since calculated concentrations are inversely proportional to dn/dc. We used dn/dc of 0.191 mL/g for N-86-10 based on measurements prior to fractionation. Since dn/dc varies with molecular weight for oligomers according to dn/d$c = a - b/M_n$ (Chance, Baniukiewicz, Mintz, & Ver Strate, 1995; Itakura et al., 2004; Podešva, Procházka, & Medin, 1995), it is possible that this created an overestimation of mass when low molecular weight fractions are predominant. This overestimation in calculated mass was not observed for W-83-20 possibly since its molecular weight was higher than for N-86-10.

3.7. Reconstitution of molecular weight profiles from fractions

For high molecular weight chitosans (B-77-190 and W-79-145), M_n and the recovered mass from fractions F17 to F26 on G6000PW$_{XL}$ and F27 on G5000PW$_{XL}$ were used to reconstitute the molecular weight profile of the polymer before fractionation (Fig. 5). The other fractions were not used since the calculated mass in the fraction was insignificant, with high SEC–MALLS molecular weight calculation error (from Wyatt software), or the polymer peak was in the excluded volume of the analytical column. We found B-77-190 to contain significant mass in the range of M_n from 65 to 400 kDa, while for W-79-145 this range was 20 to 600 kDa. This result was particularly informative since analytical SEC of the original unfractionated chitosans provided M_n = 188 kDa for B-77-190 and M_n = 145 kDa for W-79-145 with PDIs of 1.73 and 1.83, respectively. One may have deduced from these latter PDI values that these chitosans were similar and of acceptable polydispersity, while analysis of the fractionated material revealed a larger range of molecular weight in both chitosans with W-79-145 yet larger than B-77-190, demonstrating the value of analyzing fractionated chitosans to appreciate the polydispersity of their compositions.

For low molecular weight chitosans, there were two distinct populations that required analyses. The high molecular weight peak was reconstituted from fractions F16 to F19 for W-83-20 and F17 to F21 for N-86-10, all analyzed using column G6000PW$_{XL}$. The low molecular weight main polymer component used the following fractions for W-83-20: F20 to F22 on G6000PW$_{XL}$, F23 on G5000PW$_{XL}$, F24 to F28 on G4000PW$_{XL}$, and F29 to F31 on G3000PW$_{XL}$, while for N-86-10 the following were used: F22 and F23 on G6000PW$_{XL}$, F24 to F28 on G4000PW$_{XL}$, and F29 to F32 on G3000PW$_{XL}$. Here again fractions were used when high SEC–MALLS molecular weight calculation error was low, and the polymer was not in the excluded volume.

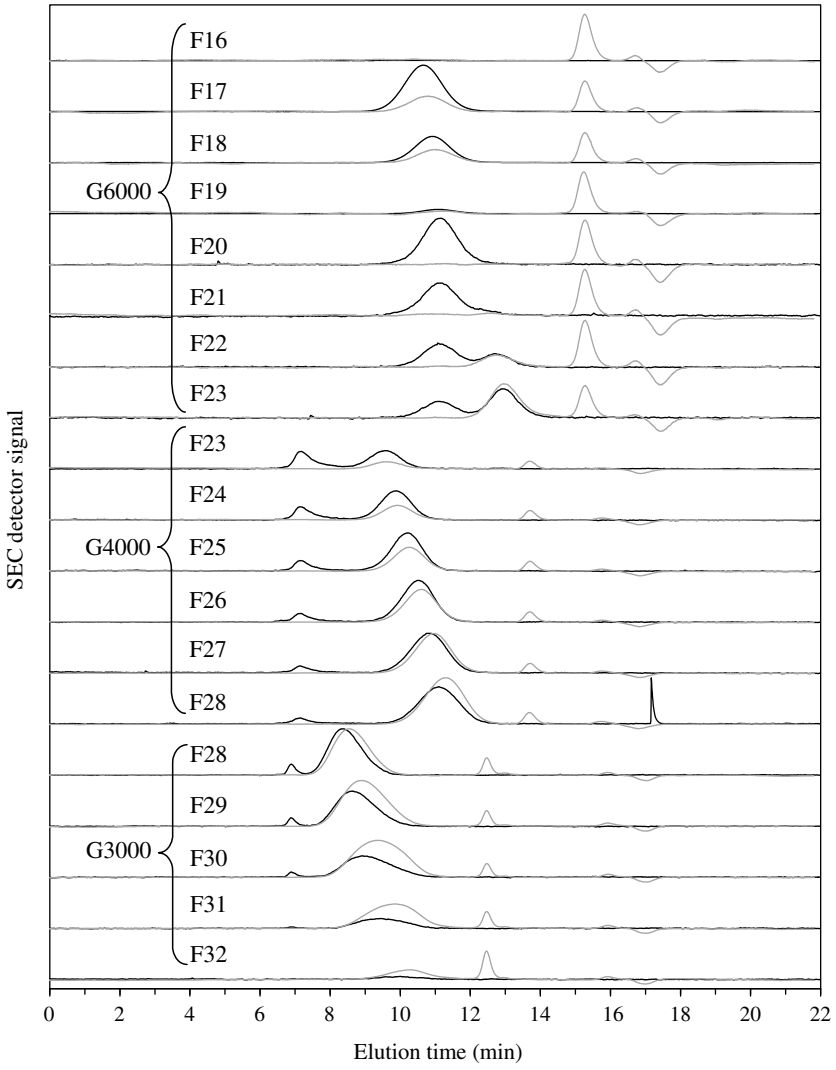

Fig. 4. SEC–MALLS traces for fractions F16 to F32 for chitosan N-86-10 (duplicate 2) in half-diluted buffer: on G3000PW$_{XL}$ (G3000, F28 to F32), G4000PW$_{XL}$ (G4000, F23 to F28), and G6000PW$_{XL}$ (G6000, F16 to F23). LS traces (90° angle) are shown in black and RI traces in grey. The one (or two) leftmost peaks on the traces are chitosan while the two rightmost peaks are solvent.

For W-83-20, we found the high molecular weight peak to contain M_n in the range of 200–2500 kDa with PDI ranging from 1.7 to 2.6 while for the main component, the low molecular weight peak, the range was 7–120 kDa with PDI ranging from 1.1 to 1.3 (Fig. 5). The unfractionated chitosan run directly on SEC with G4000PW$_{XL}$ had M_n (PDI) of 1606 kDa (1.51) for the high molecular weight peak and 19.5 kDa (1.96) for the low molecular weight peak. This fractionation procedure therefore successfully produced nearly monodisperse chitosans in the low molecular weight range (7–120 kDa) from a polydisperse starting material (PDI of 1.1–1.3 versus 1.96). Nonetheless these fractions were accompanied by a low quantity of high molecular weight chitosan in fractions F23 to F31, constituting less than 0.8 wt-% of the mass in the fraction. For N-86-10, we found the high molecular weight peak to contain M_n in the range of 115 kDa to 2700 kDa with PDI ranging from 1.0 to 2.7 and for the low molecular weight peak, the range was 4–50 kDa with PDI ranging from 1.0 to 1.4 (Fig. 5). The unfractionated chitosan run directly on SEC–MALLS with G4000PW$_{XL}$ had M_n (PDI) of 2064 kDa (1.13) for the high molecular weight peak and 9.9 kDa (1.86) for the low molecular weight peak. Here again, fractionation on G4000PW$_{XL}$ was successful in obtaining monodisperse low molecular weight fractions although many (F22 to F31) were accompanied by a high molecular weight component in low proportion compared to the main peak.

3.8. Calculation of M_n, M_w from fractions

In order to examine the consistency of molecular weights obtained after fractionation with those obtained before, we calculated M_n and M_w of the unfractionated chitosans from the M_n and M_w of the fractions with low molecular weight calculation error and without any peak in the excluded volume, and compared them with M_n and M_w directly measured on the unfractionated material. The number average molecular weight M_n is defined as:

$$M_n = \frac{\sum_j n_j M_j}{\sum_j n_j} = \frac{\sum_j m_j}{\sum_j \frac{m_j}{M_j}} \quad (3)$$

where n_j is the number of moles and m_j the mass of polymer with molecular mass M_j. Christensen and Smidsrod (Christensen, 2007) have shown that for a population of j polydisperse samples, each with average molecular mass of $M_{n,j}$ and $M_{w,j}$, the overall M_n of the entire population is found from the same equation using the M_n of each fraction, $M_{n,j}$:

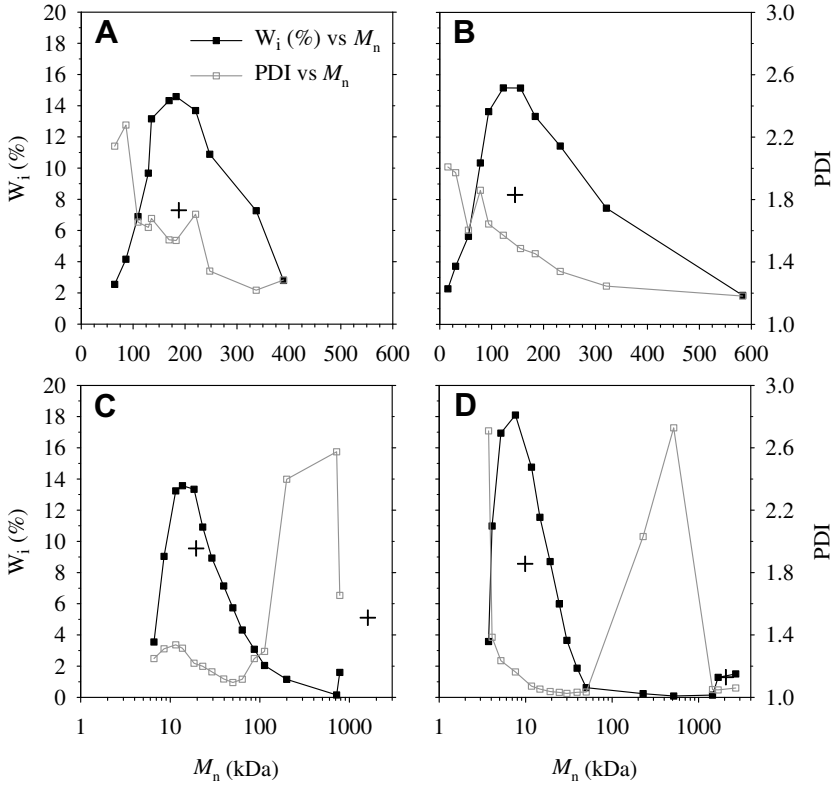

Fig. 5. Weight content, W_i (%) (solid black squares and leftmost axis for all four graphs) and PDI = M_w/M_n (open grey squares and rightmost axis for all four graphs) versus M_n (kDa) of chitosans (A) B-77-190 duplicate 2, (B) W-79-145 duplicate 2, (C) W-83-20 duplicate 1, (D) N-86-10 duplicate 2. The crosses (+) show M_n and PDI before fractionation. M_n for fraction F27 of W-83-20 (C) was offscale at 2,500,000 Da and is not shown.

$$M_n = \frac{\sum_j m_j}{\sum_j \frac{m_j}{M_j}} = \frac{\sum_j (\sum_i m_i)_j}{\sum_j \left(\sum_i \frac{m_i}{M_i}\right)_i} = \frac{\sum_j \left(\sum_i m_i\right)_j}{\sum_j \left(\left(\sum_i \frac{m_i}{M_i}\right) \times \frac{m_j}{m_j}\right)_j}$$
$$= \frac{\sum_j \left(\sum_i m_i\right)_j}{\sum_j \left(\left(\sum_i \frac{m_i}{M_i}\right) \times \frac{m_j}{\sum_i m_i}\right)_j} = \frac{\sum_j m_j}{\sum_j \left(\frac{m_j}{M_{n,j}}\right)} \quad (4)$$

Similarly, M_w of the overall population and PDI (M_w/M_n) can be obtained from the $M_{w,j}$, of each fraction:

$$M_W = \frac{\sum_j m_j M_j}{\sum_j m_j} = \frac{\sum_j (\sum_i m_i M_i)_j}{\sum_j m_j} = \frac{\sum_j \left(\left(\sum_i m_i M_i\right) \times \frac{m_j}{m_j}\right)_j}{\sum_j m_j}$$
$$= \frac{\sum_j \left(\left(\sum_i m_i M_i\right) \times \frac{m_j}{\sum_i m_i}\right)_j}{\sum_j m_j} = \frac{\sum_j (M_{W,J} \times m_j)_j}{\sum_j m_j} \quad (5)$$

The dn/dc value used to calculate mass fractions did not account for dependency on molecular weight that could occur particularly for low molecular weight fractions.

For high molecular weight chitosans, recalculated values of M_n and M_w from fractions were systematically lower, and PDI were higher or equal compared to those obtained on unfractionated material (Table 3). For B-77-190, M_w/M_n (PDI) was 325/188 (1.73) before fractionation and 289/162 (1.78) calculated from fractions. For W-79-145, M_w/M_n (PDI) were 265/145 (1.83) before fractionation and 260/107 (2.43) calculated from fractions. The reduction in M_n and corresponding increase in PDI when calculated from fractions was more significant for the more polydisperse W-79-145. Taking into account these results along with the reconstituted molecular weight profile of this chitosan (Fig. 5), it appears that direct SEC–MALLS analysis of polydisperse chitosans underestimates their polydisperse character.

For low molecular weight chitosans, a similar trend was apparent where M_n and M_w from fractions were systematically lower and PDI were higher or equal to those obtained on unfractionated material (Table 3). Here however, the presence of two distinct populations, a high and low molecular weight fraction, in the original unfractionated material amplified the differences between values obtained with fractionation versus without, and exemplified the difficulty of characterizing the molecular weight profile at all in the unfractionated case. The need to run W-83-20 and N-86-10 on the G4000PW$_{XL}$ to separate the low molecular weight component resulted in the high molecular weight component eluting in the excluded volume without being effectively separated, creating a gross overestimation of the molecular weight of the high molecular weight fraction that was 2–3 times the value determined after fractionation, e.g. 1606 versus 587 kDa for the M_n of W-83-20. Once again, taking into account the large range of molecular weights found in the molecular weight profile reconstituted from fractions (Fig. 5), it was clear that direct SEC analysis of these types of polydisperse chitosans without fractionation is problematic and can greatly underestimate its polydisperse character. Similar results were found with chitosan N-86-10.

3.9. Evolution of DDA with fractions

DDA can be determined by ^{1}H NMR. Here, the presence of acetate salt made the peak for the acetyl groups of chitosan unusable

Table 3
Average molecular weights and polydispersity ratios, recalculated for chitosans B-77-190, W-79-145, W-83-20, and N-86-10, from the molecular weight data of the fractions collected using the preparative column, and run with single PW_{XL} columns in half-diluted buffer

Chitosan	Recalculated from fractionation			From direct analytical GPC[c]		
	M_n[a]	M_w[a]	PDI[b]	M_n	M_w	PDI
B-77-190	162,100	288,600	1.78	188,100	324,700	1.73
W-79-145	107,000	260,500	2.43	145,000	265,300	1.83
W-83-20						
P1	587,500	1,674,000	2.85	1,606,000	2,428,000	1.51
P2	17,200	34,900	2.03	19,500	38,100	1.96
N-86-10						
P1	996,100	2,111,000	2.12	2,064,000	2,331,000	1.13
P2	8000	13,600	1.69	9900	18,300	1.86

P1 refers to the high molecular weight peak; P2 refers to the main polymer peak.
[a] Average of the duplicates.
[b] PDI = M_w/M_n.
[c] Columns used: guard-G6000PW_{XL}-G5000PW_{XL} for chitosans B-77-190 and W-79-145, G4000PW_{XL} for chitosans W-83-20 and N-86-10.

because their peaks overlapped. However, DDA can be determined using the peak integral of the anomeric proton H1A of the acetylated units instead of the acetyl groups themselves, given sufficient signal to noise (S/N) ratio and resolution from the neighboring peaks, water and the anomeric proton H1D of the deacetylated units. Preliminary experiments revealed selective excitation of the anomeric proton region, i.e. in a spectral width corresponding specifically to the anomeric zone, to be effective in avoiding the strong acetate peaks. A solution of W-83-20 in D_2O:DCl without acetate salts was compared to chitosan dissolved directly in half-diluted buffer, freeze-dried, and redissolved in D_2O:DCl in order to mimic fractionated samples. Analyses at 60 °C with selective excitation found a DDA of 85.0% for the solution without acetate versus 90.9% with acetate salts suggesting the analyses of the fractions overestimate DDA by 5–6%. The validity of the derived DDA of a particular fraction was accepted when the S/N ratio > 8 for both H1A and H1D peaks. For high molecular weight chitosans B-77-190 and W-79-145 this resulted in accepting DDAs from only 2 to 4 fractions, and they were found uniformly at ~90%, without significant variation with fraction number. Here again the presence of acetate resulted in an overestimation of DDA by ~10% compared to that of the starting material. For W-83-20, accepted DDAs were between 81.2% and 94.9% with no apparent relationship to M_n. These results are consistent with Ottoy et al. who found that chitosan DDA was not dependent on molecular weight at the molecular weight range of the three chitosans described above where three selected fractions with M_n of 490, 220, and 48 kDa had DDAs of 82%, 81%, and 84%, respectively (Ottoy et al., 1996a).

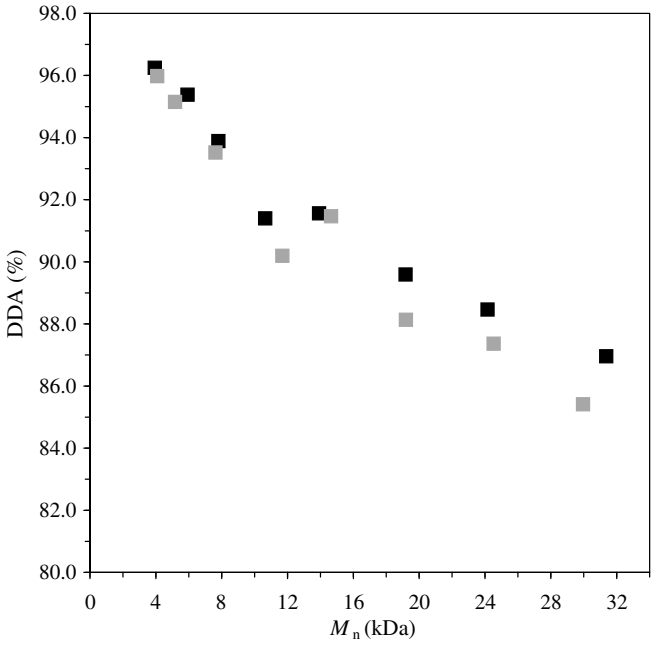

Fig. 6. Degree of deacetylation, DDA (%), of the fractions F24 to F31 of the low molecular weight chitosan N-86-10 as a function of M_n, for duplicate 1 (black) and duplicate 2 (grey).

For N-86-10 a heterogeneity in DDA was found and was linked to molecular weight where DDA increased from 87% for fraction F24 where M_n = 31 kDa to 96% for F31 where M_n = 4 kDa (Fig. 6). This heterogeneity in DDA that is M_n dependent could be due to the recovery method after degradation with nitrous acid by precipitation in concentrated sodium hydroxide, followed by centrifugation and resuspension in deionized water (Lavertu et al., 2006). Since low molecular weight chitosan with low DDA is more soluble in water, the centrifugation may have selectively recovered higher DDA components which had low molecular weight. Fractionating chitosan prior to analyses has therefore permitted the detection of this significant heterogeneity of DDA within a particular chitosan preparation. This information can be used to modify the recovery procedure and verify that the resulting product has acceptably homogeneous properties.

4. Conclusions

Accurate characterization of the molecular weight profile of chitosans from various sources and preparation methods can be problematic when applying directly SEC with a particular set of analytical columns, due to the large range of molecular weight present in most chitosans, which typically exceeds the functional molecular weight range of the SEC column(s). This usually necessitates accepting low resolution data from columns with a wide molecular weight range or introduces errors due to artefactual peak splitting when combining columns with greater resolving power. To overcome these issues, chitosans can be fractionated on a semi-preparative SEC and analytical SEC–MALLS be performed on the fractions with the appropriate single analytical column, with the fractions also characterized by ^{1}H NMR for DDA. This analysis revealed that direct SEC of chitosans can significantly underestimate polydispersity and in some cases does not provide reliable estimates of the molecular weight profile when distinct populations are present. Fractionation not only provided a greater level of detail on the molecular weight profile distribution but also served to reveal important heterogeneities in DDA of one particular chitosan. We also demonstrated that the fractionation procedure was reproducible, with low variability, and has the ability to pro-

duce homogeneous monodisperse chitosans in the molecular weight range of 5–100 kDa that are particularly useful in biomedical applications such as gene delivery.

Acknowledgements

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