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Characterization of chitosans via coupled size-exclusion chromatography and multiple-angle laser light-scattering technique

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ABSTRACT

Chitosan [(1 → 4)-2-amino-2-deoxy-β-D-glucan], a cationic biopolymer, was obtained by deacetylation of chitin samples from various sources. Nine chitosan samples differing in their degree of acetylation and molecular weight were used in this study. They were characterized by size-exclusion chromatography (SEC) coupled to a multiple-angle laser light-scattering (MALLS) photometer. The results show that accurate, reliable, and reproducible molecular weight and radius of gyration data are obtained by using SEC–MALLS. These samples were also characterized by a conventional secondary calibration technique. The molecular weights obtained by secondary calibration were found to be 2–3 times higher than those obtained using SEC–MALLS. The dissimilarity between the chitosan samples and polymer standards employed for calibration were presumed to be responsible for overestimation of the molecular weights. Therefore, we conclude that the unavailability of chitosan polymer standards underscores the need of an absolute molecular-weight determination technique, such as SEC–MALLS. This technique can be used for establishing structure–function relationships of chitosans and as a quality control tool for monitoring bulk production of chitosans.

INTRODUCTION

Chitosan [(1 → 4)-2-amino-2-deoxy-β-D-glucan], a biodegradable and biocompatible amino(poly)saccharide, has been a subject of intensive research for well over a decade[1,2]. Interest in chitosan has been sparked by this materials unique properties and numerous potential applications[2,3]. Chitosan may have a future as an antibacteriostatic/anti-fungal agent; as a gelling agent in drug delivery systems; as a film, fiber, or gel in wound dressing; and in chelating metal ions from waste streams in detoxifying chemical wastes (or other novel applications)[1–3]. These

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potential applications of chitosans are envisioned primarily as a result of its unique physicochemical properties.

The physicochemical behavior of polymer solutions[4] and the numerous other physical properties of polymers are known to be functions of their molecular-weight averages and molecular-weight distributions[5]. More specifically, the biological activity of chitosan (such as, its blood anticoagulant and wound-healing activity) are dependent on molecular weight[6]. The mechanical properties of chitosan films, gels, and fibers are also known to be a function of their molecular weights. Similar dependencies have been observed for other polymers[5]. Polymer molecular weight is also known to be an important quality-control parameter[1] in monitoring chitosan production on a commercial scale. The foregoing reasons emphasize the scientific and engineering needs for an accurate and reproducible method of determining the molecular weight and molecular-weight distribution of chitosan.

Classical techniques (such as sedimentation, static light scattering, size-exclusion chromatography, etc.) used for determining polymer molecular weights are intricate and time consuming, thus several light-scattering experiments are required to produce a Zimm plot from which molecular weight can be determined[7]. In addition, great care in sample preparation needs to be taken, as several factors (notably the presence of impurities in the sample being characterized, errors in polymer concentration measurement, etc.) affect the accuracy of the molecular-weight data obtained from these techniques. Further complications arise when characterizing chitosans, primarily due to the materials polyelectrolytic nature, strong intramolecular and intermolecular interactions[8], and a tendency for the polymer to self-aggregate in solution[9]. Generally, the aggregation process is presumed responsible for an "overestimation" in the M_w value[9] of chitosan obtained from "stand alone" static light scattering. Irrespective of these problems, the importance of chitosan has stimulated research into fundamental methods of macromolecular characterization; particularly as directed to molecular-weight determinations.

The literature indicates that researchers have frequently resorted to an indirect approach for obtaining molecular weights of chitosan; measuring intrinsic viscosity and using the Mark–Houwink equation (and its associated constants) to calculate molecular weights for chitosan[6,8]. This "indirect method" is prone to many errors as evidenced by the difference in Mark–Houwink constants (Table I). The use of viscometry as a method of determining molecular weights is an approach not usually employed by researchers working with other polymer systems. Generally, such methods as size-exclusion chromatography (SEC) or some colligative property (light scattering, osmometry, etc.) are used for this purpose. In those cases where the polymer is more difficult to characterize, combinations of methods have been developed.

Chitosans have been "characterized" in the literature by size-exclusion chromatography (SEC)[10,11]. SEC is a powerful method of obtaining macromolecular characteristics provided that the chromatograms can be calibrated. Calibration of

TABLE I

Mark–Houwink constants for chitosans [a]

Solvent	a	$K \times 10^4$
0.1 M AcOH [b]–0.2 M NaCl	0.93	18.1
0.1 M AcOH–0.02 M NaCl	1.26	0.3
0.33 M AcOH–0.3 M NaCl	1.02	34.1
0.2 M AcOH–0.1 M NaCl–4 M Urea	0.71	890.3
2% AcOH–0.2 M AcONa–equal ionic strength Cl_2CHCO_2H	0.85	12.8

[a] Abstracted from ref. 8. [b] Acetic acid.

chromatograms can be performed by "direct" or "indirect" (or secondary) means. The unavailability of "narrow"-molecular-weight chitosan standards has dictated the use of secondary calibration methods; employing "broad"-molecular-weight, water-soluble standards (such as dextrans). However, the use of these secondary standards has been shown to cause significant errors in determination of the molecular weight of chitosan[12] (that is, due to "limitations" in calibration methodologies). Therefore, it can be concluded that the most "often-used" (in the case of chitosan) SEC secondary calibration techniques exhibit inherent limitations, and thus researchers have continually sought more accurate and direct methods for determining molecular weights of chitosan.

Absolute average molecular weights of chitosans have been determined by "stand-alone" membrane osmometry[9] and static light scattering[6]. These techniques too are limited, as molecular-weight distribution (MWD) cannot be obtained from such experiments without recourse to some prior polymer fractionation (a time-consuming process); thus reintroducing the original characterizational problem (namely developing a routine method). Size-exclusion chromatography coupled to an online absolute molecular-weight determining device (such as a light-scattering photometer) is currently the best technique available[13] for quick and accurate determination of polymer molecular weights and their distributions. Only limited use of SEC coupled to online light scattering has been attempted for characterizing chitosans[14,15]. No attempt has been made to determine the accuracy and reproducibility of the molecular weight data obtained by these methods[14,15]. A systematic study of the application of SEC coupled to light scattering for chitosan characterization has not been reported.

The study reported here is an extension of the size-exclusion chromatography (SEC) reported by others[10,11], but in this study the chromatography is coupled to an online light-scattering detector (that is, an absolute molecular-weight detector). Other researchers have shown that a low-angle laser light-scattering (LALLS) photometer coupled to SEC (a technique referred to as SEC–LALLS) is a quick, efficient, and accurate technique, capable of providing polymer molecular weights, molecular-weight distributions[16], and degree of polymer branching[17]. An even more important advance is that of the online multiple-angle laser light-scattering

TABLE II

Products of enzymic hydrolysis from chitosan no. 085-260-03 (40°C, pH 4.0)

Sample [a]	Hydrolysis time (min)	Weight (mg)	Recovery (% original) [b]	Specific viscosity [c]
Q0	0	659		1.59
Q1	10	722		0.85
Q2	20	759	95	0.47
Q3	30	786	98	0.29
Q4	90	671	84	0.07
Q5	120	601	75	

[a] The source sample was obtained from Protan (Woodinville, WA) and had a degree of acetylation = 17%. [b] Recovery by precipitation, at pH 10 in 50% EtOH. [c] A 2 mg/ml sample in 0.02 M acetic acid at 23°C.

(MALLS) photometer, which is capable of determining polymer molecular size and conformation. This instrument has been recently[18] adapted to chromatographic operations and offers the advantage of providing the polymer radius of gyration information in addition to the properties listed above for the LALLS. In this sense, SEC–MALLS is a more versatile technique than that of SEC–LALLS; particularly in studies where conformational information is of importance.

This paper describes a systematic study of the application of the SEC–MALLS technique for characterizing chitosans.

MATERIALS AND METHODS

Polymers.—Chitosan samples 125-280-01, 085-260-03, and 056-552-02 were obtained from Protan Inc. (Woodinville, WA). A series of chitosan samples Q0–Q5 were prepared by enzymically hydrolyzing the source Protan sample 085-260-03. The enzyme chitosanase (EC 3.2.1.99) used in these experiments was obtained from *Penicillium islandicum* according to the methods of Fenton and Eveleigh[19]. The relevant properties of these samples are listed in Tables II and III respectively. A series of well-characterized dextran standards were purchased from Pharmacia Labs. (Piscataway, NJ). These dextran polymers were T-500, T-70, T-40, and T-10.

TABLE III

Properties of source chitosans

Sample no.	Source	Degree of acetylation [a] (%)
125-280-01	Shrimp	< 1
085-260-03	Crab	17
056-552-02	Crab	24

[a] Determined using infrared spectroscopy, according to ref. 20.

Solvent.—The solvent used was 0.333 M AcOH + 0.1 M NaOAc buffer (pH 4.2). This solvent is a good solvent for chitosans[9] and for the dissolution of dextrans. Sodium azide (8 mM) was added to the solvent to prevent microbial growth on the chromatography gel. The solvent was made up in ultrapure water ($\sigma \geq 10$ Mohm-cm) obtained by passing deionized water through a MILLI-Q Plus system (Millipore Corp., Milton, MA). Distilled water or deionized water were found to be incapable of removing "spikes" (dust particles) from the resulting SEC–MALLS chromatograms.

Instrumentation.—The DAWN model F laser light-scattering photometer (Wyatt Technology, Santa Barbara, CA) may be used in either a flow mode (in conjunction with an SEC system) or in batch mode (as a traditional static light-scattering instrument)[21]. The light-scattering measurements were carried out at 632.8 nm using a 5 mW He–Ne linearly polarized laser. Pure solvents of known Rayleigh ratio (namely, cyclohexane, toluene, and methanol) were used to calibrate the MALLS. The intensity of scattered light was measured at 15 different angles from 21 to 160°. All measurements were carried out at room temperature. Data collection and processing was effected using an AT&T 6300 PC IBM-compatible computer. The software used were ASTRA and EASI for SEC–MALLS analysis and AURORA for batch analysis, all supplied by Wyatt Tech.

SEC–MALLS.—The size-exclusion chromatographic system consisted of a Waters M6000A pump, a U6 K model injector, and a R401 differential refractometer (Waters Associates, Milford, MA) coupled to a Wyatt DAWN F multiple-angle laser light-scattering photometer (Wyatt Technology, Santa Barbara, CA). A three-column set consisting of one 300×7.5 mm Bio-Gel TSK-60 column connected in series to two 300×7.5 mm Bio-Gel TSK-50 HPLC SEC columns (Bio-Rad Lab., Richmond, CA) was used to chromatograph the samples. A schematic of the SEC–MALLS system is shown in Fig. 1.

The specific refractive-index increment [(dn/dc)] of chitosan and dextrans were determined at 632.8 nm and 25 ± 0.5°C using a Model RF-500 absolute-deviation type differential refractometer made by C.N. Wood MFG. Co., Newtown, PA. No difference in (dn/dc) with the degree of acetylation or polymerization was found for chitosans. The observed values of 0.181 for chitosan and 0.150 for dextrans are in good agreement with those reported in literature[6,8].

Sample preparation.—Chitosan and dextran samples pre-dried at 60°C and of appropriate concentrations (see below) were prepared in aqueous acetate buffer. The amount of insolubles was between 0 and 0.5% for all chitosan samples analyzed and found to have no effect on light-scattering experiments. The solvent used for either dissolving the polymer samples or as the SEC eluent were from the same batch. This was done so as to eliminate minor variations in solvent character from two different buffer batches which might affect the differential refractometer response. The SEC eluent was degassed and filtered through a 13-mm diameter in-line filter (LDC Analytical, Riviera Beach, FL) containing a 0.1-μm mixed cellulose ester membrane (Millipore corp., Bedford, MA). Polymer samples were

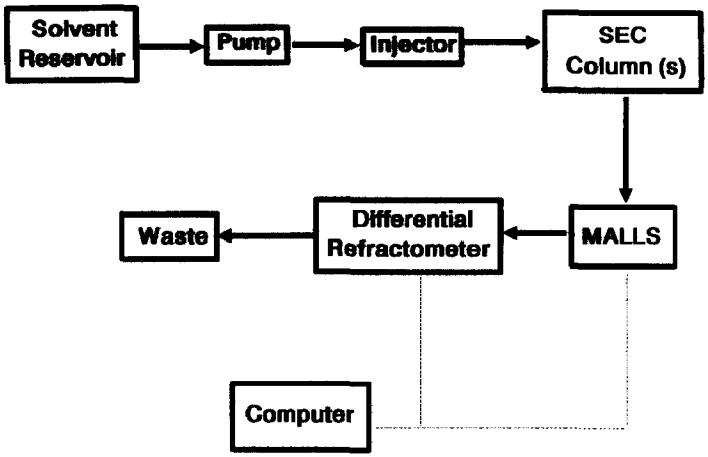

Fig. 1. Schematic representation of the SEC–MALLS system.

centrifuged at 1000*g* for 10 min prior to injection. Sample size and sample concentration were varied depending on the molecular weight (see results). Low-molecular-weight samples were found to require higher injection volumes according to established light-scattering theories. The solvent flow rate through the SEC/MALLS was maintained at 0.6 mL/min.

RESULTS AND DISCUSSION

Molecular weight and MWD using SEC–MALLS.—The main objective of this study was to determine the applicability of the SEC–MALLS technique in characterizing chitosans. The SEC–MALLS systems accuracy and reproducibility was tested using well-characterized dextran standards. Each of these samples was injected ~6 times in order to establish a comparative basis. The molecular-weight data for these dextrans obtained using the SEC–MALLS procedure show (Table IV) excellent agreement with that of the suppliers values. The difference between the two sets is ~4% for dextran T-10 and <1%for the other three samples. The reproducibility of this technique is also evident from the low standard deviations in the molecular-weight data. Sample recovery of dextrans, defined as the ratio of eluted mass (as calculated using ASTRA) to the injected mass, was very close to 100%, indicating no irreversible adsorption of polymers onto the chromatographic gel. Sample recoveries >85–90% were considered acceptable for an SEC experiment[22]. Thus, nearly ideal SEC behavior of our chromatographic system was demonstrated for dextrans and further extended to the study of chitosans.

The molecular-weight data for chitosans obtained using SEC–MALLS are shown in Tables V and VI. For these experiments, at least 5 injections of each chitosan sample were made. No systematic errors were determined over the 8-month period in which these experiments were conducted. The standard devia-

TABLE IV

Molecular weight data for dextrans

Sample	Injected mass ($\times 10^4$ g)	Sample recovery (%)	Elution volume (mL)	SEC–MALLS data (±SD)		PD [b]	Nominal mol wt [a]
				M_w	M_n		
T-500	2.2	98	23.1	498000 ±17000	298000 ±5000	1.67	500000
T-70	4.0	101	25.3	70500 ±700	56500 ±4950	1.25	70000
T-40	4.0	99	26.0	40000 ±2000	31000 ±4600	1.29	40000
T-10	10.0	100	28.7	10400	7500	1.33	10000

[a] Supplied by the manufacturer, Pharmacia Labs, Piscataway, NJ. [b] Polydispersity = M_w / M_n.

TABLE V

Molecular weight data for Q-series chitosans

Sample	Injected mass ($\times 10^4$ g)	Elution volume (mL)	SEC–MALLS data (±SD)		PD [a]	Radius of gyration $\langle R_g \rangle_z$ (nm)
			M_w	M_n		
Q0	2.0	23.4	180000 ±5000	120000 ±10000	1.50	45.6
Q1	4.5	26.2	51500 ±2100	30000 ±1400	1.72	31.8
Q2	4.5	26.9	41000 ±3000	24000 ±3700	1.71	30.2
Q3	6.0	27.7	31000 ±2900	19000 ±2000	1.63	29.3
Q4	7.2	29.4	11000 ±1000	3300 ±200	3.33	
Q5	10.5	29.9	10000 ±100	5650 ±640	1.77	

[a] Polydispersity = M_w / M_n.

TABLE VI

Molecular-weight data for source chitosan samples

Sample	Injected mass ($\times 10^4$ g)	Sample recovery (%)	Elution volume (mL)	SEC–MALLS data (±SD)		PD [a]	Radius of gyration $\langle R_g \rangle_z$ (nm)
				M_w	M_n		
Q0	2.4	90	22.3	190000 ±7000	120000 ±1000	1.58	45.8
085-260	2.5	90	21.8	250000 ±22000	145000 ±10000	1.72	49.0
056-552	2.5	80	21.3	375000 ±20000	195000 ±19000	1.92	54.6
125-280	2.5	100	21.3	350000 ±20000	196000 ±30000	1.79	54.5

[a] Polydispersity = M_w / M_n.

TABLE VII

Molecular weight, z-averaged radius of gyration $\langle R_g \rangle_z$ and second virial coefficients A_2, of dextrans and chitosans from MALLS

Sample	Static light-scattering data			SEC–MALLS data	
	M_w ($\times 10^{-5}$)	$\langle R_g \rangle_z$ (nm)	$A_2 \times 10^3$ ($cm^3\ mol/g^2$)	M_w ($\times 10^{-5}$)	$\langle R_g \rangle_z$ (nm)
T-500	4.6	33	0.292	4.9	32.3
085-260-03	2.8	46	2.827	2.5	49.0
056-552-02	3.7	197	1.550	3.8	54.6
125-280-01	4.2	67	2.337	3.5	54.5
Q0	2.2	32	3.112	1.9	45.5

tions in both M_w and M_n are well within 10% of the mean value; generally accepted as a sufficient indication of experimental consistency. The recoveries of all chitosan samples (except sample no. 056-552-02) from the SEC–MALLS system were $> 85\%$, again incidating acceptable SEC behavior. The recovery of sample no. 056-552-02 was only marginally acceptable; that is, $\sim 80\%$ recoverable. This particular sample has the highest degree of acetylation (Table III). Hydrophobic interactions between the *N*-substituted acetyl groups and the hydroxylated polyether material of the SEC gel could conceivably have caused adsorption of polymer onto the gel. However, no further attempt was made to validate this supposition.

Despite the fact that chitosan samples can be successfully chromatographed on an SEC column, an independent method to verify the accuracy of the SEC–MALLS analysis method was necessary. Classical "stand alone" light scattering (using the MALLS in batch mode without prior fractionation using SEC) was used for this purpose and compared to prior literature studies. The preliminary results (Table VII) indicate that the molecular-weight averages are comparable to the results from SEC–MALLS and the virial-coefficient data for chitosan is in good agreement with those reported in the literature[6].

The quality of light-scattering data (and hence the method of analysis) is independent on the characteristics of the polymer being assayed. Low-molecular-weight chitosan (or dextran) polymer samples require higher concentrations for "proper" light scattering characterization than high-molecular-weight chitosan (or dextran) polymer samples. This is also true for the experiments reported here (see Tables IV and V). The mass concentrations of the polymer solutions required for "appropriate" SEC–MALLS experimentation must be adjusted as these solutions exhibit general trends consistent with "classical" light-scattering experiments. Light-scattering theories specify two main reasons for adjusting the amount of injected polymer mass in such experiments. For example, it is well known that light-scattering intensity from a polymer solution (in "classical" light-scattering experiments) is dependent on both the polymer concentration and its molecular weight[7,23]. This is also the case in SEC–MALLS studies. For example, at the "tail

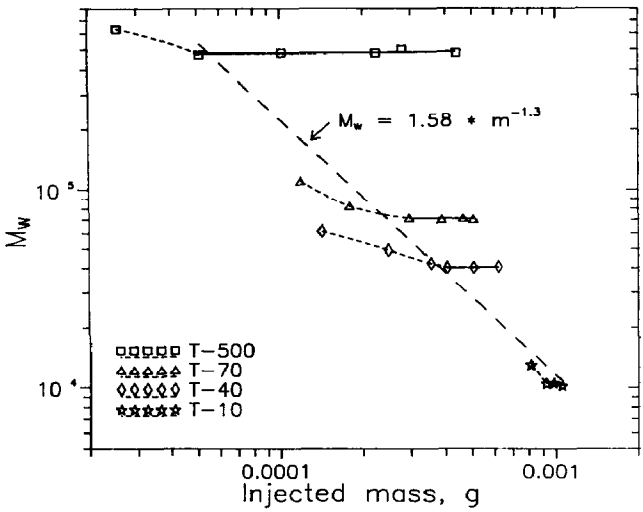

Fig. 2. Illustration of overestimation of molecular weight with low injection amounts of low-molecular-weight polymers T-70, T-40, and T-10. The dashed line indicates the minimum amount of polymer that needs to be injected into the SEC–MALLS system to obtain accurate molecular weights.

end" (trailing edge) of an SEC chromatogram, both polymer concentration and molecular weight of an eluting polymer are low. In such circumstances (namely, at these "chromatographic tails"), the intensity of scattered light will be low (that is, nearly the same value as that of the "pure" solvent) in this region of the chromatogram and the eluting polymer will go "undetected" (by the MALLSs photodetectors). This effect is particularly severe for low-M_w polymers (for example, for dextrans T-70, T-40, and T-10 and chitosan samples Q1–Q5). Similar effects are observed when the total amount of injected polymer is low (for example, as caused by small injected sample-volume or low polymer–mass concentration). In either case, this will result in an overestimation of the polymer samples molecular weight, as data from only the measured, higher-molecular-weight polymers are collected.

It was observed in this study that small amounts (~ 0.1–0.2 mg) of dextrans T-70 and T-40 injected into the SEC–MALLS system resulted in their calculated molecular weights being higher than the nominal molecular weight as reported by the manufacturer. Increasing sample concentration and/or injected sample-volume (that is, total injected mass) rectified this problem. Fig. 2 illustrates this, here SEC–MALLS-measured molecular weight is plotted against injected sample mass. As the amount of polymer injected in the SEC–MALLS increased, the M_w values decreased; eventually converging to constant values. The mass of injected polymer required for convergence to a constant molecular weight increased with decreasing nominal molecular weight for each homologous series, as shown by the dashed line in Fig. 2 (that is, the amount of T-10 needed was greater than that of T-40, which in turn was greater than that of T-70). This agrees with theoretical expectations as

the intensity of scattered light (at a fixed observational point) is proportional to the polymers molecular mass and its mass concentration; in agreement with Rayleigh's relationships. All polymer samples were tested in this manner to assure accuracy of their reported molecular weights. The molecular weights reported in Table IV are the convergent values so obtained and are in agreement with the manufacturers reported values.

It was thus established that the SEC–MALLS technique can provide accurate and reproducible M_w and MWD data for polymers spanning a molecular-weight range from 500 000 to 10 000 daltons. A minimum amount of polymer must be injected (as shown by the curve in Fig. 2) in order to obtain accurate molecular-weight values, the amount being inversely proportional to the polymer molecular weight. The data in Tables IV–VI confirm these observations.

Comparison of SEC–MALLS technique with secondary calibration.—Many researchers[10,11] have employed dextran standards as calibrants in SEC experiments and report molecular weights of polymers in terms of equivalent-dextran molecular weights. Such an indirect secondary calibration technique is prone to many errors. One most obvious error is the presumed conformational similarity between the standards and the unknown sample polymers. In other words, this technique is only valid if the two polymers behave identically in the same solvent. This is seldom true. More generally, different polymers will have different calibration curves (molecular weight vs. elution volume).

In Fig. 3, the weight-average molecular weights of the Q-series chitosans and of dextrans (determined using SEC–MALLS) are plotted against their SEC elution volumes. The slope of the calibration curve for dextrans is greater than that of the

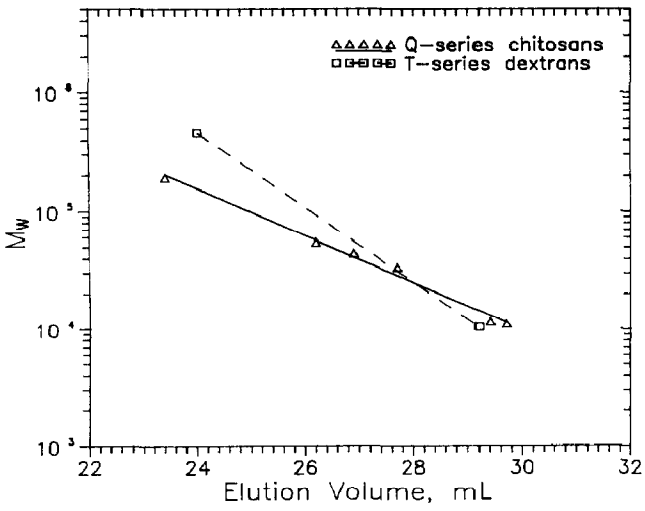

Fig. 3. A plot of log M_w (as calculated by SEC–MALLS) vs. polymer elution-volume (determined at the peak of the DRI chromatogram) for Q-series chitosans and Pharmacia dextrans (T-500, T-70, and T-10).

TABLE VIII

SEC–MALLS vs. secondary calibration for Q series chitosans

Sample	Elution volume (mL)	SEC–MALLS data			Secondary calibration data [a]		
		M_w	M_n	PD [b]	M_w	M_n	PD [b]
Q0	23.4	180000	120000	1.50	705000	321000	2.20
Q1	26.2	54000	31600	1.71	275000	77400	3.55
Q2	26.9	44000	24500	1.80	164000	31600	5.16
Q3	27.7	33000	21000	1.63	117000	20900	5.62
Q4	29.4	11500	3750	3.07	31700	7810	4.06
Q5	29.7	11000	6000	1.83	18800	5060	3.72

[a] From ref. 12. [b] Polydispersity = M_w / M_n.

Q-series chitosan samples; that is, the molecular weight of dextran is greater than that of the chitosan which elutes at the same SEC elution volume except at very low molecular weights. This suggests that, under the conditions of this assay, chitosan is present in solution as a more highly extended chain and thus sweeps out a larger hydrodynamic volume than a dextran of equivalent molecular weight. These observations suggest that using a secondary-calibration technique as described here, would result in erroneous molecular-weight values of chitosans; specifically overestimating the molecular weight (Table VIII). The last three columns in Table VIII present the molecular-weight data that would be obtained using such a secondary-calibration technique employing dextrans as standards. The values calculated by this method gives results 3–4 times higher than those obtained with SEC–MALLS. Thus use of a dextran calibration curve leads to significant overestimation of the molecular weight of chitosan. Based on these results, the use of dextrans as secondary-calibration standards is not recommended and previous reports that have used this technique should be reexamined.

Another possible source of error in using secondary-calibration techniques as the primary route for quantitating molecular weights is due to changes in polymer elution volume with changes in column loading (injected sample size). These changes are caused by such phenomena as viscous fingering, osmotic swelling, etc.; topics which are reviewed in more comprehensive articles[5,24,25]. Irrespective of the phenomenological origin of elution-volume changes, SEC–MALLS molecular-weight data are unaffected as this technique generates absolute molecular weights that are unaltered due to these complicating separation factors. Table IX illustrates this effect. In these experiments, differing amounts of chitosan no. 085-260-03 were injected in the SEC column. An increase in elution volume with higher-mass amounts of the injected sample was observed. The elution-volume increase (as much as 1.2 ml) with increased column loading will result in errors in reported molecular weights when the chromatographer relies on a procedure employing elution volume as the independent experimental measurement (for instance, secondary or universal calibration). However, as shown in Table IX, the results

TABLE IX

Effect of polymer injection mass on elution volume and calculated molecular weight for chitosan no. 085-260-03

Sample concn (% w/v)	Injected mass ($\times 10^4$ g)	Elution volume (mL)	SEC–MALLS data		
			M_w ($\times 10^{-5}$)	M_n ($\times 10^{-5}$)	PD [a]
0.49	2.0	21.5	2.5	1.3	1.92
0.49	2.5	21.5	2.4	1.5	1.58
0.52	2.0	21.3	2.8	1.6	1.77
0.52	2.6	21.5	2.7	1.4	1.90
0.52	4.0	22.5	2.4	1.5	1.60
0.52	4.7	22.7	2.2	1.4	1.60
Average			2.50	1.45	1.72
SD			0.22	0.10	

[a] Polydispersity = M_w / M_n.

obtained using the SEC–MALLS technique are unaffected by column loading. The benefit in using SEC–MALLS is once again established.

In summary, two main sources of error: (*1*) differences in conformation between the standard and sample polymers and (*2*) elution volume changes due to column overloading may cause errors in the reported molecular weights obtained using a secondary-calibration technique. The use of an absolute molecular-weight determination technique such as SEC–MALLS overcomes the pitfalls associated with all indirect calibration techniques. Its use in characterizing chitosan (as well as other polymers for which identical, narrow-molecular-weight standards are not available) is firmly established.

Polymer size and conformation using SEC–MALLS.—The advantage of MALLS over LALLS lies in its ability to provide hydrodynamic size (that is, radius of gyration) of polymers. Size estimation is critical in the study of protein denaturation[26,27], diffusion of polymers through porous media[28,29], and thickening behavior of polymer solutions[30,31]. Information on polymer conformation is important in predicting immunological behavior; thus, the antitumor activity of aqueous schizophyllan solutions against Sarcoma 180 is related to the amount of triple helices formed by the (1 → 3)-β-D-glucan relative to that of the coexisting single-helical coils in solution[32]. In studies where polymer hydrodynamic size information is needed, MALLS techniques are the a priori method of choice.

The *z*-averaged radius of gyration; heretofore denoted as $\langle R_g \rangle_z$; for chitosans as determined by SEC–MALLS are shown in Tables V and VI. The data shows the expected trend; namely $\langle R_g \rangle_z$ decreases with decrease in molecular weight. However, for the low-molecular-weight chitosan samples, Q4 and Q5 as well as for dextran samples T-70 to T-10, accurate size data cannot be obtained. This is due to the fact that the minimum size of polymer (mean-square end-to-end distance) that can be determined by light scattering[23,27] is $\sim \lambda/20$ nm; λ being the wavelength of

TABLE X

Root-mean-square end-to-end distance (RMS) and radius of gyration R_g using the Flory–Fox equation for dextrans and chitosans

Mol wt	Dextrans [a]		Chitosans [b]	
	R_g (nm)	RMS (nm)	R_g (nm)	RMS (nm)
10000	2.5	6.1	2.8	6.9
25000	3.9	9.7	5.1	12.5
50000	5.6	13.9	7.9	19.5
100000	8.1	19.9	12.4	30.5
200000	11.6	28.5	19.4	47.6
500000	18.9	45.8	35.0	85.8

[a] Mark–Houwink constants: $k = 4.01 \times 10^{-4}$; $a = 0.55$ from ref. 34. [b] Mark–Houwink constants: $k = 1.81 \times 10^{-5}$; $a = 0.93$ from ref. 35.

incident light. For the laser employed in our study, this was 30 nm. Using the Flory–Fox equation[33], the size of polymers characterized in this study was determined. Table X shows that the minimum polymer size detectable using light scattering corresponds to a molecular weight of $< 150\,000$ daltons for dextrans and $< 100\,000$ daltons for chitosans, respectively. In other words polymer samples whose weight-average molecular weights are below these limits (dextrans T-70 to T-10 and chitosans Q1–Q5); are distributed such that a larger component of these polymers consist of chains whose size is lower than the required, minimum critical size. The radius of gyration data for these polymers is thus questionable, given the constraints of the specific experimental conditions. In principle, lasers operating at lower wavelengths could be used to extend the range to lower limits. On the other hand, for the high-molecular-weight polymers employed in this study, reproducible radius of gyration data were obtained.

The inadequacy of light scattering in providing size data for polymers whose radius of gyration is ≤ 10 nm (corresponding roughly to a mean-square end-to-end distance of 30 nm, assuming a random-coil conformation) raises the possibility of a similar error in determining molecular weights of such small polymers. Yau[36] has examined this question in detail and concluded that, for obtaining accurate molecular weights, moderately low signal-to-noise ratios can be tolerated. However, very high signal-to-noise ratios are necessary to obtain accurate size data; such a high ratio is not achieved by the MALLS instrument used in the experiments reported here. The Wyatt-MALLS is thus quite capable of providing accurate molecular-weight data for the polymers characterized in this study, but its ability to determine the size of low-molecular-weight polymers ($M_w \leq 100\,000$) is questionable.

One way to probe the conformation of a polymer is by determining the scaling relationship between the polymers radius of gyration and molecular weight, as shown in eq *1*:

$$R_g = KM^{\nu} \qquad (1)$$

TABLE XI

Size and conformation data for T-500 and source chitosans

Sample	M_w ($\times 10^{-5}$)	$\langle R_g \rangle_z$ (nm)	K	ν
T-500	4.9	32.3	2.43	0.19
Q0	1.9	45.5	4.26	0.19
085-260-03	2.5	49.0	2.77	0.22
056-552-02	3.8	54.6	2.09	0.24
125-280-01	3.5	54.5	7.94	0.15

Simple geometric arguments have shown that[5], for rod-like molecules $\nu = 1$, for a sphere it is 0.33, and for random coils in a theta solvent, it is 0.5. For random coils in a good solvent, Flory[33] has shown that ν reaches an asymptotic value of 0.588. The experimental data for most randomly coiled synthetic polymers in good solvents agrees with Flory's theoretical predictions[37].

The SEC–MALLS technique determines molecular weight as well as radius of gyration of the eluting polymer sample at each slice of the chromatogram, thus allowing for evaluation of exponent ν in equation *1*. This can be calculated readily from the slope of a log–log plot of radius of gyration vs. molecular weight. The calculated values of the exponent ν for select chitosan samples and dextran T-500 are shown in Table XI. The calculated values of ν are far lower than those reported for synthetic polymers in good solvents[33,37]. The reason for this underestimation could be due to the inaccuracy in measuring polymer size close to one-twentieth the wavelength of incident light[7,23]. In addition, the polymers listed in Table XI are moderately dispersed ($M_w/M_n \leq 1.6$); and thus inaccurate determination of polymer size in the low-molecular-weight end of the polymer distribtution due to the reasons outlined previously could very well have skewed the calculated ν values. Perhaps a better way to determine the scaling relationship would involve characterizing a series of polymers with at least two orders of difference in their molecular weights. The range of molecular weight and radius of gyration for a single polymer sample may not be broad enough to calculate the exponent ν accurately. It should also be pointed out that scaling relationships (such as eq *1*) for branched polysaccharides[27] and polyelectrolytes[38] in dilute solution have not been developed. The increased short-range interactions between polysaccharide molecules[26] (due to intermolecular hydrogen bonding) and increased long-range interactions between polyelectrolytes[38] (due to residual charge) could result in scaling relationships vastly different than those primarily observed for synthetic polymers (random coils) in organic solvents. Also, $\nu = 0.25$ has been reported for branched polymers[27]. Dextran, being a branched polymer, may very well have a low ν value such as this. Thus the results obtained from the SEC–MALLS experiments may be quite close to the actual values. One possible way to resolve this question would be to use narrowly distributed polymers of high molecular weights (≥ 1 million) or alternatively, to use well characterized monodis-

persed polymer standards (such as pullulans or sodium polystyrene sulfonates) to check the capability of the SEC–MALLS technique in providing accurate scaling relationships for polysaccharides and polyelectrolytes. Clearly, additional work needs to be done in this direction.

CONCLUSIONS

Nine chitosan samples varying in molecular weight, composition (degree of acetylation), and source of origin were characterized using SEC–MALLS. The results show that this technique is capable of providing reliable molecular-weight and distribution data for polymers in the molecular weight range 10 000–500 000. The chitosan samples were also characterized by a secondary-calibration technique employing dextrans as calibrants. The molecular weights obtained using secondary calibration were 2–3 times higher than that obtained using SEC–MALLS. The dissimilarity in conformation between randomly coiled dextrans and the much stiffer worm-like chain conformation of chitosan may be responsible for this overestimation. The advantage in using an absolute molecular-weight detection technique like SEC–MALLS for characterizing chitosans as opposed to any other indirect technique is thus firmly established.

The size (radius of gyration) was also obtained using SEC–MALLS. However, the reliability of size data for low-molecular-weight polymers ($\leq 100\,000$) was found to be questionable. For polymers having a molecular weight $\geq 100\,000$, reliable size data were obtained. An attempt was made to determine the conformation of these polymers by establishing the scaling relationship between the radius of gyration and molecular weight ($R_g = KM^{\nu}$) at each slice of the SEC chromatogram. The value of ν was found to be much lower than theoretically predicted. The inability of this technique to provide accurate R_g values at the low end of the molecular weight distribution may be responsible for this underestimation.

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