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THE MOLECULAR WEIGHT OF CHITOSANS STUDIED BY LASER LIGHT-SCATTERING*

RICCARDO A. A. MUZZARELLI, CATHERINE LOUGH, AND MONICA EMANUELLI
Faculty of Medicine, University of Ancona, I-60100 Ancona (Italy)

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ABSTRACT

Laser light-scattering spectrometry was used to study the molecular weights of several chitosans, *N*-(carboxymethyl)chitosan, glycol *N*-(carboxymethyl)chitosan, and *N*-(carboxymethyl)chitosan 6-sulfate. Improved production methods permitted the obtaining of chitosans having M_w in the range of 4.10^5–7.10^5 rather than $1.2.10^5$ (usual until a few years ago). Starting from a chitosan having M_w 464,990, the increase in M_w for NCM-chitosan obtained from glyoxylic acid under reducing conditions (543,300) confirms that all of the available amino groups on chitosan can be carboxymethylated. Dramatic degradation occurs when NCM-chitosan is sulfated (M_w 16,000); moderate degradation takes place when it is 6-hydroxyethylated (M_w 316,000). The data are of use for the reproducible preparation of water-soluble chitosans to be used in the form of solutions, gels, membranes, and freeze-dried powders, in such fields as cosmetics, pharmaceuticals, and medical aids.

INTRODUCTION

The definition of the average molecular weight of polysaccharides and the understanding of its consequences on their physicochemical behavior have presented a real challenge to chemists for a number of years[1]. In the case of chitin and its derivatives, relatively little is known about their molecular weights, in spite of the obvious importance of such data for industrial uses and for research advancement in numerous fields. Although the primary structure of chitosan is a backbone of (1→4)-β-D-glucosamine residues randomly acetylated to various extents, the name chitosan is in fact a collective term for deacetylated chitins differing in terms of crystallinity, optical characteristics, degree of acetylation, impurity content, and average molecular weights[2].

Production methods and origins are mainly responsible for the differences just listed, which are encountered in various chitosans. If quality-control standards are sought for properly characterized chitosans[3], especially for use in specialty applic-

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ations, a reliable method for molecular-weight determination is desirable. Methods currently used are based on viscometric measurements, and take advantage of the Mark–Houwink equation, which requires the knowledge of two parameters, K and a. Because K and a are difficult to obtain, and differ remarkably according to the type of chitosan and the conditions of measuring the viscosity of the chitosan solutions [e.g., $8.93.10^{-2}$ and 0.71 (ref. 4); $1.28.10^{-4}$ and 0.85 (ref. 5); and $1.81.10^{-3}$–$3.04.10^{-5}$ and 0.93–1.26 (ref. 6)], the results obtained in this way, as well as by chromatography, have led to disagreements and to the declared[4] "inapplicability of the viscosimetric law".

In 1972, one of the present authors published the first report[7] on the determination of the molecular weight of chitosan by light-scattering; it was found to be $1.2.10^5$. Other industrial chitosans were found to have similar M_w values [8,9]. Those articles demonstrated that the M_w of commercial chitosans at that time was one order of magnitude lower than that of chitin (see ref. 2, p. 81).

More recently, laser light-scattering spectrometry (l.l.s.) has been applied to the study of polysaccharide molecular weights[10-12]. One of the advantages of this technique is its ability to provide absolute measurements, *i.e.*, weight-average molecular weights, without the use of any reference material. We have undertaken molecular-weight measurements both on chitosans and chitosan derivatives by l.l.s.; such values should prove useful for proper characterization of industrial chitosans. Moreover, the biological activity of chitosan and modified chitosans, such as their blood anticoagulant and wound-healing activity, depends on molecular weight. The same applies to the gel-forming ability of modified chitosans and the mechanical properties of cross-linked chitosan gels and membranes.

The following compounds were selected for reaction with chitosan and subsequent analysis by l.l.s.: glyoxylic acid[13,14], α-ketoglutaric (2-oxopentanedioic) acid[15], and the phenolic compound 3,4-dihydroxybenzaldehyde[16]. The chitosan derivatives obtained with these compounds under reducing conditions are *N*-(carboxymethyl) chitosan (NCM-chitosan), otherwise called "glycine-glucan", depending on the degree of deacetylation, "glutamate glucan", and *N*-(3,4-dihydroxybenzyl)chitosan. In addition to these derivatives, we selected two other water-soluble chitosan derivatives for characterization by l.l.s., namely glycol-NCM-chitosan and NCM-chitosan 6-sulfate.

EXPERIMENTAL

Chitosan solutions. — The chitosans used were the following: Protan chitosans from king crab, Lot nos. PTL-4657201, PTL-123, PTL-1605203, and PTL-124; Katakura Chikkarin chitosan from crab *Chionoecetes opilio*, Lot no. 26686; Rybex chitosan from Antarctic krill *Euphausia superba*; and Bioshell crab chitosan, Lot no. 5164.

The chitosans were dissolved in acetic acid (0.5% solutions in 1% acetic acid). These solutions were, in part, used for viscometry and, in part, were further diluted

with 1% acetic acid and then sodium acetate added to give a final acetate concentration of 0.2M. Dilutions were so made as to give at least four concentrations ranging from 0.1 to 1 g/dm^3. The pH of the solutions was 4.8. These diluted samples were optically clarified by filtration, in sequence, through 0.45 and 0.22 μm Sartorius Minisart membranes into cylindrical (2-cm diameter) Burkard cells, manufactured by Hellna. Measurements were preferably taken 24 h after final dilution. It was confirmed that this filtration procedure does not change either the polymer concentration or the viscosity of the solutions. In the case of direct sampling from reaction mixtures, the polymer concentrations were accurately determined by weighing a dialyzed and freeze-dried aliquot of the chitosan acetate solution.

Instrumentation. — L.l.s. measurements at 632.8 nm were performed with a Malvern System 4700-C instrument, using a He–Ne Spectra-Physics 35 mW laser, and an Olivetti M-24 computer. The software used was Automeasure®, developed by Malvern. Pure benzene was used as a standard with a Rayleigh ratio $0.136.10^{-4}$. The angular dependence of scattering was determined at 15-degree intervals between 30 and 150 degrees at 25°.

The increment in refractive index for chitosan in acetate buffer was found to be 0.160 cm^3/g at 632.8 nm, a value very close to those determined at the same wavelength for other polysaccharides, *i.e.*, 0.162 and 0.155 for alginates and xanthans, respectively[10,11], and for chitosan at 436 and 546 nm[6,7,17].

Viscometry. — Measurements were carried out with a Haake Rotovisco RV12 viscometer equipped with a programmer and a recorder; the temperature was controlled with a Haake thermostat-crysotat. The rotor used was model NV. The readings were plot of torque, *S*, *versus* test speed, *n*. The volume of the sample was 12 mL. The values were recorded at 25° on 0.5% polymer solutions in 1.0% acetic acid, 0.2M in sodium acetate.

Nuclear magnetic resonance spectroscopy. — The ^{13}C spectra were recorded at 35° with a 20-MHz Varian CFT-20 spectrometer equipped with a 10-mm probe. The solutions were prepared in D_2O (90–100 mg in 2 mL; 99.7% of D) whose pH value was adjusted to >5 by adding concentrated DCl. The experimental conditions included 90° pulses, 2k data points, 8k Fourier number, 100–300-k pulses, with the chemical shifts measured against a CH_3OH internal standard: 50.04 p.p.m. from internal Me_4Si.

Differential calorimetry. — Analyses were performed with a Perkin–Elmer DSC-2 calorimeter interfaced to a computer, in the interval 200–400° at the scan rate of 5°/min.

Modified chitosans. — The modified chitosans were prepared according to methods already reported. In addition to these, NCM-chitosan 6-sulfate was prepared by reaction of NCM-chitosan in a sulfuric acid and chlorosulfonic acid mixture (ref. 1, p. 371), and glycol-NCM-chitosan [*i.e.*, 6-(hydroxyethyl)chitosan, presumably etherified to a limited extent at the side chain alcohol group] was prepared as follows: NCM-chitosan was swollen in 42% NaOH, filtered off, and stirred with crushed ice, and the concentration of NaOH was adjusted to 14%. 2-Chloroethanol

was added dropwise to the cold slurry, and after stirring for a few hours, the mixture was dialyzed to neutral pH, and freeze-dried[18].

RESULTS AND DISCUSSION

The Zimm plots recorded by l.l.s. (see Fig. 1) showed that the various industrial chitosans examined possess different weight-average molecular weights (M_w), which fall in the range of $1.9.10^5$–$7.0.10^5$ (see Table I). In particular, the Katakura chitosan, for which we found M_w 698,340 was of the same origin as that studied by Mima *et al.*[19], who reported values of 630,000 for production conditions presumably close to those employed to produce the Katakura sample examined in the present study. For other conditions, they reported values in the range of $5.0.10^5$–$8.5.10^5$, which seem generally reasonable[20].

The good agreement between the M_w data and the viscosity measurements is shown in Fig. 2, where the linear relationship between torque and M_w recorded on the same solutions are shown for crab chitosans. Torque is related to shear stress and viscosity through coefficients specified for the instrument used; here, torque is preferred (to avoid misunderstandings). The good correspondence of the Katakura value with a previously reported value[19], the linearity of the plot, and the inclusion of the origin in the curve are indications of the general, good accuracy of the l.l.s. measurements.

The dependence of scattered light on polymer concentration has been measured for salinities ranging between 0.1 and 0.5M sodium acetate. The values of $KC/\Delta R_\vartheta$, where ΔR_ϑ is the excess Rayleigh ratio due to the polymer, and K is the optical constant containing the refractive index increment dn/dc, are plotted *versus* chitosan concentration in Fig. 3, where the influence of salinity on the l.l.s. behavior of chitosan is shown. All curves are linear, and have a common intercept at zero po-

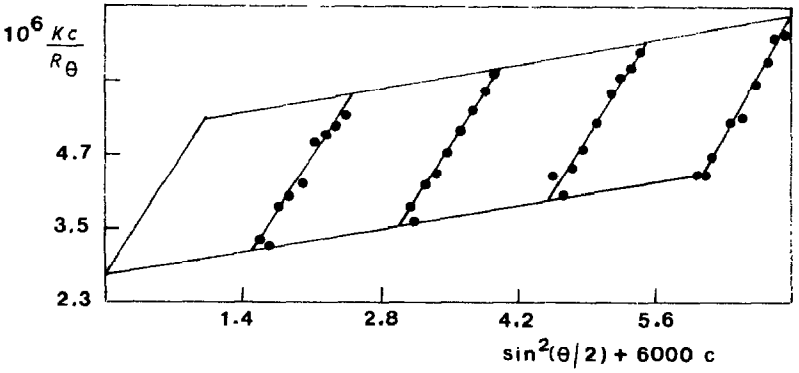

Fig. 1. Typical Zimm plot for chitosan PTL-124 solutions containing 0.25, 0.50, 0.75, and 1.0 mg of chitosan/mL. The solvent was 0.5M sodium acetate–0.5% acetic acid buffer, pH 4.8. The standard was pure benzene. Measurements were taken at 15-degree intervals between 30 and 150 degrees. The extrapolated values in this particular Zimm plot are those for the lower curve of Fig. 3.

TABLE I

PROPERTIES OF CRAB CHITOSANS[a]

Sample	*Weight-average molecular weight ± std. dev.*	*Radius of gyration (nm) ± std. dev.*	*2nd virial coefficient ($g^{-2}.mol.cm^3$)*	*Torque[b] at 512 r.p.m. (scale units)*	*Viscosity[c] (cPs)*	*Degree of deacetylation[d] (%)*	*Insolubles in chitosan (%, w/w)*
Bioshell	191,260 ± 13,390	15.3 ± 0.7	3.09×10^{-3}	40	—	86.8	—
Katakura	698,340 ± 50,970	93.7 ± 4.3	1.41×10^{-3}	120	1230	85.0	0.18
Protan 201	594,100 ± 41,580	89.9 ± 4.0	1.64×10^{-3}	100	4100	78.8	0.47
Protan 123	395,850 ± 28,900	75.9 ± 3.5	1.65×10^{-3}	65	990	79.0	<0.4
Protan 203	440,650 ± 30,810	59.9 ± 2.7	0.51×10^{-3}	65	1060	84.6	0.69
Protan 124	353,257 ± 25,800	62.6 ± 2.8	1.79×10^{-3}	59	990	80.0	$\leqq 0.4$

[a]Diluted chitosan solutions (0.1–1.0 g/L in 1% acetic acid and 0.2M acetate. [b]Measured on 0.5% chitosan solutions with the Haake Rotovisco System RV12 and rotor NV. [c]Viscosity declared by the manufacturer, as determined on 1% chitosan solution in 1% acetic acid, with an Ubbelohde viscometer. [d]Determined by first-derivative spectrophotometry, according to ref. 21.

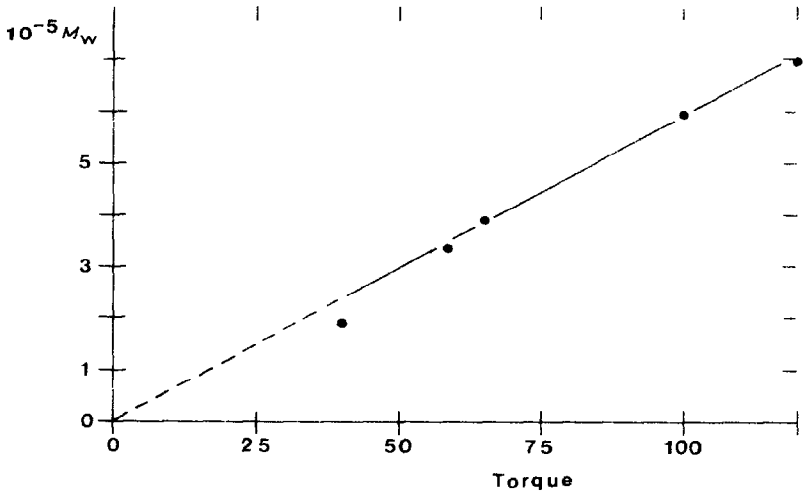

Fig. 2. M_w values for the crab chitosans listed in Table I, plotted against torque, measured as scale units at 512 r.p.m. with the Rotovisco RV12 system by using rotor NV on 0.5% chitosan solutions (1.0% acetic acid, 0.2M sodium acetate).

lymer concentration, which clearly shows that the molecular weight measurement is not affected within this salinity range. The molecular weight invariance with salinity indicates that no aggregation due to sodium acetate is observed at high salinity, with these samples at the time of the measurement (24 h after final dilution).

Modified chitosans. — For the NCM-chitosans obtained from glyoxylic acid under reducing conditions at pH 4.8, the M_w value of 545,300 was obtained, in agreement with the value calculated for the NCM-chitosan on the basis of the chitosan M_w 464,990 (see Table II). This corresponds to the complete reaction of the available free amino groups, leading to a modified polymer containing 58% of its units in the carboxymethylated form and 42% in the original acetamido form. This also confirms our finding that the glyoxylate and chitosan reaction proceeds to 100% yield[13].

The NCM-chitosan is a product that can be readily isolated and used for further derivatization. NCM-chitosan 6-sulfate and glycol-NCM-chitosan are in fact obtained from NCM-chitosan under acidic and alkaline conditions, respectively.

The relevant data (see Table II) provide evidence of chemical degradation of the polysaccharide chains, due to harsh reaction conditions. NCM-chitosan appears to be susceptible to degradation both in strongly acidic and alkaline solvents: the acidic medium necessary to produce NCM-chitosan 6-sulfate dramatically lowers its M_w from 592,530 to ~16,000. On the other hand, the reaction of the Na form of NCM-chitosan with 2-chloroethanol (leading to glycol-chitosan) appears to take place: this in view of the alterations in the C-6 zone of the ^{13}C-n.m.r. spectrum recorded for glycol-NCM-chitosan (compare Fig. 4 with Fig. 1 of ref. 14). The spectrum also shows the absence of all signals from the acetylated polymer units,

TABLE II

PROPERTIES OF KRILL CHITOSAN AND ITS WATER-SOLUBLE DERIVATIVES[a]

Chitosan or chitosan derivative	*Molecular weight*	*Radius of gyration (nm)*	*2nd virial coefficient ($g^{-2}.mol.cm^3$)*
Chitosan from krill	464,990[b]	75.0	1.20×10^{-3}
Water-soluble derivatives			
N-(Carboxymethyl)chitosan	543,300	70.2	0.83×10^{-3}
Glycol-*N*-(carboxymethyl)chitosan	316,000	62.9	—
N-(Carboxymethyl)chitosan 6-sulfate	16,000	—	—
Water-soluble Schiff bases			
Glyoxylate	1,075,000	113.8	1.80×10^{-3}
α-Ketoglutarate	592,530	83.1	0.93×10^{-3}
3,4-Dihydroxybenzaldehyde	896,490	112.9	1.39×10^{-3}

[a]Averages of at least three measurements. [b]In agreement with the value 450,000 obtained by g.p.c. (ref. 22).

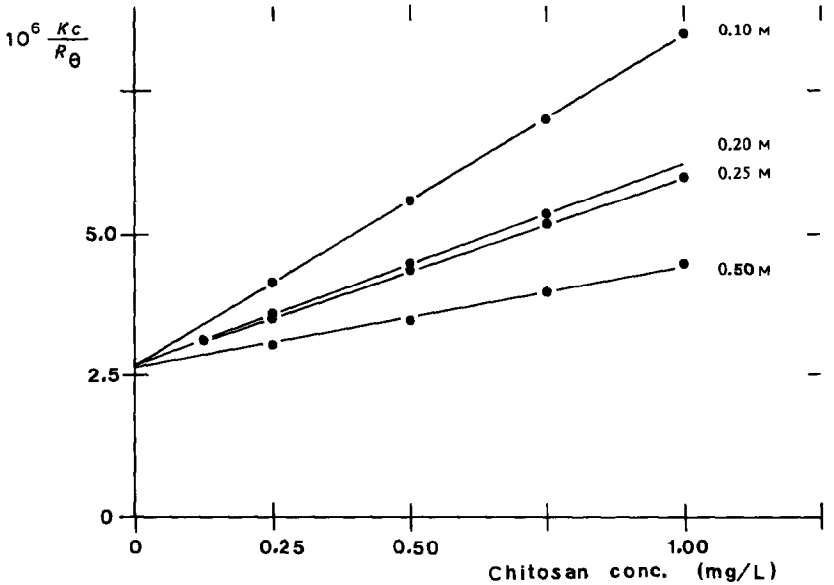

Fig. 3. Influence of salinity on the laser light-scattering behavior of crab chitosan PTL-124. Sodium acetate molarities were from 0.10 (upper curve) to 0.50 (lower curve). The extrapolated value common to all curves indicates that M_w is not influenced by the presence of the salt needed to depress the polyelectrolyte effect.

which indicates that the conditions adopted in order to obtain the sodium alcoholate form promotes the complete deacetylation of the polysaccharide. In this case, the deacetylation would mean an ~8% decrease of the M_w. The glycolation reaction is

further demonstrated by differential calorimetry, as shown in Fig. 5, where the three distinct thermal-degradation curves for krill chitosan, NCM-chitosan, and glycol-NCM-chitosan show qualitatively the different chemical nature of the three poly-

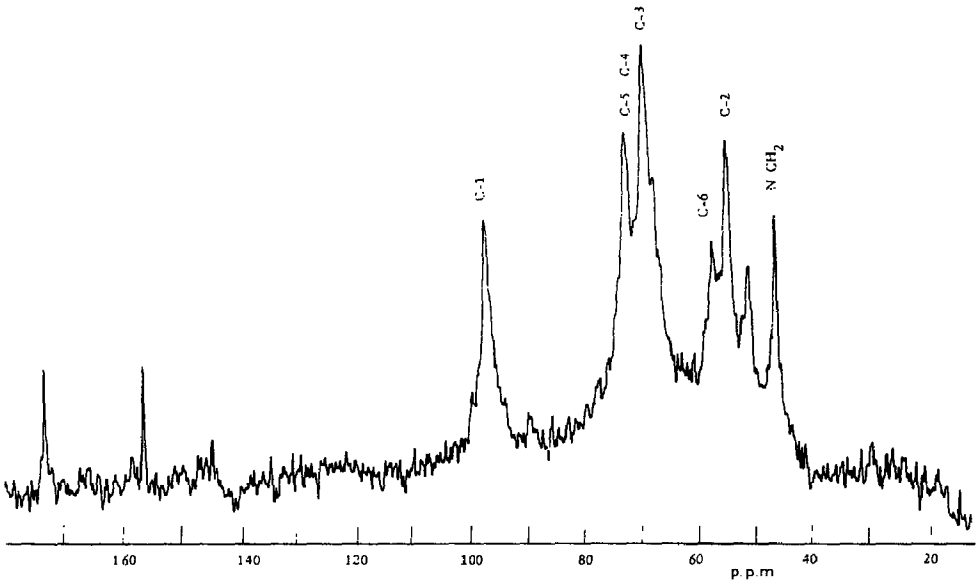

Fig. 4. ^{13}C-N.m.r. spectrum for glycol-NCM-chitosan, showing alterations in the C-6 region (compare to Fig. 1 in ref. 14).

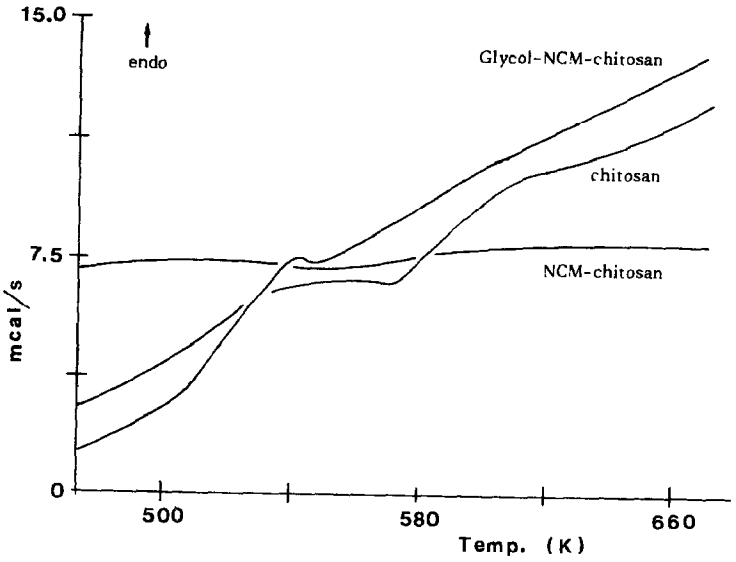

Fig. 5. Differential calorimetry curves for krill chitosan and its modified forms, NCM-chitosan and glycol-NCM-chitosan.

saccharides. Macroscopic evidence is also given by the general behavior of the product in terms of viscosity and solubility, the latter being greatly enhanced.

It is even possible to expect that the 6-(hydroxyethyl)-NCM-chitosan formed initially would further react with NaOH and 2-chloroethanol to extend the polyether side chain on C-6. This would have as a consequence a remarkable increase in the M_w. The experimental results, however, indicate a decrease in M_w, from 545,300 to 316,000, which shows that the polymer degradation due to the alkaline medium is hardly compensated for by the side-chain elongation and is a major component of the M_w decrease, in which the deacetylation reaction also takes part.

As far as Schiff bases are concerned, the data obtained in all three cases (see Table II) are much higher than expected. For the glyoxylic acid aldimine, a M_w that was nearly double was recorded, namely 1,075,000. The data relevant to the α-ketoglutaric acid product, 592,530, point to a 99% substitution, which is not in agreement with the 30% substitution measured by titration[15]. For the 3,4-dihydroxybenzaldehyde chitosan aldimine, the values are also high, 896,490, and could possibly be explained in terms of partial oxidation of the diphenol and subsequent cross-linking; however the general behavior of these three Schiff bases would indicate a tendency to association. As far as the reduced polymers are concerned, there is no measurable association for the water-soluble NCM-chitosan; the two other compounds, namely, glutamate-glucan and dihydroxybenzyl-chitosan, being insoluble, prevent further measurements in this direction. In general, the behaviour of these three Schiff bases would indicate a tendency to association which is not shared by their reduced products.

CONCLUSIONS

The weight-average molecular weight of chitosans can be readily measured by l.l.s. It appears that, during the last decade, the production technology of chitosan has improved to the point that chitosans of $M_w \sim 5.10^5$, rather than 1.10^5, can be generally obtained. Careful treatment of the raw materials yields chitosans of higher M_w.

N-(Carboxymethyl)chitosan 6-sulfate appears to be highly depolymerized, and no prospect of avoiding this degradation exists; on the other hand, stricter control of the conditions for preparation of glycol-*N*-(carboxymethyl)-chitosan can be exerted in order to keep its M_w value higher.

N-(Carboxymethyl)chitosan and glycine-glucan are confirmed to be fully substituted polysaccharides when produced in the presence of stoichiometric (or an excess of) glyoxylate. Laser light-scattering is found to be a rapid, nondestructive, and absolute analytical technique that outdates such elaborate and scarcely reliable techniques as viscometry and gel-permeation chromatography, as fas as determination of the M_w of chitosans is concerned. It is now possible to monitor the M_w data of a number of very attractive, water-soluble chitosans whose characteristic properties can only be repoduced if their molecular size can be kept under control.

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