Designation: F2602 – 13

Standard Test Method for Determining the Molar Mass of Chitosan and Chitosan Salts by Size Exclusion Chromatography with Multi-angle Light Scattering Detection (SEC-MALS)[1]

This standard is issued under the fixed designation F2602; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers the determination of the molar mass of chitosan and chitosan salts intended for use in biomedical and pharmaceutical applications as well as in tissue engineered medical products (TEMPs) by size exclusion chromatography with multi-angle laser light scattering detection (SEC-MALS). A guide for the characterization of chitosan salts has been published as Guide F2103.

1.2 Chitosan and chitosan salts used in TEMPs should be well characterized, including the molar mass and polydispersity (molar mass distribution) in order to ensure uniformity and correct functionality in the final product. This test method will assist end users in choosing the correct chitosan for their particular application. Chitosan may have utility as a scaffold or matrix material for TEMPs, in cell and tissue encapsulation applications, and in drug delivery formulations.

1.3 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

F2103 Guide for Characterization and Testing of Chitosan Salts as Starting Materials Intended for Use in Biomedical and Tissue-Engineered Medical Product Applications

2.2 *United States Pharmacopeia/National Formulary:*[3]

<621> Chromatography

2.3 *National Institute of Standards and Technology:*[4]

NIST SP811 Special Publication: Guide for the Use of the International System of Units (SI)

3. Terminology

3.1 *Definitions:*

3.1.1 *chitosan, n*—a linear polysaccharide consisting of β(1→4) linked 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-D-glucopyranose (GlcN). Chitosan is a polysaccharide derived by *N*-deacetylation of chitin.

3.1.2 *degree of deacetylation, n*—the fraction or percentage of glucosamine units (GlcN: deacetylated monomers) in a chitosan polymer molecule.

3.1.3 *molar mass average, n*—the given molar mass (M) of a chitosan will always represent an average of all of the molecules in the population. The most common ways to express the molar mass are as the *number average* ($\bar{M}_n$) and the *mass average* ($\bar{M}_w$). The two averages are defined by the following equations:

$$\bar{M}_n = \frac{\sum_i N_i M_i}{\sum_i N_i} \quad \text{and} \quad \bar{M}_w = \frac{\sum_i w_i M_i}{\sum_i w_i} = \frac{\sum_i N_i M_i^2}{\sum_i N_i M_i} \tag{1}$$

where:

N_i = number of molecules having a specific molar mass M_i, and

w_i = mass of molecules having a specific molar mass M_i.

3.1.3.1 *Discussion*—In a polydisperse molecular population the relation $\bar{M}_w > \bar{M}_n$ is always valid. The coefficient $\bar{M}_w / \bar{M}_n$ is referred to as the polydispersity index, and will typically be in the range 1.5 to 3.0 for commercial chitosans.

NOTE 1—The term molecular weight (abbreviated MW) is obsolete and should be replaced by the SI (Système Internationale) equivalent of either

[1] This test method is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.42 on Biomaterials and Biomolecules for TEMPs.

Current edition approved Aug. 1, 2013. Published September 2013. Originally approved in 2008. Last previous edition approved in 2008 as F2602–08ε1. DOI: 10.1520/F2602-13.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from United States Pharmacopeia and National Formulary, U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

[4] Available from National Institute of Standards and Technology (NIST), 100 Bureau Dr., Stop 1070, Gaithersburg, MD 20899-1070, http://physics.nist.gov/cuu/Units/bibliography.html.

relative molecular mass (M_r), which reflects the dimensionless ratio of the mass of a single molecule to an atomic mass unit (see ISO 31-8), or molar mass (M), which refers to the mass of a mole of a substance and is typically expressed as grams/mole. For polymers and other macromolecules, use of the symbols M_w, M_n, and M_z continue, referring to mass-average molar mass, number-average molar mass, and z-average molar mass, respectively. For more information regarding proper utilization of SI units, see NIST SP811.

4. Significance and Use

4.1 The degree of deacetylation of chitosan, as well at the molar mass and molar mass distribution, determines the functionality of chitosan in an application. For instance, functional and biological effects are highly dependent upon the composition and molar mass of the polymer.

4.2 This test method describes procedures for measurement of molar mass of chitosan chlorides and glutamates, and chitosan base, although it in principle applies to any chitosan salt. The measured molar mass is that for chitosan acetate, since the mobile phase contains acetate as counter ion. This value can further be converted into the corresponding molar mass for the chitosan as a base, or the parent salt form (chloride or glutamate).

4.3 Light scattering is one of very few methods available for the determination of absolute molar mass and structure, and it is applicable over the broadest range of molar masses of any method. Combining light scattering detection with size exclusion chromatography (SEC), which sorts molecules according to size, gives the ability to analyze polydisperse samples, as well as obtaining information on branching and molecular conformation. This means that both the number-average and mass-average values for molar mass and size may be obtained for most samples. Furthermore, one has the ability to calculate the distributions of the molar masses and sizes.

4.4 Multi-angle laser light scattering (MALS) is a technique where measurements of scattered light are made simultaneously over a range of different angles. MALS detection can be used to obtain information on molecular size, since this parameter is determined by the angular variation of the scattered light. Molar mass may in principle be determined by detecting scattered light at a single low angle (LALLS). However, advantages with MALS as compared to LALLS are: *(1)* less noise at larger angles, *(2)* the precision of measurements are greatly improved by detecting at several angles, and *(3)* the ability to detect angular variation allows determination of size, branching, aggregation, and molecular conformation.

4.5 Size exclusion chromatography uses columns, which are typically packed with polymer particles containing a network of uniform pores into which solute and solvent molecules can diffuse. While in the pores, molecules are effectively trapped and removed from the flow of the mobile phase. The average residence time in the pores depends upon the size of the solute molecules. Molecules that are larger than the average pore size of the packing are excluded and experience virtually no retention; these are eluted first, in the void volume of the column. Molecules, which may penetrate the pores will have a larger volume available for diffusion, they will suffer retention depending on their molecular size, with the smaller molecules eluting last.

4.6 For polyelectrolytes, dialysis against the elution buffer has been suggested, in order to eliminate Donnan-type artifacts in the molar mass determination by light scattering (**1, 2**).[5] However, in the present method, the size exclusion chromatography step preceding the light scatter detection is an efficient substitute for a dialysis step. The sample is separated on SEC columns with large excess of elution buffer for 30 to 40 min, and it is therefore in full equilibrium with the elution buffer when it reaches the MALS detector.

5. Materials

5.1 *Chemicals:*

5.1.1 Chitosan or chitosan salt sample.

5.1.2 Deionized water (Milli-Q Plus or equivalent; conductivity < 10 µS/cm).

5.1.3 CH_3COONH_4 (ammonium acetate).

5.1.4 Pullulan standards. (See Note 2.)

NOTE 2—A series of linear homopolysaccharides with sufficiently narrow dispersity to be suitable for utilization as molar mass calibration standards in aqueous eluent.

5.2 *Mobile Phase:*

5.2.1 For SEC-MALS of chitosan and chitosan salts, a mobile phase stock solution of 0.40 mol/L CH_3COONH_4 in deionized water is prepared. Adjust the pH to 4.5 using acetic acid.

5.2.2 The stock solution can be stored cool (3 to 8°C) for 6 months. Before use as a mobile phase, the stock solution is diluted 1:1 (v/v) with deionized water and passed through a 0.22 µm filter.

5.3 *Instruments:*

5.3.1 Analytical balance (0.1 mg).

5.3.2 Shaking device.

5.3.3 pH meter.

5.3.4 HPLC system with injector, pump, degassing unit.

5.3.5 Size exclusion columns: TSK-Gel PW_{XL} columns from Tosoh Biosep., for example, PW_{XL}-guard column + G6000 PW_{XL} + G5000 PW_{XL} + G3000 PW_{XL} (last in the series), or equivalent.

5.3.6 Refractive Index (RI) detector, with a known calibration constant (*dn/dV*).

5.3.7 Multiple Angle Laser Light Scattering (MALS) detector, with known calibration constant.

5.3.8 Computer with suitable software.

6. Procedure

6.1 *Preparation of Standards and Chitosan Salt Samples for SEC-MALS:*

6.1.1 Samples are prepared at a concentration suitable for injection of 200 µL of sample.

6.1.2 Dissolve all samples in deionized water at twice the required concentration for molar mass determination by shaking at about 100 min^{-1} overnight at cool temperature (3 to 8°C).

6.1.3 Dilute samples 1+1 with stock solution of mobile phase and shake gently for a few seconds.

[5] The boldface numbers in parentheses refer to a list of references at the end of this standard.

6.1.4 Pass all samples through a 0.45 µm filter, and transfer to HPLC vials.

6.1.5 Final concentration of pullulan standards of known $\bar{M}_w$ values of approximately 11 800 to 47 300, 112 000, 212 000, and 404 000 g/mol should be approximately 4, 3, 2, and 1.5 mg/mL, respectively.

6.1.6 Guidelines for final concentration of chitosans for molar mass determination are given in Table 1. If SEC-MALS data display poor reproducibility with respect to replicates, this might be an indication of column overload. In this case, less sample should be injected.

6.2 *Preparation of Chitosan Base Samples for SEC-MALS:*

6.2.1 Samples are prepared at a concentration suitable for injection of 200 µL of sample.

6.2.2 Dissolve the chitosan base in 1 % acetic acid to a 1 % solution by shaking at about 100 min^{-1} overnight at cool temperature (3 to 8°C).

6.2.3 Dilute samples mobile phase (Note—2 mol/L ammonium acetate, not stock solution) to the required concentration (Table 3) and shake gently for a few seconds.

6.2.4 Filter all samples through a 0.45 µm filter, and transfer to HPLC vials.

6.3 *Chromatography and Data Collection:*

6.3.1 The complete experimental setup of the SEC-MALS system is shown in Fig. 1. The refractive index detector is placed at the end of the solvent/sample line as it is highly sensitive to pressure changes.

6.3.2 Pullulan standards should be injected and analyzed with 2 replicates before and after all chitosan samples (total of 4 replicates). Three (3) replicates should be injected for chitosans.

6.3.3 A procedure for setting up the chromatography run and collecting the data is given below:

6.3.3.1 Use a flow rate of 0.5 mL/min.

6.3.3.2 Purge the injector with mobile phase before the sample set is run.

6.3.3.3 Purge the RI-detector for at least 30 min (at 0.5 mL/min) before start of the run.

6.3.3.4 Confirm that both the MALS detector and RI detector has a stable and low baseline level.

6.3.3.5 Define the collection set-up as follows:

(1) Inject 200 µL of sample.

(2) After a collection delay of 10 mL (20 min), data should be collected from both detectors every 2 seconds for 40 mL (80 min).

(3) Use *dn/dc* = 0.148 mL/g and 0.142 mL/g for pullulans and chitosans, respectively (relevant only for calculations).

TABLE 1 Suggestions for Concentration and Injected Mass of Chitosan Chloride Samples for SEC-MALS

Apparent Viscosity as Chitosan Chloride (mPas)	Concentration for Injection (mg/mL)	Injected Mass[A] (mg)
<10	2	0.4
10–50	1	0.2
50–100	0.75	0.15
>100	0.5	0.1

[A] Injected mass = Concentration*200 µL.

TABLE 2 Suggestions for Concentration and Injected Mass of Chitosan Glutamate Samples for SEC-MALS

Apparent Viscosity as Chitosan Glutamate (mPas)	Concentration for Injection (mg/mL)	Injected Mass[A] (mg)
<10	1.5	0.3
10–50	1	0.2
>50	0.75	0.15

[A] Injected mass = Concentration*200 µL.

TABLE 3 Suggestions for Concentration and Injected Mass of Chitosan Base Samples for SEC-MALS

Apparent Viscosity as Chitosan Acetate (mPas)	Concentration for Injection (mg/mL)	Injected Mass[A] (mg)
<100	0.75	0.15
100–500	0.5	0.1
>500	0.375	0.075

[A] Injected mass = Concentration*200 µL.

(4) Use a second virial coefficient of $2*10^{-4}$ mol.mL.g^{-2} and $5*10^{-3}$ mol.mL.g^{-2} for pullulans and chitosans, respectively (relevant only for calculations).

6.3.4 After all samples have been run, purge the injector with deionized water to wash off remaining salt from the valves.

6.4 *Data Analysis:*

6.4.1 Data analysis follows closely recommended procedures for SEC-MALS data. Generally, the chromatograms are divided into a number of volume elements, defined by the peak width, the rate of data collection and the flow rate. Concentration of sample in each volume element (c_i) is determined from the RI-detector response using known values of *dn/dc* and *dn/dV* (the RI-detector calibration constant). Furthermore, LS-detector response is divided by c, the molar mass in each volume element (M_i) is considered monodisperse, and the mass is determined from a Zimm representation of a Debye plot by extrapolation to zero angle (which is essentially a solution to Eq X2.1 in X2.2). Once the values of c_i and M_i are known, calculation of the various average molar masses is straightforward.

6.4.2 In detail, the above procedure consists of the following operations to be performed in a suitable software:

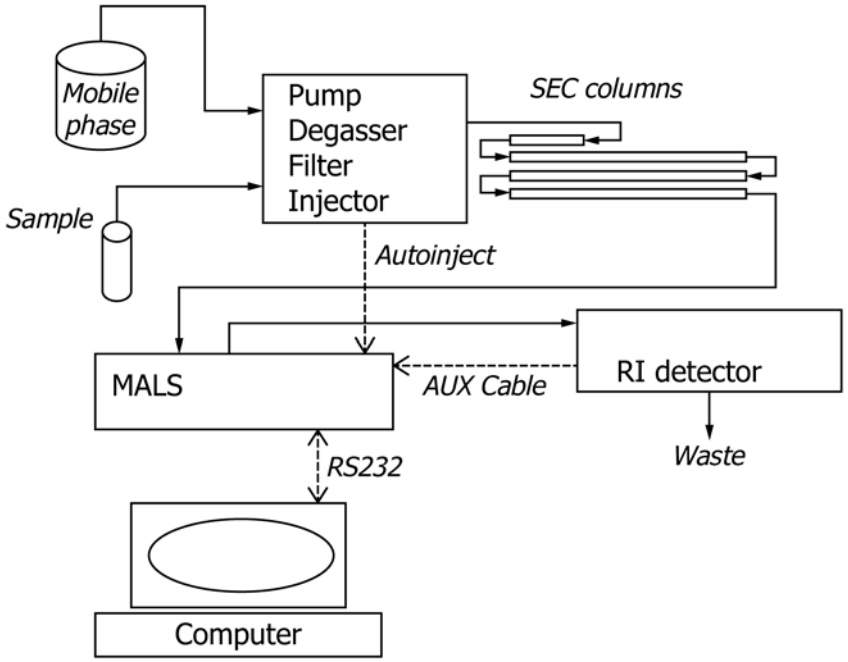
6.4.2.1 Define baselines for signals from both detectors.

6.4.2.2 Calculate inter-detector delay volume using a monodisperse low-molar mass pullulan standard.

6.4.2.3 Define the peak area of interest.

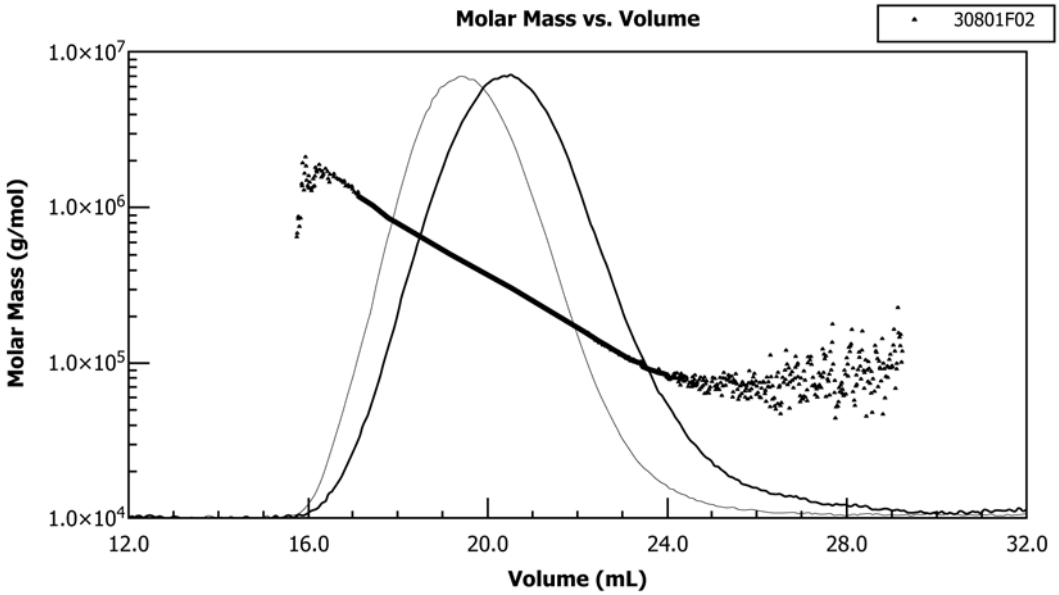
6.4.2.4 Normalize LS-detector responses to correct for different sensitivity at different angles. Normalization is performed on an isotropic scatterer (low molar mass compound) in the sample set, and is saved with the data file. For the other samples, one reads the normalization performed on an isotropic scatterer from file.

6.4.2.5 Check the goodness-of-fit of the LS-detectors using a 3-D representation of the data or a Debye-plot (in Zimm representation). Do not use LS-detector responses that are clearly non-linear.



NOTE 1—Solid lines indicate solvent/sample flow, dashed lines indicate cabling for data transfer.

FIG. 1 Complete SEC-MALS Set-Up



NOTE 1—Solid line: RI detector; dashed line: MALS detector; (♦) molar mass for each chromatographic data point.

FIG. 2 A Chromatogram of Chitosan Chloride ($\bar{M}_w$ of 360 000 g/mol, as acetate)

6.4.2.6 Perform the required calculations for determination of $\bar{M}_n$, $\bar{M}_w$ and $\bar{M}_w/\bar{M}_n$, using a Zimm representation of the Debye plot (that is, a plot of $K^*c/R(\theta)$ versus $\sin\theta^2/2$) for solving Eq X2.1.

6.4.3 *Conversion of* $\bar{M}_w$-values—The mobile phase is 0.2 mol/L ammonium acetate, and the $\bar{M}_w$ determined according to this method is that of chitosan acetate [$\bar{M}_w$(Ac)]. This mass average molar mass can be converted to that of chitosan chloride [$\bar{M}_w$ (Cl)], glutamate [$\bar{M}_w$(G)] or base [$\bar{M}_w$(B)] according to the following equations:

$$\bar{M}_w(\mathrm{G}) = \bar{M}_w(\mathrm{Ac})*(\%\mathrm{DA}*\mathrm{M}(\mathrm{G})+(1 - \%\mathrm{DA})*\mathrm{M}(\mathrm{A}))/(\%\mathrm{DA}*\mathrm{M}(\mathrm{Ac})+(1-\%\mathrm{DA})*\mathrm{M}(\mathrm{A})) \quad (2)$$

$$\bar{M}_w(\mathrm{Cl}) = \bar{M}_w(\mathrm{Ac})*(\%\mathrm{DA}*\mathrm{M}(\mathrm{Cl})+(1 - \%\mathrm{DA})*\mathrm{M}(\mathrm{A}))/(\%\mathrm{DA}*\mathrm{M}(\mathrm{Ac})+(1-\%\mathrm{DA})*\mathrm{M}(\mathrm{A})) \quad (3)$$

$$\bar{M}_w(\mathrm{B}) = \bar{M}_w(\mathrm{Ac})*(\%\mathrm{DA}*\mathrm{M}(\mathrm{B}) + (1 - \%\mathrm{DA})*\mathrm{M}(\mathrm{A}))/(\%\mathrm{DA}*\mathrm{M}(\mathrm{Ac}) + (1 - \%\mathrm{DA})*\mathrm{M}(\mathrm{A})) \quad (4)$$

where:

%DA = degree of deacetylation,
M(G) = molar mass of deacetylated chitosan residue in glutamate form = 308 g/mol,
M(Cl) = molar mass of deacetylated chitosan residue in chloride form = 197 g/mol,
M(Ac) = molar mass of deacetylated chitosan residue in acetate form = 221 g/mol,
M(B) = molar mass of uncharged deacetylated chitosan residue (base) = 161 g/mol, and
M(A) = molar mass of acetylated chitosan residue = 203 g/mol.

7. Control and Approval of Data

7.1 The mass average molar mass, $\bar{M}_w$, and $\bar{M}_w/\bar{M}_n$ for the replicates of each sample (4 replicates for pullulan standards, 3 replicates for chitosans) should be calculated. Standard deviations for $_w$ should be calculated.

7.2 For approving the data the following conditions apply:

7.2.1 *Condition 1*—$\bar{M}_w$ of pullulan standards (using at least 3 replicates) should be within ±10 % of the stated value from the manufacturer.

7.2.2 *Condition 2*—Relative standard deviation (RSD, for example, standard deviation divided by mean value) for pullulan standards should be less than ±10 %.

7.2.3 *Condition 3*—Reproducibility in the detector responses for the 3 replicates of chitosan samples should be manually evaluated. Different curve forms may indicate column overload, and reanalysis at lower concentration should be considered.

7.3 If condition 1 or 2 fails, the entire sample set needs reanalysis. The system should be inspected for possible faults before the reanalysis.

7.4 Failure of condition 3 requires reanalysis of the chitosan sample in question, only.

8. Precision and Reporting Results

8.1 The precision/relative standard error (RSE) of the method is <10 %, as shown in method validation.

8.2 Data on $\bar{M}_w$ should be reported rounded off to the nearest whole ten thousand in units of g/mol, for example 260 000 g/mol.

8.3 $\bar{M}_w$ values should be reported for chitosans as the acetate form. Additionally, for convenience, the $\bar{M}_w$ can also be reported calculated as chitosan base.

APPENDIXES

(Nonmandatory Information)

X1. RATIONALE

X1.1 The use of naturally occurring biopolymers for biomedical and pharmaceutical applications and in tissue engineered medical products (TEMPs) is increasing. This test method is designed to give guidance in characterizing chitosan and chitosan salts used in such applications.

X2. BACKGROUND

X2.1 Chitosan is a linear, binary polysaccharide consisting of β(1→4) linked 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc; acetylated unit) and 2-amino-2-deoxy-D-glucopyranose (GlcN; deacetylated unit). The two different monosaccharides differ only by the substitution at carbon 2; GlcNAc contains an *N*-acetylated amino group, whereas GlcN contains only the amino-group (it is said to be deacetylated). Thus, the degree of deacetylation (in %) is a measure of the fraction of GlcN-units in the chitosan chain.

X2.2 The principles of SEC-MALS can be summarized as follows: Samples of polymer are injected into the mobile phase and separated according to size on the SEC columns. For a given concentration *c* (g/mL) of the solute, the scattered light signal as measured by the MALS detector is proportional to *cM*, where *M* is molar mass (or the mass average molar mass, $\bar{M}_w$, for non-fractionated polydisperse samples). Using a concentration (for example, refractive index) detector to measure *c*, one may determine the molar mass in each volume fraction eluted from the columns. Solving Eq X2.1 is the heart of this analysis:

$$K*c/R(\theta) = 1/\left\{\bar{M}_w*P(\theta)\right\} + 2A_2c \quad (X2.1)$$

X2.2.1 The excess Rayleigh ratio R(θ) is the light scattered by the solution at an angle θ in excess of that scattered by pure solvent, divided by the incident light intensity. A_2 is the second virial coefficient. K* is equal to $4\pi^2 n_0^2 (dn/dc)^2/\{\lambda_0^4 N_A\}$, where n_0 is the refractive index of the solvent, λ_0 is the vacuum wavelength of incident light, and N_A is Avogadro's number. Finally, P(θ) is a form factor which depends on the structure of the scattering molecules and describes the angular dependence

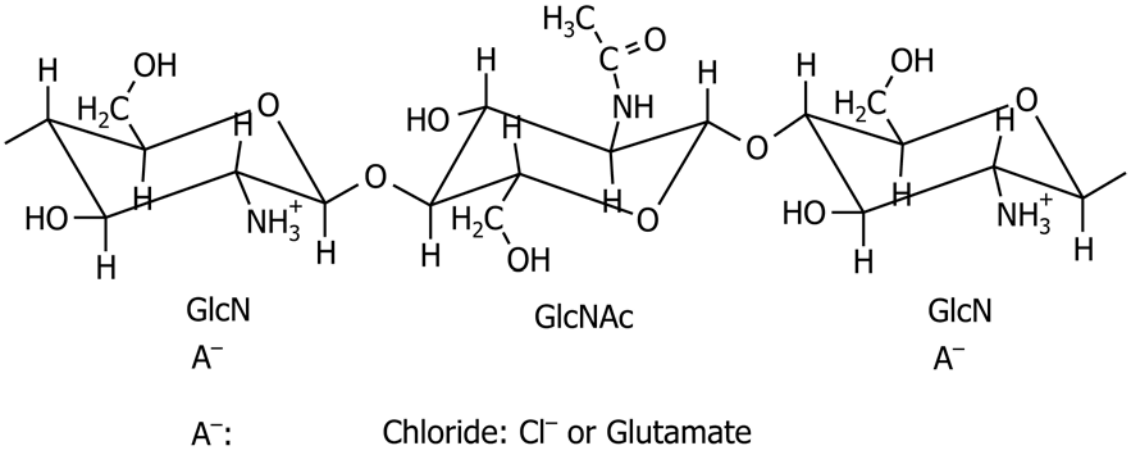


FIG. X2.1 Structure of Chitosan

of the scattered light, from which the mean square radius of the molecules may be determined.

X2.2.2 Eq X2.1 is typically solved using a Debye plot (that is, plotting $R(\theta)/(K^*c_i)$ versus $\sin^2(\theta/2)$, where the $\sin^2(\theta/2)$ term results from an expansion of $P(\theta)$), for each volume element eluted from the SEC columns assuming monodispersity within each volume element. By extrapolating the Debye plot to zero angle, the intercept yields the mass directly. The Debye plot is also commonly performed using a Zimm representation (that is, plotting $(K^*c_i)/R(\theta)$ versus $\sin^2(\theta/2)$), from which the intercept yields the inverse of the molar mass ($1/M_i$). The Zimm representation of the Debye plot may be preferable for macromolecules like alginates and chitosans, since only linear fits to zero angle are normally required. A comprehensive review of light scattering and absolute characterization of macromolecules, including experimental procedures, have been reported by P. J. Wyatt **(3)**.

X2.2.3 No molar mass standards are required in the analysis. Only a set of fundamental or measured constants (π, n_0, (dn/dc), λ_0, N_A, A_2) and a set of experimentally measured values (c, $R(\theta)$, $P(\theta)$) are required to calculate the molar mass.

X2.3 Once the sample has been fractionated and c_i and M_i have been determined in each fraction, calculation of the mass average molar mass is given by Eq X2.2:

$$\bar{M}_w = \sum_i w_i M_i / \sum_i w_i = \sum_i c_i M_i / \sum_i c_i \qquad \text{(X2.2)}$$

X3. CONSTANT VALUES

X3.1 *Calibration and Normalization of MALS-detector*—A MALS detector typically incorporates several photoreceptors in a planar circular arrangement around a flow cell, with laser light passing down the bore of the flow cell. Calibration of the MALS detector is required to establish a correct reading, typically for the 90° detector, using a well-defined isotropic scatterer (toluene is commonly employed). Calibration should be done following the instrument manufacturer's recommended procedures, and checked for consistency and agreement with instrument manufacturer's expected value. Furthermore, normalization of the other photoreceptors to the 90° detector is required to reduce errors caused by differences in sensitivities of each detector and difference in distance between the sample and each detector. Normalization is typically done using an isotropic scatterer in the same solvent as the unknown samples to be analyzed. Pullulan standards are common in use in aqueous solvents. Bovine serum albumin (BSA) is another commonly used isotropic scatterer for normalization in aqueous solvent.

X3.2 *RI-detector Calibration Constant*—In the experimental setup described herein, the RI-detector is used as an absolute concentration detector. Depending on the type of detector, one may need to calibrate the analog output of the detector to refractive index (dn/dV). Such calibration can be performed using a concentration series of samples with known refractive index increment (dn/dc), and recording the voltage output at each concentration. NaCl is commonly used for calibration of RI detectors.

X3.3 *Refractive Index Increment, dn/dc*—It has been reported that dn/dc-values are strongly dependent on the degree of deacetylation of chitosans **(4, 5)**. However, in acetate buffers, including the acetate counter ion in the chitosan concentration term, it has been shown that $(dn/dc)_\mu$ (dn/dc measured on dialyzed samples, for example, at constant chemical potential) is independent on degree of deacetylation in the range of 60 to 99 %, which encompasses most commercial chitosan preparations, with a value of 0.142 mL/g (±0.004

mL/g) (6). The uncertainty quoted for this value is based on one standard deviation for 15 replicate samples on 4 different chitosans with different degree of deacetylation, and corresponds to a relative standard error of 3 %. For pullulan in aqueous solvent, a value 0.148 mL/g is suggested according to Nordmeier (7). Values of $(dn/dc)_{\mu}$ can be determined by measuring the refractive index of a concentration series of dialyzed samples on a RI-detector with known calibration constant (*dn/dV*).

X3.4 *Second Virial Coefficient, A_2*—A_2 is a measure of macromolecular self-association and deviation from ideality of infinitely dilute solutions. Beri et al (8), found values of 2-3*10^{-3} mol.mL.g^{-2} for different chitosans in a SEC-MALS system. Anthonsen et al (9) found values of 2-7*10^{-3} mol.mL.g^{-2} by osmotic pressure measurements, depending somewhat on the degree of deacetylation. 5*10^{-3} mol.mL.g^{-2} appears to be a reasonable average value for chitosans, with an uncertainty of ±50 %. Nordmeier (7) reported second virial coefficient of 2*10^{-4} mol.mL.g^{-2} for pullulan. A_2-values can be determined by light scattering experiments in batch mode, and by constructing a classical Zimm-plot to fit and extrapolate data to zero concentration and zero angle. A_2 can also be determined in chromatography mode following the procedures published by Girod et al (10).

REFERENCES

(1) Casassa, E. F., and Eisenberg, H., *J. Phys. Chem*., 64, 1960, pp. 753–756.
(2) Hennink, W. E., van den Berg, J. W. A., and Feijen, J., *Thrombosis Research*, 45, 1987, pp. 463–475.
(3) Wyatt, P.J., *Analytica Chimica Acta*, 272, 1993, pp. 1–40.
(4) Wang, W., Bo, S., Li, S., and Qin, W., *Int. J. Biol. Macromol*., 13, 1991, pp. 281–285.
(5) Sorlier, P., Viton, C., and Domard, A., *Biomacromolecules*, 3, 2002, pp. 1336–1342.
(6) Christensen, B. E., unpublished results.
(7) Nordmeier, E., *J. Phys. Chem*., 97, 1993, pp. 5770–5785.
(8) Beri R. G., Walker, J., Reeses, E. T., and Rollings, J. E., *Carbohydrate Res*., 298, 1993, pp. 11–23.
(9) Anthonsen, M. W., Vårum, K. M., and Smidsrød, O., *Carbohydrate Polym*., 22, 1993, pp. 193–201.
(10) Girod, et al, *Journal of Chromatography A*, 943, 2001, pp. 147–152.