Designation: F2605 – 16

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

This standard is issued under the fixed designation F2605; 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 (typically expressed as grams/mole) of sodium alginate 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 alginate has been published as Guide F2064.

1.2 Alginate 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 alginate for their particular application. Alginate 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]

F2064 Guide for Characterization and Testing of Alginates as Starting Materials Intended for Use in Biomedical and Tissue Engineered Medical Product Applications

F2315 Guide for Immobilization or Encapsulation of Living Cells or Tissue in Alginate Gels

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

2.4 *ISO Standards:*[5]

ISO 31-8 Quantities and units- Part 8: Physical chemistry and molecular physics

3. Terminology

3.1 *Definitions:*

3.1.1 *alginate, n*—a polysaccharide substance extracted from brown algae, mainly occurring in the cell walls and intercellular spaces of brown seaweed and kelp. Its main function is to contribute to the strength and flexibility of the seaweed plant. Sodium alginate, and in particular calcium cross-linked alginate gels are used in tissue-engineered medical products (TEMPs) as biomedical scaffolds and matrices, for immobilizing living cells (see Guide F2315), and in drug delivery systems.

3.1.2 *molar mass average, n*—the given molar mass (Mw) of an alginate 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:

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

[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.

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[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.

[5] Available from International Organization for Standardization (ISO), ISO Central Secretariat, BIBC II, Chemin de Blandonnet 8, CP 401, 1214 Vernier, Geneva, Switzerland, http://www.iso.org.

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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.

3.1.2.1 *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 alginates.

NOTE 1—The term molecular weight (abbreviated MW) is obsolete and should be replaced by the SI (Système Internationale) equivalent of either 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 composition and sequential structure of alginate, as well as the molar mass and molar mass distribution, determines the functionality of alginate in an application. For instance, the gelling properties of an alginate are highly dependent upon the composition and molar mass of the polymer.

4.2 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 to obtain 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.

4.3 Multi-angle laser light scattering (MALS) is a technique where measurements are made simultaneously over a range of different angles and used to determine the scattering at 0°, which directly relates to molecular weight. MALS detection can be used to obtain information on molecular size, since this parameter is determined by the angular variation of the scattered light. This can be related to branching, aggregation, and molecular conformation. Molar mass can also be determined by detecting scattered light at a single low angle (LALS) and assuming that this is not significantly different from the scattering at 0°.

4.4 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 penetrate the pores will have a larger volume available for diffusion; their retention will depend on their molecular size, with the smaller molecules eluting last.

4.5 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**).[6] 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 Alginate sample.

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

5.1.3 Na_2SO_4 (sodium sulfate).

5.1.4 EDTA (ethylene diamine tetraacetic acid).

5.1.5 NaOH (1 mol/L).

5.1.6 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 *The Mobile Phase:*

5.2.1 For SEC-MALS of alginate, a mobile phase of 0.05 mol/L Na_2SO_4/0.01 mol/L EDTA in deionized water is used. Adjust pH to 6.0 using 1 mol/L NaOH.

5.2.2 Mobile phase should be prepared as a stock solution of 0.10 mol/L Na_2SO_4/0.02 mol/L EDTA in deionized water, which 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 High Performance Liquid Chromotography (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 Alginate Samples for SEC-MALS:*

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

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

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.

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 the final concentration of alginates 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 *Chromatography and Data Collection:*

6.2.1 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.

6.2.2 Pullulan standards should be injected and analyzed with 2 replicates before and after all alginate samples (total of 4 replicates). 3 replicates should be injected for alginates.

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

6.2.3.1 Use a flow rate of 0.5 mL/min.

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

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

6.2.3.4 Confirm that both the MALS detector and RI detector have stable and low baseline levels.

6.2.3.5 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 0.150 mL/g for pullulans and alginates, respectively (relevant only for calculations).

(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 alginates, respectively (relevant only for calculations).

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

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

$\bar{M}_w$ (g/mol)	Apparent Viscosity (mPas)	Concentration for Injection (mg/mL)	Injected Mass[A] (mg)
<50 000	<10	4	0.8
50 000–75 000	10–20	3	0.6
75 000–100 000	20–40	2	0.4
100 000–150 000	40–100	1.5	0.3
150 000–250 000	100–300	1	0.2
>250 000	>300	0.5	0.1

[A] Injected mass = Concentration*200 µL.

6.3 *Data Analysis:*

6.3.1 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. The 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, MALS-detector response is divided by c_i, 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.

6.3.2 In detail, the above procedure consists of the following operations to be performed using suitable software:

6.3.2.1 Define baselines for signals from both detectors.

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

6.3.2.3 Define the peak area of interest.

6.3.2.4 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.

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

6.3.2.6 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.

7. Control and Approval of Data

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

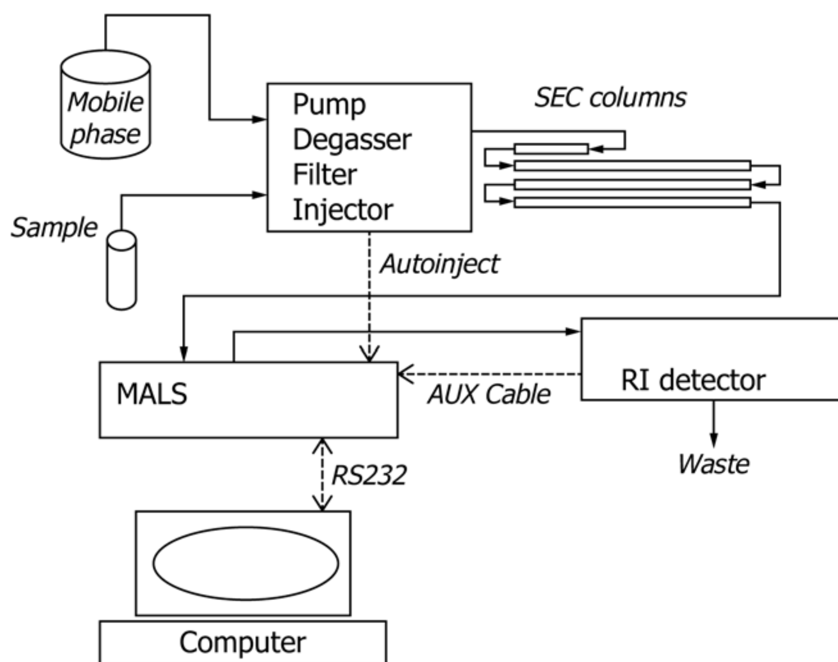
7.2 For approving the data the following conditions apply:

7.2.1 *Condition 1—*$\bar{M}_w$ of pullulan standards (using at least 3 replicates) shall 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 shall be less than ±10 %.

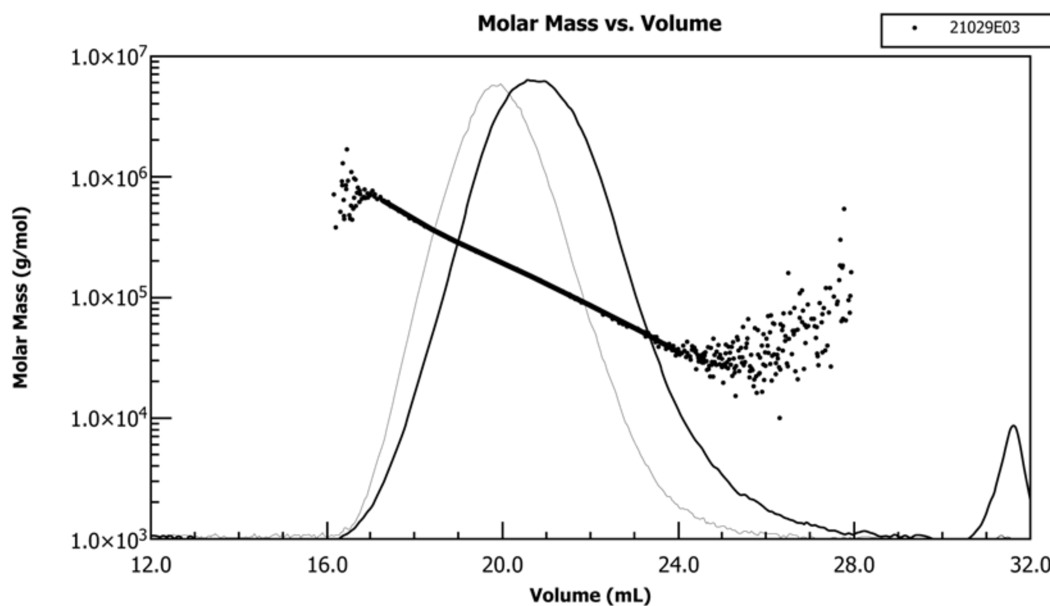
7.2.3 *Condition 3*—Reproducibility in the detector responses for the 3 replicates of alginate samples shall be manually evaluated. Different curve forms may indicate column overload, and reanalysis at lower concentration shall be considered.

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



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

FIG. 1 Complete SEC-MALS Setup



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

FIG. 2 A Chromatogram of Sodium Alginate ($\bar{M}_w$ 160 000 g/mol)

7.4 Failure of condition 3 requires reanalysis of the alginate 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, 160 000 g/mol.

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 the characterization of sodium alginate used in such applications.

X2. BACKGROUND

X2.1 Alginate is a family of non-branched binary copolymers of 1-4 glycosidically linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues. The relative amount of the two uronic acid monomers and their sequential arrangement along the polymer chain vary widely, depending on the origin of the alginate. The uronic acid residues are distributed along the polymer chain in a pattern of blocks, where homopolymeric blocks of G residues (G-blocks), homopolymeric blocks of M residues (M-blocks) and blocks with alternating sequence of M and G units (MG-blocks) co-exist. It has also been shown by nuclear magnetic resonance (NMR) spectroscopy that alginate has no regular repeating unit.

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:

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

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 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(θ)), 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 Column calibration molecular weight standards are not 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(θ), P(θ), detector calibration constant) are required to calculate the molar mass.

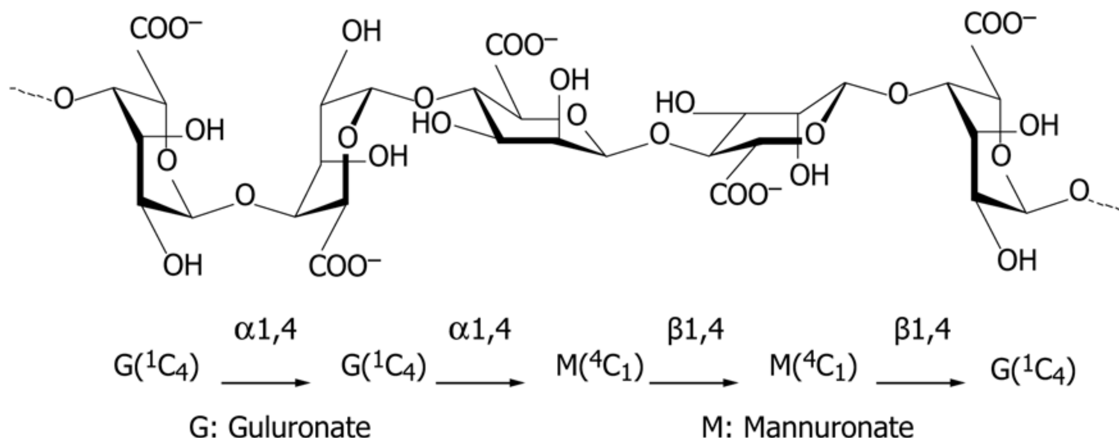


FIG. X2.1 Structure of Alginate

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. Calibration of the MALS detector is required to establish a correct reading, typically for the 90° detector, using a well-defined isotropic scatterer (toluene or a NIST-traceable molecular weight standard can be used). Calibration should be done following the instrument manufacturer's recommended procedures, and checked for consistency and agreement with the 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 the 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 commonly used 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*—Martinsen et al (**4**) found a value of *dn/dc* of 0.150 mL/g for sodium alginate in aqueous 0.1M NaCl at 633 nm. No uncertainty was reported for this value, but it was reported to be in accordance with previous literature data. For pullulan in aqueous solvent, a value 0.148 mL/g is suggested according to Nordmeier (**5**). Values of $(dn/dc)_\mu$ (*dn/dc* measured on dialyzed samples, for example, at constant chemical potential) can be determined by measuring the refractive index of a concentration series of 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. Martinsen et al (**4**) found values of $1.36*10^{-3}$ mol.mL.g^{-2} to $7*10^{-3}$ mol.mL.g^{-2} using wide-angle and low-angle laser light-scattering on sodium alginates of different sources and chemical composition. A value of $5*10^{-3}$ mol.mL.g^{-2} appears to be a reasonable average value for sodium alginates, with an uncertainty of ±50 %. Nordmeier (**5**) 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 (**6**).

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