SEC-MALS USER TRAINING/CHROMATOGRAPHY LAB

Motivation & Introduction

Size exclusion chromatography with multi-angle light scattering is used for the determination of absolute molecular weight (MW). A concentration detector, such as refractive index (RI) or ultraviolet (UV), is required for all SEC and SEC-MALS applications. Classical SEC uses RI or UV detection and polymeric standards to establish the retention times of polymers with known MW to create a calibration curve. This is traditionally used to estimate the MW of samples, but can produce errors when the standards do not accurately represent the shape, size, or interactions of your samples with the column's stationary phase. The combination of SEC with MALs uses the relationship between light scattering and molar mass (Rayleigh ratio), which allows the determination of the absolute molecular weight without the use of standards (as shown in the equation below).

$$\textbf{Rayleigh ratio - } I(\theta) \propto R(\theta) = K^{*} M c P(\theta)$$

- $I(\theta)$ = intensity of light scattered
- $R(\theta)$ = Rayleigh ratio
- $K^{*} = \frac{4\pi^{2} n_{0}^{2}}{N_{A} \lambda_{0}^{4}} \left(\frac{dn}{dc}\right)^{2}$ = optical contrast constant
    - n_{0} = solvent refractive index
    - N_{A} = Avogadro's number
    - N_{A} = vacuum wavelength of incident light
    - $\frac{dn}{dc}$ = specific refractive index increment in mL/g
- M = molar mass in g/mol
- c = solute concentration (w/v)
- $P(\theta)$ = form factor or scattering function, relates angular variation in scattering intensity to the mean square radius of the particle

Based on the variables in the Rayleigh ratio, only the specific refractive index increment (*dn/dc*) is unknown and needs to be calculated. The *dn/dc* can be determined by calculating the change in refractive index per the change in concentration of solute. Using a differential RI detector, the optical contrast constant (K^{*}) can be established.

Application of SEC-MALS requires assessment of solubility, solvent, column compatibility, and determination of the *dn/dc*. The *dn/dc* value is also used to determine the concentration of any particles eluting through the SEC column based on the RI response to calculate MW at each point throughout an

elution profile. Thus, *dn/dc* is one of the most important values to determine, especially since it is unique for every analyte and solvent combination. MW distributions (M_n, M_w, M_z) can be determined since MW can be calculated at each data point from the chromatogram. Weight average molar mass (M_w) takes into account the weight of a polymer chain in determining the total molecular weight average. Number average molar mass (M_n) takes into account the number of chains that make up the MW. The z-average MW (M_z) emphasizes the contribution of larger MW polymer chains by using the third moment of molar mass, which can be useful to predict mechanical properties. M_z is less frequently used compared to M_w and M_n since the dispersity (D or traditionally known as polydispersity index (PDI)) can be determined from the ratio of M_w to M_n.

$$M_w = \frac{\sum N_i M_i^2}{\sum N_i M_i} \qquad M_n = \frac{\sum N_i M_i}{\sum N_i} \qquad M_z = \frac{\sum N_i M^{n+1}}{\sum N_i M_i^n} \qquad D = \frac{M_w}{M_n}$$

M_i is the molecular weight of a chain, and N_i is the number of chains of that molecular weight. In the M_z calculation, $n = 2$ produces the z-average MW, and greater values will produce increasing series of molecular weights.

So, if we measure the intensity of scattered light, calculate the concentration of eluting particles, and know the *dn/dc* of said particles, the full range of molecular weight distributions and dispersity can be calculated.

Objectives & Outcomes

- Students will gain theoretical and operational knowledge of SEC-MALS and relationship to *dn/dc* and MW determination
- Determine batch *dn/dc* of the sample of their interest (approved by the instructor) or polystyrene using a batch system (manual injection) with the RI detector.
    - Interim report with *dn/dc* data analysis and evaluation of instrument repeatability
- Analyze samples, normalization standard, and polystyrene calibration with SEC-MALS-RI and compare MW determination based on traditional SEC retention time calibration and SEC-MALS theoretical relationship.
    - Final compiled report with *dn/dc* and MW distributions including Mn, Mw, and Mz and D (dispersity).

Preparation for the lab includes the following items described below in detail:

a) **Lab sign up**. The two parts of the lab must be performed in close sequence to utilize the same solvents and samples. Sign up with the instructor or upon agreement with the operator.

b) **Take-home prelab test.** Before the lab, students are expected to complete the Home to Take Lab Test. The results of this test have to be provided at the beginning of the lab and will be discussed and reviewed with the instructor. **Unsatisfactory preparation/knowledge** will result in a cancellation of a lab appointment, loss of 5 pts, and the lab will be deferred until a new time slot is available. *The test must be handwritten. The study materials include this lab guide and the other supporting materials posted with this assignment, which should be read prior to the lab.*

c) **Use of lab notebook and personal protective equipment**. Students are expected to have their own laboratory notebook with page numbers (handwritten page numbers are acceptable). Safety glasses must be worn at all times in the lab (students should use their own; however, optionally, they may use those available in the lab).

d) **Chemical structures** of the analyte must be drawn, and solvents suitable for solubilization and for the analysis considered, including safety considerations. Column compatibility must be verified for both the solvent and the analyte of choice.

Safety

While most of this work is done on a small scale, safety precaution has to be taken when working with solvents. You are required to check SDS for the utilization of any solvent or analyte you will be working with. In particular, pay attention to THF, which in this lab is used without preservative and which, if too old, could produce explosive peroxides. For this reason, the preservative (butylated hydroxytoluene) is always added to the waste solution, ensuring a final concentration of 100 ppm. Furthermore, one has to verify that the expiration date of any solution is not past 1 year without being stabilized. HPLC systems operate under high pressure; thus, it is extremely important to maintain good eye protection when near instruments in case of any leak.

Lab Assignment Instructions

The lab assignment is performed on an individual basis (Chem 543). Sign-up is required to ensure the availability of the operator and instrument. The take-home quiz will need to be submitted before the first lab session. Missing submission, unsatisfactory, or lack of pertinent knowledge will require rescheduling of the lab session or loss of points. Students will need to provide a record of all measurements to complete the training, along with the report in the form of a PowerPoint presentation

The data processing must be accomplished **on an individual basis** in room 343 using Astra software (computers 2 and 3) with the support of video training. Although students may discuss (and this is encouraged) their data, the processing files cannot be duplicated even if students worked on the same analytes. *Submission of the same or nearly the same files will be considered plagiarism, and students will be dismissed from the lab with a zero score and no further access to the SEC lab.*

The lab will be performed in two modules, including:

- The determination of *dn/dc* values using the RI batch system, followed by the interim report. Only after the approval of the interim report (based on the quality of the data) will students be permitted to continue to the second module (Lab I).
- The determination of MW using SEC-MALS will be compared to the determination of MW based on retention (SEC-UV) and will be presented in a final report (Lab II).

Instrumentation

SEC-MALS analysis will be performed with an Agilent HPLC 1220 Infinity with a variable wavelength UV detector combined with a Waters (Wyatt Technology) Dawn Neon 18 angle multiangle light scattering

detector and Optilab Neon differential refractive index detector. Unless noted, an Agilent PLgel Minimix-D SEC column will be used for separation with a mass range of 200-400,000 Da.

The setup for *dn/dc* determination will consist of an HPLC pump, a manual Wyatt high-pressure injection system (WISH), and the RI.

Materials

Solvents and standards will be provided by the instructor. Samples will likely be dissolved in unstabilized THF or DMF, which will also be used for analyses. Batch *dn/dc* will require 5 samples prepared in a concentration between 0.2-1.5 mg/mL each in 8-10 mL of solvent in test tubes. The same solvent will be used to equilibrate the column and detectors. Samples for SEC-MALS analysis will be prepared at 1 mg/mL in autosampler vials.

- Solvent (must be HPLC grade, 500 mL with 100 mL for sample preparation)
- Sample/polystyrene standards
- Test tubes
- 3 mL glass syringe
- Syringe filters 0.2 µm (PTFE is required for THF, but nylon can be used for DMF and DMSO)
- Autosampler vials

Lab I: determination of dn/dc

Sample Preparation

For *dn/dc* experiment, students will prepare their samples in their desired solvent (upon confirmation with the operator). If you are using polystyrene, the solvent will be DMF. 7 wide-mouth test tubes (12 mL) are required for the 5 samples and 2 blank solvent tubes. Prepare samples and record preparation in your notebook. Record weights of samples and solvents to determine volume from density. Label each sample with your initials, notebook number, page number, and sample number (MC-2187-1). *Any unlabeled or improperly labeled samples will be discarded.*

Test tubes: weigh the tube, sample, and solvent to have accurate concentration calculations.

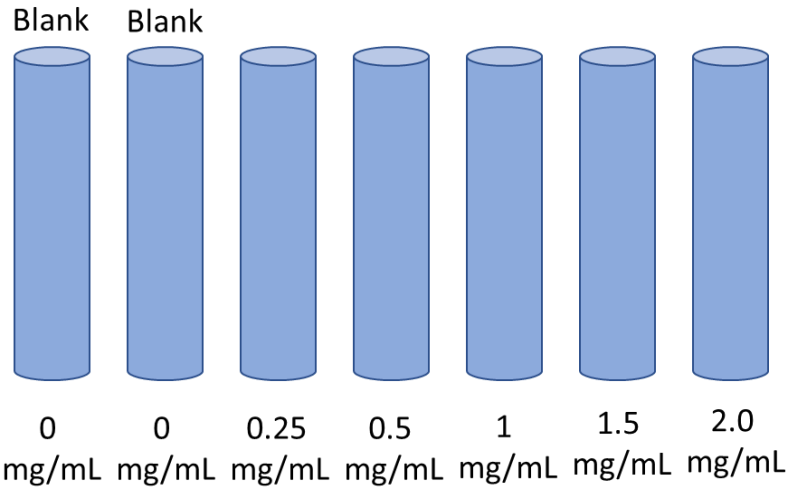
- Prepare Samples – it is *extremely important to dissolve samples in the exact same solvent* as the mobile phase, so you must draw solvent for your sample prep directly from your mobile phase bottle if you did not set some aside earlier.

  Many GPC solvents are harsh chemicals that can dissolve plastics and cause bodily injury or damage to instruments. Be sure to understand your chemicals and their compatibility and miscibility with solvents in the system. *Do not mix immiscible solvents inside the instruments!*

  - Test tube, samples, and solvents should be weighed, and the concentration should be calculated by density. If volatile solvents are used, a single sample (stock) can be prepared at a time, and each following sample can be a single-step dilution from the stock. Do not use serial dilution; this will introduce more errors.

- Each dn/dc plot should contain a minimum of **5 concentrations** with duplicate analysis at ~2-3 mL injections. Thus, 8-10 mL of each sample may be enough for two sets of analysis, and maybe an extra injection for any errors.

Set aside a minimum of 70 mL of mobile phase to prepare your 5 samples as well as 2 blanks. Samples should be prepared



Determination of Batch *dn/dc*

Follow all standard operating procedures (SOP) for batch *dn/dc* operation beyond the overview here:

- Prepare mobile phase solvent for experiment (500 mL)
- Set aside 100 mL for sample prep
- Prime HPLC pump
- Plumb detectors
- Purge RI and WISH injector
- Prepare samples and blanks for injections (follow instructions in Sample Preparation section above)
- Create *dn/dc* method utilizing ASTRA software in the lab
- Begin analysis and inject blank followed by samples of increasing concentrations, followed by a repeat of the blank. **Be sure to inject 2 different concentrations twice to check for injection/instrument repeatability.**
- Analyze data in ASTRA to determine *dn/dc* and create an interim report following the video instructions provided by the instructor (ASTRA is available in the computer lab on computers 2 and 3)

Interim Report (PowerPoint & Excel)

1. The report should include a title page (slide 1) showing the structure of the target analyte and its molecular formula, and the author.

2. The interim report should also contain all material related to the samples used, published *dn/dc*, as well as sample preparation and concentrations, and description of instrumental setup (slides 2 and 3).
3. Data analysis for the interim report should include the baseline collection from the RI detector showing the graphs with plateaus generated from each injection during the experiment, peak selection with corresponding concentrations, and the plot of *dn/dc* from the RI (Slide 4 – 3 graphs).
4. The dn/dc should be calculated manually to match ASTRA calculation. Include the graph created in excel along with the LINEST calculations (Slide 5)
    a. Export data to excel
    b. Average the dRI response for each peak with the same exact timeframes used in the ASTRA software
    c. Plot the concentration vs the average dRI and perform LINEST function and report slope and errors in slope
5. The final slide should give a short explanation of how *dn/dc* is calculated and why it is needed for MW calculations (Slide 6).
6. Attach the excel file. All Excel data must be well-labeled and organized with linked data and functions to follow calculations. Attach the Excel file with the interim report.

LAB II: SEC-MALS

Sample Preparation *SEC-MALS*

For the SEC-MALS experiment, the same solvent and sample will be used, but a new preparation will be needed directly before analysis (unless SEC-MALS is performed soon after batch dn/dc, which leftover sample can be used). For each of the 4 samples listed below, weigh an autosampler vial with lid and record, then add the sample and weigh, finally add solvent and weigh after closing the crimp cap and dissolving completely for best concentration determination.

Four vials need to be prepared for SEC-MALS analysis at roughly 1 mL: blank, normalization standard, PS calibration mix, and sample.

Vial 1 – Blank solvent

Vial 2 – Normalization 30 kDa PS 1.5 mg/mL

Vial 3 – PS Cal. Mix in single vial (0.5 mg/mL dilution from stock mixtures MC3007-1, 3, 5, 7)

270 kDa PS – MC3007-1
38 kDa PS – MC3007-3
5 kDa PS – MC3007-5
0.5 kDa PS – MC3007-7

Vial 4 – Sample 1 mg/mL (if expected MW is lower than 20kDa use higher concentration if possible)

SEC-MALS Analysis with SEC-UV calibration

Follow all standard operating procedures (SOP) for SEC-MALS operation beyond the brief instructions here:

- Equilibrate the SEC column overnight
- Clean the inline filter
- Plumb instruments and ensure no leaks
- Purge the RI and LS detectors
- Prepare samples right before analysis in the exact mobile phase
    - Sample - 1 mL at 1 mg/mL in auto sampler vials
    - Normalization standard 30 kDa PS – 1.5 mg/mL auto sampler vial
    - Mixture of 4 PS standards for SEC-UV calibration – size dependent concentration.
- Determine ideal UV wavelength for analysis based on solvent cutoff
- Create ASTRA method for collection
- Setup sequence
    - Blanks – until a good baseline is established
    - Normalization and PS standards
    - Samples
    - PS standards
    - Blanks
- Perform analysis and monitor back pressure in the system
- Analyze data in ASTRA and prepare results for the final report

Final Report (PowerPoint)

The final report is comprehensive, including the component of the interim report, which determined the *dn/dc* value and was revised based on the feedback for the interim report. After the SEC-MALS analysis is completed, instrument normalization, SEC-UV calibration, and determination of MW by light scattering should be reported with all chromatograms included (blanks also). Each slide should contain a brief conclusion stating what the data or procedure shown demonstrates.

Normalization (1-2 slides)

Attach the chromatogram of the normalization standard and how it is used to normalize the light scattering instrument. Perform detector alignment and band broadening with the normalization standard. Insert the detector values for the new normalization, alignment, and broadening. Insert an explanation of what the normalization standard does for normalization, alignment, and broadening. Also, include what kind of analyte is required for normalization.

SEC-UV Calibration (1-2 slides)

The chromatogram of the PS standard mixture should be included with the calibration curve determined from the retention time of each PS standard. Use the light scattering intensity to calculate the MW of the PS standards and explain a reason why the MW does not match the theoretical. The calibration curve should be calculated from ASTRA following the provided video tutorial and used to calculate the relative molecular weight of the analyzed samples as well.

MW via MALS (2-3 slides)

The complete chromatograms for the samples analyzed should be included. After completing the normalization steps, the light scattering intensity can be used to calculate the Mn (number average MW), Mw (weight average MW), Mz (z-average) and D (dispersity), and all should be reported. The difference in these three values should be explained, such as why Mw is always equal to or higher than Mn. Using your determined *dn/dc* values, report how much the MW values change when using the online *dn/dc* estimated value.

In ASTRA, the "results fitting" graph should be included in the report to visually represent the D of your sample. Using the ASTRA "molar mass and radius from LS" procedure, attach the Debye plot for the slice representing the peak molecular weight (top of the highest peak in the elution profile). Referring to the x and y axes in the Debye plot, explain how this is used to calculate the MW of your sample (refer to the Rayleigh ratio).

Lab Checkout

At the end of this lab assignment, each student will be responsible for returning the cleaned syringes and any materials to the instructor. All disposable materials must be discarded, and glassware must be washed with an appropriate solvent. Each student needs to demonstrate proper use of the laboratory notebook to the instructor.

Grading

The grading will be based on:

1. Prelab test (5 points) – see section below
2. Intermediate report (10 points) submitted as Excel and PowerPoint files see section "Interim report" for specific requirements
3. Final Report (60 points), consisting of the PowerPoint presentation and supported by Excel file sheet. The final report is comprehensive thus including interim report slides. The final score will be based on the results and data processing. Consideration will be given to proofreading and language expressions, clarity of the presentation, graphical presentation of Tables and/or Figures with clear labeling and captions, and appropriate references to them within the text (see sections "Interim report" and "Final report" for specific requirements).
4. Lab checkout and demonstration of effective use of the lab notebook (5 points)