SEC-MALS: Draganova Lab Getting Started Guide to Size Exclusion Chromatography with Multi-Angle Light Scattering

The Draganova Lab thanks Dr. Kelly Arnett and the Center for Macromolecular Interactions (CMI) Core at Harvard Medical School for modification of their current protocols.

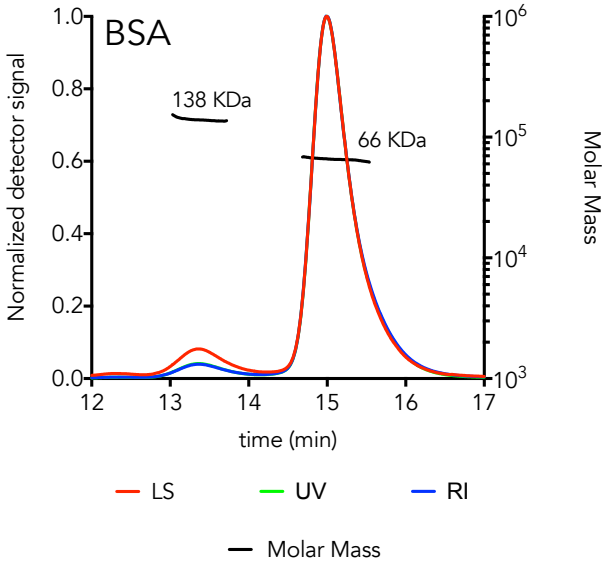
Introduction

Size exclusion chromatography with multi-angle static light scattering (SEC-MALS) is used to measure macromolecular mass in solution. Size-exclusion chromatography (SEC) separates molecules based on hydrodynamic volume. Mass estimate from SEC depends on similarity in shape to a set of reference standards for accurate mass determination and fail for elongated or sticky proteins. Multi-Angle Light Scattering (MALS) is used to accurately measure the intensity of scattered light. Light scattering intensity is proportional to weight-averaged molar mass in solution. Combining SEC, MALS and concentration detectors in a SEC-MALS experiment allows for more accurate mass measurements than SEC or MALS alone. MALS can also be used to determine oligomeric state and polydispersity of macromolecular samples.

$$I(\theta) \propto M_w \cdot C \cdot (dn/dc)^2$$

Weight-averaged Molar Mass

$$M_w = \Sigma n_i M_i^2 / \Sigma n_i M_i$$



Dynamic Light Scattering (DLS) measures the time-dependent fluctuations in scattered light. These fluctuations are directly related to rate of diffusion of the molecule through the solvent and can be used to measure hydrodynamic radius (R_h) of the particles in solution. Peaks that separate by SEC but that have the same mass will have different shapes and R_h.

Protein Conjugate Analysis. By using two concentration detectors (RI and UV), the molar mass and weight-fraction of a modifier can be determined. This can be used to measure protein and modifier masses of integral membrane proteins in detergent micelles and of glycosylated proteins.

THE BOTTOM LINE:

- You must be trained to use this instrument. Email neha.mohanty@emory.edu to set up training. You will gain access to the Outlook calendar after training – this calendar is where you can book future uses. Users will typically book the instrument for a week at a time, because of the time-consuming nature of equilibrating (and disconnecting) columns.
- You must come with at least 2L of FILTERED running buffer (0.1um filter preferred) prepared. See the section on "Assay Buffers" for more info.
- Please make sure your samples are prepared, filtered (if possible), and ready to go when you are ready to use the instrument.
    - We recommend preparing your samples in your running buffer to minimize RI peaks.
- You will need BSA as a standard. We prefer the Pierce BSA ampules from ThermoFisher (catalog #23209) as they do not need to be filtered and the concentration is known.
    - If you prefer not to buy the ampules, you must filter your prepared BSA sample and spec it beforehand (you will need to know exact concentration).
- If you will be performing multiple experiments, we require you purchase and use your own column to prevent sample contamination occurring either within our own column or in the system. The first time you use a column, it will need an extra day of column prep (before standard column equilibration). Please plan accordingly. See "Required Supplies" section under "Analytical SEC column" for more information.
- Note that equilibration times vary based on column storage – please plan accordingly:
    - If your column is stored in running buffer, it only needs 30 minutes to equilibrate (at 0.5 mL/min).
    - If your column is stored in sodium azide (recommended) or water, it will need to equilibrate overnight (at 0.1 mL/min).
- Our standard SEC methods have a column flow rate of 0.5 mL/min. If this is not compatible with your column, please let Neha know. We will show you how to set up a custom method.
- When you are done using the instrument, **it must be in recycle mode**. You MUST fill out the "Light Scattering Instrument Log" in the Biochemistry Intranet.
    - Link: https://inside.med.emory.edu/departments/biochemistry/lab-services-equipment/light-scattering-log.html
    - Failure to fill out this form will result in loss of access to the instrument.

Instrument Overview

The Draganova lab has an SEC-MALS system with light scattering detectors (MALS and DLS) and refractive index (RI) detector from Wyatt and liquid chromatography system and UV detector from Agilent. For particles not amenable to SEC separation, the Draganova lab also has a field-flow fractionation system from Wyatt (user-designed protocol). We also have composition gradient (CG) MALS for assessment of protein binding interactions in solution (separate protocol).

MALS/DLS

- Wyatt Dawn Multi-Angle Light Scattering (MALS) detector
- Wyatt Optilab Refractive Index Detector
- Wyatt In-line QELS detector for Dynamic Light Scattering (DLS)
- Wyatt microCuvette for bulk DLS

Chromatography

- Agilent 1260 Infinity II Isocratic Liquid Chromatography System
- Agilent 1260 Infinity II Autosampler
- Agilent 1260 Infinity II Fraction Collector
- Agilent variable wavelength UV detector
- Inline solvent degasser

SEC-MALS Applications

- Weight-averaged molar mass (M_w) of particles separable by SEC
- Oligomerization state
- Mass contribution from two components
    - Protein and detergent micelle
    - Protein and glycan
- Radius of hydration (R_h)
- RMS radius (R_g) for large particles (> 10 nm)

Key Features

- 18-angle Dawn static light scattering detector for mass measurements for a wide size range and R_g measurements
- UV and RI detectors for concentration measurements in two orthogonal ways.
    - Allows for deconvolution of mass contribution of two components.
- In-line DLS detector for R_h measurements of particles for which R_g cannot be determined.
- 100 sample autosampler for minimal protein loss and automated data collection.

Required Supplies

- Sample filters, 0.02-0.2 µm filters (recommended)
- Autosampler vials:
    - 2 mL Screw Top Vials (250 uL dead volume!): Agilent – Part Number 5182-0716
        - https://www.agilent.com/store/en_US/Prod-5182-0716/5182-0716
    - 2 mL Screw Caps: Agilent – Part Number 5190-7024
        - https://www.agilent.com/store/productDetail.jsp?catalogId=5190-7024
- Analytical SEC Column:

- NOTE: All users must provide their own SEC columns for experiments. Columns must be compatible with Agilent.
- Make sure that the column is compatible with your sample. Some detergents and membrane proteins will require special columns designed for hydrophobic samples and buffers.
- We recommend the following for hydrophilic proteins:
  - Wyatt WTC-030S5
    - This column can accommodate flow rates up to 1 mL/min and buffer pH from 3 - 8.5. The molar mass range for the column is 5 kDa to 1250 kDa. At 1 mL/min, each injection will take ~25 minutes.
  - Wyatt WTC-030N5
    - This column is the same as the one above, but it's a narrow bore column. It can only accommodate flow rates up to 0.4 mL/min and is only recommended if you have very limited sample volume as the loading volume is much lower than the above standard bore column.
  - Shodex OHpak LB-804
    - Accommodates flow rates up to 1 mL/min, pH from 3 – 10 and molar masses from 5 kDa to 400 kDa.

Sample Preparation

Assay Buffers

Running buffer should always be compatible with both the SEC column and the protein sample. **Make sure you know the buffer compatibility of the SEC column you are using.**

- **Prepare sufficient buffer (2L minimum)**
- Recommended Buffer: 1X PBS or HBS (25 mM HEPES pH 7-7.5, 150 mM NaCl), filtered.
    - It is recommended to 0.02% sodium azide to your buffer (if compatible with your sample) to prevent growth in your column and in the system.
    - Let us know if you are unable to add sodium azide and we will discuss a workaround
- Some buffer components (e.g. glycerol – absolute max 10%) will require customization of the solvent profile. Take note of the absolute refractive index (ARI) of the solvent after equilibration and before data collection.
- Mild detergents such as SDS are okay.
- Salt should be limited in the system to avoid salting out issues (discussed below). If you need a buffer composition that vastly differs from what is described above, please consult with the Draganova Lab prior to proceeding.
- **Regardless of the composition, all buffers MUST be filtered – 0.1 um filter recommended.**

Samples

- **This SEC-MALS system is only for purified proteins.** Injecting unpurified samples into the machine can result in contamination that can take weeks to resolve.
    - The sample should be prepared in running buffer to minimize an RI peak due to sample solvent. This is particularly important for samples that run near the solvent peak.
- Know the UV extinction coefficient (in ml/mg•cm) and concentration (in mg/ml) of your protein.
    - Concentration should be accurately measured to assess column recovery.
- Filter (0.45 um filters are good) or centrifuge (16,000 x *g* for 10 min if your sample cannot be filtered) samples before use.
    - Protein aggregates can damage your column and clog the instrument.
- Protein heterogeneity can be analyzed via dynamic light scattering (in the DynaPro plate reader).
- Recommended protein concentration to inject varies depending on protein mass:
    - Scattering is proportional to mass
    - Larger proteins require less sample than smaller proteins
    - Typical range: 5 – 500 µg/injection (this is column dependent but in general, 200 µg is a lot of protein for most columns)
    - When starting out, aim for 100 µg – an overloaded column will show peak tailing - if this happens, reduce your concentration and try again
- Sample volume:
    - Maximum injection volume: 100 µl
    - Minimum injection volume: 1 uL
    - Glass autosampler vial with low volume insert has 10 µl dead volume. Fill vial with at least 110 µl for a 100 µl injection
    - Glass autosampler vial without an insert has a 250 uL dead volume. Fill the vial with at least 350 uL for a 100 uL injection.

- Always run a BSA standard prior to sample injection for each new column connection.
    - BSA (~66 kDa monomeric)
        - The Pierce BSA ampules from Thermo are ideal for a standard (catalog #23209)
            - Do not filter the BSA from Thermo
        - 100 µl at 2 mg/ml always gives a good light scattering signal – max recommendation – this should give the highest S/N without tailing

Getting Started

Resources

All protocols are available on the Draganova Lab website. Wyatt Technology website is also a great resource for literature and videos.

Experimental Design Tips

- The mass calculated from MALS is the weight-averaged molar mass and in a polydisperse sample, is shifted toward the mass of higher mass particles in the mixture.
- Even if you cannot see peaks at the void in the UV signal, there will almost always be some soluble aggregates in your sample that will be in the void peak.
- Choose a column such that your major peak of interest is in the center of the column, neither near the void peak nor near the solvent peak (comes out at the end of the column).
    - Any overlap between your peak of interest and the void peak will be hard to interpret, due to contributions to the weight-averaged mass from scattering of aggregates.
    - A solvent peak will be visible in the refractive index signal due to mismatch (even minor) between the running buffer and the sample buffer. If using RI as the concentration source, the solvent peak must be baseline resolved from sample peaks.

General Care and Maintenance

- Treat the SEC columns and chromatography system with care.
    - Avoid rapid pressure changes when a column is attached by changing flow rates gradually **(using a maximum flow gradient to 0.1 ml/min^2).**
    - Always **filter** or centrifuge samples before loading.
        - BSA ampules from ThermoFisher are the only exception (see above).
    - Only load well-behaved protein samples (sticky proteins can clog the instrument).
    - Use filtered buffer with at least 100 mM salt and a pH appropriate to the column.
- Store the system in 50:50 water:isopropanol when not in use.
- Salting out is a major problem in light scattering equipment. Therefore, when introducing new solvents and/or buffers into the system, follow a matching system:
    - If in 50:50 50:50 water:isopropanol, you must switch to water first
    - After water, one can switch to buffer
    - Going directly from solvent to buffer can result in pressure errors and salting out (salt buildup in the lines and on the detectors) depending on solvent/buffer composition
- Follow recommended start-up and shut-down sequences.

Autosampler Info:

- The autosampler has two trays: P1 (left) and P2 (right).
- Vial positions are indicated by rows (1-11) and columns (A-F).
- All vial locations are specified with a "Plate-Vial Position"notation. For example, "P1-A1" is Plate 1 (P1), left-most column (A), bottom-most row (1).
    - You will need to know vial locations for your runs.
- Vial P1-A1 should always be a full vial (1.5 mL) of MilliQ water (used throughout the runs to rinse the injection needle)

Startup

1. Use the shared Outlook Calendar to book time for the Light Scattering Equipment.
    a. You MUST book the time slots you need to use the equipment!
    b. When booking, please include the following:
        i. Under "New Event": lab name and the name of the person using the equipment.
        ii. Indicate the time frame you expect to use the instrument.
        iii. Under notes, you can include other pertinent information.
2. Login to the computer:
    a. Username: select your lab from the list of users on the bottom left of the screen.
        i. If your lab does not have an account listed, please let Neha know (neha.mohanty@emory.edu).
    b. Password: wyatt123
        i. Same for all accounts
3. The system should have been left running at a low flow in water. Right click Eclipse widget and select "Control". Click "Flows Off".
4. Move the lines into the correct buffers:
    a. Line A1: move from water to your running buffer (e.g. PBS)
        i. The recycle and autosampler lines should also be moved into running buffer. Secure the lines with a piece of tape to the top of the bottle to prevent them from popping out.
    b. Line A2: move from 50:50 to filtered MilliQ water.
        i. Make sure to rinse the line with DI water before placing the line into the water bottle.
5. Update the solvent volume in the computer: This volume always corresponds to whatever solvent is in Line A1 (right now should be your running buffer).
    a. Right click on the Eclipse widget (computer) and click "Manage Solvent".
    b. Update the "Current Volume" in the computer to be however much running buffer you have *(should start this step with 1L minimum. 2L is recommended for multiple days of sample injections).*

System Solvent Exchange (water → buffer)

6. Purge the pump to remove any air from line A1.
    a. Open the doors on the pump compartment.
    b. Right click on Iso. Pump widget on computer and select "Method".
    c. In the "Solvents" section, select "1". This will purge the line that is now in the running buffer.
    d. Open the purge valve by turning the black knob counterclockwise until it is loose (~270 degree turn).
    e. On the computer, set the flow rate to 3 mL/min and let the system purge for at least 3 minutes.
        i. Watch the purge line (clear tubing coming out of the purge valve) to ensure the solvent is moving through. You will know the line is properly purged when there are no more air bubbles moving through it.
    f. Change the flow rate back to 0 mL/min. Close the purge valve by turning the black knob clockwise until it is tight.
        i. **Important**: If you see air bubbles in the purge line when you are closing the purge valve, you need to purge longer. If this happens, reopen the purge valve. Change the flow rate back to 3 mL/min and allow the system to purge for another 5 minutes. Then, change the flow rate back to 0 mL/min and close the purge valve again.

7. Complete solvent exchange to take system out of filtered water and put into running buffer.
    a. In the tabs at the top of the VISION Run Software, select "Sequence". Click "New Sequence" from the drop-down.
    b. Click "Append".
    c. Insert the following information:
        i. Vial: P1-A1. (**make sure this has at least 1 mL MilliQ water!)**
        ii. Volume: 100 uL
        iii. Under VISION RUN method, select "**Complete Solvent Exchange 1**".
        iv. Under the "Operator and Project" heading at the top of the Sequence window, go to the "Signal Selection" drop-down and click "Default".
        v. The sequence and data need to be saved before the sequence can be ran:
            1. Under the "Data Output & Method" heading, click the "…" next to EMDF Data Path. Select where you would like the data file saved.
            2. Click "Save Sequence" at the bottom right of the screen. Select where you would like the sequence saved.
        vi. Click "Run Sequence". Allow the Solvent Exchange to complete.

8. Flush Eclipse valves:
    a. Right click on the Eclipse widget and select "Eclipse Flush…".
    b. Start at the top and perform each of the flushing steps.
        i. Each one takes about ~2-3 min.
        ii. Note: When selecting the Rheodyne Flush, it will tell you to bypass your column. Your column should not be connected yet –select ok.

1. Sometimes the Rheodyne Flush gives an error during the flush – this is normal. Select ok and run the Rheodyne Flush again.
    a. If this happens, you will need to clear the error following the flushes: hover over the Eclipse widget and select "On".

iii. Note: When selecting the FC Port flush, a pop-up will appear asking to add tubing. You can ignore this – select "Yes" and the flush will start.

Connect the column

9. Attach the SEC column:
    a. Right click on the Iso. Pump widget and select "Method".
    b. In the "Solvents" section, select "**2"**. This will allow filtered water to go through the tubing so a wet connection can be established.
        i. Iso Pump flow rate should be at 0 mL/min (this is to avoid running solvent through the system).
    c. Right click the Eclipse widget and select "Control".
    d. On the "Separation Device" drop-down, select "Column-B".
        i. Should automatically switch to SEC mode (a column image should appear on the Eclipse equipment display).
    e. Open the column compartment. There is a spacer and some extra tubing connecting the inlet and outlet tubing. Remove both the spacer and extra tubing.
        i. Keep the spacer and extra tubing in the small square container next to the Agilent (says "Keep Spacer Here" inside!).
    f. Remove the endcaps from your column.
    g. The column should be labeled with its flow direction. Orient the column so the flow is directed from left to right (green tubing at the column inlet and orange tubing at the column outlet).
    h. Set "Column Flow" to 0.1 mL/min.
    i. Attach the inlet tubing to the top of the column using a wet connection.
        i. Let the green tubing drip into the left side of the column before screwing the line fitting in – the column head should overflow a little when the tubing is screwed in.
    j. Allow water to exit the bottom of the column, and then attach the orange outlet tubing to the bottom of the column.
        i. You want to be very careful not to introduce air when attaching the column!
    k. Feed the lines of the tubing through the little slits on each side of the column compartment to secure it. Sit the column in the indented space.
    l. Allow the column to continue to flow for ~2-3 mins to check there are no leaks in the column connection.
    m. Close the column compartment.
    n. Set the "Column Flow" back to 0 mL/min.

Column equilibration

10. Equilibrate the column with at least 8-12 column volumes of running buffer.
    a. Click the "Sequence" tab in the top left corner and click "New Sequence" from drop-down menu.

b. Select "Append".
c. Under "VISION RUN Method" drop-down, select the correct option for your column:
    i. If your column was stored in buffer, it only needs 60 minutes to equilibrate (at 0.5 mL/min).
        1. Select "EQUIL SEC 0.5 mL_min" in the drop-down.
    ii. If your column was stored in sodium azide or water, it needs to equilibrate overnight (minimum 12 h at 0.1 mL/min).
        1. Select "EQUIL SEC 0.1 mL_min" in the drop-down.
            a. Method is automatically set to run for 20 h.
    iii. Important: If your column is not compatible at either 0.1 mL/min or 0.5 mL/min, please let us know during the training as the method will need to be modified.
d. Set the Signal Selection as "Default", and select where you would like your EMDF Data Path saved. Save the sequence and run.
    i. Note: After hitting "Run Sequence", the sequence table might say "not ready" as it ramps up to the correct column flow rate. The run will automatically start when it reaches the intended flow rate.
e. On the RI detector, the purge valve should be "ON," indicating that the valve is open, and the reference cell is being equilibrated.
f. Check the absolute refractive index (ARI) on the Optilab panel to check buffer exchange progress.
    i. aRI of buffer should be similar to water (~ 1.33) if fully exchanged at end of run
        1. If you have a detergent, your RI will be different from water
        2. RI ~1.36 for isopropanol alcohol

Data Collection

- Prior to data collection, two control runs are performed: blank/water and BSA
  - Blank injection: gives time for lamps to warm up and RI baseline to settle.
  - BSA: used as a standard to ensure the system is running properly. If your sample data isn't as expected, refer to the BSA injection to rule out system errors/issues.
- **Note**: Each time you connect your column (even if it has been used before), you will need to run a BSA injection prior to any sample injections.
  - You do NOT need to run a BSA standard before EACH injection however (ex: if you leave your column connected overnight, you don't need to run another BSA injection prior to injecting more samples the next day).

Important: Pre-existing methods have a column flow rate of 0.5 mL/min. If your column is not compatible with this flow rate, please let us know during the training as the methods will need to be modified.

1. Check system readiness and prepare for data collection.
   a. Record the absolute refractive index (aRI) for your solvent – you will need to report this value on the "Light Scattering Instrument Log" when you are done using the instrument (more details at the end).
   b. On the DAWN, turn on the laser. On the Optilab, turn on the LED and turn off the purge. Allow signal to stabilize (1-5 mins).
   c. Check that LS detector 11 voltage signal is stable ~0.01-0.02 V, with noise ~10^{-4} V (on DAWN).
   d. Check that the RI signal is stable with drift < $5x10^{-8}$/min.
2. Load your blank (sample buffer) into a vial. Place the blank into the autosampler. Note the tray location.
3. Load BSA (preferably the recommended Thermo ampule) into a vial. Place the BSA vial into the autosampler. Note the tray location.
4. Load your filtered sample into a vial. Place the vial into the autosampler. Note the tray location.
5. Run a sequence with a blank injection, then BSA injection, and then sample injection.
   a. Click the "Sequence" tab in the top left corner and select "New Sequence" from the drop-down menu.
   b. Select "Append" 3 times – 3 rows should appear.
   c. The first row will be the blank injection.
      i. Vial: Input the vial containing your blank.
      ii. Volume: 100 uL
      iii. Sample Name: Enter "Blank".
      iv. VISION RUN Method: "SEC BLANK 0.5 mL_min"'
      v. ASTRA Method: click on the "…" in the cell. Expand the "Method Builder" folder. Expand the "User" folder. Select "SEC UV+LS+RI". Hit ok.
   d. The second row will be the BSA injection:
      i. Vial: Input the vial containing BSA sample.
      ii. Volume: 50 uL
      iii. Sample Name: Enter "BSA".

iv. VISION RUN Method: "SEC BSA 0.5 mL_min"
v. ASTRA Method: click on the "…" in the cell. Expand the "Method Builder" folder. Expand the "User" folder. Select "SEC UV+LS+RI". Hit ok.
vi. Enter the following values (specific to BSA):
1. Conc: 2 mg/mL
2. dn/dc: 0.1850
3. UV Ext: 6.670E-001
4. A2: 0

e. The third row will be your sample injection:
i. Vial: Input the vial containing your sample.
ii. Volume: Input the volume of your sample you want injected.
iii. VISION RUN Method: "SEC Sample 0.5 mL_Min"
iv. ASTRA Method: click on the "…" in the cell. Expand the "Method Builder" folder. Expand the "User" folder. Select "SEC UV+LS+RI". Hit ok.
v. Sample Name: Enter the name of your sample.
vi. Enter the concentration, dn/dc, UV Ext. values as applies to your sample.
1. You will likely not know the dn/dc but it is typically ~0.185 for proteins
2. This can be calculated at a different time if you think yours is different

f. As done before, set the Signal Selection as "Default" and select where you would like your EMDF Data Path saved.

6. Save the sequence. Run sequence.
7. After the sequence has finished, MANUALLY:
a. Turn OFF the UV lamp (press off on MWD widget on computer).
b. Turn OFF the laser (on DAWN).
c. Turn ON the purge (on OptiLab)
8. The system will still be running at the same column flow rate after the sequence has ended.
a. If you will be using the system again tomorrow and need to leave it overnight, please do the following:
i. Right click on the Eclipse widget and select "Control". Set the column flow to 0.1 mL/min. Hit "Apply".
1. MAKE SURE THE RECYCLE BOX IS NOT CHECKED!!!
ii. VERY important: **You must have enough buffer to run the system overnight** – 150 mL used per 24 hours at 0.1 mL/min. Air in the system will cause significant disruption to instrument operation.
b. If you will not be using the system again (or do not have enough buffer to run overnight), proceed to "Column Removal" and "Shutdown".

Column Removal:

1. Equilibrate column into Storage buffer (this is where you can switch your running buffer with buffer + azide for storage, if needed).
a. Right click on Eclipse widget and click "Control".
1) Set column flow rate to 0.5 mL/min, if not already set.
(1) Uncheck recycle box if it is checked.
b. Purge the RI detector.

1) On the Optilab, make sure "Purge" is turned ON so the reference cell is open and can be washed out.

c. Once the column flow rate has reached 0.5 mL/min, run Comet.
   1) On the DAWN, click the timer icon next to the "COMET" slider. Set Comet to start running in 1 minute, and to run for 60 minutes.

2. Once Comet has finished, remove column:
   a. Right click on Eclipse widget and click "Control". Set column flow rate to 0.1 ml/min.
   b. Open the column compartment.
      1) Remove column and replace with spacer and the extra piece of tubing.
      2) Cap the column ends.
      3) Keep column flow going for ~1-2 minutes to ensure there are no leaks.
   c. Secure the spacer and extra tubing in the indented grooves (the same way you did with the column when connecting). Close the compartment.
   d. Set flow rate to 0 mL/min.

Leaving the System: Solvent Exchange (running buffer → water)

3. Move lines into the correct buffers:
   a. Move Line A2 from filtered MilliQ water to 50:50 IPA:water.
   b. Move Line A1 from your running buffer to the filtered MilliQ water (please ensure there is at LEAST 1L of water in the bottle).
      1) Make sure you move the autosampler and recycle lines into the water as well! Secure lines with tape to the top of the bottle (otherwise they may pop out).
4. Update the solvent volume (right click Eclipse widget → Manage Solvent → update based on volume of MilliQ water).
5. Purge Line A1 – done the same way as Startup Step 11. Make sure "1" is selected under Isopump "Method" widget.
6. Select Sequence tab in the upper left corner and select "New Sequence" from the drop down.
   a. Select Append. Input the following:
      1) Vial: P1-A1
      2) Volume: 100 uL
      3) Vision RUN Method: Complete Solvent Exchange 1
   b. As before, set the Signal Selection as "Default". Select where you want the EMDF Data Path saved.
   c. Save the sequence. Run the sequence.
7. Flush the Eclipse valves.
   a. Right click on the Eclipse widget and select "Eclipse Flush".
   b. Start at the top and perform each of the flushing steps.
8. Ensure the system is flowing at a low flow rate:
   a. Right click on the Eclipse widget and select "Control". Set the column flow to 0.1 mL/min. Hit "Apply". MAKE SURE THE RECYCLE BOX IS CHECKED!!!
9. Please be sure to remove all of your vials from the autosampler and take your buffers with you. Please do not leave anything at the instrument when finished.

Fill out "Light Scattering Instrument Log" in the Biochemistry Intranet.

- Link: https://inside.med.emory.edu/departments/biochemistry/lab-services-equipment/light-scattering-log.html
- Failure to fill out this form will result in loss of access to the instrument.

Data Analysis

1. Open ASTRA. Go to the File tab, select Open, select Experiment, and find your run.
2. **Parameters**
   a. Expand the Procedures folder on the left side. Open up **Basic Collection** (double-click).
      i) Examine the peaks.
      ii) Check the noise in the light scattering detectors (particularly LS 11 and LS 12).
   b. Double-click **Despiking** from the Procedures drop-down.
      i) Check the despiking and change despiking level if needed (Normal despiking is usually fine).
         (1) If level needs to be changed, select the proper level from the drop-down tab on the top left corner.
      ii) Click ok if you make changes – this will close the window.
   c. Double-click **Peaks** from the Procedures drop-down.
      i) Delete any existing peaks that were saved with the method.
      ii) Create peaks by dragging across the peak area.
      iii) Click ok.
   d. Double-click **Baselines** from the Procedures drop-down.
      i) Check the baseline for LS 11 (90 degree detector).
      ii) Use auto-baseline calculation or modify baseline manually as necessary for each detector.
      iii) Running a few minutes past the solvent peak on the RI detector with simplify the baseline calls.
      iv) Click ok.
   e. Double-click **Molar Mass and Radius from LS** from the Procedures drop-down.
      i) Expand the enabled detectors folder.
      ii) In the control graph, deselect detectors that are not contributing light scattering signal (UV and dRI).
      iii) Reference the calculated dn/dc value for your sample against standard dn/dc values:
         (1) Protein: 0.185 ml/g
         (2) Glycan: 0.145 ml/g
         (3) DNA: 0.17 ml/g
         (4) Detergents: check supplier for value.
      iv) Click ok.
   f. If you want a more accurate calculated dn/dc value, you can do either of the following:
      i) On-line: Use the UV as a concentration source (if you know the extinction coefficient)
         (1) Double-click on the Configuration page
         (2) Select "UV" from the Concentration Source dropdown menu
         (3) Right-click on the Experiment name and select "Apply Method"
         (4) Navigate to "System/Methods/RI Measurement"
         (5) Select "dndc from UV." This will use the amount of mass detected by the UV to calculate the dn/dc for the RI data.
         (6) You can then reprocess your data using this calculated dn/dc value.
      ii) Batch: Make a solution of very well-known concentration with your sample of interest.
         (1) Use the Wyatt protocol for determining dn/dc in batch.

3. **Examine data**.
   a. Examine peak calls and Molar Mass calculations in graph (in Molar Mass and Radius from LS tab again), modify peak calls as necessary.
   b. Examine results in the EASI graph and EASI table (on left panel).
      i) For the complete list of values, you can analyze, select the drop-down next to "Scalars".
   c. **For user samples, proceed to *Save and Export Results*** (below).
4. **Save and Export Results**.
   a. Save data.
   b. Export graphs.
      i) Right-click on any graph.
      ii) Click Edit → Export.
      iii) Choose export parameters.

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