SEC-MALS Getting Started Guidelines

Getting Started
You will need to bring:

- running buffer (500 ml-1L for 1 day)
- samples for analysis, in running buffer
- BSA, in buffer without reducing agent (used to normalize detectors and align peaks)
- pipettes and tips (to fill the autosampler sample vials and to make dilutions)
- your column (if you have one)

Samples

*maximum injection volume: 100 µl
*minimum injection volume: 5 µl
*typical protein amount: 50-500 µg
*typical protein volumes: 20-120 µl (add 10+ µl for autosampler dead volume)*

- The limiting factor in SEC-MALS analysis the light scattering of the molecules being tested. The amount of sample needed depends on the size of the molecule, as large molecules scatter more light than small molecules.
  - 100 µg of BSA (67 KDa) gives a very good light scattering signal
- Samples should be prepared in running buffer to minimize the RI peak due to sample solvent (this is particularly important for samples running near the solvent peak)
  - it’s good practice to run a standard size-exclusion chromatography purification just before analytical SEC-MALS to buffer exchange and ensure protein is well-behaved. **This is a requirement if you are using a shared CMI column (don’t put junk on the column!)**
- Samples must be filtered or centrifuged prior to injection
- Know the concentration (in mg/ml) of your protein
- Know the UV extinction coefficient in mL/mg•cm

Running Buffers

- Most standard aqueous SEC running buffers should be fine
  - some buffer components (eg. glycerol) will require customization of the solvent profile (especially if measuring dynamic light scattering)
  - buffer should be chosen to ensure that the sample is well-behaved
- **Make sure you know the buffer compatibility of the SEC column you are using**
  - recommended: 25 mM HEPES pH 7-7.5, 150 mM NaCl, 0.02% NaN₃ (filtered)
  - The CMI Tosoh G4SWxl and G2SWxl columns are stable at **pH 2.5-7.5** (and MAY NOT be run at pH above 7.5)
  - The CMI Agilent AdvanceBio 300 column is stable at pH 2.5-8.6
SEC-MALS Standard Protocol

Start-up Sequence

1. Before you start, book time on the PPMS calendar
2. Login to the computer using your PPMS credentials (eCommons ID and password)
3. The Agilent chromatography system will usually be on, but if not, then:
   a. turn on the pump
   b. turn on everything else
   c. Open chromatography software using the Agilent SEC (online) shortcut on the desktop
4. To change buffers, purge the pump:
   a. open purge valve on pump (by turning knob ¼ turn to the left)
   b. purge at 5 ml/min for at least 5 min (there should be no backpressure if the purge valve is open)
   c. stop flow
   d. close purge valve
5. If necessary, change the column under low flow (~0.1 ml/min)
   • If using a new column (or your own column), disconnect the MALS and RI detectors from the column and equilibrate with water or buffer for at least 4 column volumes before reattaching the detectors
6. Change flow rate gradually to the target flow rate (in 0.1 ml/min increments) to minimize shedding
   a. target flow rate is typically 0.5 ml/min for analytical Si-based SEC columns
   b. to change flow rate, either:
      i) right click the pump module and change flow rate manually
      ii) double-click the appropriate ramp method in the CMIsharedMethods_SEC-MALS folder (eg. RAMP_100ul_min_2min)
7. Clean MALS flow cell using Comet for 10 min (System tab on MALS)
8. Open Purge on RI detector during equilibration
9. After the system has reached the target flow rate open or write a method.
   a. Tosoh G4SWxl Method
      i) Flow rate: 0.5 ml/min
      ii) duration: 35 min
      iii) max pressure limit 36 bar
   b. Tosoh G2SWxl Method
      i) Flow rate: 0.5 ml/min
      ii) duration: 35 min
      iii) max pressure limit 60 bar
10. Equilibrate column with several (4-8) column volumes buffer until:
    a. MALS detector 11 is stable with V ~0.02 with drift ~10^{-4}
    b. RI signal is stable with drift <5x10^{-8}/min
11. After equilibration, go to the RI detector
    a. record the absolute refractive index (aRI) for your solvent
    b. turn off purge and zero the baseline
SEC-MALS Method

1. Open ASTRA software for MALS data collection
2. For each combination of column, buffer and flow rate, a new normalization run should be performed (this should be a well-behaved monodisperse, small macromolecule, such as BSA, 50 µl at 2 mg/ml in your running buffer)
3. Write a MALTS method (it's easiest to start from an existing method)
   a. File → New → Experiment from Method
      eg. 0,5ml-min_35min_25C
4. expand configuration menu
5. double-click generic pump → set the flow rate
6. expand generic pump and click solvent → select best match to solvent (usually PBS) or create a solvent profile
7. double-click injector → set injection volume
8. expand injector and click sample → fill in:
   a. name
   b. dn/dc: 0.185 (for protein)
   c. UV extinction coefficient in mL/mg•cm
   d. concentration of sample
9. expand procedures
10. double-click basic collection → set duration
11. Save
12. After the system has equilibrated, click Run button
    The software will wait for the autosampler signal and then start collecting UV, MALS and RI signals.
13. Go to the Agilent OpenLab Instrument Control Panel
14. Go the Sample Entry Tab
15. create method entry by double-clicking sample position or [+ ] button
    a. set autossample vial position (eg. 1)
    b. select method (click the … to select from methods in the CMIsharedMethods_SEC-MALS folder) *the method flow rate must match the equilibration flow rate to avoid shedding during the run
    c. set injection volume (maximum 100 µl)
16. Add sample to Queue, which will execute the first run

Shutdown Sequence

1. After completing experiments, the chromatography system will still be running at the flow rate of your experiment
2. Decrease flow rate gradually (0.1ml/min increments) to 0 ml/min
3. Change the solvent to Milli-Q Water with 0.02% NaN₃
4. **Purge the pump (and degasser) at least 5 min at 5ml/min** before equilibrating to water to ensure that all salt is removed from the pump and the degasser unit
5. Increase flow rate gradually (0.1 ml/min increments) to equilibration flow rate (0.5 ml/min)

6. Equilibrate the column in at least 3 column volumes for 1 hour or more
   • shared columns should be store in 0.02% NaN₃, unless indicated otherwise
   • your column may be stored in 20% ethanol or the buffer of your choice, but
     you'll need to wash first with water to remove salt and return the system to
     Water+NaN₃ afterward

7. Run Comet on the MALS detector for 1 hour during equilibration to clean the flow cell

8. Purge RI detector

9. Turn off the MALS laser and RI LED

10. Decrease flow rate gradually (0.1 ml/min increments) to 0 ml/min

11. Empty the waste!

SEC-MALS Data Analysis

1. Go to Astra Software and open experiment, if necessary (it will be open after data collection)

2. Parameters
   a. Basic Collection
      i. check the peaks
      ii. check the noise in the light scattering detectors (especially low angle
          detectors LS2-4)
   b. Despiking
      i. check the despiking (Normal despiking is usually fine)
      ii. click ok if you make changes
   c. Baselines
      i. check the baseline for each detector
      ii. use auto-baseline calculation or modify 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
   d. Peaks
      i. delete any existing peaks that were saved with the method
      ii. create peaks by dragging across the peak area
      iii. click ok
   e. Molar Mass and Radius from LS
      i. expand the enabled detectors folder
      ii. deselect detector that are not contributing light scattering signal
      iii. click ok

3. Normalization, Alignment and Band
   • These procedures should be performed using a small monodisperse standard
     such as BSA (DO NOT use your protein for these procedures)
Should be performed once for each combination of column, buffer and flow rate,

a. Align Signals
   i. right-click Configuration
   ii. select alignment
   iii. highlight peak (eg. BSA monomer peak)
   iv. click Align Signals button (top-left)
   v. click ok (bottom-left) to apply and close the window

b. Normalize Detectors
   i. right-click Configuration
   ii. select Normalize
   iii. chose which peak to use for normalization (default peak 1)
   iv. click Normalize (or import from another file)
   v. software will prompt peak selection if none have been selected
   vi. click ok (bottom-left) to apply and close the window

c. Band Broadening
   i. right-click Configuration
   ii. select Band Broadening
   iii. reset the broading terms in the table at the bottom
   iv. select the a region in the center of the peak (not the entire peak)
   v. click Perform Fit (top-left)
   vi. click ok (bottom-left) to apply and close the window

4. Examine peak calls and Molar Mass calculations in graph, modify peak calls as necessary
5. Examine results
6. Logoff from PPMS!
   • rates are based on booked and real time usage

Book time and Report Problems through the PPMS system:
https://ppms.us/hms-cmi

Contact cmi@hms.harvard.edu with questions.

CMI Users must bring all their own materials and supplies (including pipettes, tips and gloves). Please respect adjacent research labs by staying out of their space.

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