SEC-MALS: CMI Guide to Protein Conjugate Analysis

dn/dc Sample Preparation dn/dc Data Collection Protein Conjugate Analysis

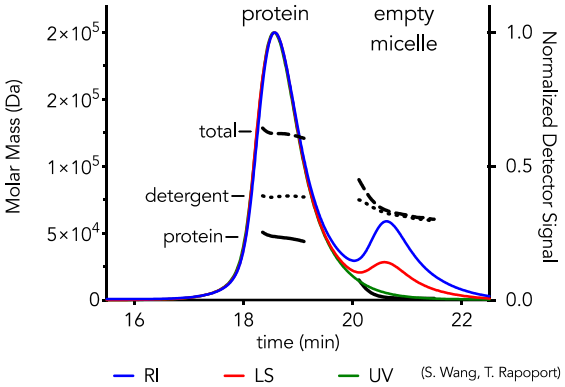
Introduction

MALS calculations are dependent on a good estimate of the refractive index increment (dn/dc) of the sample. All proteins have a dn/dc near to 0.185. However, common modifiers, such as glycans or detergent micelles, have different dn/dc values. To calculate accurate masses from MALS data for modified proteins, the fractional mass contribution of the protein and the modifier must be deconvoluted. A method called Protein Conjugate Analysis is applied to SEC-MALS data to perform these calculations. Protein Conjugate Analysis requires that two concentration detectors (RI and UV) are used simultaneously during the MALS data collection and that the dn/dc, and UV extinction coefficient of each component are known. With sufficient pure material (e.g. detergent), dn/dc values can be determined using a batch refractive index experiment.

$$\left(\frac{dn}{dc}\right)_{conjugate} = \overbrace{\left(\frac{dn}{dc}\right)_p \cdot X_p}^{\text{protein}} + \overbrace{\left(\frac{dn}{dc}\right)_m \cdot (1\text{-}X_p)}^{\text{modifier}}$$

$$\epsilon_{conjugate} = \epsilon_p \cdot X_p + \epsilon_m \cdot (1\text{-}X_p)$$

X_p = weight fraction protein
dn/dc = refractive index increment (ml/g)
ϵ = UV extinction coefficient (mg/ml•cm)



Standard dn/dc values

- Protein 0.185 ml/g
- Glycan 0.145 ml/g
- DNA 0.17 ml/g
- Detergents Variable (check supplier for value or measure it yourself, see below)

References

Strop, P., & Brunger, A. T. (2005). Refractive index-based determination of detergent concentration and its application to the study of membrane proteins. *Protein Science: a Publication of the Protein Society*, *14*(8), 2207-2211.

Barer, R., Ross, K. F. A., & Tkaczyk, S. (1953). Refractometry of Living Cells, *171*(4356), 720 EP —724.

Pasternack, S. G., Veis, A., & Breen, M. (1974). Solvent-dependent changes in proteoglycan subunit conformation in aqueous guanidine hydrochloride solutions. *The Journal of Biological Chemistry*, *249*(7), 2206–2211.

Instrument Overview

The CMI has a Wyatt WISH module, a high pressure manual injection system with 1 ml injection loop. This module is attached downstream of the chromatography pump and upstream of the refractive index detector to control batch measurements of differential refractive index.

Required Supplies

- Syringe(s) for filling the injection loop.

dn/dc Sample Preparation

Assay Buffers

- Water or Buffer is needed for the pump and to dissolve and dilute sample.
- Running buffer and Dilution buffer are typically the same (but can differ).
    - Water is often used for both the pump and to dissolve sample.
    - Prepare at least 200 ml of Running buffer.
    - If different from Running buffer, prepare sufficient Dilution buffer to dissolve and dilute your samples and for two blank injections (to normalize the RI signal).

Samples

- Typically detergent, but could be performed for any sample type (even protein) provided you have sufficient quantity of pure sample.
- Accurate concentration measurements are critical for dn/dc measurements
    - Collect replicate data (2-3 replicates).
    - Weigh your sample out independently for each replicate.
- Prepare least 2 ml of 6-7 concentrations of your sample, in replicate.
    - Use a high concentration of ~5 mg/ml and a low concentration of ~0.1 mg/ml.
    - Prepare a concentration series (e.g. 5, 2.5, 1.25, 0.625, 0.3125, … mg/ml).

dn/dc Data Collection

1. Book time on the PPMS calendar before you start.
2. Login to the computer using your PPMS credentials (eCommons ID and password).
3. Attach the Wyatt WISH module to the Agilent chromatography system and to the Optilab TrEX RI detector.
    a. Make sure there is NO COLUMN inline on the HPLC system
    b. Attach the outlet on from HPLC system UV detector (or other convenient location downstream of the pump) to the inlet on WISH.
    c. Attach the outlet on WISH to the inlet on the Optilab TrEx.
4. Equilibrate the RI detector:
    a. Purge pump on the Agilent chromatography system with water (or buffer) for 5 min at 5 ml/min flow rate. (Open pump purge valve, Purge, Close pump purge valve).
    b. Turn on the flow at 0.5 ml/min.
    c. Rinse the WISH module in both inject and load positions for several minutes.
    d. Open RI purge valve.
    e. Rinse the RI detector while the purge valve is open for at least 30 min.

   f. Close the RI purge valve.
   g. Zero the RI detector.
5. Open Astra software.
6. Write a Batch method.
   a. File → New → Experiment from Method.
      i) Go to System → Method → RI Measurement → Batch (determine dn/dn).
      ii) Or Find a batch dn/dc method in the user methods folder.
   b. Open Basic Collection (double click).
   c. Set calculated duration to 60 min (or longer).
7. Start Method in Astra:
   a. Click Run button to start method.
   b. Click OK when prompted (after pump has started).
8. Inject the buffer for several minutes to establish a baseline.
9. Inject samples (from low to high concentration):
   a. In the load position, inject the sample into WISH module (must use over 1 ml to ensure that the loop is filled).
   b. Turn valve to the inject position for sample injection.
   c. After injection is complete (or nearly complete, turn valve to load position.
   d. Repeat injections for each concentration.
   e. Run duplicate measurements, either in two experiments, or in one continuous experiment.
10. Inject the buffer again for 5-10 minutes.
11. Stop the Method once finished.
12. Draw Baseline:
   a. Open Baselines.
   b. Draw a baseline between the two regions of buffer injection.
   c. Click OK.
13. Select Peaks:
   a. Open Peaks.
   b. Click and drag through a flat region to define each peak (usually the 2nd half of the injection peak).
   c. Enter the concentration in mg/ml for each injection.
   d. Click OK.
14. Extract dn/dc:
   a. Open dn/dc from RI.
   b. Examine the linear fit.
   c. Get the dn/dc value.
15. Wash system
16. Return system to Storage buffer.
   a. Turn off RI LED.
   b. Purge the RI detector.
      i) Turn Purge "ON" on the RI detector LCD screen to open the reference cell so it is washed during equilibration.
      ii) Leave the purge valve ON at all times, except during data collection

c. Stop flow in HPLC Manager.
d. Purge the Pump:
   i) Change the solvent to storage buffer (Filtered water with 0.02 % NaN_3).
   ii) **Open the purge valve on the Agilent Infinity 1260 isocratic pump**.
   iii) Go to HPLC manager.
   iv) Set the maximum flow gradient to 100 ml/min^2.
   v) Set the flow rate to 5 ml/min.
   vi) Run at least 25 ml buffer through degasser and pump (5 min).
   vii) Close the purge valve on the Agilent pump.
e. Equilibrate system in storage buffer:
   i) Set equilibration flow rate (0.5 ml/min for most shared columns).
   ii) Set flow to 0 ml/min.
   iii) Set **maximum flow gradient to 0.1ml/min^2**.

17. Shutdown
    a. Clean up in and around the instrument.
    b. Close ASTRA.
    c. Logoff from PPMS

Protein Conjugate Analysis

1. Open Protein Conjugate Analysis Method.
   a. Open a SEC-MALS data file in ASTRA
   b. Right click on the file name of the open file in ASTRA.
   c. Select Apply Method.
   d. Go to System → Method → Light Scattering → With QELS → Protein. Conjugate.
   e. Click Create.
2. Edit Peaks.
   a. Open Peaks.
   b. Expand Protein Conjugate Analysis.
   c. Enter modifier dn/dc value for each peak (and UV if non-zero).
   d. Click OK.
3. View data.
   a. Open EASI Graph.
   b. Display Protein Conjugate.
   c. Open EASI Table to view summary data for all open files.
4. View Report.
   a. Open Report (detailed).
   b. Protein mass, modifier mass and conjugate (the total) mass are reported.

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

- rates are based on *booked and real time usage*

Contact cmi@hms.harvard.edu with questions.

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