**LSU Second Day Overview**

**Second Day Emphasis:**

1. Using flowing separation systems and the DAWN or the miniDAWN to determine molar masses and molar mass distributions;
2. The difference between a Zimm plot (batch) and a Debye plot (flowing);
3. Using ASTRA software to assess the quality of MALS data;
4. Using ASTRA software to process MALS data;
5. Selection of processing parameters to include plotting and fit formalism;
6. Polymer sample information from MALS with on-line separation: distributions, conformations, branching, etc.

**SEC-MALS Analysis Start-Up Guide**

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| **Action** | **References** | **Notes** |
| 1. Confirm that your instruments are connected to your computer. | LSU Manual, Chapter 3 | Use the Diagnostic manager to view your instruments in ASTRA or start a short data acquisition to test. |
| 1. Confirm that all fluid connections between your HPLC, column and instruments are set up properly and auxiliary connections and autoinject connections are made. | LSU Manual, Chapter 8 | Keep fluid connections as short as possible. Auxiliary signals can be viewed on the front panel of your Wyatt instrument. |
| 1. Check that your MALS calibration constant has been determined within the last 12 months. | LSU manual, Chapter 3, Astra user’s guide p 8-15. | The validity of the calibration constant can also be checked by confirming that the correct molar mass is obtained for the validation standard. |
| 1. Ensure that your HPLC system is clean:   Check baseline levels and noise on your 90° LS detector at flow rate with the column connected: *Aqueous (PBS):* baseline 0.009-0.02V , noise less than 100 μV, ideally less than 50 μV. *Organic (THF):* baseline 0.025-0.029V , noise less than 50 μV, ideally less than 20 μV. | LSU manual, Chapter 2 | Flush a new column overnight directly to waste before connecting to your MALS setup.  If your baseline noise is too high, try to locate the source of the noise: column, mobile phase, HPLC contamination, dirty LS cell by isolating the corresponding components or replacing buffer, filters and tubing, if necessary. |
| 1. Perform a blank (buffer or solvent) injection. |  | This will identify any “ghost” peaks or contamination of your column. |
| 1. Inject validation standard:  * BSA for aqueous protein work * BSA or aqueous polymer for aqueous polymer work * 30kDa Polystyrene for organic mobile phase | LSU manual, Chapter 7 |  |

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| 1. Check system constants, such as Normalization, Alignment, and Band Broadening. | LSU manual, Chapter 7 | |  |
| 1. Check molar mass from your concentration detector. Use molar mass from both RI and UV, if present. |  | If the molar masses are correct, your system is ready for analysis. | |
| 1. Check mass recovery |  | Prepare your validation standard in the exact concentration. Pierce BSA ampoules have a concentration of 2 mg/mL and the mass recovery across all peaks should be ~ 95%. | |
| 1. Save your validation experiment as a method. Use this method for all future data acquisitions. | LSU manual, chapter 7, ASTRA user’s guide, Chapter 6. | Your method will contain all the correct system constants. If your method includes processing parameters, make sure to check baselines and peaks settings and adjust values for dn/dc, UV extinction coefficients, concentration and other sample parameters, if necessary! | |
| 1. Periodically check your HPLC-MALS performance with your validation standard. |  | A weekly interval is recommended for medium throughput labs, more checks may be appropriate for higher use.  A check is also recommended if anything in the hardware setup was changed or the system has not been in use for a while. | |

Parameters to Check during ASTRA Data Analysis

1. ***Constants:***

* Calibration constant of DAWN or miniDAWN;
* Normalization coefficients of DAWN or miniDAWN;
* Calibration constant of third RI and/or UV detector;
* Delay volume between DAWN/miniDAWN and RI/UV;
* Band broadening terms;
* Dn/dc value;
* For UV: extinction coefficient; UV Response Factor; AUX channel
* Solvent entered in ASTRA;
* Flow rate.

1. ***Other Parameters:***

* LS overlay plot to check normalization & stray light;
* Baseline settings – check for ***all*** detectors;
* Baseline noise;
* Peak region – is it the region of interest;
* Proper LS detectors selected;
* Fitting formalism (Zimm, Debye, Berry, Random Coil);
* Proper polynomial fit order – (1st, 2nd, etc order);
* Results fitting (if fitting is used: is the fit a good fit?);
* Molar mass vs. elution volume plot;
* Mass recovery for a well known sample of known concentration.

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| **Minimum Amount of Sample Required** | |
| **SEC-MALS** | |
| **OrbitStack.jpg1100-3[1]** |  |
| For s/n = 5, noise = 20 μV, with a monodisperse sample using one standard SEC column.  Example: 0.6 μg for 100 kDa protein |

### Sensitivity of MALS

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| **Minimum Concentration required** | |
| **Batch with Flow Cell** | |
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| For s/n = 5, noise = 20 μV, with a monodisperse sample using one standard SEC column.  Example: 0.6 μg/mL for 100 kDa protein  **Minimum volume required: 300-500 μL** |

**Sample Quiz Questions:**

***1. Which scattering angles are most affected by particulate matter in the mobile phase?***

The lower scattering angles are most affected by particulate matter if the flowing system (mobile phase, columns, FFF channel, or injector) is not clean. Care must be taken to get the flowing system clean and keep it clean to reduce the baseline noise - especially at the lower scattering angles.

***2. What is the lower limit for determination of the rms radius by light scattering?***

Approximately 10 nm.

***3. In the conformation plot (log(rms) vs log (M)), what should the slope be if the polymer is a sphere?***

Approximately 0.3. Random coils have conformation parameters of 0.5 to 0.6. Rigid rods have conformation parameters of 1.

***4. What assumptions are required to perform an on-line dn/dc determination?***

1) The sample concentration is known,  
2) the volume injected is known,   
3) the flow rate is known, and   
4) 100% of the sample injected elutes from the separation system under a single clean   
 peak.

***5. If only the 90 degree scattering detector were used to process the MALS data from a high molar mass polymer sample, would the computed molar mass be greater or less than the “true” molar mass?***

The computed molar mass would be less than the “true” molar mass. To determine the “true” molar mass the scattering data must be fit over the full angular profile.

***6. If you only have a detector at one scattering angle (such as LALLS or single 90° detector), can you determine the radius of the polymer?***

No! You must know the angular dependence of the scattering to determine radius, requiring multiple angles.

***7. In MALS why do we need to enter the flow rate? How is the flow rate used in ASTRA?***

ASTRA needs the flow rate to compute the “computed mass (g)” of the sample. The concentration of the sample in each data slice (collection interval) is determined from the RI or the UV detector signal. ASTRA needs the flow rate to compute the volume of mobile phase in each data slice in order to compute the actual mass (grams) of the polymer eluting during each data slice.

***8. How can you tell if your flow cell is getting dirty?***

The most straightforward way is to monitor the baseline voltage of the 90-degree detector. As the cell widows get dirty the sustained baseline voltage will gradually increase.

***9. How can you tell if you have excessive stray light in your flow cell?***

Use a LS Overlay plot to overlay the light scattering signals for the peak from an isotropic scatterer. If there is little stray light, system clean - windows clean, all light scattering peaks will overlay perfectly.

***10. How can you check to see if your normalization is correct?***

Use the 3D plot to look at the relative scattering as a function of the scattering angle or use the Debye plot to compare the response of each angle with the fit line.

***11. Why is band broadening between the LS and the RI signal a potential problem?***

To compute the molar mass of each data slice, ASTRA needs to know the concentration (from the RI signal) and the scattering intensity of each data slice as the polymer elutes. Broadening of the RI peak relative to the LS peak will distort the concentration measured at each slice resulting in an erroneous molar mass determination for that slice. This effect is devastating when using an in-line viscometer.

***12. If the size of a significant portion of a polydisperse polymer sample exceeds the upper size limit of your SEC column, will the measured molar mass be correct?***

The *weight average* molar mass will be correct. The number average and the z average molar mass values will be incorrect since these values are computed based upon the assumption that the polymer in each slice is monodisperse.

***13. If the size of a significant portion of a polydisperse polymer sample exceeds the upper size limit of your SEC column, will the measured rms radius be correct?***

The *z-average* rms radius will be correct. The number average and weight average rms radius will be incorrect since these values are computed based upon the assumption that the polymer in each slice is monodisperse.