



Live Webinar Q&A Sheet: Separation and Characterization of Macromolecular and Supramolecular Structures by Light Scattering

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Q: *Can the shape factor, R_g over R_h , indicate shapes other than homogeneous sphere and spherical shell?*

A: Yes, the shape factor, indicating the particle shape and compactness of the structure, has been reported for different macromolecular conformations and particle shapes; for example, random coil conformation (1.78), helical structure, star architecture (1,33), and for dendritic polymers. For particles, shape factor is known, beside for homogeneous sphere and spherical shell, also for prolate (1.36 to 2.24) and oblate (0.875 to 0.987) ellipsoids of different axial ratio (between 1 and 100), for stiff rod-like shape particles (2-2,36), and disks.

Q: *In which cases would you prefer SEC over FFF for use with light scattering to characterize macromolecules, and when would you prefer FFF?*

A: The drawback of SEC is its limited working range. Therefore, for larger macromolecules of few million Da or for nanoparticles AF4 is preferred over SEC since AF4 has larger dynamic range from few nanometers up to a micrometer. For example, there are only few SEC columns on the market, available for separation of high molar mass species (few MDa).

- In addition, for macromolecules of high molecular weight that are intended to be separated by SEC, there are issues with degradation due to hydrodynamic shear forces, which is not the case in AF4.
- SEC can also remove aggregates; either they are filtered by frits or column packing material itself.
- Additionally, the mobile phase for SEC usually has to be adjusted to allow separation by size. So, the chemical composition of the mobile phase is frequently different from the chemical



composition of the protein formulation buffer. Thus, the mobile phases used in SEC (can contain organic co-solvents or has extreme pH) can cause dissociation of aggregates that are otherwise irreversible in the formulation buffer. On the other hand, in AF4 we can usually apply the same buffer solution for protein storage as well as for a mobile phase.

- Biopharmaceutical formulations frequently contain stabilizing agents that improve stabilization of protein drug during production and storage. Most widely used surfactant that protects proteins against mechanical stress-induced aggregation is polysorbate 80 (Tween 80). However, in SEC the degree of association of this surfactant was found to be dependent on injection number. The degree of association was found to decrease with increasing number of successive injections, which was not observed in AF4. Such dynamic dissociation of the surfactant micelles in SEC prevents quantification of protein aggregates in storage buffer solution.
- Moreover, the advantage of AF4 is that it can separate inhomogenous samples, which is usually not the case for SEC.
- On the other hand, SEC is preferred for smaller size macromolecules, where AF4 encounters issues with low sample mass recovery due to the passage of the sample through the membrane or when low mass recovery is due to adsorption of the sample on the AF4 membrane.
- SEC is invaluable also for determination of molecular weight characteristics of polymers that are molecularly dissolved only in organic solvents of high polarity (HFIP, DMSO, DMF, etc).

Q: *What information do you need to know about conjugates such as PEGylated proteins or miktoarm copolymers in order to determine their composition by multi-detector conjugate analysis?*

A: For a complex two-component system the best results are obtained if only one component of the two-component system is UV active at the chosen wave-length of the UV-detector, while the other component is invisible at this wavelength. So, for determination of chemical composition by a multi-detection system we need to know the values of specific refractive index increments (dn/dc) and specific extinction coefficients of both constituents of the two-component system. It should be noted that accurate determination of delay volumes between the detectors is also important.

Q: *Can multi-detector conjugate analysis be carried out on large unilamellar vesicles (LUVs) or lipid droplets (LDs) to determine if they are encapsulating genetic material, a protein or drug compound?*



A: In the case of LUV and LD usually the amount of the sample loaded in the channel is small since these particles scatter light intensely. So we get nice LS signal, but the intensity of the RI detector response can be too low, especially when small amounts of samples are available, like in the case of exosomes. Additionally, the contribution of low molecular weight drugs to the total LS intensity will be most probably too low.

In our lab we try to determine the amount of protein pre-pores and pores formed on artificial LD and LUV by a multi-detection system, however, the intensity of the UV-signal due to the protein component was too low, which means that other more sensitive concentration detector should be used instead of the UV-detector to monitor the small amount of the protein component, e.g. fluorescence detector.

Nevertheless, the loading capacity of the low molecular weight drugs with high specific extinction coefficient as well as proteins or DNA can be evaluated by comparing a ratio of the intensities of the two concentration detectors if the signal responses of both concentration detectors are high enough and they also differ enough for both constituents. If only the drug is UV-active at a chosen wavelength of the UV detector, its amount can be determined solely by the UV detector.

Q: *Are there any ways to overcome the flow-rate dependent error in hydrodynamic radius?*

A: Underestimated hydrodynamic size of large particles determined in flow mode is connected to incorrect calculation method of diffusion coefficient from the correlation function. Namely, when the flow is applied to a system of particles undergoing Brownian motion, the additional translational component affects the particle movement. For the system of particles, exhibiting both random Brownian and uniform translation motions, Chowdhury in 1984 (Chowdhury, D. P.; Sorensen, C. M.; Taylor, T. W.; Merklin, J. F.; Lester, T. W. Appl. Opt. 1984, 23, 4149-4154) proposed the so-called modified time correlation function, which takes into account both components. They showed that by using such modified correlation function the accuracy of particle sizing in flow mode can be significantly improved. The improvement in calculation is the most effective for smaller particles and less for the larger ones.

The first term of this modified correlation function is related to the diffusional information and strongly depends on the particle size, while the second term is related to translational information that is associated with the passage time of the particle through the laser beam. This term is independent on particle size, but, it strongly depends on the rate of the passage flow at the detector.



As far as I know Wyatt Company has already developed a flow cell that extends the range of accurate DLS measurements up to 300 nm in size for a flow rates up to 0.3 mL/min.

Q: *What detector angle is your DLS detector attached to?*

A: In our case, DLS module is embedded at angle 99° of the MALS detector.

Q: *Can we use Kratky plots for the structural analysis even in the SEC-MALS system or it is only possible in FFF-MALS system?*

A: Unfortunately, the sizes of macromolecules and particles coming out from SEC (up to 20-30 nm) are too low to obtain the Kratky plots from which the particle shape can be inferred.

Q: *Is there any formula for having a correction factor to adjust for flow rate effect in Rh calculation via QELS?*

A: We found out that that the error in particle size depends solely on particle size and detector flow rate, but not on the type of the particle studied (at least for the spherical particles). So theoretically it can work, but I do not recommend such approach.

Q: *Some commercial equipment based on light scattering provide complete particle size distributions, in some cases showing multimodalities. Is the technique capable of providing such detailed multimodal distributions considering the inherent ill-conditioned nature of the data inversion for polydisperse samples?*

A: AF4 can provide size distribution of polydisperse particle samples (from a few nanometers to a few micrometers). It is superior over DLS (size distribution by DLS often does not represent real situation) and NTA (blind for particles smaller than 30 nm).



Q: Would it be possible to characterize the miktoarm copolymers based on the ratio of R_g/R_h , as you described in the first part of your presentation?

A: Based on the ratio of R_g/R_h it is possible to discriminate stars from their linear analogues, but I am not sure that we can discriminate the stars containing different number of arms.

Q: What's the smallest particle size that AF4 can separate?

A: The lower size limit depends on the membrane cut-off (size of the membrane's pores) that is mounted on the bottom channel plate. Usually the membrane of 10 kDa cut-off is used, but you can go down to the membranes of 5 kDa and 2 kDa cut-off.

Q: Can we determine the particle size for metal-organic frameworks called MOFs?

A: They can be determined if they are in the form of suspension and if they are of sizes below few micrometers.

Q: When using WyattQELS in flow DLS measurement does it have the same dependency on flow rate as FFF4?

A: If the software in WyattQELS uses conventional correlation function that does not take into account translational component you have the same dependency of R_h on the flow rate as in the case of DLS module in AF4.

Q: Are the methods that you talked about suitable for molecules of molecular weight below 10K?

A: Multi-detection system works as long as you have satisfactory intensities of all three detectors (LS, UV, and RI). Since the molar masses of macromolecules (polymers and peptides) can be measured down to 200 Da I do not see any problems with using a multi detection system. However, the physical dimensions (R_g) of macromolecules up to 10 kDa cannot be determined, and therefore also the shape factor can not be used to infer on macromolecular conformation.

Q: What is the maximum size for SEC characterization? What is the artifact with SEC above this size limit?

A: Maximum size depends on the pore-size of the SEC column packing material. Above the maximum size limit, the solute is excluded and does not penetrate into the pores of the stationary phase, meaning that you do not have any separation of your sample by size.



Q: *Is there any chance for aggregation of proteins/conjugates in focusing step of FFF?*

A: If there is a chance for aggregation, it most probably depends on protein type. I recommend to check this by measuring the samples at different focusing times. If this is an issue it is expected that degree of protein / conjugate aggregation increases with focusing time, which was however not observed in our case.