MOSBRI Molecular-Scale Biophysics Research Infrastructure Basic Level School

Size exclusion chromatography coupled with a static light scattering detector (SEC-SLS)

Introduction - Guidelines:

A**nalytical size exclusion chromatography (SEC),** also known as gel filtration, is currently the standard column-based chromatography technique to quantify protein oligomers. SEC separates molecules according to their Stokes or hydrodynamic radius R_H, with larger sized molecular species eluting before smaller ones from the column. Aggregates, contaminants and potentially different oligomeric states of the protein of interest can be readily separated and quantified via online concentration detection. Depending on the manufacturer and the matrix, each column has a specific range of separation that is defined in terms of the molecular mass of the proteins or sugar chains. One should keep in mind that the molecular mass is not identical to the hydrodynamic size and that there may not even be a direct correlation, e.g. for non-globular proteins. The column should be chosen for its ability to elute the protein of interest at a volume situated well between the upper and lower limit ensuring ideal separation (see Note 1). For quantitative, i.e. analytical, measurements, columns should be used that usually have a smaller diameter than the ones used for preparative purposes. In order to select the best column and ensure correct handling, one should read the manufacturer instruction, which gives the molecular weight limit, pressure limit, the volume of the column, the void volume, and the recommended flow rate. In order to determine the absolute molecular mass of each eluted species an online **Static Light Scattering (SLS)** detector, in combination with a UV or a refractive index (RI) detector, must be coupled to the SEC system (SEC-SLS). Currently, different types of SLS systems are available on the market, e.g. **Multi Angle Light Scattering (MALS)** detectors and **Low Angle Light Scattering (LALS)** detectors. MALS detectors measure the intensity of the scattered light at different angles θ followed by extrapolation of the intensity to $\theta = 0°$, which allows to calculate the radius of gyration (R_G). The main reason for this extrapolation is to compensate for the angular dependence of light scattering intensity for molecules larger than $1/20^{th}$ of the laser wavelength (i.e. $R_G = 10 - 15$ nm). LALS detectors measure the scattered light at a small angle (e.g. 7° for some systems), assuming that the difference in intensity between 7° and 0° is negligible, even for molecules with a molecular mass of 10 MDa. Thus, the main difference between MALS and LALS detectors is the way they obtain light scattering intensity at angle $\theta = 0°$. If the sample is pure and homogeneous SEC will give one sharp and symmetric peak in the elution profile/chromatogram associated with molecular mass of the target protein. Polydispersity will be immediately obvious if there is more than one peak. However, one should keep in mind that the sample will be diluted during the SEC run, that SEC is usually not an equilibrium method, and that SEC will apply shear forces on the sample that may disturb protein oligomers/complexes. Finally, it is to mention that, although the gel filtration resins are generally “inert”, some proteins interact with them, falsifying results or making SEC even impossible.

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Columns are usually stored in 20 % (v/v) ethanol. Therefore the column must first be washed extensively with water (at least 2 column volumes) as ethanol may precipitate salt in the buffer if the column is directly equilibrate with the buffer. Subsequently, it is equilibrated with the buffer of interest until the baseline is stable (at least 2 column volumes). The buffer should be stable over time as any change such as oxidation will lead to a drift in the baseline of the refractometer. The buffer should contain at least 50 mM of salt to avoid interactions with the column matrix and 0.1 % (w/v) NaN_3 to prevent microbial growth. All solutions must be freshly filtered and degassed to avoid background scattering and an unstable baseline. In line with the column, the refractometer and the light scattering detectors allow the measurement of the molecular mass of all the peaks of interest.

Protocol:

1. Switch on the instrument and equilibrate at specific temperature.
2. Centrifuge on bench centrifuge full speed 15 min to remove particles > 100 nm in hydrodynamic radius.
3. Purge the system with water.
4. Connect the column to the SEC-SLS / SEC-MALS system.
5. Choose the flow rate and pressure limit according to the column manufacturer's instruction.
6. Equilibrate the system with at least 2 column volumes of water equilibrated at the desired temperature.
7. Purge the system with the sample buffer.
8. Equilibrate the system with at least 2 column volumes of the buffer.
9. Purge the refractometer extensively until the baseline remains stable for one column run. (Negligible drift can be compensated by the software baseline correction).
10. Turn on the light scattering laser and wait for stability of the detector signal. Before collecting data, all the detectors available should be stable over a period of $>$ 20 min.
11. Collect data for sample. Repeat as necessary. Inject a standard if required.
12. After data collection is complete, check that the detectors have returned to reasonable baselines. If not, additional flushing or column cleaning may be needed.
13. Flush system and column with water. Store the column in 20 % ethanol. To store the system, see the manufacturer recommendations (20 % Ethanol, 10 % Acetone, 10 % Methanol etc.)

Note 1: Care should be taken to choose the column as an elongated 60 kDa protein may behave on the column like a globular 160 kDa protein. Be aware that SEC columns are often described by their separative power on globular protein. Test runs with small injection volumes of the protein of interest may be performed to choose a good column setup.