Protein Applications for Advanced Multi-Detector Size-Exclusion Chromatography

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Size-exclusion chromatography (SEC) or gel permeation chromatography (GPC) is frequently used in bioscience laboratories to characterise purified and recombinant proteins. This technique has also been used to measure molecular weight or to resolve mixtures of monomers, oligomers and higher order aggregates. Using this method, molecular weight is calculated by creating a calibration curve of standard globular proteins and comparing the elution time of an unknown to the standards. This is limited in that it is based on a stable relationship between molecular weight, size, shape or hydrodynamic volume. This relationship varies significantly between proteins introducing unquantifiable inaccuracies in all measurements made in this way.

Modern analytical systems are capable of more than just sample detection with a single concentration detector. The use of multiple detectors including refractive index (RI), ultraviolet (UV), light scattering (LS) and viscometry (IV) allows extensive characterisation of protein samples in a more absolute way than was previously possible. RI and UV both allow accurate concentration measurements to be made. A combination of these allows conjugation analyses to be performed. Light scattering detectors allow the measurement of molecular weight without the need for column calibration. Intrinsic viscosity is a measure of molecular density and enables structural changes to be assessed. A combination of light scattering and intrinsic viscosity allows size (Rh) to be calculated. This is called tetra detection. A typical tetra detector analytical SEC system is shown in the schematic in Figure 1.

This article describes some common applications for which an advanced analytical SEC system is used to characterise proteins beyond the capability of a single detector system.

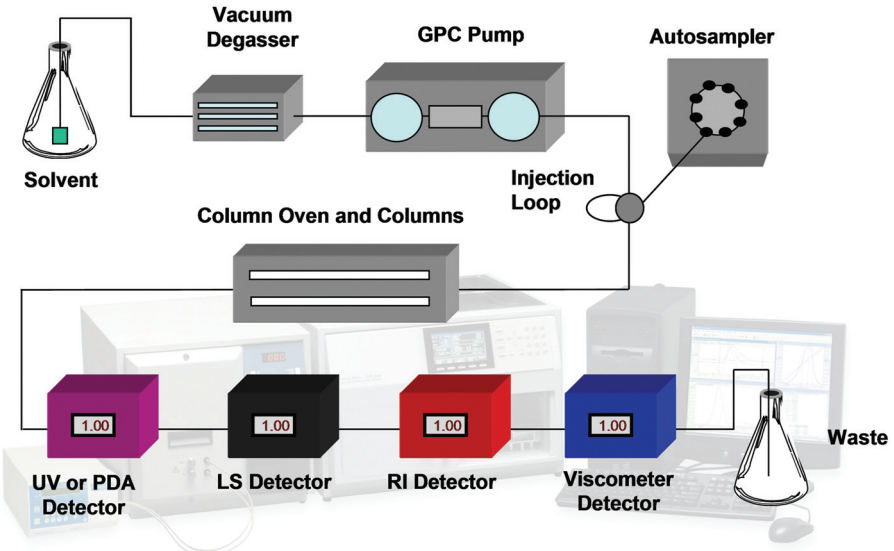


Figure 1: Schematic of a tetra-detector analytical SEC system.

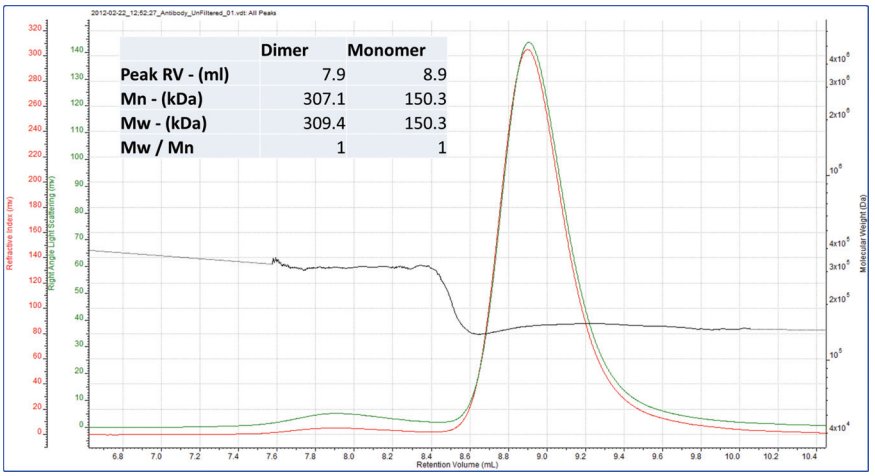


Figure 2: SEC measurement of IgG showing monomer and dimer molecular weights.

Molecular Weight

Individual proteins have a very well defined molecular weight. Once synthesised within a cell, and following any post-processing, such as cleavage or glycosylation, a protein's molecular weight will be fixed with minimal variation.

Measuring the molecular weight of a novel protein is interesting in itself. Different proteins can have significantly different molecular weights, so measuring the molecular weight of a purified protein is an excellent way of confirming that the purified sample contains the protein of interest. During production of a recombinant protein, confirming the molecular weight to be the expected value is a good indicator that it is being produced correctly by the host cell line.

Measuring molecular weight is therefore of interest both academically and practically as an indicator of product or sample quality.

Figure 2 shows an example of an antibody (IgG), a protein commonly used in biopharmaceutical drugs. Its molecular weight has been measured as 150 kDa and is constant across the peak. A very small secondary peak can also be observed and its molecular weight has been measured to be slightly over 300 kDa. The molecular weight suggests that this small second peak is a stable dimer present in the sample. With this knowledge, the dimer can be deemed acceptable to product quality or purified out if required.

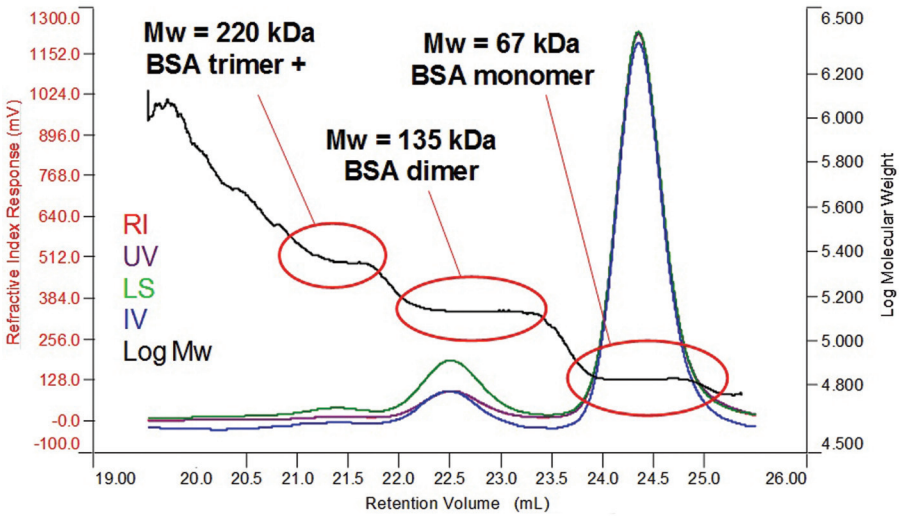


Figure 3: The molecular weight of each peak has been measured in this BSA sample. The molecular weights correspond to the monomer and dimer. The molecular weight of the third peak is slightly above that of the trimer suggesting it contains this as well as higher order oligomers.

Oligomerisation State

The activity of any protein is defined by the final structure or conformation that it takes. Quaternary structure defines the final complex formed when multiple protein chains come together to form a single unit. This too can be assessed by analytical SEC. Proteins are individually monodisperse meaning their molecular weights are stable across a peak as they elute from a SEC column. As multiple proteins come together to form oligomers, the measured molecular weight will increase discretely resulting in step changes of molecular weight within the chromatogram.

In the case of homo-oligomers, when a number of identical proteins come together, the measured molecular weight will be a multiple of the monomer molecular weight allowing the oligomer number to be determined via a simple calculation. In Figure 3 it can be seen that the molecular weight of BSA increases in steps between each peak. Each of the molecular weights corresponds to a multiple of the monomer molecular weight, in this case, two and three fold indicating the presence of dimers and trimers. In the case of the trimer, the slightly higher than expected molecular weight and that fact that it is increasing at earlier retention volumes within the peak suggests this peak also contains some larger oligomers. The molecular weight continues to tail upwards identifying the presence of some higher order aggregates. The oligomers, which will have some activity, are clearly distinguishable from the higher order aggregates (which will be inactive) by the stable molecular weights across the peak. The proportion of each can, of course, be individually measured by the RI or UV detector to determine the overall composition of the oligomer mixture.

Aggregate Quantification

Protein aggregation is a common result of many sample treatments, prolonged storage or freeze/thaw action. The structure of a protein is held together by Van der Waals forces, hydrogen bonds and hydrophobic interactions. Changing conditions can disrupt the delicate balance that holds the structure together to reveal buried regions of the polypeptide chain. These regions can interact with those on other proteins to form larger complexes of misfolded proteins. Hydrophobic regions are especially susceptible to this effect. Aggregates are often strongly held together by these forces meaning that their formation is irreversible and that they continue to grow. The activity of the proteins will be lost and they may reach the point when they are no longer soluble.

Protein aggregates tend to have a very high molecular weight and are often very polydisperse, as the complexes formed are very unlikely to become stable structures of predictable sizes.

In SEC, aggregate peaks can sometimes appear in the void volume of the column as

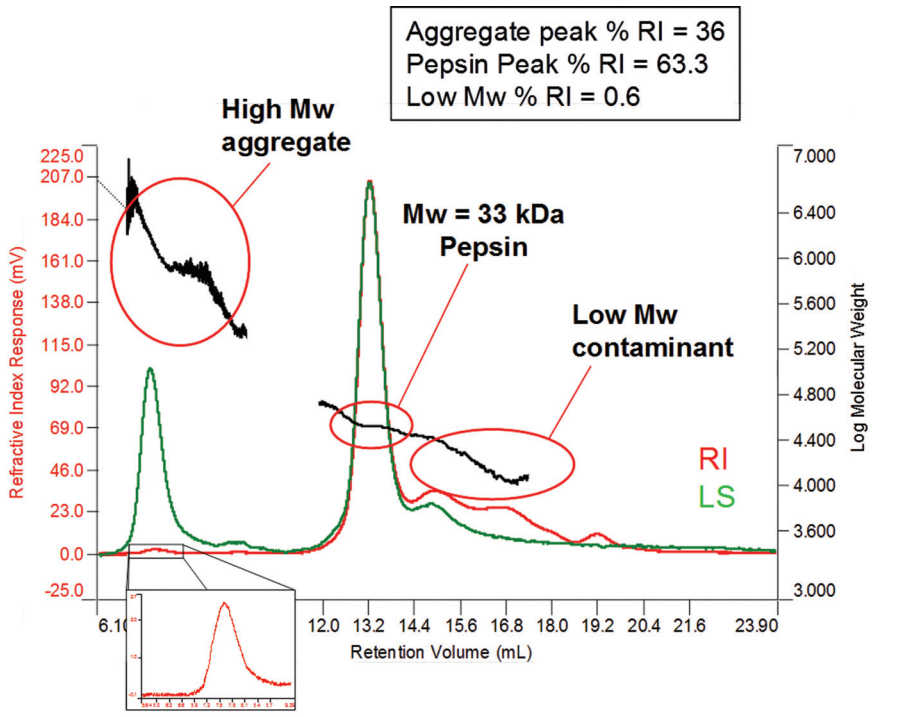
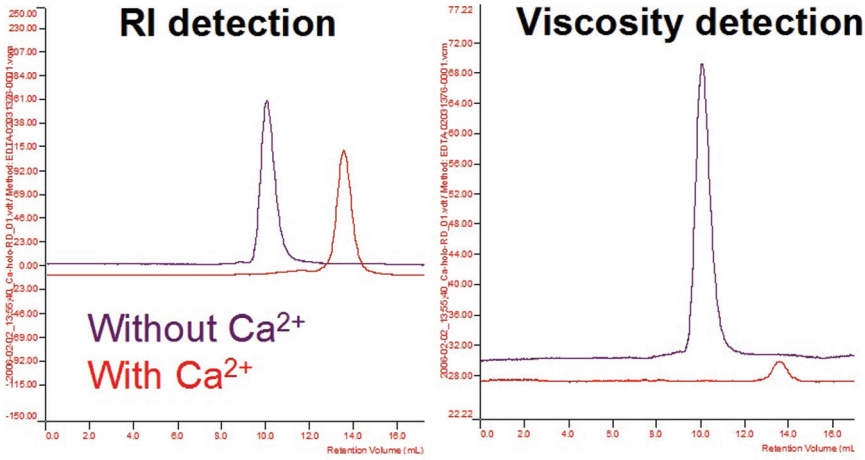


Figure 4: The light scattering detector clearly identifies the high molecular weight aggregate while the concentration detector, in this case RI, can be used to calculate the proportion of the sample making up this material.



Sample	Mw (kDa)	ή (dl/g)	Rh (nm)
With Ca	77.5	0.0531	4.0
Without Ca	78.9	0.5134	8.6

Figure 5: Intrinsic viscosity for anylate cyclase toxin changes dramatically in the presence and absence of calcium.

they are too large to penetrate any of the pores in the packing material. Their molecular weight may have limited meaning given their high polydispersity but it can, nevertheless, be measured along with their concentration using refractive index or UV.

The relative sensitivity of light scattering and concentration detectors means that in the case of very small amounts of very high molecular weight material, the concentration may be below the limit of the concentration detector, in which case the light scattering effectively acts as a 'first responder' to the formation of aggregates. If their concentration is high enough, then their concentration can be measured. Figure 4 shows a sample of pepsin with a small amount of aggregated material. It's large molecular weight and high polydispersity are typical of aggregated proteins. The small RI peak allows the concentration to be determined as a proportion of the total material and is seen to make up 0.6% of the sample. In this particular sample, some lower molecular weight material has also been identified, making up 36% of the sample.

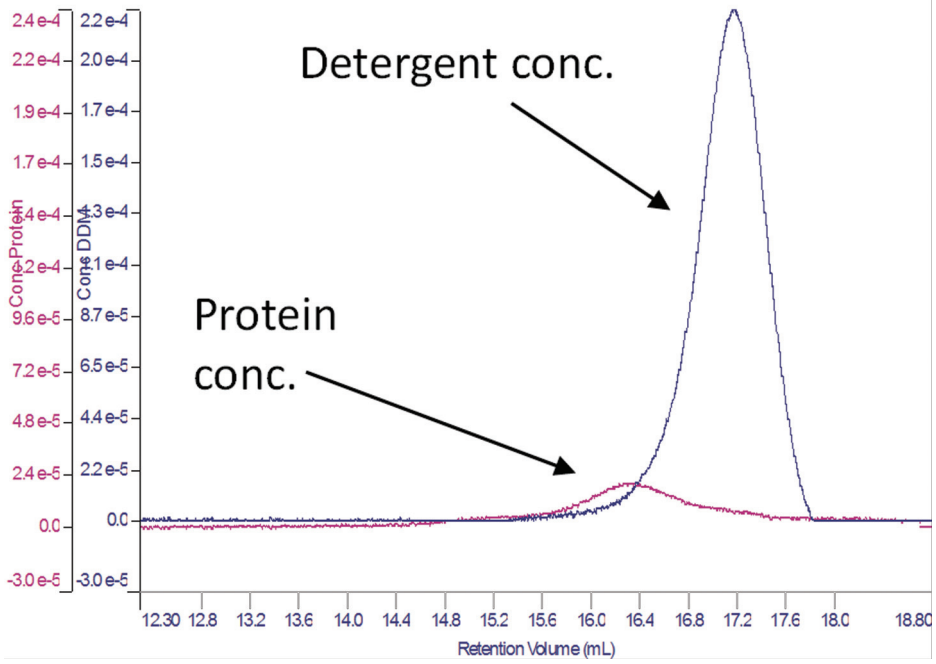


Figure 6: Derived chromatogram showing the calculated concentration of protein and detergent.

Size and structural information

Intrinsic viscosity is inversely proportional to molecular density (or partial specific volume). This can be measured by a viscometer. A combination of this and the molecular weight data from light scattering also allow a molecular size to be determined. These two values are very useful for assessing structural changes between samples. A change in size and intrinsic viscosity with a constant molecular weight is a clear indication of a change in structure and, from an increase or decrease in density, an idea of the structural change can be reached.

For proteins, structure determines function and changes in or loss of structure result in changes in or loss of activity. Proteins will change their conformation under different conditions and this can be assessed using this technique. Figure 5 shows adenylate cyclase toxin which changes its size and intrinsic viscosity (IV or ή) significantly with a constant molecular weight in the presence or absence of calcium. In the absence of calcium ions, the protein is unfolded with high IV and a larger size. Upon addition of calcium, the protein assumes its native conformation with a smaller size and a considerably lower IV. It is worth noting that the change in IV is much greater than the change in size showing its higher sensitivity to these changes.

Protein conjugates

When two different materials are eluting from a column, combining two concentration detectors, in this case, RI and UV, allows the concentration of each component to be measured at the same time if the response factors of each detector to each component is known. For RI and UV, these response factors are the dn/dc and the dA/dc. This is particularly interesting for conjugated proteins where it is useful to know how much of a second material (e.g. membrane lipids or PEG) is conjugated with the protein of interest.

Studies of membrane proteins are becoming increasingly frequent in molecular biology research. Crystallisation is one of the main goals of this research in order to characterise the structure of a protein, However, crystallisation of these proteins has proven difficult. Crystallisation of a membrane protein can depend on many factors such as protein purity and the detergent concentration or type; removal of too much of the detergent component of a membrane

protein complex can lead to degradation of the protein and reduce the likelihood of crystallisation. Characterising and optimising the proportion of protein and detergent in a purified membrane protein sample can provide valuable insight into the likelihood of crystallisation and the protein content of the protein detergent complex (PDC).

Figure 6 shows a derived chromatogram from a study of a membrane protein which has been purified in the presence of detergent lipids acting as a surrogate membrane for the protein to bind to. The derived chromatogram shows the calculated protein and detergent concentrations which have been calculated using a combination of the RI and UV detectors. From the analysis, the mass of the complex, protein and detergent can all be calculated identifying the protein as a monomer conjugated with detergent. As can be seen in the figure, there is also a large excess of free detergent.

Conclusion

Analytical SEC has undergone significant development since its inception. While measurements of molecular weight and purification were the original goals of the technique, analytical measurements can now be performed in a more direct way. Furthermore, concurrent measurements of concentration, intrinsic viscosity, size and conjugation can also be performed making advanced analytical SEC an invaluable tool for many bioscience laboratories. In combination, these techniques turn SEC into a very powerful tool for the characterisation of protein samples.