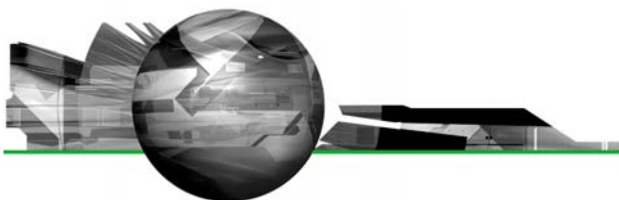


Protein and polymer molecular size by GPC/SEC

Introduction

The main goal of most Gel Permeation/Size Exclusion Chromatography (GPC/SEC) experiments is to determine the molecular weight distribution (MWD) of the sample or compare molecular weights (MW) of several samples. However, in many cases the MW alone will not explain sample differences. In the particular case of biologically important molecules such as proteins or polysaccharides, the molecular size may be of equal or even greater importance than the MW to the researcher. Indeed, in the case of proteins, the MW is often known accurately before the experiment and the information required is purely the size of the protein and the amounts and sizes of any associated aggregates. There are two measurements of molecular size in common usage and these are the radius of gyration (R_g) and the hydrodynamic radius (R_H). In simple terms, R_g is a mathematically defined dimension describing the distribution of mass centres in the molecule, whereas R_H is a phenomenological property of the molecule. This means that for practical purposes, R_H is a much more useful measurement, particularly for biologically important molecules.

Radius of Gyration

Direct determination of Rg can only be achieved by measuring the change in scattered light intensity with observation angle. The lower size limit for all detectors, even under favourable conditions, is 12-15 nm. This means that almost all proteins and many condensation polymers cannot be measured. At the upper end of the size scale, the problem of fitting the non-linear data means that many large molecules, such as polysaccharides will give erroneous data (both MW and size) with angular dependant detectors. A good estimate of R_g can be made from viscometry data without these upper and lower limits by utilizing the Flory-Fox equation linking MW and IV to R_g.

$$M[\eta] = 6^{3/2}\Phi_0 R_g^3$$

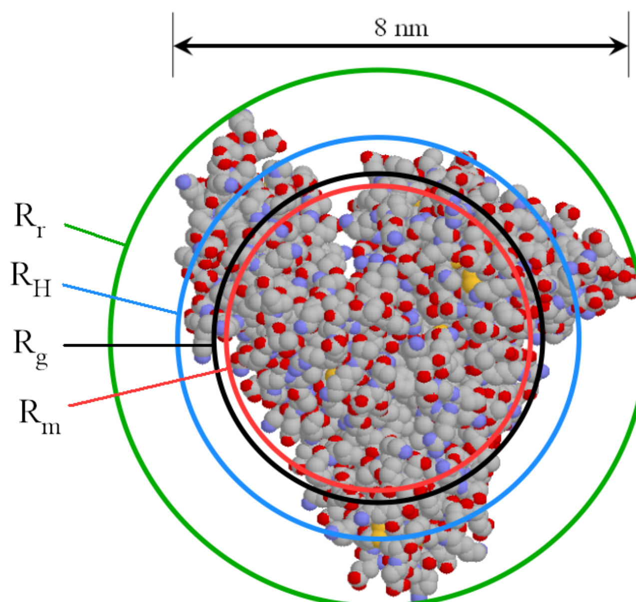
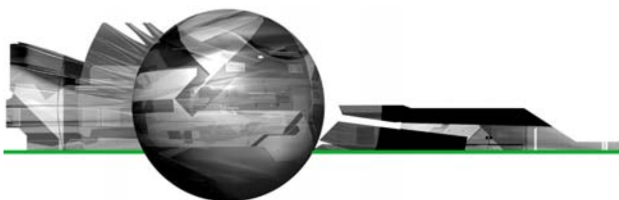


Figure 1: Radius of rotation, hydrodynamic radius, radius of gyration and mass radius which is the radius of a sphere with the same partial specific volume as the protein..

Hydrodynamic Radius

The R_H can be determined in two ways. The first method is by Dynamic Light Scattering (DLS), which is generally used as a batch technique to measure the average size in the whole sample.

A very accurate, precise and, most importantly, practical method of obtaining R_H of all components in a sample after separation is by triple detection SEC. By the use of online light scattering and viscometer detectors, it is straightforward to determine the MW and intrinsic viscosity (IV or [η]) of the polymer or protein at any point on the chromatogram with a high degree of accuracy and precision. This then allows the simple determination of R_H at any point. The equation below shows the relationship between R_H, MW and IV. The figure 1 also shows



the relationship between Rh and Rg for BSA.

$$M[\eta] = \frac{4}{3}\pi\nu N_A R_H^3$$

Instrumentation and conditions

To demonstrate the use of triple detection SEC in determining accurate molecular sizes the following instruments and conditions were used. A Viscotek Model 302 triple detector array (TDA) equipped with the following detectors; light scattering, differential viscometer, and refractive index. Two 30 cm mixed bed columns in series were used to separate the BSA. The eluent was 0.5 M $LiNO_3$ flowing at 0.6 mL/min. The samples used were dextran T70 (Pharmacia) at a concentration of ~3 mg/mL with a100 µL injection and bovine serum albumin (Sigma) at a concentration of ~3 mg/mL with a100 µL injection.

Results and Conclusions

The data were all calculated using Viscotek OmniSEC software. The results from the dextran are shown in figure 2 and from the bovine albumin in Table 1. For the dextran, the Rh was measured across the whole of the peak, ranging from 3.38 nm to 11.03 nm. Note the excellent signal-to-noise on all three detectors which ensures the quality of the calculated size data. At the high end of the distribution the slight curvature in the Rh plot is noted due to the branching in the dextran.
The viscometer data can be further utilised to quantify this branching. When this triple detection technique is applied to proteins it can differentiate clearly between the protein and its oligomeric states as shown in Table 1.

Here the bulk of each peak has been taken to give an average for the monomer, dimer and trimer. The software can give a continuous measurement of size versus molecular weight or retention volume, as in the dextran example but since proteins have discrete molecular weight values for each oligomeric state, there is no continuous distribution. It is clear that triple detection technique provides the most convenient and reliable way to get accurate size values from SEC. It is the only technique which has no upper or lower size limit and is applicable for all molecules that can be analysed by SEC.

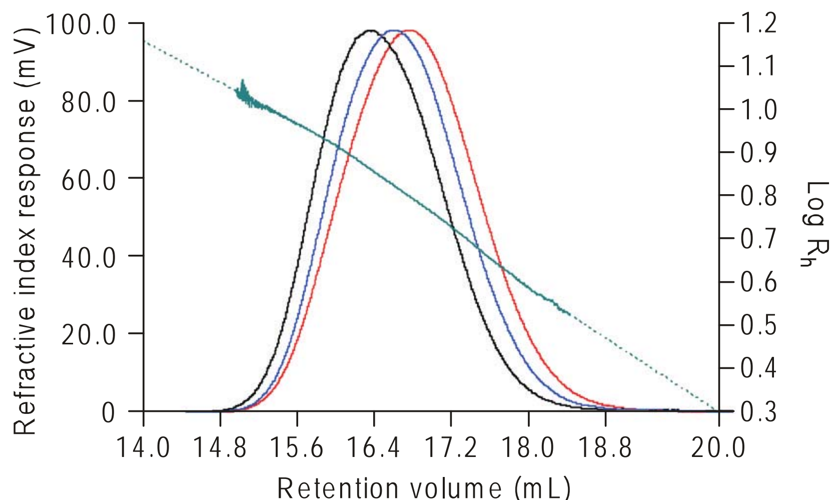


Figure 2: Triple chromatogram of dextran T70 overlayed with the calculated hydrodynamic radius. Red=RI, Blue=Viscometer, Black=LALS

Table 1: Molecular Weight, Intrinsic Viscosity and Rh for BSA.

	Monomer	Dimer	Trimer
MW (Da)	66,400	133,000	201,000
IV (dL/g)	0.056	0.071	0.095
Rh (nm)	3.88	5.32	6.69

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