Determination of Molecular Size in Polymers and Proteins by GPC/SEC

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Introduction

The main goal of most Gel Permeation/Size Exclusion Chromatography (GPC/SEC) experiments is to determine the molecular weight distribution (MW) of the sample or compare MWs 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 may be 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 (Rg) and the Hydrodynamic Radius (Rh). In simple terms, Rg is a mathematically defined dimension describing the distribution of mass centres in the molecule, whereas Rh is a phenomenological property of the molecule. This means that for practical purposes, Rh 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. In GPC/SEC this places a severe limitation on the sizes that can be measured accurately using a multi-angle light-scattering instrument (MALS). The lower size limit for all MALS detectors, even under favourable conditions, is 10-15 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 MALS. A good estimate of Rg can be made from viscometry data without these upper and lower limits by utilising the Flory-Fox equation as explained below.

Hydrodynamic Radius

The Rh can be determined in two ways. The first method is by Dynamic Light Scattering (DLS/QELS), which is not really suitable for on-line GPC/SEC experimentation because one of the fundamentals of the method is a quiescent polymer solution. Clearly this is not the case in a flowing GPC/SEC detector. Many assumptions, which compromise the data, have to be made to overcome this.

By far a more accurate, precise and, most importantly, practical method of obtaining Rh is by Triple Detection GPC. By the use of on-line light scattering and viscometer detectors, it is straightforward to determine the MW and intrinsic viscosity (IV) 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 \( R_g \) at any point. The equation on the left of figure 1 shows the relationship between \( R_h \), \( R_g \), MW and IV. The diagram also shows the relationship between \( R_h \) and \( R_g \) for random coil polymers. On the right of Figure 1 is the Flory-Fox equation, linking MW and IV to \( R_g \). Of course, this equation can also be used in reverse to determine an estimate of \( R_g \) from IV and MW measurements.

**Instrumentation and conditions**

To demonstrate the use of Triple Detection GPC in determining accurate molecular sizes the following instruments and conditions were used:

Viscotek Model 302 TDA equipped with the following detectors:

- Low Angle Light Scattering
- Differential Viscometer
- Refractive Index

Columns: 2 x 30 cm ViscoGel GMPWxl

Eluent: 0.5 M LiNO3, 0.6 mL/min

The samples used were:
1. Dextran T70 (Pharmacia) at a concentration of ~3 mg/mL with a 100 µL injection.
2. Bovine albumen (Sigma) at a concentration of ~3 mg/mL with a 100 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 \( R_h \) 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 (>16 mL), you can clearly see the slight curvature in the \( R_h \) plot 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 aggregated 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. Of course, the software can give you a continuous measurement of size versus molecular weight or retention volume, as in the dextran example.

It is clear that this technique provides the most convenient and reliable way to get accurate size values from GPC/SEC. It is the only technique which has no upper or lower limit and is applicable for all molecules that can be analysed by GPC/SEC. The use of the low angle light scattering also means that the molecular weights are directly determined without the fitting or calculating of other GPC/SEC techniques.

**Table 1: MW, IV and \( R_h \) data for bovine serum albumen (BSA).**

<table>
<thead>
<tr>
<th></th>
<th>Monomer</th>
<th>Dimer</th>
<th>Trimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight (MW) D</td>
<td>68100</td>
<td>144200</td>
<td>198660</td>
</tr>
<tr>
<td>Intrinsic Viscosity (IV) dL/g</td>
<td>0.046</td>
<td>0.065</td>
<td>0.072</td>
</tr>
<tr>
<td>Hydrodynamic Radius (( R_h )) nm</td>
<td>3.68</td>
<td>5.30</td>
<td>6.10</td>
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