Combined use of SEC/GPC, rotational, microfluidics and micro rheology for characterization of Hyaluronic acid solutions

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ABSTRACT

Rheology and Size Exclusion Chromatography (SEC/GPC) are both well-established methods for the characterization of polysaccharide solutions. SEC targets characterization on a molecular scale whereas rheology offers insights into properties on a more macroscopic scale. Linking the results of independent characterization techniques, results in a much more complete picture of the samples under investigation.

INTRODUCTION

Size exclusion chromatography focus on measurements of polymer characteristics like molecular weight and molecular weight distribution but also gives information about radius of hydration and radius of gyration obtained using GPC/SEC with triple detection, including refractive index (RI), and multi angle light scattering (MALS) detectors with a viscometer detector (Malvern Viscotek TDA-GPCMax with SEC-MALS 20). For the understanding of dilute polymer solutions rheology micro-rheology measurements are performed to get data in the high frequency range. Combining the two technologies gives insight into the complexity of sample structure. Increasing the concentration, the polymer molecules start to entangle, sample has reached the critical overlap concentration c*. Further increase in concentration leads to highly entangled systems where c> c*as pictured in Fig. 1.

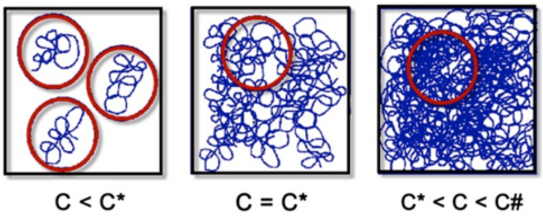

Figure 1. Critical overlap concentration, c*, as function of polymer concentration, showing effect of coil overlap.

Higher polymer concentrations are measured using conventional rotational techniques to focus on lower frequencies and shear rates but to gain information about higher shear rates microfluidics technique is used.

DILUTE POLYMER SOLUTIONS

Determination of molecular structure using GPC/SEC

GPC/SEC is determined on very dilute polymer systems in solution.

When a macromolecule is irradiated by an incident beam of light photons are absorbed and then re-emitted or scattered in all directions. The intensity of the scattered light is proportional to the MW of the polymer, a relationship described by the Rayleigh equation (Eq. 1)

$$\frac{KC}{R_\theta} = \left(\frac{1}{Mw} + 2A_2C\right)\frac{1}{P_\theta} \qquad (1)$$

The simplified Rayleigh equation shows how the intensity of scattered light at a given angle, Rɵ is related to molecular weight Mw. A_2 is the second virial coefficient and C is concentration. Pɵ is a complex term that approaches a value of 1 when ɵ is zero. The Rayleigh equation becomes relatively simple to apply when the term Pɵ, which describes the angular dependence of the scatted light, tends to 1. This occurs when ɵ becomes zero i.e. the scattered light is measured an angle of zero degrees to the incident beam. Unfortunately, measuring at an angle of 0^o is not practical as the incident beam dwarves the signal from the scattered light, preventing an accurate reading.

Small molecules, less than around 10-15 nm, scatter light isotropically, so intensity measurements are independent of the angle at which the measurement is made. Larger molecules scatter anisotropically, a phenomenon that must be accounted for to access accurate MW for this class of molecules, using the Rayleigh equation. Three different approaches to static light scattering (SLS) have been developed to address this issue: right angle light scattering (RALS); low angle light scattering (LALS); and multi-angle light scattering (MALS). With RALS, scattering intensity is measured at 90^o to the incident beam. This gives an excellent signal to noise ratio. However a RALS detector takes no account of anisotropic scattering, it simply assumes that the scattering intensity at 0^o is the same as at 90^o. This approach is good for small molecules but give unpredictably inaccurate results for anisotropic scatters. LALS is based on the measurement of scattered light at an angle very close to 0^o to eliminate the error associated with anisotropic scattering. This works for all molecules but the signal to noise ratio becomes challenging for smaller molecules. Combining RALS/LALS technology can therefore work well to determine small and large molecules simultaneously. MALS measures at several angles and uses a model to measure data at 0 angle.

Three different hyaluronic acid (HA) samples are measured with RI/IV/MALS technology to determine the structural information from GPC/SEC measurements and the results are summerized in Table 1.

Table 1. Measurement data from GPC/SEC analysis of three HA samples

Sample ID	MW (kDa)	Rg (nm)	Rh (nm)	IV	C*
1	263	45	29	6.47	4.8
2	483	49	36	7.85	6.8
3	333	47	32	7.02	1.6

Fig. 2 is a Mark Houwink log-log graph of the intrinsic viscosity measured with the viscosity detector in the Viscotek TDA versus molecular weight measured by MALS in the GPC experiment.

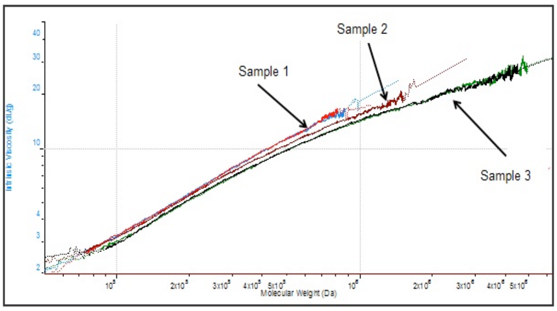

Figure 2. Mark Houwink plot of three HA samples from GPC/SEC analysis showing intrinsic viscosity (IV) vs. MW.

Constructing a Mark-Houwink plot (see Eq. 2) for the three samples provides detailed insight into their structure (Figure 2). An MH plot is a log-log graph of intrinsic viscosity vs. molecular weight. Its *y* intercept provides information about the flexibility of the backbone while the gradient is associated with the degree of branching. A material with a high degree of branching tends to have a shallower slope than one with a more linear structure. Construction of an MH plot relies on having MW and IV data, underlying the value of triple detection.

$$[\eta] = K \times Mw^{\alpha} \tag{2}$$

The linear structure of sample 1 is reflected in the gradient of the MH curve which is close to 1 across the whole molecular weight range. In contrast the gradient of the other samples becomes shallower as molecular weight increases. At any given molecular weight, most especially in the high molecular weight range, both sample 2 and 3 has a lower intrinsic viscosity than sample 1.

The IV is inversely proportional to the molecular density of the sample, so these deviations towards lower intrinsic viscosity provide insight into the impact sample differences from chemical modifications. The cross-linking in the sample 2 is responsible for its high molecular density. The fact that the IV of sample 3 is higher than that of the sample 2 at any given molecular weight suggests that this sample has a substantially lower degree of cross linking. In the lower MW region the sample 3 and sample 1 plot overlay one another but there is divergence in the higher MW region as a result of this branching.

Micro rheology of dilute polymer solutions

As mentioned above the rheology of the polymer solutions is dependent on the concentration of polymer in solution and its structural behaviour on the critical overlap concentration. This behaviour is related to the R_g in Eq. 3

$$c^* = \frac{Mw}{{R_g}^3 N_A} \tag{3}$$

At low concentrations, the polymer coils have sufficient space to avoid interaction. In these dilute systems the viscoelasticity of the sample is therefore governed by the properties of a single coil multiplied by the number of coils present. It is the properties of the coil itself and how the surrounding liquid flows through it that determines behaviour. The Rouse-Zimm model describes behaviour in this regime.[1,2]

As the concentration of polymer coils increases the coils become prone to entanglement since there becomes insufficient space for them to remain discrete. The concentration at which this happens is referred to as the critical overlap concentration (c*) and can be calculated from measurements of the radius of gyration (Rg)[3], a measure of the size of the polymer molecule or from IV data.[1]

Above c* the rheological behaviour of the system is no longer governed by the properties of single coils, but rather by interactions between multiple coils. These interactions result in a sharp rise in viscosity and the onset of more substantial elasticity. (Fig. 1). The frequency or timescale over which shear is applied defines whether viscous or elastic behaviour is dominant.

Micro rheology is based on the principle that the movement of dispersed probe particles within a suspension or solution is related to the viscosity and viscoelasticity of the system. Dynamic light scattering (DLS) is one of the techniques that can be used to track the movement of probe particles. DLS micro rheology can be implemented with much smaller sample volumes than are typically needed for a mechanical rotational rheometer and is well-suited to polymer solution characterization.[4]

To investigate the rheological properties of the sample 1, 2 and 3 from the GPC/SEC analysis, 5mg/ml samples of these samples were analysed using DLS micro rheology (Zetasizer Nano ZSP, Malvern, UK). Fig. 3 shows the resulting frequency sweeps from micro rheology measurements.

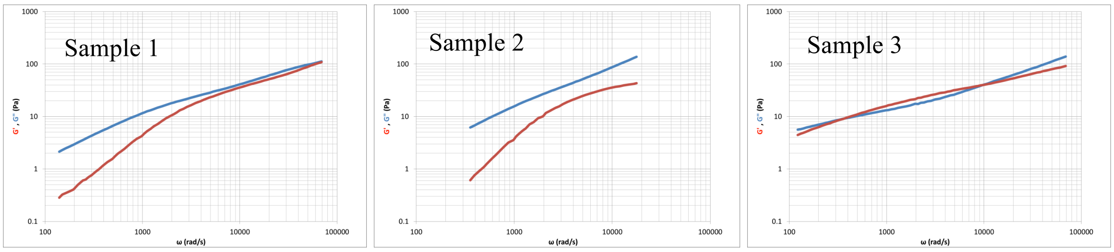

Figure 3. Elastic modulus, G' (red) and viscous modulus G'' (blue) as function of angular frequency

At 5mg/ml the concentration of the tested solutions is below the c* for sample 2, which has the highest c* (see table 1) of the three samples. The flow profile for sample 2 shows that correspondingly, it exhibits the most dilute solution behaviour. G'' is dominant at all frequencies meaning that the solutions will flow and has limited elasticity.

Sample 1 has a lower c*, much closer to the test concentration. For this solution G' is a lot higher than for the sample 2 and at higher frequencies G' and G'' become close and parallel with a gradient of 2/3 when plotted logarithmically. The shape of this profile is associated with classical Rouse-Zimm dilute solution behaviour^2.

In contrast, the profile for sample 3, which has the lowest c*, shows clear evidence of a 'rubbery plateau', a region where G' exceeds G". This behaviour is associated with the chain entanglement that occurs at polymer concentrations above c*.

CONCENTRATED POLYMER SOLUTIONS IN RHOTATIONAL RHEOMETRY

Concentrated polymer solutions are more suitable to measure suing conventional rheometry. Four different concentration, all above the critical overlap concentration, is measured in viscometry (table of shear rates at equilibrium conditions) and in a frequency sweep for a linear hyaluronic acid sample. The samples where measured using a Malvern Kinexus Pro+ rotational rheometer, equipped with a Peltier Plate Cartridge that keeps sample at a constant temperature of 25°C. The concentrations measured are 0.35, 0.5, 0.75 to 1 w/v %. Viscosity versus shear rate results are modelled using the Cross model. These results are presented in Fig. 4.

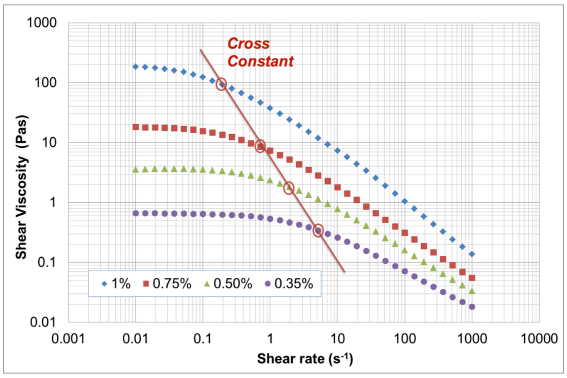

Figure 4. Viscosity vs. shear rate for four concentrations of HA solutions

At low shear rates a constant shear viscosity plateau termed the zero shear viscosity (η_0) is observed. In this region the disruption of the entangled polymer coils happens so slowly that re-entanglement can occur simultaneously due to Brownian motion. As a result there is no net change in the extent of entanglement and viscosity remains constant. In contrast, at higher shear rates the rate of disruption exceeds the rate of re-entanglement and the solution becomes less viscous. Shear thinning is observed. This transition to shear thinning behaviour happens at lower shear rates for more concentrated solutions, because they are prone to greater entanglement.

For the frequency sweep data the results for the four concentrations are shown in Fig. 5.

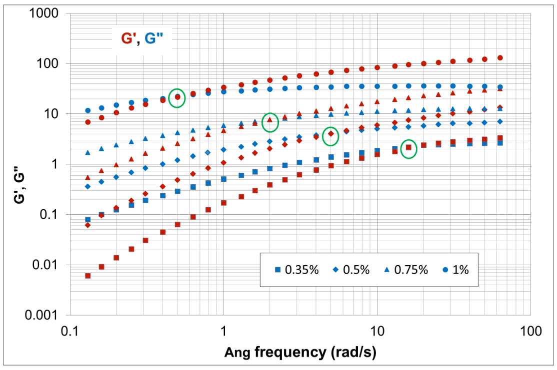

Figure 5. Elastic and viscous modulus vs. angular frequency, ω, for four concentrations of HA solutions.

The crossover frequency at which rubbery behaviour establishes is an important value since it can be used to determine the longest relaxation time of the polymer τ_{max}. Relaxation time is simply taken as the inverse of the angular frequency at the crossover point, circled in green in Fig. 5. The longest relaxation time tends to increase with MW and concentration and is a measure of how long it takes for polymer molecules within the solution to disentangle. From a practical perspective the key factor defining material behaviour is the time of the applied stress relative to the relaxation time of the material, as defined by Deborah number (De)[5] defined in Eq. 4, :

$$De = \tau_{max}/t \text{ or } De = \omega\tau_{max} \qquad (4)$$

When De > 1 solid like behaviour dominates and for De < 1, liquid like behaviour dominates. The relevant timescale will depend on the application, so for example the time for an eye blink may be on the order of 0.05s or 20 rad/s, while the eye surgery operation, the time scale of experiment is slow, around 5s or 0.2 rad/s up to very quick movements of 0.1s or 10 rad/s. These are important time scales for eye operations and the functionality of eye drops. Since the eye should operate without the eyelid scratching the eye lens the sample should have an elastic behaviour at time scales of an eye blink. For the eye surgery the sample must on short time scales be elastic to avoid contact with the lens while the operation is performed within a single drop of HA acid solution. At the same time the viscosity must be high enough at longer time scales to stabilize the movement of the surgery tool.

MICROFLUIDICS RHEOLOGY

Microfluidics rheometry[6] or viscometry is the measurement of the viscosity in a micro capillary slot die equipped with pressure transducers to measure the pressure drop over the die. A schematic representation of a microfluidics channel is shown in Fig. 6.

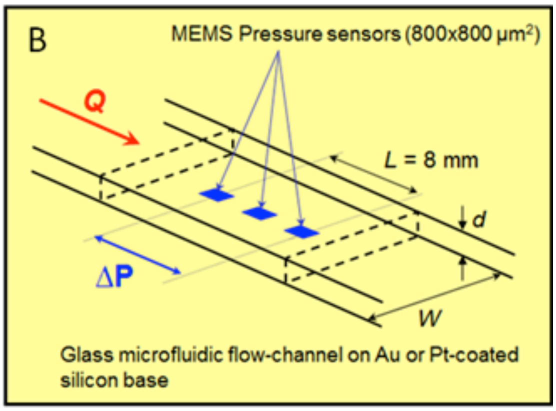

Figure 6. Microfluidic flow channel with MEMS (MicroElectroMechanical System) pressure sensors for measurement of high shear rate viscosity

The sample is pressed through the slot die and the volumetric flow rate measured is proportional to the shear rate and the pressure drop over the slot is proportional to the shear stress. The viscosity for non-Newtonian samples can be corrected for non-Newtonian flow behaviour. Viscosity and shear rate range are selected by using different type of MEMS sensor, having a different channel depth (from 200μm to 50 μm) and pressure range (from 10kPa to 2MPa).

COMBINING MEASUREMENT TECHNIQUES

For a high molecular weight HPAM (Hydrolized PolyAcrylamide) polymer solution in low concentration of 0.1 % w/v, viscosity was determined using a Malvern Kinexus Pro + rheometer and Microfluidics rheometer mVROC i from Malvern instruments. The data are shown in Fig. 7 over 9.5 decades in shear rate.

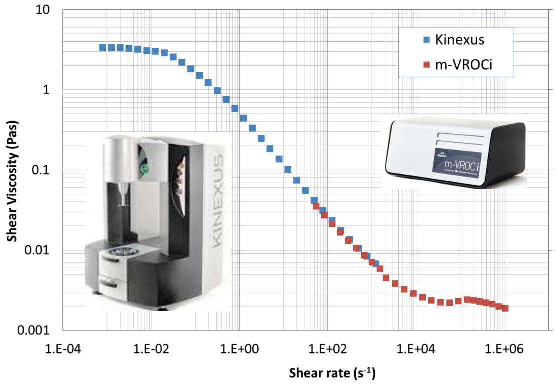

Figure 7. Viscosity vs. shear rate measured with a rotational rheometer and microfluidics technology for the high shear rate applications (red is microfluidics data and blue is rotational rheometry data)

High shear rate applications are becoming more important since industry is aiming for higher productivity in processes and short time scales corresponds commonly to high shear rates. Interest is also coming from the development of newer methods for ink jet printing as well as 3D printing. Other areas where high shear rates are utilised is in coating process and tertiary oil recovery

For the same solution frequency sweep using a Malvern Kinexus rheometer and micro rheology were used to determine a large frequency range to fully characterize the elastic and viscous behaviour as shown in Fig. 8.

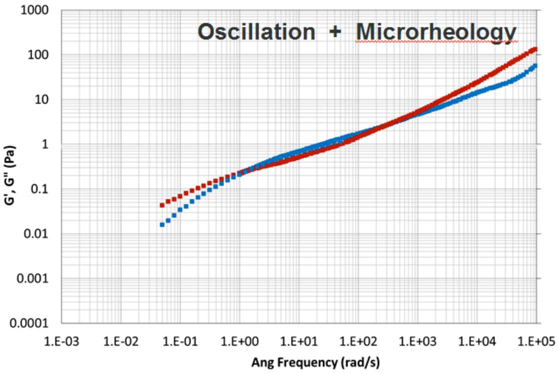

Figure 8. Rotational rheology and micro rheology used for large frequency range characterization of a polymer solution.

From Fig. 7 and 8 its clear that microfluidics, micro rheology and rotational rheometry are complementary techniques for the characterisation of dilute polymer solutions. The knowledge from MW characterisation and Rg measurements from GPC/SEC experiments gives further insight into the chemistry and structure of polymer solutions that are crucial for the understanding of flow properties of polymer solutions.

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