GPC/SEC eBook Series

GPC/SEC Applications

What you should know when you need to analyze polymers, biopolymers, and proteins

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Trusted Answers

Contents

About this eBook series

GPC/SEC *Tips & Tricks* articles have been published in more than 60 editions of LC/GC's digital magazine *The Column* over the course of 10 years. These Tips & Tricks are designed to support GPC/SEC users in their daily work, providing comprehensive overviews on different aspects of this powerful technique.

To have all published topics at a glance, we created this series of eBooks.

The topics of these eBooks will cover:

- GPC/SEC theory and background
- GPC/SEC columns
- GPC/SEC detection
- GPC/SEC troubleshooting
- GPC/SEC applications

Each eBook contains 5 to 8 different Tips & Tricks publications that have been updated with the latest information, new examples, and figures.

To allow new users to GPC/SEC a continued reading experience, content has been edited, resulting in some differences compared to the original publications.

Nevertheless, the original spirit is maintained. So, the publications are independent references that allow users to read only the dedicated publication of interest.

Introduction to GPC/SEC Applications

GPC/SEC is the established separation technique in all modern laboratories working with classical polymeric materials, construction products, electronic chemicals, pharmaceutical excipients, nutritional (hydro)colloids, biotherapeutics, and many more.

The success story of GPC/SEC has multiple reasons:

- GPC/SEC delivers results that allow predicting product properties and application behavior directly from the chromatography lab.
- It is a versatile technique with applications in R&D, product development, quality assurance, and (online, at-line) production control.
- It is a mature separation technique that is well understood.
- Instrumentation and software can be scaled from simple systems/applications to hyphenated systems with information-rich detectors in a macromolecular chromatography data system (MCDS) environment.
- It is a perfect fit for regulated laboratories in pharma, food, cosmetic, and related industries with established partners providing optimized hardware, software, quality support, and expert service engineers to cover the complete system life cycle including comprehensive qualification services.

All sections in this eBook of the GPC/SEC series describe various interesting GPC/SEC applications in detail.

1.1. Product registration and REACH

A big advantage of polymers/macromolecules is that they can be considered as nontoxic, even when produced from toxic monomers.[1] Due to their large size and the fact that reactive functional groups are reacting during polymerization, polymers are usually considered to be (bio) chemically inert.

Of concern, however, are unreacted monomers, oligomers, adjuvants, and additives, which are added to improve the processibility, the stability, and the quality of the final product. Leaching of these components can possibly cause severe issues.

Registration agencies therefore require comprehensive characterization with detailed molar mass information and, for some products, also extractables and leachables studies.

The molar mass of a polymer product is traditionally measured using GPC/SEC and the results are of utmost importance as the molar mass can influence which registration category will apply for a new product.

For example, the molar mass determines whether new products fall into the category “polymer of low concern” (PLC) or if a product is subject for registration or not (such as EU REACH).

Accordingly, the generation of detailed and appropriate molar mass data, which satisfies regulatory guidelines, is a key component of successful registration.

REACH

REACH (registration, evaluation, authorization, and restriction of chemicals) is an EU regulation that entered into force on 1 June 2007. It addresses the production of chemicals and their impacts on human health and the environment. For Europe, all polymers are exempt from registration and evaluation under REACH.

A polymer is defined using three criteria:

- The molecules must be distributed over a molecular weight range.
- The weight percentage of molecules containing three monomer units or above should exceed 50% (3M + 1 rule).
- The weight percentage of any molecule of the same molecular weight shall not exceed 50%.

Different REACH-like regulations around the world have adopted this definition, although the regulatory requirements about the consequences may differ. For example, polymers are not exempt for China-REACH.

The preferred method to determine whether a substance falls under the definition of a polymer is GPC/SEC with referral to the Organisation for Economic Co-operation and Development (OECD) TG 118.[2]

If the molar mass of a product is sufficiently low, GPC/SEC separations often show an oligomeric profile as shown in the chromatogram in Figure 1. Identification of the single peaks is, for example, possible by overlaying the data with at least one known reference material. Afterwards, the single peaks can be evaluated with respect to molar mass and weight percentage. The example shows a polyol with a molar mass distribution (criterion 1 met) and eight automatically identified peaks for chains with 1 to 8+ repetition units. For higher molar masses (longer chains, 8+ repetition units), the resolution of GPC/SEC was not sufficient to resolve this into single peaks. This behavior is very typical for GPC/SEC—for higher molar masses, broader peaks are observed.

Table 1 shows the numerical results for the identified eight species and a comparison with REACH polymer definition criteria.

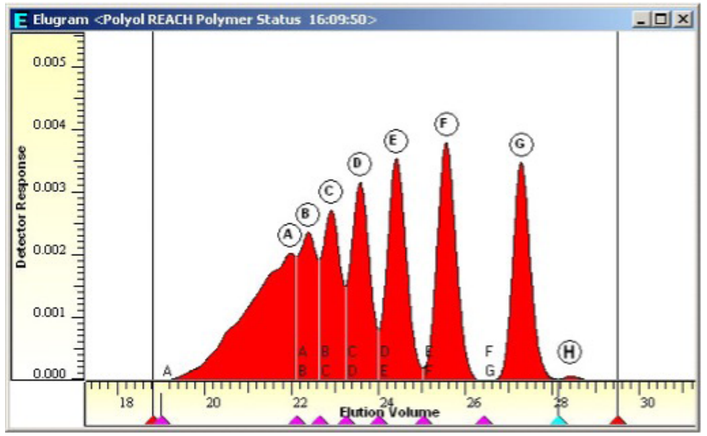

Figure 1. Chromatogram of a polyol with 8+ automatically identified single chains with 1 to 8+ repetition units.

Example Polyol	Content [%]	Molar Mass M_p [Da]*	Comment	Polymer?
Peak H	0.20	200	R-M, R-M-M	Yes, less than 50% below (3M + 1)
Peak G	13.4	490		
Peak F	15.7	790	R-M-M-M	Yes, no individual chain > 50%
Peak E	14.8	1,090		
Peak D	13.3	1,400		
Peak C	11.0	1,810		Yes, molar mass distribution
Peak B	9.30	2,190		
Peak A	22.3	2,550		

* Polystyrene equivalents

Table 1. Molar mass and weight percentage results for the peaks from Figure 1 show that this product is a polymer based on REACH criteria.

By comparing the REACH criteria with the GPC/SEC results, it is proven that all three REACH criteria are met. Consequently, the substance is a polymer according to the definition of REACH.

Polymers of low concern

Product registrations often require the M_w (weight average molecular weight) and the mass fractions below defined molar mass, such as 500 or 1,000 Da. This is required to classify a product as a polymer of low concern (PLC). Unfortunately, the definitions for PLCs in different countries can vary as well as the required actions, nevertheless GPC/SEC is able to provide the required information.

In addition to the area percentage values for each well separated peak, GPC/SEC results include the molar mass distribution, which allows you to determine M_w and to deduce the specific fractions. The molar mass distribution is obtained from the chromatogram when a calibration curve is available (or more precise, when the molar mass information for each elution volume is available).[3] Due to the special transformations required in GPC/SEC analysis, it is important to ensure that the results for registration are taken from a true molar mass distribution and not from a chromatogram where only the elution volume is replaced by the molar mass.[4]

Figure 2 shows an example for a sample with a broad molar mass distribution (no resolution into single peaks) with graphical molar mass distribution and molar mass averages as well as the fractions below 500 Da and above 1,000 Da. According to the definition, for example, in the US and China, this sample is a polymer of low concern as the M_w is above 10,000 Da, less than 2% are below 500 Da, and less than 5% are below 1,000 Da. For a polymer of M_w between 1,000 Da and 10,000 Da, the fraction below 500 Da should be below 10% and the fraction below 1,000 Da below 25%, respectively.

There are some general exclusions for polymers, which are generally not exempt. Cationic or potentially cationic polymers, high molecular weight water absorbing polymers, and polymers that degrade, decompose, or depolymerize are not eligible for exemption. Reactive groups can also be a problem with the consequence that a polymer would not be exempt from regulations, even if the mass results indicate that it is a polymer of low concern.

Also, the mass fraction for every molar mass slice (sometimes referred to as slice list) can be required for product registration. A slice list can easily be created once the molar mass distribution is available. From this slice list, the required parameters discussed above can be determined directly.

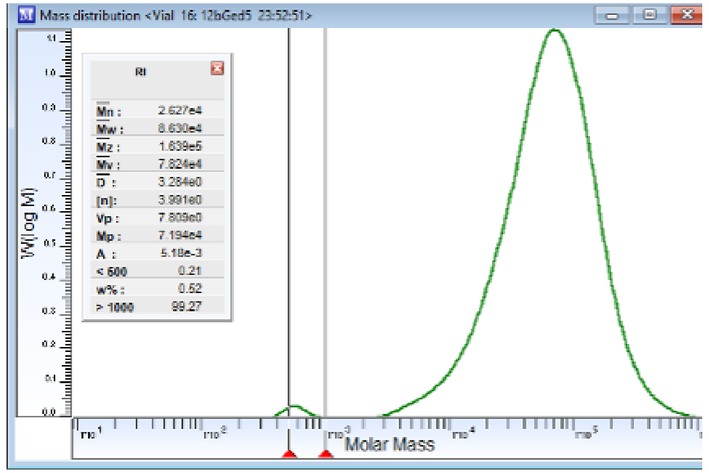

Figure 2. Molar mass distribution with settings to determine if a product is a polymer of low concern.

Measurement uncertainty and influence of analytical parameters

Analytical scientists producing data for product registrations should reflect at least two important parts of GPC/SEC analysis.

First, analysts must make the right choice for calibration. GPC/SEC, as a relative method, always requires a calibration, either using reference materials or an additional detector that allows you to measure the molar mass directly.[5] In case of registrations, where the low molar mass parts are of highest concern, mass spectrometry (such as ESI-MS or MALDI), can be a valuable addition for products where no chemically matching reference materials are available.[6] If chemically matching reference materials are available, it is recommended to prefer these materials over maybe more common materials.

Partially related to calibration is the second part to be reviewed, the measurement uncertainty.[7] Quality of raw data, analytical conditions, and calibration quality influence the results, and the measurement uncertainty can help identify random error sources to improve the quality of the data.

Figure 3 shows an example of a molar mass distribution where the fraction below 1,000 Da is slightly above the 5%. Here, data review is particularly important as the results are close to the boundary for a PLC classification. A more detailed analysis showed that both calibration quality (here, not enough data points in the low molar mass area) and the quality of raw data (here, detector noise) were the main sources of uncertainty and should be improved to gain better confidence in the data. Especially in such critical cases, it is important to have a sound, well-developed, documented method and maybe the support of additional methods and data to enable the company to make the right decisions and to produce trustworthy, precise, and accurate data.

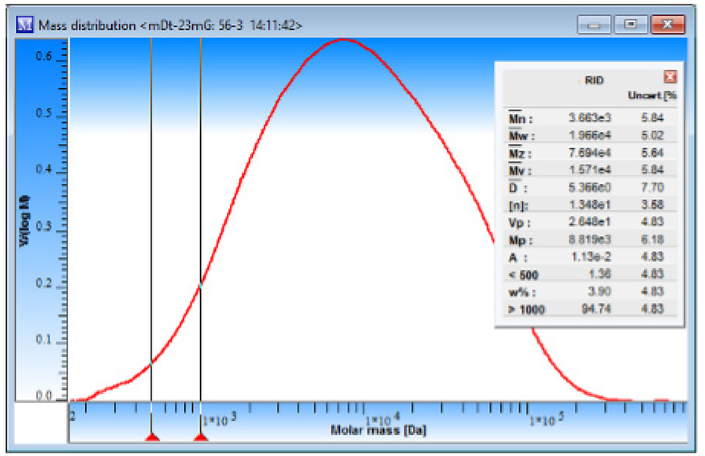

Figure 3. Molar mass distribution determined for a polymer product that does not fulfill the molar mass requirements for a polymer of low concern (fraction < 1,000 Da is above 5%).

Summary

- The molar mass of a product determines which registration category will apply. GPC/SEC data are therefore crucial for product registrations.
- Molar mass averages are often not sufficient. Detailed analysis is required especially in the low molar mass area.
- Registration data can only be obtained from true molar mass distributions where the y-axis w (log M) is correctly converted. Results for fractions could otherwise be wrong.
- Determination of result uncertainty can be important to ensure that the submitted results are of high analytical quality.

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Tips & Tricks: GPC/SEC Product Registration and REACH was originally published in The Column, June 2017, by author Daniela Held.

1.2. Quantify and get more than molar mass averages

GPC/SEC is a liquid chromatographic (LC) fractionating technique that separates molecules based on their size in solution. The fractionation occurs in the pores of particles, which have been packed into a separation column. Molecules of larger sizes cannot enter the pores and therefore elute first, while smaller sizes penetrate the pores and elute later.

A typical GPC/SEC calibration curve (compare Figure 1), where the logarithm of the molar mass is plotted versus the elution volume, illustrates this behavior nicely and helps distinguish between three regions.

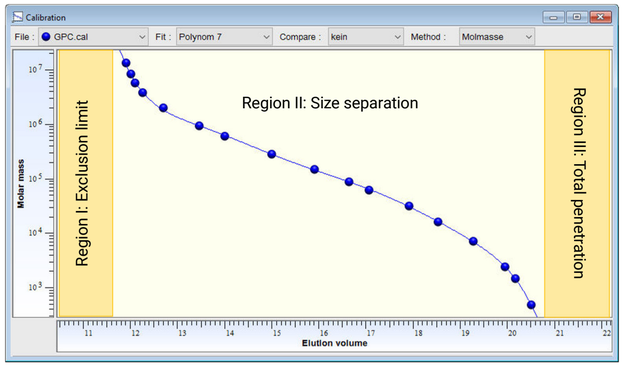

Figure 1. GPC/SEC calibration curve with three distinct regions.

In region I, the region between the injection and the exclusion limit, nothing can elute. After the exclusion limit, where molecules (independent of their size) elute at the same volume as they are too large to enter any pores, efficient size separation is possible—region II starts. The molecules can now enter some larger pores while they are excluded from smaller others. Here, the molar mass distribution can be accurately determined. Region II ends at the limit of total penetration. At this limit, the molecules in solution are so small that all of them can penetrate all pores. This is the start of region III, which is the most interesting region for learning more about low molar mass fractions or additives.

In theory, it should be impossible to separate molecules such as toluene from butylated hydroxytoluene (BHT) or acetone if the sole driving force for separation is interaction-free size exclusion. However, quite often it is observed that there is a separation.

Figure 2 shows the overlay of chromatograms of BHT, toluene, and acetone obtained using a column with a medium molar mass separation range. There is a (baseline) separation, which cannot be explained by the size difference of the molecules alone. Therefore, the separation must result from enthalpic interactions between the analyte and the stationary phase, indicating that a mixed-mode mechanism is operating. This means that both molecular size and interaction of the molecules with the stationary phase contribute to the elution behavior.

Although interactions should be strongly avoided when determining molar masses of polymers by GPC/SEC, this mixed separation can be advantageous, as it allows for the identification and even quantification of low molar substances.

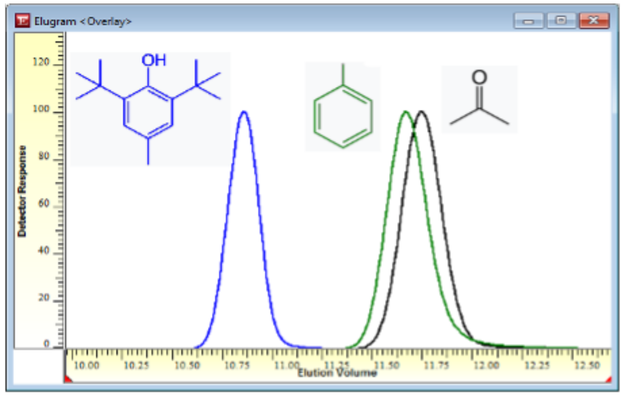

Figure 2. Overlay of BHT (blue), toluene (green), and acetone (black). Although the molecules fit into all pores a separation is observed (retention by interaction).

Identification

Figure 3 shows a chromatogram of a QC sample with a broad polymer peak and different peaks eluting in the low molar mass range (high elution volumes) and close to the total penetration limit. The first step is to distinguish between sample peaks and typical system peaks. This can be done by measuring a blank sample. A blank is pure mobile phase (ideally taken from the mobile phase bottle) that has been treated in the same way as the sample.

An overlay of the blank injection with the sample shows directly which peaks are system peaks. In the example of Figure 3, the two peaks F and G could be identified as system peaks.[1] Peak E also does not belong to the sample, this peak results from the added internal flow marker BHT.[2] The sample itself contains a polymer part (peak A) with a long tail. In the tailing area, three peaks (B, C, and D) should be further characterized.

How can peaks be identified?

Here, several strategies can be applied.

If there is already a suspect (for example, educts from production process, residual monomer, solvent, initiator, or additives are known) and the pure substances are available, they can be prepared and injected as separate samples. Assigning the peaks can be done by overlaying the chromatograms or comparing the elution volumes. It is also possible to spike the original sample with the suspected substance and then monitor an increase in the respective peak area.

However, relying only on the peak position might be not sufficient. Verification can be performed by advanced detection techniques or fraction collection followed by offline characterization. This is also recommended when there is no information about potential additives or contaminants

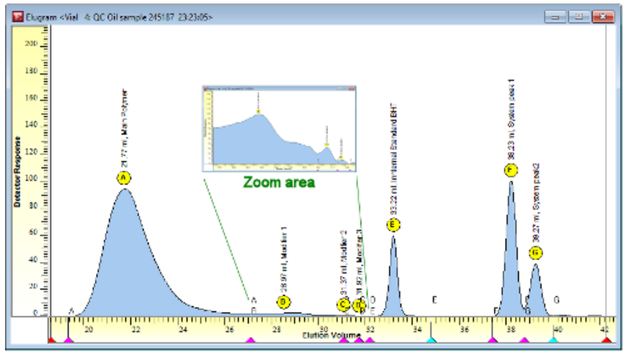

Figure 3. Chromatogram of an oil sample. Peaks F and G are system peaks. Peak E is the internal flow marker BHT. The figure inset shows a zoom of the low molar mass fractions, where peaks B, C, and D should be identified and quantified.

An easy and cost-effective approach is the use of additional UV detection. Often GPC/SEC systems already have two different types of concentration detectors—the more universal refractive index (RI) detector and the more specific UV detector (DAD/PDA). If the substance is UV active, the UV wavelength can be chosen to specifically detect the substance. If a DAD/PDA is available, the spectra can also be used for identification.

More complex efforts require hyphenating GPC/SEC with online mass spectrometry, NMR, or (for a limited number of solvents) FTIR detection.[3]

A practical approach is fractionation and offline analysis. As GPC/SEC is a nondestructive technique, it is possible to collect fractions and then apply spectroscopic or spectrometric techniques offline to characterize the fractions. If the amount of collected fraction is not sufficient, repeated fractionations can be performed. Analytical GPC/SEC systems can easily be upgraded to semipreparative systems by replacing the analytical column with a preparative column, so that higher amounts can be injected. Automation is easily possible by adding a fraction collector.

An elegant way to hyphenate GPC/SEC with FTIR connection is the use of an online collection module. This FTIR interface is connected as the last device of the system. A heated nozzle evaporates the solvent as the effluent leaves the column and the nonvolatile components are deposited on a rotating germanium disk, resulting in a special separation of the different components. After the run is finished, the trace on the germanium disk is analyzed spot by spot using an FTIR spectrometer. A similar approach is possible for MALDI instruments.

Problematic for both approaches, fraction collection with FTIR or MALDI hyphenation, are nonvolatile additives. For example, salts are sometimes required to ensure proper GPC/SEC separation. Replacement by volatile salts is needed.[4]

Quantification

Figure 4 shows detailed example results for the high molar mass peak and the three low molar mass peaks. Here the absolute molar mass distribution of the polymer peak is determined using GPC/SEC with light scattering detection. Simultaneously, the RI detector signal alone is used to determine the relative peak areas (composition results). Therefore, within just one run, the sample is fully characterized.

Quantification in such a setup is also possible. Just like in HPLC analysis, the area percentage of each peak is easily determined. Absolute concentrations can also be determine. Once the peaks are identified, the pure substances can be injected at known concentrations to determine the RI detector response factors for each peak.

Once the response factors are known, they can be used to determine the concentration of each peak.

Please note that this detector calibration differs from the typical GPC/SEC calibration. While the detector calibration requires injecting a pure substance with known concentrations, GPC/SEC calibration requires the injection of several standards with known molar masses to yield a calibration curve like the one shown in Figure 1.[5]

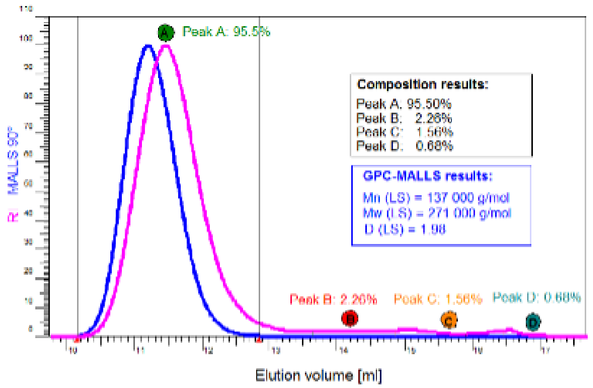

Figure 4. Absolute molar masses for peak A (GPC/SEC with light scattering detection) and the relative amount of four identified peaks

Summary

- GPC/SEC is a nondestructive fractionation technique.
- Two different types of information can be obtained simultaneously, the peak area (for example, in percent) and the molar mass distribution.
- Overlays can help to identify peaks if there are hints from production/processing. Special detection (such as UV (spectra), FTIR, or MS) can be applied either online or offline to identify peaks after fractionation.
- Detector response factors can be determined analog to typical HPLC analysis. These factors allow for concentration determination using the measured peak area.

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1.3. Protein analysis with size exclusion chromatography

In contrast to synthetic macromolecules and many biopolymers, most proteins do not exhibit a molar mass distribution[1] but are uniform in molar mass. Nevertheless, fractionating techniques are also useful for protein samples as they can identify or monitor the presence of aggregation.

Traditionally, size exclusion chromatography can be used to separate stable aggregates from monomers or fragments and, with advanced detection such as light scattering or mass spectrometry, also determine their molar mass and size.

For protein mixtures with a molar mass distribution (such as gelatins), GPC/SEC allows for the determination of the complete molar mass distribution and gives access to a variety of quality- and safety-related results.

A major challenge for GPC/SEC protein analysis is the development of a robust method that eliminates undesired interactions with the stationary phase used in the separation column.

What are the advantages/limitations of GPC/SEC for protein analysis?

A major advantage of GPC/SEC is that it is a nondestructive separation technique. It fractionates samples based on the sizes of macromolecules present in the sample. GPC/SEC is ideal to reduce the complexity of a sample before applying advanced detection techniques such as mass spectrometry, light scattering, or viscometry. The reduction in complexity eases data evaluation and interpretation as compared to direct analysis of nonfractionated heterogeneous samples. Advanced detection using two or more detectors can often be used in an online method. If this approach is not possible, then the GPC/SEC technique allows for the collection of sample fractions to be used for further offline characterization.

In GPC/SEC, proteins can be analyzed under native conditions in solution without denaturation, which is desirable as conformations and protein-protein interactions remain intact. Many GPC/SEC separations therefore preserve the biological activities of the macromolecules.

However, the conditions for true GPC/SEC separations, based only on the size of the protein in solution, can be difficult to achieve. Interactions between the sample and the stationary phase are often encountered, resulting in unexpected peak shapes, unstable retention times, and/or poor recovery. As different proteins exhibit different shapes (for example globular, rod-like, or flexible chains), their sizes in solution often do not correlate directly with molar mass, which then requires the need for more advanced detection methods for accurate molar mass determination.

A thorough method development for protein analysis therefore comprises:

- Picking the right column (or column combination) with an appropriate, noninteracting stationary phase, particle size, and porosity
- Adjusting the pH and ionic strength of the mobile phase
- Selecting the best detection option for a series of samples

It is also an essential requirement to evaluate the chromatographic recovery of all components and aggregates present in the sample.

Pitfalls in method development

Proteins need to be analyzed in aqueous solutions and are therefore a subcategory of aqueous GPC/SEC. All general method development tips for aqueous GPC/SEC analysis are applicable to protein analysis method development as well. Therefore, in this section only the specific requirements for proteins will be discussed.

An advantage of proteins, when compared to synthetic polymers or large biomolecules, is that proteins are usually small in size and the size distribution within a sample is relatively narrow. This allows the use of GPC/SEC columns packed with small particles of high efficiency. In addition, their narrow distribution enables the use of columns with very shallow calibration curves (high resolution in a narrow molar mass range). To achieve this, silica-based GPC/SEC columns are preferred for most protein applications analyses.

A disadvantage of analyzing proteins from a chromatographic point of view is that they have many functional/charged groups and may possess larger hydrophobic segments or sequences. These two characteristics make it difficult to develop a true GPC/SEC method with no (or at least minimized) electrostatic and hydrophobic interaction between column stationary phase and the protein. Interaction can result in some degree of adsorption, which results in reduced recovery (or even complete adsorption with no peaks detectable), shifted elution times, or distorted peak shapes (such as tailing).

To avoid or minimize interactions, the pH value, the ionic strength/salt content, and the amount of potential organic modifiers need to be adjusted. However, the concentration of the salts or modifier added is limited as it also influences the solubility of the sample. If it is not possible to develop a stable GPC/SEC method, a change of the stationary phase (from the silica column to a polymer-based column) should be investigated

A good starting point for method development is running the protein at its isoelectric point, pI. The pI of a protein is the pH value at which positive and negative charges, resulting from the different amino acids comprising the protein, are balanced. Neither the positively charged ammonium nor the negatively charged carboxyl groups dominate. An ideal GPC/SEC method for every single protein would be to run with an aqueous solution with a pH matching the pI.

To avoid the cumbersome approach of needing to have a method for every single protein, the pH of the mobile phases is chosen to be close to pI and additional polyelectrolytes, monovalent salts such as sodium chloride or potassium chloride, are added. The electrolyte will help with shielding residual charges thereby further reducing the undesired interaction between the proteins and the stationary phase.

Another parameter to be considered is the hydrophobicity of the protein. Most important is the polarity of the different R groups in the protein. Based on the R group, an amino acid can be classified as charged, hydrophilic, or hydrophobic. Proteins containing many amino acids such as leucine, alanine, and valine are rather hydrophobic and cannot be analyzed under the same conditions as hydrophilic or charged proteins containing amino acids such as serine, arginine, or histidine.

Figure 1 compares the influence of the ionic strength on the chromatograms of proteins with different hydrophobicity. A hydrophilic protein such as chymotrypsinogen A should be measured at high ionic strength. Bovin serum albumin (BSA), a protein that is neither hydrophobic nor hydrophilic, can be measured at high and low ionic strengths. And, hydrophobic amino acids such as alanine or substituted alanines should be analyzed at low ionic strengths. If the ionic strength is not adjusted based on the polarity of the protein, the separation will fail.

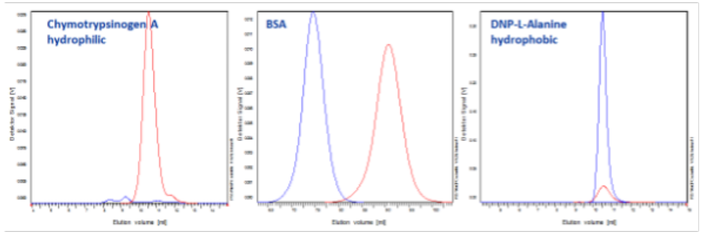

Figure 1. Chromatograms of different proteins/amino acids with different hydrophobicity (blue: low, red: high ionic strength).

Unexpected peak shapes (for example tailing) can occur, which have been shown to be the result of using standard LC equipment and detector cells that have wettable components made of stainless steel. To minimize metal-protein adducts or undesired protein interactions, the use of bio-inert or biocompatible chromatographic systems and column hardware should be investigated. Such systems are also of advantage when aggressive mobile phase conditions are required, such as extreme pH values or high salt content. Operationally, the use of bacteriocides or bacteriostats will help to eliminate bacterial contamination. Without this, algae growth can occur in aqueous mobile phases within a few hours and may cause severe problems.

Detection options

Proteins can be detected using UV detectors. This is highly convenient as UV detectors are easy to use, linear in response over a wide range, sensitive, and baseline stable. Each UV wavelength range has its advantages. 280 nm is often selected as a standard wavelength. Near UV or longer wavelengths (380–315 nm) detect aromatic amino acids, such as tryptophan. Higher sensitivity is provided by selecting a wavelength in the far UV range (280–200 nm), such as 220 nm, where the amide peptide bond has a strong absorbance.

There are also many setups where two different wavelengths are recorded simultaneously to measure the protein concentration. The combination of this dual wavelength detection approach has been proposed for purity investigations, where the lower wavelength provides the sensitivity for the low abundant species, while the higher wavelength provides a higher linear range for the major species (the monomer for example).[2]

To overcome the limitation of size-based GPC/SEC separations, either mass spectrometry (MS) or light scattering (LS) detection can be used to determine the molar mass directly. Both methods should be recognized as complementary techniques, as they offer solutions in completely different application fields. MS is applicable when there is the requirement to determine very specific details about a limited number of individual species in the low to medium molar mass range. If MS is applicable, it is by far the most precise and accurate way to determine the molar masses. However, when sample complexity and molar masses increase, the complexity of the resulting mass spectrum makes it virtually impossible to interpret the data and assign the results. Fortunately, in these cases LS can be used, as this technique is well suited for analyzing polydisperse, high molar mass samples. Another advantage is that LS is very sensitive to high molar masses, even at low concentrations. Therefore, LS can often detect higher aggregates with higher sensitivity than any other detector.

Operationally, the greatest challenge when hyphenating GPC/SEC with MS is the composition of the required mobile phase. Mobile phases in GPC/SEC often contain high concentrations of nonvolatile, MS-incompatible salts leading to problems with contamination of the mass spectrometer and ion suppression. A hyphenated approach is therefore much easier to achieve with LS. The detection is performed in solution, eliminating the need to evaporate the mobile phase and producing information that is irreversibly lost with MS detection when the proteins are ionized and vaporized.

There are many LS detectors on the market that essentially differ in the number of angles used to simultaneously acquire the scattering intensities.[3]

Most globular proteins are so small in dimension that 90° light scattering is normally sufficient to measure the molar mass. Unfortunately, with a single angle it is not possible to measure the size (radius of gyration). To obtain this value requires the use of multi-angle light scattering detection (MALS) or other techniques, such as dynamic light scattering (DLS, also known as QUELS) to measure the hydrodynamic radius (Rh).

The most common detector in GPC/SEC for synthetic polymers, the refractive index detector (RI), is also used for protein analysis. However, an RI is mainly used in combination with a LS detector to determine online the refractive index increment, dn/dc. This sample related parameter strongly influences the accuracy of light scattering results. It also depends on many experimental settings including the LS detector wavelength, the solvent, etc. However, for general screening purposes, often an averaged dn/dc of 0.185 mL/g can be applied for many proteins, but care should be taken as the dn/dc can vary significantly with the protein type.[4]

Other GPC/SEC detection options can be used. For example, some proteins can be analyzed with fluorescence detectors, which can offer improved sensitivity and/or selectivity.

The use of online viscometers is becoming more common, for example, to distinguish between denaturation and aggregation. Viscometers can detect density differences and help to identify structure changes. Therefore, the multidetection approach, which is already quite common for the GPC/SEC analysis of synthetic polymers, is also becoming more popular for protein analysis.

How do the results look?

Figure 2 shows the raw data and results for a UV-LS setup that has been used to investigate protein aggregation. The combined analysis using the UV trace and light scattering trace gives direct access to the molar masses of dimer and monomer without column calibration. As the obtained molar mass of the smaller peak is approximately twice the molar mass of the larger peak, this is an indication for dimerization. Analyzing the peak areas of the UV trace also allows the dimer content to be determined in this example to around 10%.

Figure 3 shows an overlay of the chromatograms obtained for a full-length monoclonal antibody (mAb) and antibody fragments analyzed on the same set of columns. The red curve shows the UV signal of the full-length antibody and its dimers, while the blue curve shows the UV signal of antibody fragments and their high-level aggregates.

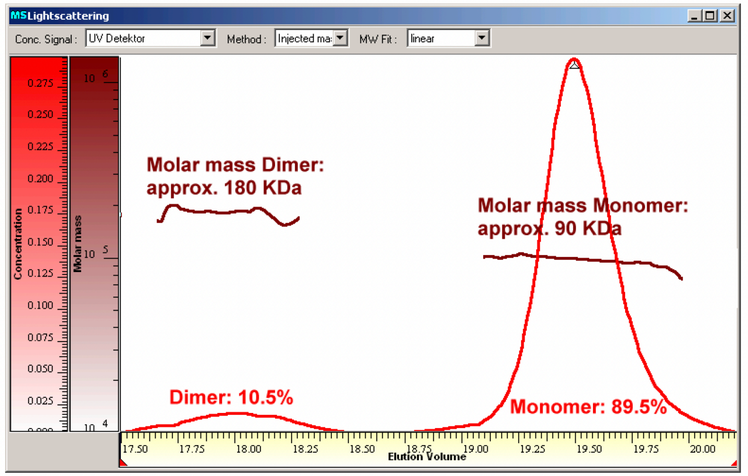

Figure 2. Raw data and results for a UV-LS setup used to investigate protein aggregation. The red curve shows the concentration for monomer and dimer, while the dark red lines show the online measured molar mass at every elution volume.

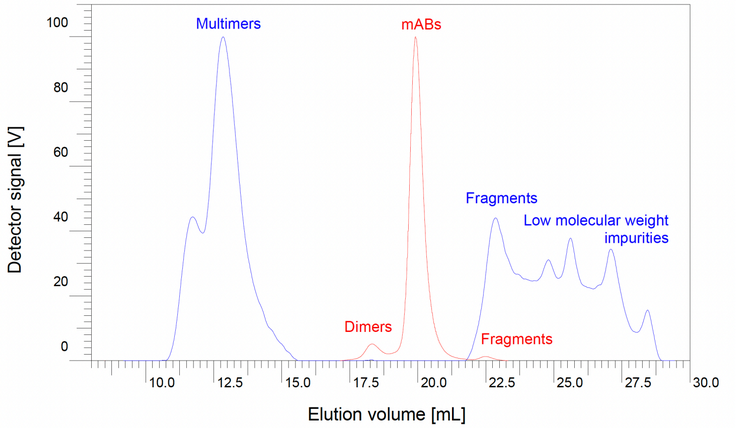

Figure 3. Overlay of the chromatograms of a monoclonal antibody (mAb) (red, UV trace) and the fragments (blue, UV trace) measured on the same column set.

Figure 4 shows the results for a protein exhibiting a molar mass distribution. In the overlay, three different gelatins can be clearly differentiated, and the molar mass averages and the molar mass distribution can be easily determined.

Summary

- GPC/SEC is a powerful technique to investigate protein aggregation. It allows separating proteins under native conditions based on their sizes in solution.
- Size-based separation conditions typically require mobile phases with adjusted ionic strengths. Method development should also include recovery investigations.
- Advanced detection options (MS or LS as complimentary techniques) can help to overcome GPC/SEC limitations and measure the molar masses directly.
- Other typical GPC/SEC detectors such as RI or viscometer can provide dn/dc (required for LS evaluation) or structural information.

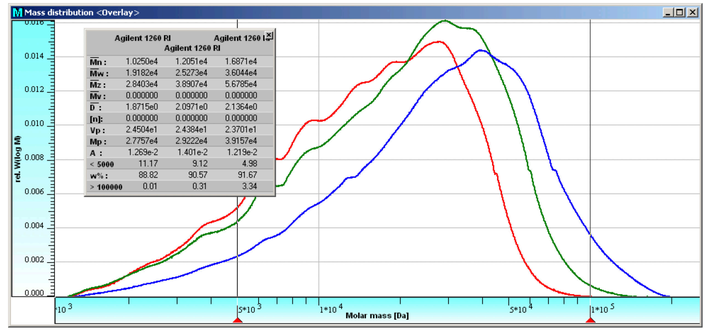

Figure 4. Overlay of the molar mass distributions of three different gelatins: silver 140 bloom (red), gold 180 bloom (green), and platinum 240 bloom (blue).

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1.4. Calibration using broad standards

For polymeric materials, where no narrow distribution molar mass standards of the same chemical type are available, reliable and true molar masses cannot be determined with just a concentration detector. However, reliable and true molar masses can be obtained by using well characterized samples of the same polymer type with broad molar mass distribution(s). This approach does not require additional instrumentation and therefore provides a cost-effective alternative to the more expensive application of molar mass sensitive detectors, such as online light scattering or online viscometry detectors.

Introduction

Gel permeation chromatography/size exclusion chromatography (GPC/SEC) is an easy and robust method to determine molar masses and molar mass distributions (MMD) of macromolecules. The separation is based on the size of macromolecules in solution. The larger the macromolecule, the earlier it elutes from the column.

The primary information in GPC/SEC is the chromatogram showing the detector signal as a function of elution volume. To derive the molar mass distribution, the chromatogram must be converted using a calibration curve, which relates the elution volume to the molar mass of the eluting polymer. Such calibration curves are usually established using narrow distribution polymer standards. However, chemically different polymers of the same molar mass often exhibit very different sizes in solution. Therefore, correct molar masses are obtained only if the chemical structure of the calibrant matches the structure of the sample.

Unfortunately, for many important polymers, such as polyamides, polyesters, or polyolefins, narrow standards are not commercially available. To obtain true molar masses, alternative methods are required. The use of a molar mass sensitive detector is one alternative. It, however, requires additional and expensive instrumentation, more precise and accurate sample preparation, more time, and a higher level of knowledge of data evaluation. Especially for quality control laboratories, these limitations are crucial. Here, fast, robust, and easy-to-use calibration methods are required. A suitable, more cost-effective solution is the use of broadly distributed standards.

Calibration using broad samples—how does it work?

For a narrow distribution polymer, the peak maximum of the chromatogram can be easily identified and assigned. This is not the case for a broad distribution sample. Even if a value for the M_p is known, the peak maximum can be difficult to identify precisely, as it can depend on the resolution of the separation columns. Therefore, it is not recommended to produce a calibration curve by simply plotting the molar mass values versus the elution volume at peak maximum.

The generally accepted concept of universal calibration[1] in GPC/SEC results in the following relation between the calibration curve constructed with narrow distribution calibrants and the analyte:

$$\log M_{analyte} = A + B \times \log M_{calibrant}$$

This assumes that both, calibrant and analyte, elute free of interaction with the stationary phase.

The parameter A shifts the calibration curve vertically relative to the calibration curve produced with the narrow distribution calibrants, while a change in B varies the slope of the calibration curve (compare Figure 1). The parameters A and B are related to the Mark-Houwink parameters of the analyte and calibrant in the same solvent.[2–5] Due to the effect of the parameters A and B on the calibration curve, the MMD and consequently the molar mass averages calculated for a given chromatogram change as a function of the parameters as well.

This is shown in Table 1, where two chromatograms of broad distribution polylactic acid (PLA) samples were evaluated using a polystyrene calibration curve (base calibration) and different sets of A and B parameters. Application of the polystyrene calibration curve overestimates the true molar mass values (M_w and M_n) by approximately a factor of three. Changing the parameters brings the molar mass values closer to their true values. However, both parameters need to be changed to get reliable results for different samples. For the optimized parameter set for A and B, given in the last line, good agreement with the reference values from light scattering is observed.

Table 1 also explains how the parameters A and B can be determined from the chromatograms and known values of molar masses of broad distribution standards. To obtain A and B, they are systematically varied until the best agreement between the known molar masses and the ones calculated from the respective chromatograms using the base calibration is obtained.

Fortunately, this variation is done in an automated fashion with most modern GPC/SEC software packages. With the calculated values of the parameters A and B, a suitable calibration curve for unknown samples of the same structure as the analyte can be easily derived from the base calibration.

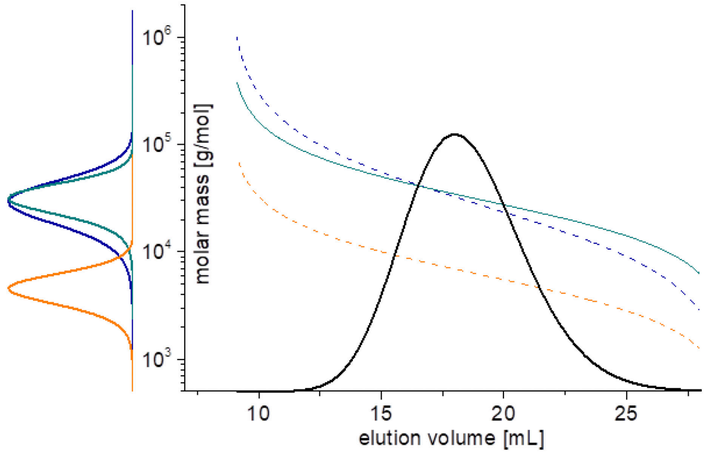

Figure 1. Effect of parameters A and B on the calibration curve and therefore the MMD of the analyte.
- Turquoise: Original (base) calibration curve and MMD (left) derived therefrom
- Blue: Calibration curve and MMD derived from turquoise curve by changing parameter B (slope)
- Orange: Calibration curve and MWD derived from blue curve by changing parameter A (vertical shift).

	PLA-Sample 1 M_w [g/mol]	PLA-Sample 2 M_w [g/mol]
Reference value from light scattering	37,000	500,000
Polystyrene calibration (A = B = 1)	82,300	1,247,000
A = 0.5; B = 1	35,400	580,000
A = 0.5; B = 8	3,790	355,000
Optimized values from software A = 0.717; B = 0.957	36,950	500,700

Table 1. Effect of variation of parameters A and B on the molar masses derived from a polystyrene calibration curve and a chromatogram of a broad distribution PLA sample

In contrast to other approaches for broad standard calibration, the previously described approach benefits from the fact that the base calibration reveals the pore size distribution of the column packing and therefore the true shape of the calibration curve (which is sigmoidal or nonlinear in shape in most cases), the exclusion volume, and separation limit of the base calibration curve. An additional advantage is that more than one sample can be used to create the calibration curve. This increases accuracy and allows a wider molar mass range to be covered without extrapolation.

Process to establish a calibration curve using broad standards

Establishing a calibration curve for the aforementioned procedure requires:

1. A conventional calibration curve (base calibration) established using narrow distribution standards of arbitrary chemical structure, such as polystyrene for many organic solvents or pullulan or PEO/PEG for aqueous applications
2. At least one broad distribution sample with known M_w and M_n and of the same chemical structure as the analytes to be characterized; if two or more broad distribution samples are used, one molar mass average, such as M_w or M_n, per sample is sufficient

Then, the procedure is straightforward:

- Run the narrow and the broad distribution standards on your GPC/SEC system.
- Establish the base calibration using the molar masses (M_p) and peak maxima of the narrow distribution calibrants.[6]
- Use a software tool (e.g. Agilent WinGPC or Agilent OpenLab CDS GPC/SEC) to shift the calibration curve and to adjust the slope so that the calibration provides correct results for the broad distribution sample. This means that the software internally determines the optimized parameters A and B using the chromatograms of the broad standards and by applying the known molar mass averages.
- Save this new calibration curve so that it can be used to determine the complete molar mass distribution and the true molar mass averages of samples with unknown molar masses.

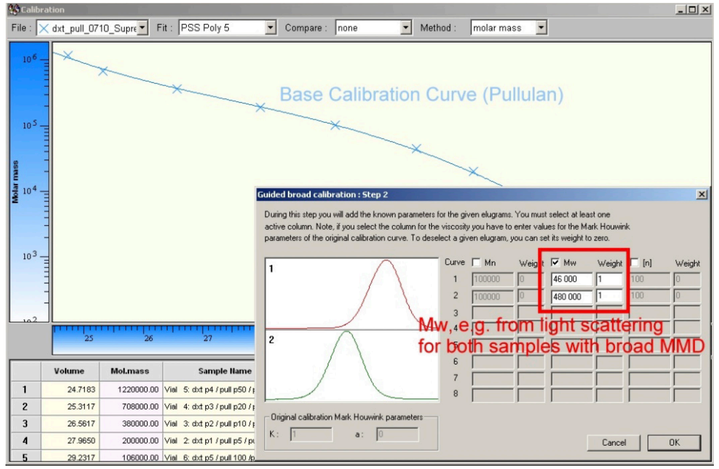

Figure 2. Base calibration and chromatograms of two broad distribution samples with known M_w. To increase accuracy and molar mass range, up to eight different samples can be used.

Figure 2 shows the chromatograms of the broad distribution standards as well as what is required to use the broad standards calibration concept. To increase the accuracy of the calibration, more than one broad distribution standard should be used. If a wide molar mass range needs to be covered, the use of several samples is recommended.

Figure 3 shows a comparison of the conventional/base calibration (blue) and the resulting calibration curve (red) after determination of A and B. This calibration curve is then used for the evaluation of unknown samples, and it provides true molar masses for all samples of this type.

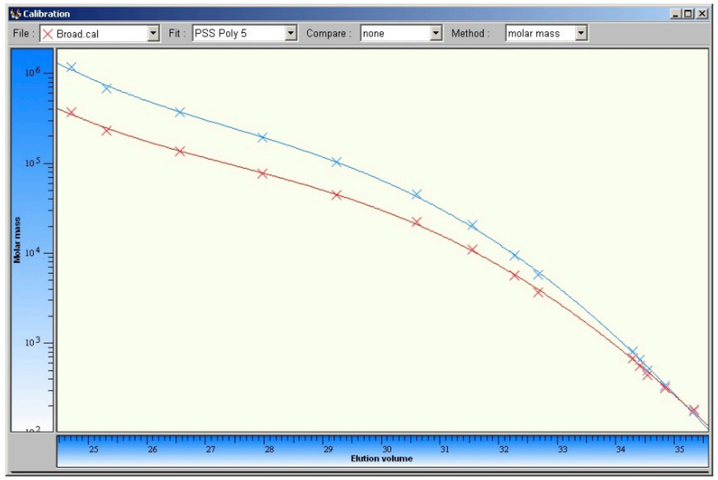

Figure 3. Pullulan base calibration curve (blue) compared to the calibration curve using the optimized A and B values from the samples with the broad molar mass distribution (red).

How to obtain broad standards

A limited number of broad standards with a variety of chemical structures are available from suppliers. If no broad standards are commercially available, sample testing laboratories can determine true molar mass values using batch measurements obtained from absolute techniques, for example by light scattering for M_w or NMR or osmometry for M_n. Online determination using multidetection GPC/SEC can also solve this problem

Summary

- Calibration using broad standards is a fast and easy-to-use method for quality control laboratories eliminating the need of expensive advanced detection.
- Standards with a broad molar mass distribution with known Mw and Mn values can be easily obtained from contract analysis laboratories using batch methods. This allows scientists to measure precise and accurate molar masses worldwide.

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1.5. The art of analyzing high molar mass samples

GPC/SEC is a method that can be used for a variety of samples with molar masses ranging from only a few hundred up to several million Daltons. While the analysis of low molar mass samples is straightforward, more care and understanding are required to analyze high molar mass samples in the range of several million Daltons.

Sample preparation

The analysis of macromolecules requires patience. This is especially true for high molar mass samples. The dissolution time required by macromolecules is significant and can be, in the worst cases, up to several weeks. The dissolution time depends on many parameters such as sample, solvent, molar mass, polydispersity, chain chemistry, crystallinity, composition, and stereochemistry. As a rule of thumb, the higher the molar masses of a material and narrower it's distribution, the more time is required for complete and reproducible dissolution.

There are not many options to speed up the polymer dissolution process. The use of ultrasonic devices is not recommended, as this will most probably result in degradation of the sample. For ultrahigh molar mass samples, even the use of a magnetic stirrer bar might result in chain scission. Therefore, the only option is to wait until the sample is dissolved into single isolated chains. If the dissolution process takes several weeks/days, stabilized solvent should be used. Keeping the dissolution container in a dark environment is also often recommended. At most, only use occasional gentle swirling of the dissolution container to help to homogenize the solution.

While filtering a sample solution through a membrane filter is generally recommended if samples contain gels or particles, care must be taken for high molar mass samples. The pore size of the filter needs to be adjusted to avoid sample degradation. If possible, filtration of high molar mass sample solutions should be avoided.

Compared to low molar mass samples, reduced concentrations should also be used. High molar mass molecules need room to expand to their true hydrodynamic volume without interference from other chains. As an example, the exact same mass of a high molar mass material could be injected onto a column set for analysis with a wide range of injection volume/concentration options. Using two options as an example, firstly, we could inject the sample with a 10 µL volume at 1.0% w/v or as 100 µL volume at 0.1% w/v.

For the first example, it is extremely unlikely that we would get accurate and reproducible molar mass values for a high molar mass material due to the very high value of viscosity of the solution and the fact that the high molar mass molecules, as noted above, would not be at their true hydrodynamic volumes. In practice, it is likely that you would observe the sample peak eluting at increasingly later elution times (see Figure 1) or as a much broader peak eluting from the column.

Molar Mass Range	Concentration
100–10,000	2 mg/mL (0.2%)
10,000–1,000,000	1–2 mg/mL (0.1–0.2%)
> 1,000,000	0.5 mg/mL or less (0.05%)

The effect of sample concentration on the elution volume increases with molar mass. The table summarizes recommended sample concentrations based on molar mass. These recommendations are for samples with narrow molar mass distributions. For samples with a high polydispersity (broad molar mass distribution), higher concentrations are possible.

Figure 1 shows the elution volume and peak shape change for a reference material with a molar mass of 500,000 Da compared to that for a molar mass of 5,000 Da. While there is a small effect for the low molar mass sample, the elution volume and the peak shape of the high molar mass change significantly.

The change in elution volume is especially problematic when the GPC/SEC system is calibrated with narrow distribution molar mass standards, where the peak position of a sample will be compared to that of a reference standard. The change in peak shape (indicating the lack of separation) affects all kinds of calibrations, even the ones with online viscometers, light scattering detectors, or triple detection systems.

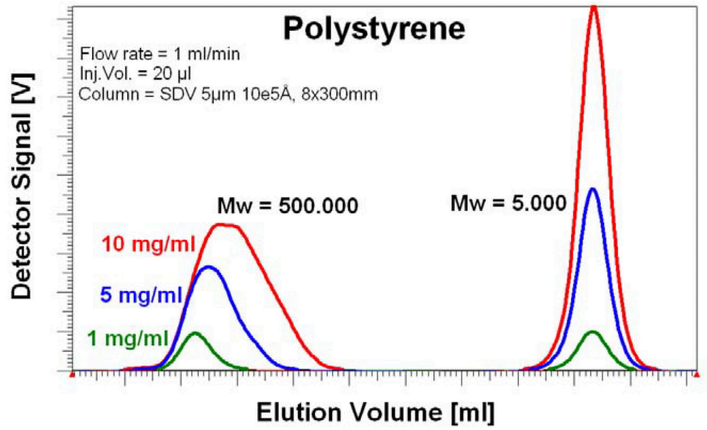

Figure 1. Overlay of chromatograms of two polystyrenes at three concentrations each. There is only a minor effect for the 5,000 Da sample, but the elution volume and even the peak shape of the 500,000 Da sample is highly affected by concentration.

Chromatographic conditions

The following rules apply for all samples to be analyzed with GPC/SEC:

- The separation of higher molar masses requires separation columns with larger pores.
- Larger particle sizes are generally used for higher molecular weights to avoid shear degradation of the samples.

However, for samples of several million Dalton molar mass, additional experimental conditions need to be considered.

The most important condition to optimize is the flow rate of the system. Due to their extremely low diffusion coefficients, polymers will show significant peak broadening or unexpected peak shapes when run at too high flow rates. Reducing the flow rate to 0.25 mL/min or even less will result in more realistic peak shapes for high molar mass samples. Another factor to be considered is shear induced stretch of the long polymer chains.

Figure 2 shows the overlay of a high molar mass sample measured at 0.5 mL/min and at 0.25 mL/min. The curve for 0.50 mL/min is shifted to artificially higher elution volumes, and again the peak shape changes.

An online light scattering detector is a good detector to visualize incorrect chromatographic conditions. Since these detectors are often required to overcome the lack of available ultrahigh molar mass calibration standards, many setups already include a device.

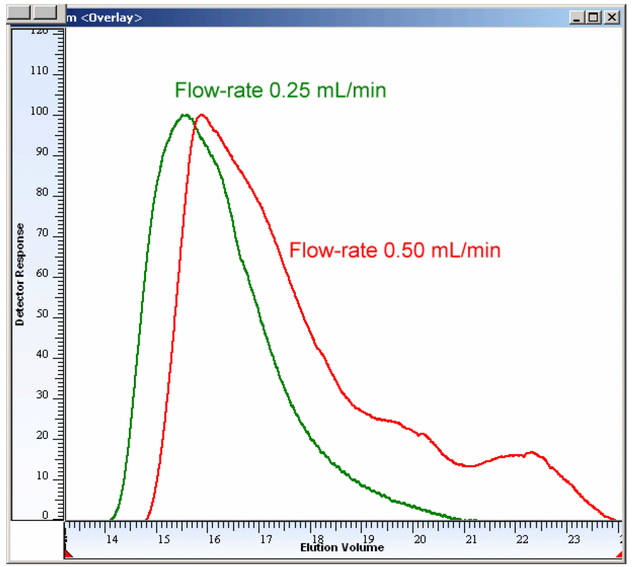

Figure 2. Overlay of chromatograms for a sample of 3.8 million Da obtained at 0.5 mL/min flow rate (red) and 0.25 mL/min (green). Reducing the flow rate to 0.1 mL/min can further improve results.

Chromatographic condition

Figure 3 shows the multi-angle light scattering results for a high molar mass sample at the flow rate of 0.5 mL/min. The measured molar mass essentially stays constant for a wide elution volume range. This is due to inefficient separation, resulting from incorrect chromatographic conditions being used.

Other practical considerations when analyzing high molar masses are detection limit and sample viscosity:

- Since low concentrations need to be used, quite often the S/N ratio for concentration detectors is low. However, instead of increasing the sample concentration, it is better to increase the elution volume for the injection. This will also increase the overall injected mass and therefore improve the signals. Please note that this is not recommended for lower molar masses—here, it is better to increase the concentration and to inject a small volume.
- If an autosampler is being used and the sample viscosity is high due to the presence of the high molar masses, a reduction in the draw speed of the autosampler syringe is recommended. This will increase the reproducibility of the injections.

Good practice for the analysis of high molar masses

- Use low concentrations; if required, increase the injection volume.
- Be patient and allow enough time for complete sample dissolution.
- If possible, avoid sample filtration. If this is not possible, use appropriate pore size media.
- Use low flow rates (0.25–0.1 mL/min) and reduce the autosampler syringe draw speed.
- Use columns with large particle size packing materials and larger pores. In case of suspicious shoulders in the chromatogram, check the exclusion limit of the columns.

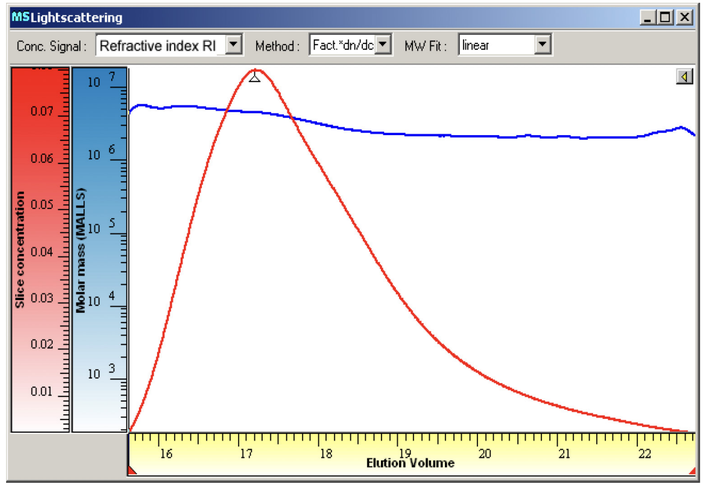

Figure 3. Slice concentration (red) and online determined molar mass (MALS, blue) for a sample run with a too high flow rate (0.5 mL/min).

1.6. Branching analysis

An advantage of polymeric materials is that their physical properties can be tailored for a specific application by adjusting many parameters. Besides composition, average molar mass and the width of the molar mass distribution, another key parameter to control application properties, is branching. Chromatography and advanced detection can help to characterize branched molecules.

Branching requires at least a single branch point where three or more chains are connected. Branching can occur as an undesired side reaction during synthesis, or it can be introduced deliberately to optimize the physical properties of the material. Different routes exist to synthesize defined structures, such as star-shaped or comb-shaped polymers.

The properties of branched polymers differ significantly from linear ones with respect to (melt) viscosity, glass transition temperature, the coefficient of bulk thermal expansion, solubility, etc. The property change depends on the parameters such as type of branching, length of the branches, and branching density.

The characterization of complex polymer mixtures comprising not only a molar mass distribution, but branching distribution as well, represents a real challenge. Depending on the type of branching there are various detection and separation options that provide deeper insight.

GPC/SEC hyphenated with online viscometry[1] (or less suitably, multi-angle light scattering) can be used to characterize defined structures, such as star- or comb-shaped polymers, or to investigate long-chain branching. High-temperature GPC (HT-GPC) with infrared (IR) detection can be used to investigate short-chain branching in polyolefins.[2]

For polymer samples exhibiting broad molar mass distributions for the number of branches and backbones that vary in branching density, the resolution of the size-based separation in GPC/SEC might not be sufficient to fully resolve the structures. Therefore, alternative separation methods such as interaction polymer chromatography (for example gradient polymer HPLC, TGIC) or two-dimensional chromatography should be applied.[3]

GPC/SEC-viscometry

A limitation of GPC/SEC is that it separates only based on the size of the molecule in solution. The consequences for branched samples are that conventional calibration with reference materials will underestimate the molar mass of the branched samples. A solution here is the use of molar mass sensitive detectors such as online viscometers or light scattering detectors. The viscometer can then be used for structure analysis and for molar mass determination based on universal calibration.

An online viscometer is classified as a molar mass sensitive detector, however, the signal intensity is dependent on the viscosity rather than on molar mass. Viscometers provide direct access to the density of the molecules in solution. While the setup of such instrumentation (in most cases, also with a light scattering detector) is very common, understanding the results and limitations requires more experience.

Important for branching analysis is the Mark-Houwink plot, where the logarithm of the intrinsic viscosity (for example obtained using online viscometry) is plotted versus the logarithm of the molar mass obtained using universal calibration (or light scattering detection). The slope of the Mark-Houwink plot—the Mark-Houwink exponent α—is dependent on the shape of the molecule in solution. If there is no molar mass dependence of the intrinsic viscosity (solid sphere) a slope of 0 is expected. On the other hand, the Mark-Houwink exponent of rigid rods is 2. Typical random coil polymers exhibit Mark-Houwink exponents in the range of 0.5 to 0.8, depending on solvent quality.

Branching analysis for a given polymer can be straightforward if the data generated can be compared to a linear chain of identical chemical structure and molar mass. Figure 1 shows Mark-Houwink plots for different polyethylene samples. A HT-GPC instrument equipped with an online viscometer was used to generate this plot.

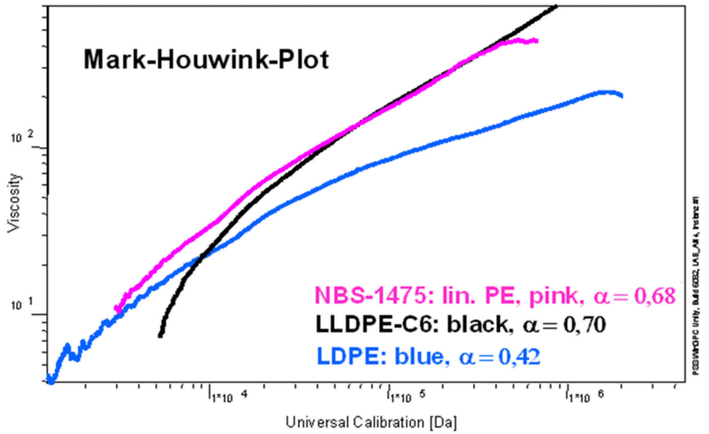

Figure 1. Mark-Houwink plot overlay for three different samples: linear NBS-1475 (pink), linear low-density polyethylene (LLDPE, black) with short-chain branching, and low-density polyethylene (LDPE, blue) with long-chain branching.

While the Mark-Houwink plot of the linear low-density polyethylene (LLDPE) that only has short-chain branching nearly superimposes with the one of the linear sample, the low-density polyethylene (LDPE) that comprises long-chain branching deviates significantly. At the same molecular weight, the LDPE chains reveal a significantly lower intrinsic viscosity as compared to the linear sample. This is a consequence of branches being present. The deviation increases with increasing branching density. By extrapolating the Mark-Houwink plots of the branched and linear polymer to a common intercept, it is possible to detect the molar mass at which branching first occurs. By taking the ratio of the intrinsic viscosity of the branched and linear polymer at the same molar mass, it is possible to determine the contraction factor g', from which conclusions on the number of branches can be deduced.

Figure 2 shows the GPC/SEC-viscometry results for a poly(tert-butyl acrylate), PtBuA, star polymer. Star polymers are relatively simple branched polymers as they consist of several arms (linear chains) connected to a central core.

The star polymer was synthesized using the arm-first approach. PtBuA precursor arms of narrow molar mass distribution have been coupled using a small amount of a bifunctional crosslinker to form the core. This means that two star polymers with different molar masses differ by the number of arms (with approximately the same length) attached to the core.

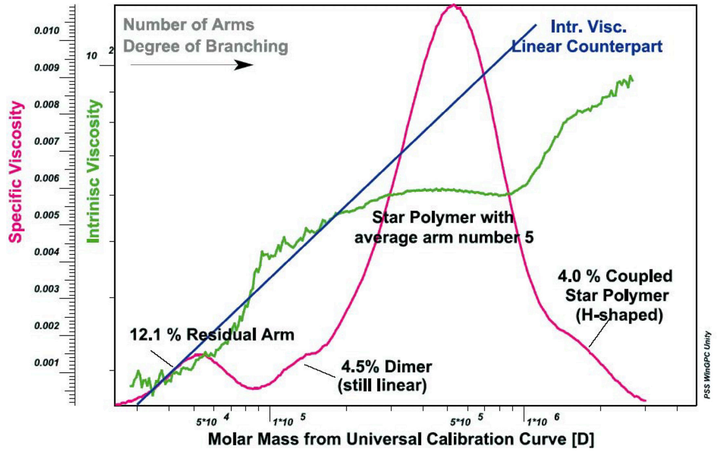

Figure 2. Mark-Houwink plot of an arm-first star polymer (molar mass increase by combining arms). The structure change (random coil to a dense sphere) is reflected by a maximum in intrinsic viscosity.

The Mark-Houwink plot reveals a maximum for the intrinsic viscosity, which has also been observed for dendrimers. Starting with a linear precursor, the coupling of two linear chains forms a still linear molecule, the dimer. Further reaction of precursor molecules with the core leads to three-arm and higher arm star polymers. Here, the increase of the intrinsic viscosity with molar mass is counterbalanced by the decreasing intrinsic viscosity due to an increasing segment density with increasing number of arms for the branched structures. This variation in molecular structure can be nicely monitored from the viscosity measured using an online viscometer. Of note is the sudden change of intrinsic viscosity occurring at approximately twice the molecular weight of the peak maximum. The drastic increase in viscosity is most probably due to the formation of H-shaped molecules as coupling of two stars via an arm occurs.

In contrast to the synthetic route described above, star polymers can also be synthesized using the core first approach, such as using a multifunctional initiator. In this case the observations and results would be different. There would be no structural change as the molar mass increase of the star would result from the growth of the arms. For such star polymers, the Mark-Houwink plots are expected to be as shown in Figure 3. For each star polymer, the Mark-Houwink plot will be shifted in parallel to lower viscosities at the same molar mass. Analysis would yield the same Mark-Houwink α, but a reduced Mark-Houwink K (intercept). The shift to lower intrinsic viscosities increases with increasing number of arms.

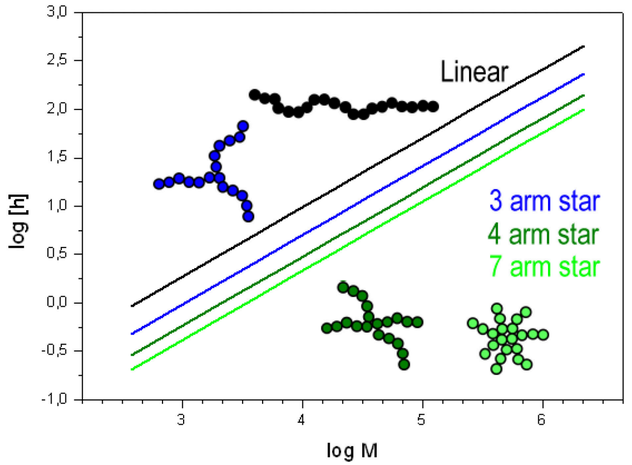

Figure 3. Schematic Mark-Houwink plots for star polymers synthesized via core-first. Arms are started from a multifunctional initiator core, and the molar mass increase is due to adding monomer units to the arms.

Advanced separation techniques

A limitation of the technique of GPC/SEC, as mentioned previously, is that it separates only based on the size of the molecule in solution. Even with mass sensitive detectors, such as viscometers and light scattering detectors, there are situations where we need to change the analytical strategy for branched polymers.

In cases where the hydrodynamic volume increases only slightly with molar mass, such as for arm-first star polymers, the resolution of size-based separations is limited. In this case interaction polymer chromatography (IPC, separation is based on chemical composition) can be used as a complementary technique. Figure 4 shows the chromatogram of a gradient separation of an arm-first star polymer with high resolution even for stars with higher arm numbers.

If samples exhibit broad molar mass distributions besides structural heterogeneity (for example branched and linear chains being present), the risk of coelution increases. In that case, branched molecules of higher molar mass having the same hydrodynamic size as linear chains of lower molar mass elute at the same retention volume. Consequently, the fractions eluting from the column cannot be regarded to be monodisperse any longer.

Comprehensive characterization of branched polymers might be possible when two-dimensional (2D) separations are applied. 2D combines two independent separation techniques to generate contour plots, which can also be used to quantify the different species. Figure 5 shows an example where linear and comb-shaped molecules of different composition have been successfully separated.

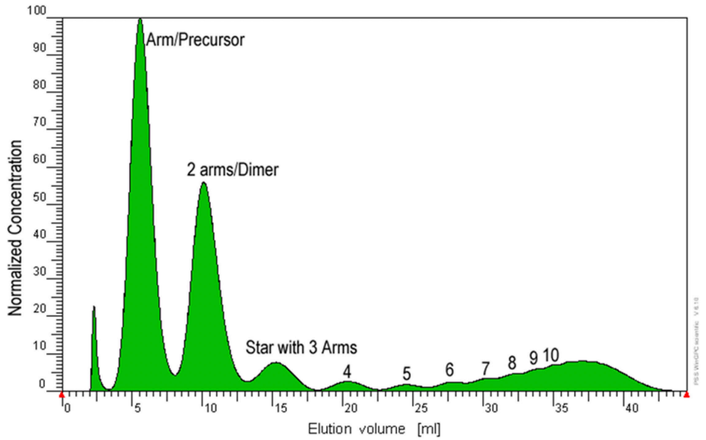

Figure 4. Separation of a star-branched polymer by interaction polymer chromatography (IPC).

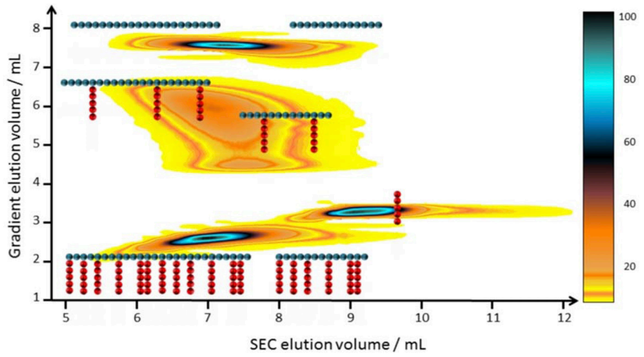

Figure 5. Separation of a star-branched polymer by interaction polymer chromatography (IPC).

Summary

- Branching can occur as a side reaction in polymerizations or can be introduced to tailor application properties.
- Online viscometers provide access to the density of a molecule and can help characterize branched molecules as they monitor structural changes with molar mass.
- Polyethylenes exhibiting long-chain branches can be analyzed by online viscometry, while information on short-chain branching can be gained by FTIR detection.
- Coelution of branched and linear molecules can be a problem. In such cases, advanced separation techniques or two-dimensional chromatography can be applied

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1.7. GPC/SEC for membrane filter analysis

Membranes or membrane filters in the form of thin films or disks are fabricated from porous polymeric or inorganic materials with specific pore sizes and distributions. With advances in membrane technology, the membrane filtration field has rapidly expanded in response to various applications, for example the increasing need for contaminant removal and water treatment solutions, retaining particles and microorganisms that exceed a certain size, or the removal of or the estimation of large soluble macromolecules in a solution. Obviously, for quality control and reproducibility requirements, there is a need to know the size of the pores and the retention behavior in a porous membrane material.

Membrane performance parameters, such as retention behavior, pore size, and molar mass cut-off, can be determined using standard chromatography equipment. This membrane characterization method is based on the selection, preparation, and ultimately the filtration of a reference material of known size or molecular weight followed by the subsequent GPC/SEC analysis.[1]

As GPC/SEC is the standard technique to characterize soluble macromolecules in solution and separates macromolecules based on differences in their size, it can be used to compare the unfiltered and the filtered solution to learn more about the filter capabilities of membranes.

An advantage of this approach is that membranes are tested under field operating conditions. This is especially important if swelling or nonpermanent porosity must be considered for the membrane quality.

Reference material selection and preparation

A reference material of known size or molecular weight is selected to prepare a stock solution.[1] Depending if an organic or an aqueous solvent is used for the filtration process, polystyrenes, dextrans, or pullulans can be used.

If the material exhibits a broad molar mass/size distribution (large polydispersity index, PDI), a single reference material is sufficient to cover the complete pore size range of the membrane in the wet state. Using a reference material where the large PDI is a product of the synthesis process is preferred over mixing several samples with a narrow molar mass distribution, as the broad molar mass distribution will be smooth, and the materials are less expensive.

The molar mass/size range of the reference material must be chosen in a way that some portions of it should be retained by the membrane while others will pass through.

The reference material is then dissolved in the desired solvent to obtain the stock solution. This stock solution is filtered through the test membrane, applying proper filtration conditions (cross flow, back pressure, etc.).

Molecules smaller than the membrane pores will be able to penetrate through the membrane into the filtrate, while larger molecules will remain on the feed side (retentate).

The same stock solution can be used to test several membranes allowing for an easy quality control or comparison of different membrane types.

Sample analysis by GPC/SEC

Important membrane properties can be obtained by a specific comparison of the concentration profiles of the stock solution, filtrate, and (optional, not required by some approaches) retentate. These collected samples will be measured using a standard GPC/SEC chromatography system comprising an isocratic pump, an injection system, and preferably a refractive index (RI) detector to be able to detect how much has passed through the membrane.

As an example, an overlay of chromatograms of a stock solution consisting of three dextran standards with different molar masses and a filtrate is shown in Figure 1. If a dextran with a broad molar mass distribution had been used, the chromatogram would not show maxima and minima but appear as a steady distribution.

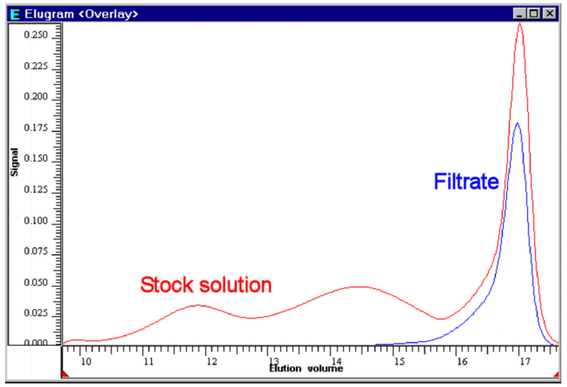

Figure 1. Overlay of the GPC/SEC chromatograms of a stock solution consisting of three dextrans with different molar masses (red) and the filtrate (blue).

By overlaying the data of stock solution and filtrate, it is clearly seen that the membrane held back most of the higher molar masses/sizes that elute in GPC/SEC at lower elution volume. A percentage of the smaller molecules eluted (compare peak around 17 mL elution volume).

In all cases, where chromatograms are compared, a reproducible elution is of utmost importance. Using an internal standard or flow marker can help to identify any nonreproducible problems at an early stage. Correction using the flow marker increases the data and result quality.[2]

Results and membrane parameters

The next step is to quantify and to obtain important membrane parameters, such as:

- Sieve curve and retention efficiency
- Average pore size and pore size distribution
- Molar mass cut-off
- Size selectivity
- Pore accessibility

Curves, referred to as sieve curves, can now be generated based on the chromatograms of the stock solution and the filtrate as shown in Figure 1.

The values for the sieve curves (S) are calculated from the stock solution (cS), the filtrate (cF), and (not required by all applications) the retentate (cR) using:

$$S = 1 - (cF / cS)$$

However, other approaches/equations have been described in the literature and can of course also be applied to the data.

Several membranes can be characterized in a sequence, and their sieve curves can be easily compared.

Sieve curve molar masses can be determined by applying a conventional calibration curve constructed using standards of the same type as the stock solution reference material.[3]

In addition, the molar mass cut-off of the membrane can be estimated. This molar mass value defines the retention of a given amount of sample. Typically, 90% is used as the molar mass cut-off. However, additional retention parameters can be useful to answer specific questions or for in-depth comparison of different membranes. From the molar masses, pore sizes can also be calculated using the R_g-M relationship with parameters, for example, taken from literature.

Figure 2 shows the results for three different regenerated cellulose membranes including the 90% cut-off results and the average pore size. All three membranes show different filtration characteristics, and even small property differences can be visualized with the overlaid data. The selectivity of a membrane, D, can also be calculated. Typically, this is determined at 25% and 75% retention. In the case of ideal selectivity, where D = 1, the membrane would only allow a single size to pass through the pore. If no selectivity is achieved and the selectivity parameter D = ∞, all sizes could pass through the membrane

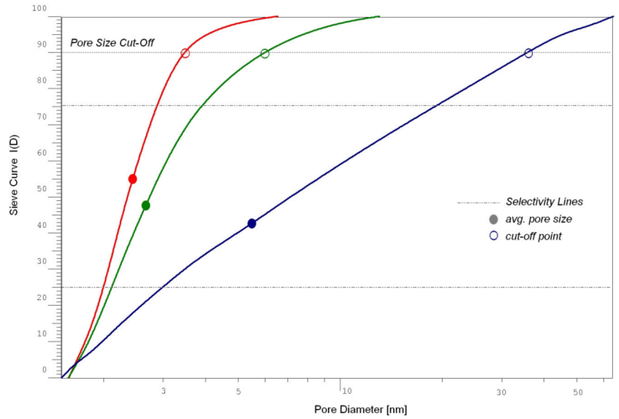

Figure 2. Overlay of the sieve curves of three different plane membranes from regenerated celluloses.

Summary

- Standard GPC/SEC equipment can be used to characterize membranes. For this, stock solutions, filtrates, and retentates obtained by a filtration experiment are analyzed as GPC/SEC samples.
- The use of a flow marker increases the data quality.
- Sieve curves can be calculated from the generated GPC/SEC chromatograms.
- Molar masses can be determined using a calibration curve, and pore sizes can be calculated using the R_g-M relationship.

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Glossary

BHT	Butylated hydroxytoluene
Da	Dalton (E g/mol)
DAD	Diode array detector
DMAc	Dimethylacetamide
DMF	Dimethylformamide
dn/dc	Refractive index increment
ELSD	Evaporative light scattering detector
ESI-MS	Electrospray ionization mass spectrometry
GPC	Gel permeation chromatography
LAC	Liquid adsorption chromatography
LC	Liquid chromatography
LiBr	Lithium bromide
LiCl	Lithium chloride
LS	Light scattering
MALS	Multi-angle light scattering
M_n	Number-average molar mass
Mobile phase	Liquid phase used in a chromatography system
M_w	Weight-average molar mass
M_z	z-average molar mass
MALDI-TOF	Matrix-assisted laser desorption/ ionization time-of-flight
NaN_3	Sodium azide
QC	Quality control
PDI	Polydispersity index ($D = M_w / M_n$)
PLA	Polylactic acid
PLC	Polymer of low concern
PMMA	Polymethyl methacrylate
PS	Polystyrene
R_g	Radius of gyration
REACH	Registration, evaluation, authorization, and restriction of chemicals
RI	Refractive index (detection/detector)
SEC	Size exclusion chromatography
Solvent	Liquid in which a solute is dissolved to create a solution
Stationary phase	Solid phase in a separation device on which materials will be separated
TCB	Trichlorobenzene
THF	Tetrahydrofuran
UV	Ultraviolet (detection/detector)
Ve	Elution volume

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