Investigation of Protein Dynamics

Seperation Method in Combination with Light Scattering Detection

The combination of GPC/GFC/SEC separation with light scattering detection is a powerful method to study the interactions of proteins and protein-ligand complexes. Comprehensive results are obtained when selecting highly efficient columns for sample fractionation in combination with comprehensive detection techniques like multi-angle light scattering detection (MALLS). This paper describes strategies for column selection and method hyphenation and shows results which would be much more cumbersome and time consuming to obtain otherwise.

The human genome has been almost completely sequenced, but it is still not well understood how gene(s) express themselves in humans and animals. E.g. the genome of a beautiful butterfly is 100 % identical to that of the caterpillar (larva stage) but they are extremely different species otherwise (fig. 1). The same is true for the about 80,000 proteins of the human genome. The chemical composition (amino acids) of the proteins are well known, but scientists still do not really understand how the proteins perform their functions (fig. 2). Sequence analysis was an important first step in what seems still to be a long journey to a better and comprehensive understanding of molecular-biological processes. Currently chemists, biologists and related scientists strive to elucidate the complex interactions between proteins and between proteins and ligands. Even those interactions which are basically understood require additional scientific investigation in order to relate them to biological processes and employ them in medical and pharmacological applications. Only after we understand the dynamics of biological functions of the proteins and their respective interactions we will be able to understand the "biology of life".

Protein Characterization by GPC-MALLS

One of the prospective methods to identify protein functions is the investigation of protein-protein interactions and potential ligands on a molecular level. Currently a number of techniques are used for this kind of research. Size-exclusion chromatography (SEC; also known as gel permeation chromatography or gel filtration: GPC/GFC) in combination with multi-angle light scattering (MALLS) detection is used increasingly, due to the number of practical and fundamental advantages:

- The combination of GFC/GPC/SEC separation with MALLS detection allows for the simultaneous measurement of the molecular weights and of the molecular size of proteins in solution.
- GPC-MALLS is non-invasive and non-destructive: samples need not be derivatized by radioactive or fluorescent labels. It is possible to recover proteins after the experiment for subsequent investigation by other (e.g. destructive) techniques.
- GPC-MALLS is an online method which requires significantly less sample amounts and sample preparation as compared to batch experiments.
- The detection of either individual proteins as well as protein-protein clusters can be performed selectively at low levels by this technique.
- The analytical conditions can be identical to the native protein environment. The samples can either be isolated or investigated in a sample cocktail (e.g. together with ligands).
- GFC/GPC/SEC experiments can be performed under or close to equilibrium conditions for proteins. This is of outmost importance when quantifying protein-protein interactions or when measuring protein dimensions.
- In comparison to other techniques GPC-MALLS allows to vary experimental parameters quickly and reliably (e.g. pH, temperature, ionic strength).

Therefore the hyphenation of GFC/GPC/SEC separation with MALLS detection is a very attractive technique for protein characterization, especially since investigations can be done under physiological conditions. To exploit the potential of the GPC-MALLS combination to its fullest extent, the specific experimental parameters of both techniques have to be optimized.

Successful GPC-MALLS Hyphenation

GFC/GPC/SEC is a well established chromatographic technique available in many laboratories to characterize macromolecules. Molecules are separated by their effective size in solution. The fractionation process is performed in one or a series of columns which are filled with highly crosslinked, spherical particles with permanent macro porosity. The separation is governed by entropy changes of the solute between the mobile phase and the stationary phase and therefore not very sensitive to temperature fluctuations. Large molecules (high molecular weight) elute first from the columns while small molecules (low molar mass) will be retained longer. The properties of the analytical fractions (e.g. concentration, molar mass, size, aggregation state) will be determined in a series of online detectors (fig. 3). The combination of different detection techniques leads to a significant increase in information content in GPC analysis [1].

Many detection techniques in GFC/GPC/SEC are independent analytical methods. Initially they have been used successfully in batch experiments without any pre-fractionation of the samples under investigation. This is also true for static light scattering which is employed in MALLS detectors.

Fig. 1 a + b: Butterflies and caterpillars are very different species but have identical genome, indicating the need to understand gene expression.

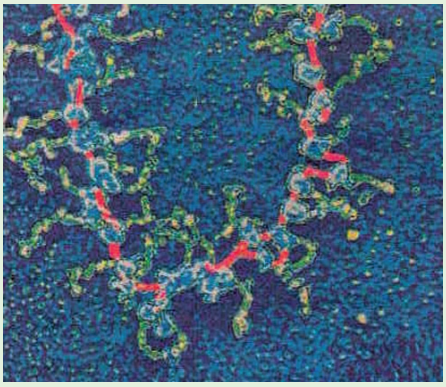


Fig. 2: molecular image of cell showing proteins (green), ribosomes (blue) and mRNA (red)

The important advantage of hyphenated GFC/GPC/SEC experiments lies in the fact that not only a bulk property can be measured (as in the case of batch experiments), but the individual properties of the entire ensemble of macromolecules can be determined in one run while the sample travels through the GPC system. This means that not only the average (bulk) property of the whole sample, but the complete property distribution and its fractions can be measured quickly and reliably.

In order to measure property distributions the GFC/GPC/SEC separation has to be performed properly and with good separation power. This is only the case when the GPC column separates according to size and adsorptive and other non-exclusion effects are absent. In that case there is no or minimal interaction between the stationary phase (column packing) and the analyte/sample. This can be a non-trivial task, especially when samples contain many and different functional groups or charges. Therefore the proper choice of the GPC columns and eluent plays a vital role especially when working with proteins, peptides and nucleic acids. Expert advice can be very useful and save lots of time, energy and frustration.

Column Selection for Proteins

GPC columns used in protein characterization have to meet a number of specific requirements which differentiate them from GPC columns used in other applications. Efficient protein characterization is only successful if the column resolution is optimized for a narrow molecular weight range. This will ensure that proteins can be separated from their aggregates/associates with highest efficiency and purity.

Optimized protein separation columns match the pore size distribution of the column packing with the optimal size range for the protein separation. In such cases the resulting calibration curve will show a small slope and a large separation volume is available for highest resolution, purity and capacity [2].

Column packings with the best results for protein separations are either based on modified silicas or special polymer based packings based on acrylate/methacrylate copolymer networks which are also powerful general use columns for aqueous GPC separations.

Silica based materials, as they are used in PSS Proteema columns, are extremely well suited for protein separations and are available in three molecular weight ranges between 300 g/mol and 1 x 106 g/mol (designated as 100 Å, 300 Å and 1000 Å columns respectively). Silica based columns can only be operated in neutral or acidic conditions, however. Some proteins depending on their isoelectric point require caustic mobile phases. In such cases PSS SUPREMA columns can be used (up to pH 12) and show high stability and performance [2]. Interaction-free chromatographic conditions can be ensured when proteins are analyzed in buffers with the addition of special electrolytes.

MALLS Detection

After the sample has been fractionated by size in the GPC columns the macromolecules enter the light scattering (MALLS) and RI flow cells. In the MALLS detector the excess scattered light is measured at several observation angles (fig. 4). Important parameters like molar mass, size and structural information of the macromolecules can be determined directly from the scattered light intensities without relying on calibration [3]. In comparison to light scattering batch measurements, i. e. without previous GPC separation, this experiment renders not only average parameters (e. g. weight average molecular weigh, z-average radius), but additionally information on the distribution of those parameters in the polydisperse sample. Any light scattering technique requires the accurate knowledge of the specific refractive index increment, dn/dc, which can be regarded as the sample contract factor. Several on-line and off-line techniques for dn/dc determination are available [4].

The light scattering instrument used in this work has a number of specific design advantages making measurements especially easy and precise:

- The compact cylindrical cell design has a small internal volume with homogeneous flow profile eliminating band broadening.
- The light scattering intensity is measured directly inside the LS cell to increase sensitivity

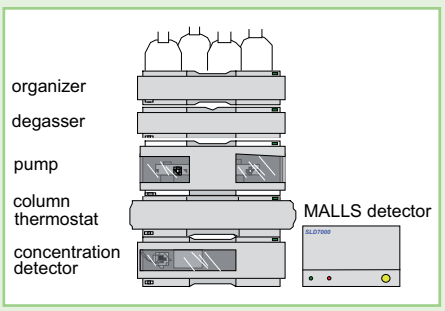


Fig. 3: Setup of a GPC/SEC system with multi-angle light scattering (MALLS)

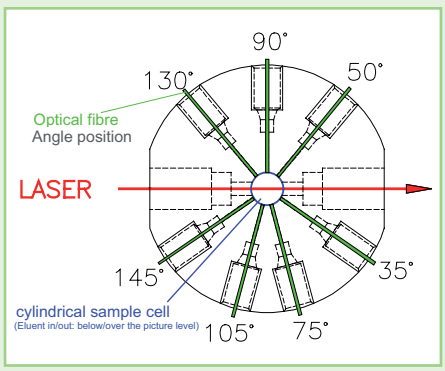
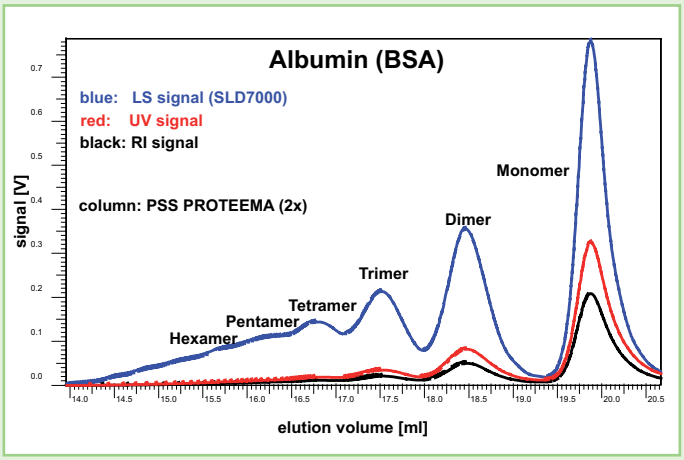


Fig. 4: Patented design [5] of the PSS SLD 7000 light scattering flow cell with simultaneous 7-angle measurement. The cell has cylindrical design with uncompromised measurement of light scattering intensities directly inside the sample solution (top view). Chromatographic flow in this drawing is from bottom to top which prevents artifacts from trapped bubbles and debris.



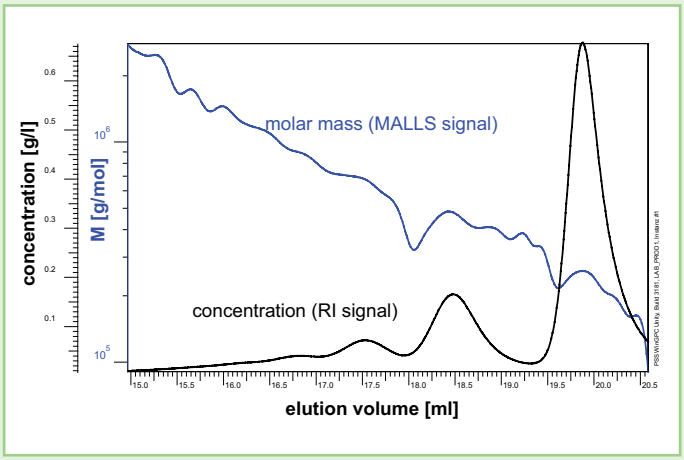


Fig. 5a + b: GPC/SEC of protein associates with effective columns separation and concentration (RI and UV) and MALLS (blue trace) detection (raw data shown in (a)) and molar mass (blue trace in (b)) of all protein associates measured independently by multi-angle light scattering.

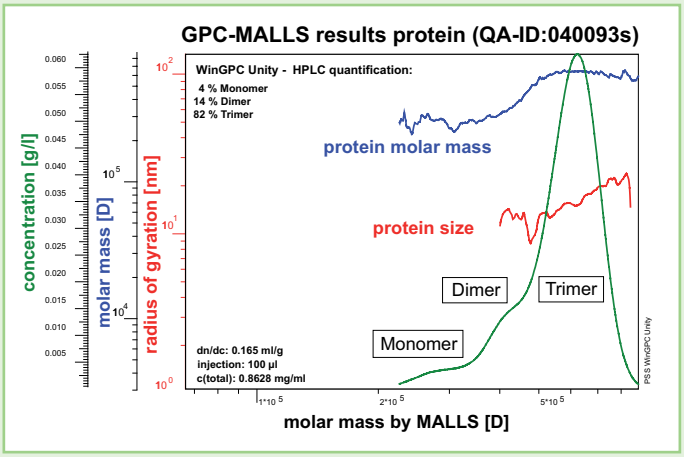


Fig. 6: Simultaneous measurement of concentration, molar mass, size (radius of gyration) and aggregation state for associated protein using GPC-MALLS.

and to eliminate unwanted refraction effects. The observation angle is always identical to the scattering angle.

- Since the cell flow is always from bottom to top no time intensive cell cleaning is required. The cell design forces a constant purging of air bubbles, particles or solvent and sample contaminations making instrument operation easy and low care.

Results and Discussion

The instrument used in this work consisted of an Agilent 1100 GPC/SEC system with:

- Agilent RI and PSS SLD7000 MALLS detector.
- PSS GPC/SEC columns: Suprema 5 µm; 8 x 300 mm; 100 Å, 1000 Å und 10000 Å; or Proteema 5 µm, 8 x 300 mm; 100 Å, 300 Å as indicated in the text.
- All samples were analyzed at 25 °C using aqueous salt and buffers as mobile phase.
- PSS WinGPC Unity with MALLS software module was used for data capture and processing.

Figure 5a shows the formation of associates of a BSA protein as seen by the RI and MALLS detector. The associated are only detected up to the trimer in the RI detector while the molar mass sensitive MALLS detector sees protein species starting from monomer (67 kDa) up to the heptamer (469 kDa).

In figure 5b the molar mass measured in each fraction by the MALLS detector is plotted and shows a very good correlation with the association states of the native protein. The measured molar mass decreases with elution time demonstrating that the column separation really is based on size which is a precondition for proper GPC-MALLS data analysis and correct result interpretation.

This example demonstrates clearly how complex protein association can be and that a perfect match of separation and detection is necessary to discover the otherwise hidden protein structures. Batch mode light scattering is not able to give the same detail of information. It would only show a slight increase in the average BSA molecular weight. In order to determine the formation of protein associates the separation step using a proper GPC/SEC column with high resolution in the investigated molar mass regime is necessary.

Figure 6 shows the results of a GPC/SEC MALLS experiment of another protein with higher molecular weight. Unimer and corresponding protein associates can be separated and molar masses and size (radius of gyration, Rg) can be determined online. The measured Rg in the range of about 12–17 nm with a molar mass of about 600 kDa indicated that the proteins in solution are still in their globular state.

Conclusion and Outlook

GPC/SEC in combination with MALLS detection is very efficient method to determine molar masses and investigate protein structure. Optimization of separation and detection towards the special requirements of protein analysis allow for the qualitative and quantitative description of protein-protein interactions in GPC-MALLS hyphenation experiments. Accurate molecular weights of unimer and associates can be measured in an absolute manner by MALLS. A properly setup GPC/SEC is necessary to obtain best results. PSS Suprema und PSS Proteema GPC/SEC columns have shown to be well suited and reliable in such cases.

In the future protein interaction and protein dimensions will be investigated as a function of temperature, pH, ionic strength in presence of other proteins or ligands. GPC-MALLS results will be compared and correlated with other techniques like dynamic light scattering and GFC/GPC/SEC with viscometry detection which will lead to other important properties like hydrodynamic radii.

References

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