Characterization of Protein Oligomers by Multi-angle Light Scattering

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1 Introduction
Numerous methods for characterizing oligomerization of proteins exist, with each of them having its advantages and limitations. Here, we focus on multi-angle light scattering (MALS), which is one of the most efficient methods for studying the oligomerization of soluble proteins in their native form in solution. MALS can provide many important parameters such as the exact molar mass and size of the protein of interest, its hydrodynamic radius, and additional structural information. Studying protein oligomerization using Light scattering (LS) methods is combined in many cases with chromatographic methods, resulting in accurate characterization of this dynamic process with minimal measurement-related interferences. Here, we describe several light scattering-based techniques combined with separation methods, focusing on the more common method of size exclusion chromatography – multi-angle light scattering (SEC-MALS) and two additional and in some cases complementary methods, ion exchange chromatography and flow field fractionation combined with MALS.

1 INTRODUCTION
Protein oligomerization is a fundamental process in cell biology, where two or more polypeptide chains are interacting, usually in a noncovalent way, to form the active form of the protein. Proteins can form homo or hetero-oligomers. Over 35% of the proteins in the cell are oligomers and over half of them are homodimers or homotetramers (Figure 1).\(^1,2\) Protein oligomerization is at the basis of a large variety of cellular processes\(^3\): Oligomers provide diversity and specificity of many pathways by regulation or activation, including gene expression, activity of enzymes, ion channels, receptors, and cell–cell adhesion processes\(^4–7\). Oligomerization allows proteins to form large structures without increasing the genome size. Smaller surface area of the monomer in a complex can offer protection against denaturation and provide stability\(^1,4,6,8–9\). This stability allows proteins to remain stable even in extreme environments. For example, some hyperthermostable proteins form large oligomers compared to their mesophilic homologs\(^10\). The oligomerization process is dynamic, and in many cases, the protein exists in equilibrium between several oligomeric states that possess different activities. Oligomers may undergo reversible transitions between different conformations, which account for their cooperative binding properties and allosteric mechanisms. For example, binding of the oxygen ligand to hemoglobin causes a change in the conformation of the other subunits, which act cooperatively to adapt an optimized conformation for binding additional oxygen ligands\(^11\). The transition between oligomerization states or conformations is part of the regulation of protein activity. Protein oligomerization can also be regulated by the binding of ATP, metal cofactors or small ligands and partner proteins or peptides\(^12–14\). For example, we developed in our lab the 'shiftide' concept, in which peptides shift the oligomerization equilibrium of a protein to a desired oligomeric state. They do so by preferential binding to a specific oligomeric state, resulting in stabilization of this
protein oligomerization is generally benefi-
cial in health, uncontrolled oligomerization followed
by aggregation can lead to disease. Examples include
several neurodegenerative diseases such as Parkinson’s
and Alzheimer’s. In these diseases, small intermediate
soluble oligomers were found to be toxic. For example,
some oligomeric species of Amyloid-β protein found
in Alzheimer disease are small and soluble enough to
diffuse through the brain parenchyma and affect synaptic
structure and function.\(^{(14,15)}\)

Another classification of oligomers is based on the
quantitative and thermodynamic characteristics of
subunits association, such as the binding affinity and
kinetics between the subunits.\(^{(22)}\) These parameters are
determined by the dissociation constant \(K_d\), which can
range from the subnanomolar/nanomolar range in the
case of strong binding to micromolar or even millimolar
in cases of medium to weak binding between the oligomer
subunits. In the case of a weak binding (high \(K_d\)),
the oligomerization state of the protein is dependent on the
protein concentration and the environmental parameters
such as temperature and pH.\(^{(17)}\) This means that some
oligomers are sensitive to forces applied in common
experiments or to the experimental conditions. This
makes quantitative studies of protein oligomerization far
from trivial.

### 2 METHODS FOR STUDYING PROTEIN
OLIGOMERIZATION

Protein oligomerization can be investigated using a
variety of techniques.\(^{(18-20)}\) Molecular weight-based
methods such as mass spectrometry (MS) and analytical
ultracentrifugation (AUC) are commonly used for this
purpose. One of the most useful methods for quantita-
tive measurement of protein mass and oligomerization
analysis is MS. When protein complexes are examined,
standard MS methods result in their dissociation under
experimental conditions. To keep the complex during
the MS experiment, native MS is usually used combined
with a variety of different strategies like denaturing,
tandem and ion-mobility MS.\(^{(21,22)}\) This means studying
the protein or protein assembly in its native state as it is
prior to the mass analysis. Just before the transition to
gas phase using electrospray ionization (ESI), the protein
is maintained in aqueous, native solution (as opposed
to the common MS analysis which performed in organic
solvents).\(^{(23)}\) This is in contrast to standard MS analysis,
which is carried out in the gas phase, under nonequi-
librium and generally nonnative conditions. In both
cases, the MS detectors are sensitive to salts and high
concentration of detergents, so these additives need to be
removed before MS analysis and thus protein complexes
and unstable proteins cannot be analyzed.\(^{(22,24)}\) In addi-
tion, a homogeneous sample is needed for uninterrupted
MS measurement. Therefore, MS is limited in its ability
for detecting noncovalent protein–protein complexes
and determination of the tertiary or quaternary struc-
ture of a protein under native, equilibrium conditions in
solution.\(^{(25,26)}\)

AUC is another useful quantitative method for char-
acterizing protein oligomerization. Because this method
relies on the fundamental laws of gravitation, AUC can
be used to analyze the solution behavior of a variety
of molecules in a wide range of solvents and solute
concentrations.\(^{(27)}\) However, it has serious limitations
as a tool for routine use because the measurement
procedure is time consuming and the analysis of the
data is complicated. AUC instruments are not widely
available due to the reasons above and their high cost.
Another method used for characterizing the molecular
mass of proteins is Polyacrylamide gel electrophoresis
(PAGE). In its most commonly used form (SDS–PAGE),
the denaturing agent sodium dodecyl sulfate alters the
high-order structure of the protein. When combined with cross-linking reagents, protein oligomerization can be studied using SDS-PAGE. However, this method is qualitative and not quantitative. The cross-linking reaction is sometimes not specific and binds neighboring molecules that do not interact, yielding artificial protein oligomers that lack biological significance. Moreover, cross-linking of biological systems drives them out of equilibrium, stabilizing only a particular oligomeric form. Alternatively, native gel experiments can be used. However, this method is more complicated, indirect, and time-consuming while still providing only qualitative information. It is also difficult to optimize and not very reliable in many cases. LS methods are highly useful for the determination of the molecular mass and oligomerization states of macromolecules. They are applicable over a broad range of molecular weights and variety of solutions. The most significant advantage of the LS method is that various parameters can be measured in solution in a noninvasive manner.

3 LIGHT SCATTERING OF MACROMOLECULES

The measurement of LS of a protein or protein oligomer in solution can provide a lot of information regarding the mass and shape of the protein. Light causes a partial separation of charge when collides with a particle. In the limit where the wavelength of the light is much longer than the physical dimension of the particle, Rayleigh scattering occurs (Figure 2).

The separated charges produce a dipole field, which becomes a source of electromagnetic radiation emitted at the same frequency but different angle than that of the incident light, or the light passing through the solution without molecular interactions. This electromagnetic field is equivalent to the intensity of the measured scattered light (Equation 1).

\[ I_{\text{scattered}} \propto |E|^2 \]  

The more polarizable the particle, the light is separating the charges more easily and thus radiation will increase, resulting in increased scattering. There are two common types of light scattering used for protein studies: static light scattering (SLS) and dynamic light scattering (DLS).

4 STATIC LIGHT SCATTERING

In SLS, the averaged intensity of scattered light is detected over time. Thus, running the sample through the system results in a peak of the LS signal, which is correlated to the concentration of the protein in each fraction of the sample.

In a typical SLS experiment, a high-intensity monochromatic light, most often a laser, is passed through the solution of interest and scattered upon interaction with the measured particles. The electric field of the polarized light beam is measured. Measuring the scattered intensity at 0° scattering angle is the ideal way to obtain the molar mass because at this angle the relationship between the molar mass, concentration, and intensity of scattered light is simple (Equation 4). However, this is impossible as the scattered intensity at this angle is experimentally disrupted by stray light coming from the light source. Alternatively, measuring the LS at a low angle (3–10°) can result in approximate values of the mass. In MALS systems, the scattering is measured in multiple angles >0° using multiple detectors, for example the ‘DAWN HELEOS II’ MALS detector by Wyatt technologies incorporates detectors at eighteen different angles. The precise mass is extrapolated from the higher angle data. Accordingly, the SLS is measured either by low-angle light scattering (LALS) detector (Figure 3a) or MALS system (Figure 3b), which results in a more accurate weight distribution. Fixed-angle (90°) measurement is the simplest experiment and it can give an estimated diffusion coefficient \( D_t \) value. If a more reliable value is needed, variable-angle system is preferred.

Figure 2 Schematic illustration of Rayleigh scattering. The interaction between the light (indicated in black) and particle causes internal vibrations in the same frequency as the electromagnetic radiation of the light. These vibrations scatter some of the light in their direction (red arrows).
The overall measured intensity carries information about the molar mass (Equation 2), while the angular dependence within the horizontal plane carries information about the size of the macromolecule (Equation 4).

4.1 The Dependence of the Static Light Scattering Intensity on the Protein Molar Mass and Concentration

The intensity of scattered light depends on the polarizability of the solute. The polarizability is expressed by the change in the refractive index (RI) of the solution (Δn) with the change in molecular concentration (ΔC). This parameter is called the specific RI increment dn/dC. For proteins, the dn/dC is determined by the amino acids sequence with an average value of 0.186 mL g⁻¹ in aqueous solution, although it can differ in some cases, depending on the amino acid sequence of the protein and the parameters of the solution. This parameter can be extrapolated by measuring the RI in a set of different protein concentrations using a RI detector. When the concentration and the specific RI increment (dn/dC) is known, the molar mass of the protein can be calculated by the measured intensity of the LS (Equation 2).

\[ I(0)_{\text{scattered}} \propto M_w C \left( \frac{dn}{dC} \right)^2 \] (2)

C is the protein concentration (in g mL⁻¹ or mol L⁻¹, depending on the software used for the analysis), which is known and controlled by the user, dn/dC is the RI increment (in mL g⁻¹) that can be measured as mentioned above and \( M_w \) is the molar mass (in g mol⁻¹) that can be calculated from this proportion (Equation 2). The only parameter required for calculating the molecular weight is \( M_w / C \). The scattered light signal is proportional to \( M_w C \). Thus, high concentrations may be required for low molecular weights (Mw < 5000 Da) in order to produce detectable LS signal. The accuracy of mass determination is high as long as the peaks of the tested products are well resolved and integrated and the dn/dC value is accurate. In addition, in order to detect the scattered light at multiple different angles, the wavelength of light should be at least 50 times greater than the size of the scattering macromolecular (e.g. 10 nm for 660 nm wavelength projected light) in order to obtain Rayleigh scattering (isotropic) and not angle-dependent scattering (anisotropic).

4.2 The Dependence of Static Light Scattering Intensity on the Protein Size

Intramolecular interference of particles with size larger than 10 nm (for 660 nm wavelength projected light) leads to anisotropic behavior when decrease in the scattering intensity occurs as the scattering angle increases. Therefore, information about the size and structure can be retrieved from the angular dependence of the scattering intensity alone (Figure 4). The size of the measured particle is defined by \( r_g \), which is the radius of gyration or root mean square (RMS) radius, defined as the mass distribution around the center of mass, weighted by the square of the distance from the center of mass (Equation 3):

\[ \langle R^2 \rangle_g = \frac{\sum r_i^2 m_i}{M} \] (3)

where \( r_i \) is the distance of element \( m_i \) from the center of mass of the molecule with a total mass \( M \). The angular variation of the scattered light is directly related to the size of the molecule by the Rayleigh–Gans–Debye (RGD) equation. Accordingly, the \( r_g \) can be determined only by
the scattering intensity in each angle (Equation 4):

\[ I(\theta)_{\text{scattered}} \propto R(\theta) = kM_w C \left( \frac{dn}{dC} \right)^2 P(\theta)[1 - 2A_2M_wCP(\theta)] \]

\[ k = \left( \frac{4\pi^2n_0^2}{N_A\lambda_0^4} \right) \]

\[ P(\theta) \] is the function that relates the angular variation in terms of scattering intensity to the radius of gyration \( r_g \) of the particle.

When \( P(0^\circ) = 1 \). The second virial coefficient \( (A_2) \) is a thermodynamic term which indicates the nonspecific solvent–solute interactions in (mol/mL)g⁻². The second virial coefficient may be neglected if the concentration is low enough. This is often the case when light scattering is connected to a separation method since the sample is diluted considerably during separation due to band broadening and polydispersity. The measured \( r_g \) may be plotted against the correspondingly measured molar mass to determine the conformation of the sample. For example, two proteins with the same molar mass can either show angular dependency if the protein is more extended with higher \( r_g \) values (>10 nm) or without angular dependency if the protein is globular and small (<10 nm) (Figure 4).

5 DYNAMIC LIGHT SCATTERING

As opposed to SLS, where the light intensity itself is detected at each time point, in DLS, the fluctuations in the intensity of the scattered light within a defined and confined point in space are measured within a short time differential. Random Brownian motion of the scattered macromolecules results in randomness in both, the number of molecules which located within the small volume studied (where the light meets the solution), and the phase of the light scattered from each particle. Smaller particles are moving faster in solution, resulting in more fluctuations in the LS intensity due to rapid changes in the phase of the scattered light and the movement of the particles into and out of the light source. This leads to time-dependent fluctuations in the measured light intensity, which correlates to the diffusion coefficient of the macromolecules. Thus, DLS is used to determine the translational diffusion coefficient \( (D_t) \) that can, in turn, be used to calculate the hydrodynamic radius \( (r_h) \) of the particle (Equation 7). \( r_h \) is defined as the radius of a sphere with the same diffusion coefficient as the measured particle. In dynamic (or Quasi-elastic) light scattering (DLS/QELS) detectors, these fluctuations are measured by a fast photon counter. The measured fluctuations in the LS intensity given in specific time \( (\tau) \) are quantified by the autocorrelation function, which reports how quickly, on average, the light intensity changes with time (Figure 5). The autocorrelation function is defined as:

\[ G^2(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2} \]
original value and the brackets (<> indicate averaging over all \( t \). The correlation function can be analyzed by the equation\(^{(38)}\):

\[
g^{(2)}(\tau) = 1 + \beta e^{-2Dtq^2\tau}
\]

where \( \beta = \langle (I^2) - \langle I \rangle^2 \rangle / \langle I \rangle^2 \) is the amplitude of the correlation function related to the magnitude of the fluctuations in the LS intensity. \( D_t \) is the diffusion coefficient, \( q = \frac{4n_0}{\lambda_0} \sin \frac{\theta}{2} \) is the magnitude of the scattering vector (\( n_0 \) is the RI of the solution, \( \lambda_0 \) is the wavelength of the light source, \( \theta \) is the scattering angle).

Finally, the hydrodynamic radius \( r_h \) can be calculated by the following Stokes-Einstein equation\(^{(38)}\):

\[
r_h = \frac{kT}{6\pi\eta D_t}
\]

where \( k \) is Boltzmann’s constant, \( T \) is the temperature in K, and \( \eta \) is the solvent viscosity. This equation is based on the assumption that the particle is spherical like globular proteins. Different shape models can be used for better fitting of the autocorrelation function. For example, a rod-like shape can be used as a model for alpha-helical proteins.
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6 LIGHT SCATTERING INTENSITY IN OLIGOMERIZATION PROCESS

The observed intensity of the LS depends on coherent and incoherent superposition of the light emitted from a scattering particle and can be used to directly determine the molar mass (Equation 2). When the protein is monomeric, separated scattering centers within each monomer have different Brownian motions but when the monomers oligomerize into one large oligomeric particle, they are moving together. When the centers are separated (monomeric state), incoherency in the measured scattering is observed. This results from phase relationship changes with time between the scattered light from each center. When the particles oligomerize, there is a definite phase relation between the light scattered from each center and the scattered light adds coherently, resulting in increased scattering. For example, the scattering intensity following dimerization is four times higher compared to that of the monomers. However, since the number of particles is only half, the final observed LS intensity doubles (Figure 7).

Structural information can be obtained from the LS dependency on the scattering angle. For example, two particles with the same mass can have a different dependency on the scattering angle if they have different \( r_g \) values. An extended particle can be viewed as having many isotropic scattering centers that cause a larger destructive interference compared to that of a globular particle, leading to more incoherent scattering and to decrease in the intensity (Figure 4).

Light scattering methods are commonly used for calculating the molar mass of proteins and protein oligomers in solution, but these methods are restricted to the characterization of homogenous samples where only one oligomeric state is present. In many cases of protein oligomers, the sample is in equilibrium between several oligomeric species, which cannot be defined by a simple light scattering detecting system. Thus, coupling between chromatographic separation and LS methods is used for optimizing the oligomerization analysis for multispecies systems.

7 CHROMATOGRAPHIC SEPARATION COUPLED WITH MULTI-ANGLE LIGHT SCATTERING

A MALS system can contain SLS and/or DLS detectors, in parallel to other detectors used in the chromatographic system. The UV absorbance detection at 280 nm is the most common strategy for the determination of protein concentration during the separation profile. For polymers without a UV chromophore, the RI can be used as a measure for the concentration. The RI detector is used in protein analysis to measure the RI increment \( dn/dC \) as mentioned earlier. An example of size exclusion chromatography (SEC)-MALS system is presented in Figure 8.

7.1 Size Exclusion Chromatography – Multi-angle Light Scattering

The most common method for characterizing the oligomerization of macromolecules is size exclusion chromatography (SEC) combined with a MALS detector. In SEC, the separation of proteins with different...
Figure 7  Schematic illustration of LS intensity in correlation to the dimerization process. Dimerization of the particles induces better coherency of the scattered light than the monomers, resulting in higher intensity of LS signal. E is the electromagnetic field induced by the LS of each scattering center.

Figure 8  Scheme of size exclusion chromatography – multi-angle light scattering system. All chromatographic MALS systems contain a degasser for preventing bubbles formation, a pump that controls the flow of the buffer in the system and an injection valve for sample loading. Any separation column can be used for sample separation. Here a size exclusion column is shown. Then, the separated fractions are analyzed by the MALS detectors (UV, LS, RI).
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Molar mass of WT
Molar mass of L344A

43±1 kDa
13±1 kDa
27.3±0.5 kDa

Monomer
Tetramer

43.0±0.3 kDa

Tetramer
Dimer

Elution volume (mL)

12.0
10.5
11.5
11.0
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0

Molar mass (kDa)

Normalized intensity

1

LS of L344A p53 CTD
LS of WT p53 CTD

Figure 9 SEC-MALS chromatogram of WT p53 CTD (293–393) and L344A p53 CTD (293–393). 0.5 mg of both proteins were tested using Superdex 75 increase gel filtration column. The Tetramer of the WT p53 CTD, with \( r_h \) of 4.50±0.02 nm, eluted between the monomer and dimer of the mutated p53 CTD that have \( r_h \) values of 3.75±0.03 nm and 4.81±0.08 nm respectively. The \( r_h \) of the oligomers correlates with their elution profiles. LS (red and gray) intensities are normalized. The molar mass of each peak of the WT (dark red) and mutated p53 CTD (black) was calculated by MALS detector.

The hydrodynamic sizes is based on their partial exclusion from the pores of the stationary phase. It is a widely used semi-quantitative analytical tool for estimating molar masses of proteins.\(^{(41)}\) However, it is often inaccurate because the retention time of the macromolecule depends not only on its mass but also on its hydrodynamic radius. Two molecules with the same molar mass will elute at different retention times if one of them is globular, with smaller \( r_h \), and the other is extended. In addition, possible interactions with the stationary phase can result in different retention times for macromolecules with the same mass. The mass determination using SEC is based on a calibration curve of different protein markers. Thus, the structural differences between the markers themselves and the measured protein can result in a misleading calculation of the protein mass.

An example for the advantages of SEC-MALS is an experiment comparing the elution profiles of WT p53 C-terminal domain (CTD, residues 293–393), which forms tetramers, with the mutant L344A p53 CTD (293–393), which cannot undergo tetramerization.\(^{(42)}\) SEC experiments can lead to wrong conclusions because the elution volume of the WT tetramer is between the elution volumes of the mutated monomer and dimer (Figure 9). Combining SEC with MALS, UV and DLS detectors can overcome the limitations of SEC and provide a better analysis of the molar mass, hydrodynamic radius and oligomeric states of proteins in native solution. Measuring the hydrodynamic radius of the peaks explains the SEC results; it revealed that the \( r_h \) of the monomer and dimer of the mutated p53 CTD are 3.75±0.03 nm and 4.81±0.08 nm, respectively, while the \( r_h \) of the WT tetramer is between them with \( r_h = 4.50±0.02 \) nm (Figure 9). The simple operation and data processing within 1 h or less make SEC-MALS practical for product development and quality control in therapeutic manufacture processes.\(^{(25)}\)

The LS signal is highly sensitive to particles present in the solution. The experimental conditions required for a successful SEC-MALS measurement include using a particle-free mobile phase in order to achieve a clean baseline LS signal. The source of the unwanted particles can be for example the column packing material ("column shedding") or the mobile phase itself. Thus, several actions are recommended prior to beginning the experiment: (i) Aqueous mobile phase should be filtered with a 0.1-micro filter after adding salts and before the beginning of the experiment, in order to remove possible particles from the solvents. (ii) The solvent should be pumped through one binary pump (and not a combination of several pumps) in order to avoid fluctuations in the RI detector. (iii) A long (overnight) equilibration of the column is recommended to encourage particle release during column wash and not during the measurement. (iv) Changing or stopping the solvent flow causes particle shedding, so from the first initiation of the flow, the flow rate should be increased or decreased gradually if needed, at a rate of 0.1 mL min\(^{-1}\) per min or less.
Normalization of the system is required for adjusting the signals of all the light detectors to the 90° detector signal. The normalization is performed with an isotropic scatterer \( (r_g < 10 \, \text{nm}) \), soluble in the same mobile phase, which is analyzed under the same conditions planned for the experiment. Afterward, alignment of the system is needed in order to correct the volume contained in the tubes between the LS and concentration detectors. This mechanical segregation of the detectors causes a shift in the detected peaks which needs to be aligned for an accurate mass analysis. Finally, Band broadening correction of the peaks is required for correction of the sample dispersity that occurs during transfer between the UV, MALS, and RI detectors. These equilibration steps (normalization, alignment, and band broadening) should be performed whenever a change is introduced into the system. Such changes may include, for example, a change in the system volume resulting from replacement of the column or one of the system tubes, or a change in the flow rate. Occasionally, the system equilibration needs to be rechecked. Accordingly, before running the sample, validation standards should be examined under the same desired experimental conditions, including the same column and flow rate that will be used for the sample analysis. In aqueous solutions, BSA can be used as a standard because it is monodispersed and can serve as an isotropic scatterer. Example for the analysis of BSA is presented in Figure 10. The resolution of the peaks can be optimized if the SEC separation is performed with ultra-high-performance liquid chromatography (UHPLC) system.\(^{(43)}\)

Despite its advantages, SEC-MALS has several limitations: (i) This technique is based only on size separation, so molecules with the same size cannot be separated and properly analyzed. In addition, most of the analytical SEC columns have limited separation ability due to their short length, which can result in overlapping peaks and make the SEC-MALS analysis much more difficult in such cases. (ii) SEC columns often release particles from the stationary phase. These particles interfere with the light scattering measurements. This requires extensive equilibration of the SEC-MALS. (iii) Separation in analytical SEC is highly influenced by the injection volume and is limited to around four percent of the column volume. This limitation does not exist in other chromatographic techniques. Therefore, in order to obtain a high enough LS signal for MALS analysis, relatively high protein concentrations may be required (mainly for small macromolecules). (iv) The presence of aggregates in the protein peak can alter the calculated molar mass because of the highly intense light scattering signal of the aggregates.\(^{(44)}\) Table 1 compares between SEC and SEC-MALS, highlighting the advantages of coupling the MALS detector to the SEC column.

### 7.2 Ion Exchange – Multi-angle Light Scattering

Ion Exchange (IEX) is separation technique based on the surface charge of the proteins and their ionic interactions with the support matrix.\(^{(45,46)}\) Anion exchange (AIEX) matrices can bind negatively charged proteins and cation exchange (CIEX) matrices bind positively charged proteins. Elution of the proteins is achieved by a linear

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**Figure 10** SEC-MALS of BSA. 0.5 mg BSA was injected to Superdex 200 increase SEC column in 20 mM Tris–HCl buffer pH = 8 with 50 mM NaCl. The UV (blue), RI (green) and LS (red) intensities are normalized.
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Table 1 Comparison between SEC and SEC-MALS

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salt or pH gradient. IEX chromatography is mostly used as an additional purification step for separating the protein of interest from the host cell proteins, aggregates, and other contaminants. IEX columns can separate between different oligomeric states of a protein, protein isoforms, and modified proteins such as glycoproteins. As opposed to SEC, the resolution can be optimized in IEX chromatography by changing several parameters such as the salt composition and concentration, the gradient slope, pH of the buffer and type of ligands and matrix.

Coupling a MALS detector to IEX columns (IEX-MALS) makes it possible to use IEX not only as a separation technique but also as a quantitative analysis technique. For example, the analysis of the Afifavidin protein using IEX-MALS showed a dynamic oligomeric shift upon biotin binding, from octamers in the apo form of the protein into dimers. IEX-MALS can be used not only for analysis of pure samples but also for heterogeneous protein samples. In addition, there is no restriction of the loaded sample volume, in opposite to SEC-MALS. Because the proteins bind to the resin of the column, the loaded volume is unlimited and can be extended up to the maximal amount according to the capacity of the column. Finally, the particle shedding effect is less common in IEX columns because the packed particles of the stationary phase are larger and more stable, leading to a very short equilibration time. This allows quick changes in the experimental conditions, which allow faster and more efficient optimization.

Combining IEX with MALS provides an excellent additional tool for protein characterization and can solve the limitations of SEC-MALS. Two examples for better analysis of protein oligomerization using IEX-MALS compared to SEC-MALS is the extracellular matrix protein Fibronectin and the mutant variant of the Hoefavidin protein. Both proteins eluted from SEC columns as one asymmetric and heterogeneous peak but as several well-defined peaks in AIEX column, which enabled better analysis of the molar masses by MALS.

When designing an IEX-MALS experiment, the isoelectric point (pI) of the protein is required in order to decide which column and which buffer to use in the experiment. For proteins with pI higher than 7, a CIEX chromatography is preferred, with a pH buffer lower than the pI. For proteins with pI lower than 7, an AIEX chromatography is preferred with a pH buffer higher than the pI. Similar to SEC-MALS, normalization and alignment corrections are required in IEX-MALS measurements. These may be performed using any globular isotropic scatterer protein for both AIEX or CIEX. For example, BSA has a pI of 4.7 (in water at 25°C), so it can serve as a validation standard for AIEX-MALS measurements. The same standard BSA run can be used for CIEX-MALS if the system and experimental conditions are the same (including the tubes and flow rate) and the column volume is similar.

The first separation in IEX-MALS experiments is usually based on a linear gradient of salt or pH (Figure 11), which is optimized later by a step of specific salt percentage or pH that results in better separation and resolution of the peaks, as presented. The change in the salt concentration during an IEX-MALS experiment leads to a change of the RI of the solution, and therefore also the RI increment (dn/dC) changes. The RI signal of the salt can be subtracted from the RI measured in the experiment for baseline correction. This can be obtained by measuring the RI of the buffer only (without protein), under exactly the same conditions, and using this run for baseline correction. Significant changes in the salt concentration can affect the detectors normalization of the MALS and can introduce some errors, mainly in the calculation of r_g. This can be solved by using a more than three angles MALS instrument. The correction of the dn/dC value can be performed by the
following equation:

$$\frac{dn}{dC} = \frac{n(\text{protein}) - n(\text{solvent})}{\bar{v}}$$  \hspace{1cm} (8)

$\bar{v}$ is the specific protein volume. An average value for proteins is $0.73 \text{ mL g}^{-1}$.\(^{(56)}\) Increase of 0.00085 in $n(\text{solvent})$, equals to increase of 85 mM NaCl, leading to a decrease of 0.0011 in $dn/dC$.\(^{(57)}\) The changes in RI can be prevented if the two mixed solvents (A and B) are isorefractive. Solvents are considered isorefractive if the difference in their RI is <0.025 units.\(^{(58)}\)

### 7.3 Field-flow Field Fractionation – Multi-angle Light Scattering

In field-flow field fractionation (F4), the separation is based on the Brownian motion and the specific diffusion coefficient of the measured particles.\(^{(59)}\) The sample is running through a thin channel (50–300 μm) by laminar parabolic flow. A perpendicular flow is applied to the channel, going through the channel and out through a semi-permeable membrane, driving the particles towards the channel wall (accumulation wall). Particles with different hydrodynamic radii have different diffusion coefficients and thus obtain different mean distances from the channel wall (<10 μm). Thus, different particles are eluted at different retention times.\(^{(59-61)}\) Smaller species elute first, and larger species elute last based on their hydrodynamic radius (Figure 12). Asymmetrical flow FFF (AF4) is distinct from F4 in the channel setup, revealing only one permeable wall, so that the solution can leave the channel solely via the accumulation wall to generate a cross-flow. This leads to a continuous decrease in the flow velocity of the axial flow while approaching the outlet channel. To compensate for this undesired effect, a trapezoidal channel geometry was invented and represents the favored system today.\(^{(62)}\) Example of separation dependency on the cross-flow is presented with the human serum albumin (HSA). With no cross-flow, the HSA is eluting as one peak in subsequent UV280 detection. The higher the cross-flow, the more efficient the separation is, and the HSA is separated into monomer, dimer, trimer, and higher oligomer fractions.\(^{(62)}\)

An AF4 experiment includes three stages: sample injection, sample focusing, and fractionation. The injection sample volume range between 10 and 100 μL, while the AF4 channel capacities contain between 200 and 1000 μL. Injection of a 100 μL sample takes a considerable part of the channel volume but the focusing step that follows the injection is driving the sample into a narrow position in steady-state equilibrium levels before the beginning of the elution. This results in optimized fractionation quality.\(^{(62)}\) AF4 has several advantages: (i) Variety of mobile phases can be used for different analytes. (ii) Wide
Figure 12 Scheme of F4 system. The sample is injected into thin channel with laminar parabolic flow (black arrows), which is subjected to perpendicular flow (blue arrows) that goes through the channel and out through a semi-permeable membrane (yellow). The crossflow is driving the particles in the sample toward the bottom of the channel (accumulation wall), while the parabolic flow is pushing them out through the outlet pore toward the detector. Small particles (orange) with small $r_h$ and diffusion constant will elute first where larger particles (red) will elute later.

Figure 13 Schematic illustration of the sample focusing process. The main flow in the channel enters from both the inlet and outlet pores and balanced together at a junction point, very close to the injection pore. The crossflow permeates through the membrane and exits from the channel. When the sample is injected, the flow pushes the particles toward the channel bottom wall resulting in a narrow band from which proper separation is possible.

Separation range from small proteins (2 nm) to large particles (100 μm). The lower limit is defined by the cutoff of the accumulation wall membrane, usually 10 kDa. This eliminates the need for multiple columns. Lack of interactions between the analyte and stationary phase, which also minimizes changes in the protein native structure or degradation caused by shear stress. Sample injection is typically performed under focusing conditions that concentrate the sample and enable working with smaller sample amounts. The channel flow is subjected through the inlet and outlet pores, meeting at a junction close to the sample injection pore, going through the bottom membrane. As the sample injected and enters the separation channel, it is focused into a thin band and is concentrated on the surface of the semipermeable membrane lining at the bottom of the channel (Figure 13).

8 CONCLUSIONS

SLS is based on two basic principles: (i) the amount of light scattering is directly proportional to the protein...
Table 2  Comparing the three chromatography methods coupled to MALS

<table>
<thead>
<tr>
<th>Principle of separation</th>
<th>SEC-MALS</th>
<th>IEX-MALS</th>
<th>AF4-MALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass and shape</td>
<td>Mass and shape</td>
<td>Charge</td>
<td>Hydrodynamic radius</td>
</tr>
<tr>
<td>Restricted. Only</td>
<td>Restricted. Only</td>
<td>Varied. Both column and buffer conditions can be changed. For example: Different gradients and steps, gradient slope, pH or salt gradient, salts, buffers, resin particle size, matrices, ligand and its charge (CIEX/AIEX) and column length.</td>
<td>Varied. Flow ratio Representative. Different separation channels, sample volume, flow ratio and rates.</td>
</tr>
<tr>
<td>Improvement of selectivity and resolution</td>
<td>Restricted. Only column-related parameters can be changed for increased resolution. For example: fractionation ranges, resin particle size, matrix or column length.</td>
<td>Varied. Both column and buffer conditions can be changed. For example: Different gradients and steps, gradient slope, pH or salt gradient, salts, buffers, resin particle size, matrices, ligand and its charge (CIEX/AIEX) and column length.</td>
<td>Varied. Flow ratio Representative. Different separation channels, sample volume, flow ratio and rates.</td>
</tr>
<tr>
<td>Sample volume and concentration</td>
<td>Limited volume and concentrated sample</td>
<td>Unlimited volume. Either diluted or concentrated sample</td>
<td>Unlimited volume (from μL to mL).</td>
</tr>
<tr>
<td>Running buffers</td>
<td>Unlimited</td>
<td>Only conditions that allow binding</td>
<td>Unlimited</td>
</tr>
<tr>
<td>Flexibility of changing parameters during the run</td>
<td>Not flexible</td>
<td>Flexible</td>
<td>Partially flexible</td>
</tr>
<tr>
<td>Time require for equilibration</td>
<td>Long because of column shedding</td>
<td>Short</td>
<td>Short</td>
</tr>
<tr>
<td>Analysis using the RI signal</td>
<td>Easy to perform (no change in buffer composition)</td>
<td>More difficult, since the RI signal changes during salt or pH gradients. Requires high sample concentration</td>
<td>Easy to perform (no change in buffer composition)</td>
</tr>
<tr>
<td>Difficult analysis options</td>
<td>Not recommended</td>
<td>Optional. Large amount of protein can be loaded on the column for a better signal.</td>
<td>Optional. Large amount of protein can be loaded for better signal.</td>
</tr>
<tr>
<td>Analysis of small proteins</td>
<td>Not recommended</td>
<td>Optional.</td>
<td>Not recommended</td>
</tr>
<tr>
<td>Analysis of mixtures of proteins with similar molecular weight</td>
<td>Not recommended</td>
<td>Optional.</td>
<td>Not recommended</td>
</tr>
<tr>
<td>Complexity of experiment</td>
<td>Easy</td>
<td>Requires prior optimization or knowledge of conditions</td>
<td>Complex. Requires optimization.</td>
</tr>
</tbody>
</table>
and analysis were performed using the ASTRA 6.1 software (Wyatt Technology). The RI of the solvent was defined as 1.331 and the viscosity was defined as 0.8945 cP (common parameters for PBS buffer at 658.9 nm). dn/dc (RI increment) value for all samples was defined as 0.185 mL·g⁻¹. The columns used were Superdex 200 Increase 10/300 GL (GE) and Superdex 75 10/300 GL (GE).

ACKNOWLEDGMENTS

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ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIEX</td>
<td>Anion Exchange</td>
</tr>
<tr>
<td>AUC</td>
<td>Analytical Ultracentrifugation</td>
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<tr>
<td>CIEX</td>
<td>Cation Exchange</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
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<tr>
<td>GF</td>
<td>Gel Filtration</td>
</tr>
<tr>
<td>HSA</td>
<td>Human Serum Albumin</td>
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<tr>
<td>ISF</td>
<td>Israel Science Foundation</td>
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<tr>
<td>LALS</td>
<td>Low-angle Light Scattering</td>
</tr>
<tr>
<td>LS</td>
<td>Light Scattering</td>
</tr>
<tr>
<td>MALs</td>
<td>Multi-angle Light Scattering</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>RGD</td>
<td>Rayleigh–Gans–Debye</td>
</tr>
<tr>
<td>RI</td>
<td>Refractive Index</td>
</tr>
<tr>
<td>RMS</td>
<td>Root Mean Square</td>
</tr>
<tr>
<td>SEC-MALS</td>
<td>Size Exclusion Chromatography – Multi-angle Light Scattering</td>
</tr>
<tr>
<td>SEC</td>
<td>Size Exclusion Chromatography</td>
</tr>
<tr>
<td>SLS</td>
<td>Static Light Scattering</td>
</tr>
<tr>
<td>UHPLC</td>
<td>Ultra-high-performance Liquid Chromatography</td>
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<td>WT</td>
<td>Wild Type</td>
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