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Static light scattering to characterize membrane proteins in detergent solution

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ABSTRACT

Determination of the oligomeric state or the subunit stoichiometry of integral membrane proteins in detergent solution is notoriously difficult, because the amount of detergent (and lipid) associated with the proteins is usually not known. Only two classical methods (sedimentation equilibrium centrifugation and static light scattering) can measure directly the absolute molecular mass of a protein present in a protein/detergent micelle, without any assumption on the amount of detergent bound, or the shape of the proteins. Here the theoretical background and practical aspects of static light scattering analysis of membrane proteins are reviewed using a number of examples from our lab to highlight potential pitfalls. A brief comparison with sedimentation equilibrium centrifugation is given and a detailed protocol of how we perform light scattering analyses is provided.

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1. Introduction

Determination of the oligomeric state or the subunit stoichiometry of integral membrane proteins in detergent solution is notoriously difficult, because the amount of detergent (and lipid) associated with the proteins is usually not known. The mass ratio of protein to detergent in the protein/detergent micelle can vary dramatically depending on both the protein and the nature of the detergent [1–3] (Table 1). Consequently, methods that infer the molecular mass of proteins (and thereby the subunit stoichiometry) from hydrodynamic properties are not suitable for membrane proteins in detergent micelles. For example, gel filtration or size exclusion chromatography (SEC) is used frequently for molecular mass determinations. The elution volume of a protein from a SEC column depends on the size of the protein in an approximate relation to the Stokes radius [4], with larger molecules eluting earlier than smaller ones. Once a SEC column has been calibrated with standard proteins of known molecular mass and Stokes radius (globular soluble proteins), the elution volumes may be used to estimate the unknown Stokes radii of other proteins. Provided that the proteins have a similar shape (globular) as the standard proteins, the molecular masses are then inferred from the sizes, a procedure that works because the partial specific volumes of all soluble proteins are roughly the same. The method does not work for membrane proteins because of the associated detergent molecules. At best an estimate of the size of the protein/detergent complex would be obtained, but because of the unknown amount of detergent bound to the protein this is uninformative with regard

to the protein molecular mass. To put it bluntly: the elution volume of a membrane from a SEC column should never be used to determine its subunit stoichiometry or oligomeric state, unless the amount of detergent bound is carefully investigated as presented for example by Bamber et al. [1]. Similar limitations apply to all techniques that infer molecular masses from the hydrodynamic properties of a protein, such as sedimentation velocity measurements, native gel electrophoresis, dynamic light scattering and Fluorescence Correlation Spectroscopy. There are, however, two classical methods that—if carried out carefully—can measure directly the absolute molecular mass of a protein present in a protein/detergent micelle, without any assumption about the amount of detergent bound, or the shape of the proteins. These two methods are sedimentation equilibrium centrifugation and static light scattering. Here we will illustrate the use of the latter technique. Because several groups have reviewed the method of static light scattering for protein analysis in recent years [5–11] we will focus specifically on issues related to the analysis of membrane proteins. The relevant theory of static light scattering for membrane protein analysis will be presented, followed by an overview of practical aspects illustrated by a number of examples from our lab. Next, a brief comparison with sedimentation equilibrium centrifugation will be given, and finally a detailed protocol of how we perform scattering light scattering analyses.

2. Theoretical background

2.1. Soluble proteins

To understand how the amount of detergent in the protein/detergent micelles is accounted for in a static light scattering

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Table 1
Associated amounts of detergents with membrane proteins

Detergent ^a	AAC3 (M_{protein} 36 kDa) ^c		YiiP (M_{protein} 68 kDa) ^d	
	Detergent mass (kDa)	δ^b	Detergent mass (kDa)	δ^b
DM	52	1.45	167	2.45
UDM	73	2.03	191	2.81
DDM	86	2.39	233	3.40

^a *n*-decyl- β -D-maltopyranoside (DM), *n*-undecyl- β -D-maltopyranoside (UM), *n*-dodecyl- β -D-maltopyranoside (DDM).

^b Mass ratio detergent:protein (g/g).

^c The yeast mitochondrial ADP/ATP carrier AAC3 (monomeric, 36 kDa). Data taken from [1].

^d The *E. coli* metal transporter YiiP (dimeric, 68 kDa). Data taken from [2].

analysis it is helpful to briefly describe the way the molecular masses of soluble proteins are determined by the technique. Static light scattering of a macromolecular solution is described by the classical Rayleigh relationship [12]:

$$\Delta LS = \left(\frac{I_\theta}{I_0} \right)_{\text{solution}} - \left(\frac{I_\theta}{I_0} \right)_{\text{buffer}} = K(\text{dn/dc})^2 M_W C \quad (1)$$

in which:

- I_θ/I_0 is the ratio of the intensities of the light scattered at angle θ and the incident light. θ is the angle between the direction of the incident and scattered light. ΔLS (Light Scattering) is the excess of light scattered at a given angle θ by the solution containing the scattering protein (subscript 'solution') compared to the light scattered by the buffer without the protein (subscript 'buffer').
- M_W is the molecular mass of the scattering protein, which is to be determined. The subscript 'W' indicates that it is the weight-averaged molecular mass. The weight-averaged molecular mass is equal to the molecular mass for monodisperse samples only, as will be discussed below.
- c is the concentration of the protein (in mg/ml) and can be measured directly using a concentration sensitive detector. For most proteins, the concentration may be determined by measuring the absorption at 280 nm (UV_{280}) if the extinction coefficient is known (A_{280} in $\text{ml mg}^{-1} \text{cm}^{-1}$, see under 'detailed protocol' for information on how to determine protein extinction coefficients):

$$c = \frac{\Delta UV_{280}}{A_{280}} = \frac{UV_{280 \text{ solution}} - UV_{280 \text{ buffer}}}{A_{280}} \text{ at a 1 cm pathlength} \quad (2)$$

- dn/dc is the specific refractive index increment of the protein, which relates changes in the refractive index of the solution to the protein concentration. For the majority of soluble proteins dn/dc has approximately the same value (around 0.187 ml/g, the precise value depending on the buffer composition [13,14]) and is constant over a wide range of the protein concentrations. dn/dc may be determined experimentally using both a differential refractometer, which measures the difference in refractive index (ΔRI) between the macromolecular solution and the buffer, and a concentration sensitive detector:

$$\frac{\text{dn}}{\text{dc}} = \frac{\Delta RI}{(\Delta UV_{280}/A_{280})} = \frac{(RI_{\text{solution}} - RI_{\text{buffer}})A_{280}}{\Delta UV_{280}} \quad (3)$$

If dn/dc is known (from literature or measurement), there is an alternative to Eq. (2) for determination of the protein concentration using the differential refractometer:

$$c = \frac{\Delta RI}{(\text{dn/dc})} \quad (2a)$$

- K is a constant, which depends on the refractive index of the solution without macromolecule (n), the wavelength of the light used (λ_0), the angle between the incident and scattered light (θ) and the distance between the scattering molecule and the detector (r); N_A is Avogadro's number:

$$K = \frac{2\pi^2 n^2}{\lambda_0^4 N_A} \left(\frac{1 + \cos^2 \theta}{r^2} \right) \quad (4)$$

Eq. (1) is an approximation, which holds as long as the solution behaves as ideal, which is a valid assumption for protein concentrations below 0.1 mg/ml (a condition generally met in the experiments described here [6]). Furthermore, the relation holds only if the proteins are small compared to the wavelength of the light (in our case 658 nm) in order not to cause an angular dependency of the light scattering. This condition is satisfied by globular proteins with molecular masses up to several million Dalton, usually well within the range of proteins complexes studied [5,6].

Because dn/dc is known for soluble proteins (or can be measured according to Eq. (3)), their molecular masses may be determined by Eq. (1) using an experimental setup with two detectors: a LS detector and a concentration sensitive detector, which can be either the UV detector (using Eq. (2)) or the RI detector (using Eq. (2a)):

$$M_W = \frac{\Delta LS}{K \left(\frac{\text{dn}}{\text{dc}} \right)^2 \left(\frac{\Delta UV_{280}}{A_{280}} \right)} = \frac{A_{280} \Delta LS}{K \left(\frac{\text{dn}}{\text{dc}} \right)^2 \Delta UV_{280}} \quad (5)$$

or:

$$M_W = \frac{\Delta LS}{K \left(\frac{\text{dn}}{\text{dc}} \right)^2 \left(\frac{\Delta RI}{(\text{dn/dc})} \right)} = \frac{\Delta LS}{K \left(\frac{\text{dn}}{\text{dc}} \right) \Delta RI} \quad (5a)$$

The method that uses Eqs. (5) and (5a) to determine the molecular mass of a protein is known as the *two-detector method* [8,9]. The method can be used to determine the molecular mass of any macromolecule as long as dn/dc is known.

2.2. Membrane proteins in detergent micelles

For membrane proteins in detergent micelles the situation is different because dn/dc is not known:

$$M_{W,\text{complex}} = \frac{\Delta LS}{K(\text{dn/dc})_{\text{complex}}^2 c_{\text{complex}}} \quad (6)$$

in which

- the subscript 'complex' indicates that the quantity relates to the complete protein/detergent micelle.

$$M_{W,\text{complex}} = (1 + \delta) M_{W,\text{protein}} \quad (7)$$

where $M_{W,\text{protein}}$ is the molecular mass (weight-averaged) of the protein component of the protein/detergent micelle (which is what we want to determine), and δ is the amount of detergent associated with the protein in the mixed micelle (in g/g). δ is not known usually.

$$c_{\text{complex}} = (1 + \delta) c_{\text{protein}} \quad (8)$$

c_{complex} is the concentration of the protein/detergent micelle (in mg/ml) and can be measured directly only if the value of δ is known, e.g. by using a concentration sensitive detector in an expression analogous to Eq. (2):

$$c_{\text{complex}} = \frac{\Delta UV_{280}}{A_{280,\text{complex}}} = \frac{\Delta UV_{280}}{\left(\left(\frac{1}{1+\delta}\right)A_{280,\text{protein}} + \left(\frac{\delta}{1+\delta}\right)A_{280,\text{detergent}}\right)} \quad (9)$$

- c_{protein} in Eq. (8) is the concentration of the protein only (weight/volume), excluding the contribution from the detergent. It is often possible to measure c_{protein} directly using an absorption measurement by choosing a wavelength at which the protein but not the detergent absorbs. For many frequently used detergents such as the alkyl maltoside series 280 nm is a good choice, because these detergents are transparent at this wavelength. Combining Eqs. (8) and (9):

$$c_{\text{protein}} = \frac{\Delta UV_{280}}{A_{280,\text{protein}}} \text{ if } A_{280,\text{detergent}} = 0 \quad (10)$$

$$\bullet (dn/dc)_{\text{complex}} = \left(\frac{1}{1+\delta}\right)(dn/dc)_{\text{protein}} + \left(\frac{\delta}{1+\delta}\right)(dn/dc)_{\text{detergent}} \quad (11)$$

The specific refractive index increment of proteins $(dn/dc)_{\text{protein}}$ and detergents $(dn/dc)_{\text{detergent}}$ are usually different, and therefore $(dn/dc)_{\text{complex}}$ can be measured directly only if the amount of detergent in the micelle (δ) is known in an expression analogous to Eq. (3):

$$\begin{aligned} \left(\frac{dn}{dc}\right)_{\text{complex}} &= \frac{A_{280,\text{complex}} \Delta RI}{\Delta UV_{280}} \\ &= \frac{\Delta RI}{\Delta UV_{280}} \left(\left(\frac{1}{1+\delta}\right)A_{280,\text{protein}} + \left(\frac{\delta}{1+\delta}\right)A_{280,\text{detergent}} \right) \end{aligned} \quad (12)$$

Clearly, because the value of δ is usually not known, it is not possible to directly determine the molecular mass of the total protein/detergent complex according to Eq. (6). Fortunately, we are primarily interested in the molecular mass of the protein only ($M_{W,\text{protein}}$) in the protein/detergent micelle, because that will directly reveal the oligomeric state or subunit stoichiometry. To obtain an expression for $M_{W,\text{protein}}$, Eqs. (7), (8), and (11) are inserted into Eq. (6), which is then solved for $M_{W,\text{protein}}$:

$$M_{W,\text{protein}} = \frac{\Delta LS}{K(dn/dc)_{\text{apparent}}^2 c_{\text{protein}}} \quad (13)$$

in which:

- c_{protein} is the concentration of the protein only (weight/volume), excluding the contribution from the detergent (which can be measured directly by Eq. (10)).
 - $(dn/dc)_{\text{apparent}}$ is the apparent refractive index increment, which describes how the refractive index of the solution changes as function of the protein concentration.
 - $\left(\frac{dn}{dc}\right)_{\text{apparent}} = \left(\frac{dn}{dc}\right)_{\text{protein}} + \delta \left(\frac{dn}{dc}\right)_{\text{detergent}}$ (14)
- $(dn/dc)_{\text{apparent}}$ can be measured directly if the detergent does not absorb light at 280 nm:

$$\left(\frac{dn}{dc}\right)_{\text{apparent}} = \frac{\Delta RI}{c_{\text{protein}}} = \frac{A_{280,\text{protein}} * \Delta RI}{\Delta UV_{280}} \quad (15)$$

Inserting Eqs. (10) and (15) in Eq. (13) yields an expression for the protein molecular mass in terms of measurable quantities:

$$\begin{aligned} M_{W,\text{protein}} &= \frac{\Delta LS}{K(A_{280,\text{protein}} \Delta RI / \Delta UV_{280})^2 (\Delta UV_{280} / A_{280,\text{protein}})} \\ &= \frac{\Delta LS * \Delta UV_{280}}{K * A_{280,\text{protein}} (\Delta RI)^2} \end{aligned} \quad (16)$$

Eq. (16) is used to calculate directly the molecular mass of the protein in the protein/detergent micelle. The method is known as the *three-detector method* [7,9]. It is important to note that

the three-detector method allows for determination of the protein molecular mass without any prior knowledge of the dn/dc values of the protein or detergent components in the mixed micelle. The only requirement is that the detergent does not absorb light at the wavelength used for the protein concentration determination (280 nm). The three-detector method also works if in addition to the detergent other molecules are associated with the membrane protein, such as co-purified lipids or attached carbohydrate moieties [7,9]. The only requirement is that these compounds do not interfere with the protein concentration determination, in other words that the absorption measurement at 280 nm is not affected. The unknown amount of detergent (δ) bound to the protein in the protein/detergent micelle may be obtained by combining the Eqs. (14) and (15), if $(dn/dc)_{\text{protein}}$ and $(dn/dc)_{\text{detergent}}$ are known from literature or from measurement (and there are no other compounds associated with the membrane protein).

There are also alternative ways for the calculations, one of which will be described here and is referred to as the *ASTRA method*, because the analysis is done by the frequently used commercial software package ASTRA (Wyatt technologies). The ASTRA method starts with the determination of the value of δ from the refractive index and UV absorption data by combining Eqs. (11) and (12). The method requires that $(dn/dc)_{\text{protein}}$ and $(dn/dc)_{\text{detergent}}$ as well as $A_{280,\text{protein}}$ and $A_{280,\text{detergent}}$ are known:

$$\begin{aligned} \frac{1}{1+\delta} \left(\frac{dn}{dc}\right)_{\text{protein}} + \frac{\delta}{1+\delta} \left(\frac{dn}{dc}\right)_{\text{detergent}} \\ = \frac{\Delta RI}{\Delta UV_{280}} \left(\left(\frac{1}{1+\delta}\right)A_{280,\text{protein}} + \left(\frac{\delta}{1+\delta}\right)A_{280,\text{detergent}} \right) \end{aligned} \quad (17)$$

The equation is solved for δ , the value of which is needed for calculation of $(dn/dc)_{\text{complex}}$ (by Eqs. (11) or (12)). $(dn/dc)_{\text{complex}}$ is used in the two-detector method of Eq. (5a) to determine the molecular mass of the protein/detergent micelle ($M_{W,\text{complex}}$). The value of δ is subsequently used again to determine the contributions of protein and detergent to the molecular mass of the complex using Eq. (7). The disadvantage of the ASTRA method is that the $(dn/dc)_{\text{protein}}$ and $(dn/dc)_{\text{detergent}}$ need to be known in advance. The advantage of the ASTRA method is that in principle it can also deal with detergents that absorb at 280 nm.

Regardless of the calculation method, information is obtained on the molecular mass of the protein in the protein detergent micelle, exactly the information needed.

3. Why couple to size exclusion chromatography?

The measurements of ΔLS , ΔRI and ΔUV could be made in a cuvette in batch, using a solution without the protein as reference to determine the baseline values of the UV, RI and LS signals. However, usually the detectors are connected in-line with a chromatography system and all three signals are measured continuously during a size exclusion chromatography (SEC) run. The method is then referred to as SEC-MALLS (Multi Angle Laser Light Scattering) [10], SEC-LALLS (Low Angle Laser Light Scattering) [7,8] or SEC-LS (Light Scattering) [5,6]. There are two reasons why direct coupling of the measurements to SEC is preferred.

First, the molecular masses determined by light scattering methods are the weight-averaged molecular masses.

$$M_W = \frac{\sum_i c_i M_i}{\sum_i c_i} \quad (18)$$

where c_i is the concentration of component i in the solution and M_i is the molecular mass of component i . If the protein solution is not

monodisperse, e.g. because there are aggregates in the sample—even small amounts—the weight-averaged molecular mass will be different from the protein molecular mass. To illustrate this point an example is shown in Fig. 1A. A solution of the soluble protein bovine serum albumin (BSA) was analyzed by SEC coupled to the three detectors. BSA has a tendency to aggregate, which is clearly visible in the elution profile of the SEC column. The main peak elutes at ~14.5 ml, but peaks eluting earlier are indicative of aggregates. Using formula (5) and (5a) it is possible to determine the molecular mass of the protein at any position in the chromatogram, shown by the thick blue line in Fig. 1. The peaks eluting at ~14.5 ml and ~12.8 ml contain monomeric (66.4 kDa) and dimeric BSA, respectively, and the peaks eluting earlier contain higher aggregates. If the measurement had been made in batch, the weight-averaged

molecular mass of BSA would have been around 85 kDa. It is important to stress that the size exclusion chromatography step is used only to physically separate the different species; the elution volumes are not used in the calculations.

Second, the ΔLS , ΔRI and ΔUV values are the differences in light scattering, refractive index and UV absorption, respectively, between the solutions with and without the protein. It is of crucial importance that the composition of the buffer in which the macromolecule is dissolved is exactly the same as the reference solution without protein, because especially the RI measurements are very sensitive to all solutes/ions. SEC guarantees that the eluting proteins are in the exact same buffer as the reference. In addition, since SEC is an isocratic technique, it allows very accurate determination of the baselines of the three detectors, because signals are

Fig. 1. SEC-LS analysis of standard proteins. (A) SEC-LS analysis of BSA. The column was equilibrated with 20 mM HEPES/KOH, 150 mM NaCl and 0.03% DDM (pH 7.4), and the protein was dissolved in the same buffer. The chromatogram shows the detector readings of the LS, RI and UV detectors in red, green and blue, respectively. The scale for the LS detector is shown in the right-hand axis. The thick blue line indicates the calculated molecular mass of the eluting protein throughout the chromatogram (scale on the left-hand axis). (B) SEC-LS analysis of five monodisperse standard proteins (monomeric BSA (66.4 kDa), tetrameric rabbit muscle aldolase (157 kDa), tetrameric bovine liver catalase (232 kDa), monomeric hen egg ovalbumin (42.6 kDa) and monomeric bovine pancreas RNaseA (13.7 kDa)). The signals from the UV₂₈₀ detector are shown (Catalase in black; Aldolase in blue; BSA in red; Ovalbumin in brown; RNaseA in green). The column was equilibrated with 20 mM HEPES/KOH, 150 mM NaCl (pH 7.4), without detergent. The thick lines indicate the calculated molecular mass of the each eluting protein throughout the peak (scale on the left-hand axis). (C) Illustration of band broadening in the peak of BSA (from B). The chromatogram shows the detector readings of the LS, RI and UV detectors in red, green and blue, respectively. The red and blue traces almost completely superimpose. The thick black and brown lines indicate the calculated molecular mass of BSA throughout the peak using Eqs. (5a) and (5), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

measured continuously, also before and after the eluting peaks. Other chromatographic techniques such as ion exchange or affinity chromatography are less suitable for the analysis, because elution of the proteins from the column requires the buffer composition be changed, which creates gradients in the RI signal that are difficult to correct for.

4. Why use standards?

In theory, static light scattering is a technique that can determine the absolute molecular mass of macromolecules without the need to use standards. Eqs. (5), (5a) and (16) contain measurable quantities and physical constants only. Nonetheless, we always use standard proteins, both in the two- and three-detector analysis and in the ASTRA analysis.

In the three-detector method, the standard proteins (of well defined molecular mass) are used to determine experimentally the value of K in Eq. (16). In doing so we avoid the need to accurately determine the absolute refractive index of the solution (Eq. (4)), and we prevent that small errors in the calibration constants of the instruments are propagated in the molecular mass calculations [6,9,11].

In the ASTRA analysis we use standards for a different reason. The ASTRA software calculates the absolute molecular masses directly from Eq. (5a) and therefore relies not only on well-calibrated detectors, but also on accurate dn/dc values (Eq. (17)). We use standard proteins to determine experimentally $(dn/dc)_{\text{protein}}$, because the value is dependent on the buffer composition [13,14]. If the amount of attached detergent needs to be determined accurately, $(dn/dc)_{\text{detergent}}$ must be determined in the same buffer as used in the measurement too. This can be done off-line with the differential refractometer [15]. The standard proteins also provide quality controls, e.g. to detect problems in the instrument calibrations.

Standard protein must have well-defined molecular masses and must be monodisperse. Fig. 1B shows how five standard proteins (monomeric BSA (66.4 kDa, see below for a remark on binding of detergents to albumins), tetrameric rabbit muscle aldolase (157 kDa), tetrameric bovine liver catalase (232 kDa), monomeric hen egg ovalbumin (42.6 kDa) and monomeric bovine pancreas RNaseA (13.7 kDa)) behave in a SEC-LS analysis. Monodispersity of the standards is very important, and therefore we always run a preparative size exclusion column for each protein to isolate the monodisperse form, which we subsequently use as standard in an analytical run. For example, BSA clearly contains several species and is not monodisperse (Fig. 1A). To use BSA as standard we first isolate the monomeric peak (around 14.5 ml elution volume), and then use it in the SEC-LS analysis (Fig. 1B, red trace), producing a symmetrical peak. Using the ASTRA analysis of Eqs. (17) and (5a), a value for δ of zero (there was no detergent in the analysis of Fig. 1B) and a $(dn/dc)_{\text{protein}}$ value of 0.182 ml/mg, the molecular masses of the standard proteins were calculated throughout the peaks (shown by the thick lines). Clearly, the molecular masses determined by the analysis are in very good agreement with the theoretical masses (less than 5% deviation).

The molecular mass curves in Fig. 1B show a ‘frowning’ behavior through the peaks. This is caused by band broadening between the three detectors, which are connected in the order UV–LS–RI. An enlargement of the chromatogram of BSA (Fig. 1C) shows that very little band broadening takes place between the UV and RI detectors (the traces almost superimpose) because the measuring cell of the UV detector has a small volume (5 μ l), but more severe band broadening occurs between the LS (which has a volume of 70 μ l) and RI detectors, and the broadening is particularly pronounced in the tail of the peaks. To confirm that the frowning was indeed

caused by band broadening effects, we also used Eq. (5) (instead of Eq. (5a)) to calculate the molecular mass throughout the peak (Fig. 1C, black line). Eq. (5) uses the signals from the UV and LS detectors only, and the molecular mass calculation resulted in a much flatter distribution throughout the peak. The mild ‘smiling’ of the molecular mass curve is caused by a small amount of band broadening between the UV and LS detectors. It is important to realize that for determination of membrane protein molecular masses always all three detectors have to be used (Eq. (16)), and therefore the calculations will always be affected by band broadening.

How many standards should be used? The more standard proteins are used, the more accurate the value of K (in the three detector method) or the value of $(dn/dc)_{\text{protein}}$ (in the ASTRA method) will be. We routinely only use BSA and aldolase but if very accurate molecular mass determinations are needed then the use of more standards is advisable. It has been noted that albumins may not be suitable standards because they can bind detergent molecules [11]. However, binding of detergent molecules to standard proteins will not affect the determination of the value of K in the three detector method. For this method the only requirement is that the protein molecular mass is known, the sample is monodisperse, and possible attached detergent/lipid molecules do not interfere with the UV_{280} measurement. Therefore, not only albumins, but also membrane proteins with well defined molecular mass in their detergent micelle are perfectly acceptable standards. For example, Wei et al. [2] used the tetrameric glycerol channel GlpF to determine the value of K . In the ASTRA analysis, the situation is different, because the standards are used to determine the values of $(dn/dc)_{\text{protein}}$ and in this case attached detergent molecules will thwart the determination. Therefore, the applicability of albumins as standards in the ASTRA analysis in the presence of detergent needs to be validated.

The molecular mass determination of RNaseA in Fig. 1B reveals another piece of information that cannot be obtained from SEC alone: the molecular mass distribution across the eluting peak. The curve of the molecular mass throughout the RNaseA peak does not show the frowning behavior, which is observed for the other standard proteins and caused by band broadening. Instead the weight averaged molecular weight decreases from 16 kDa at 17.7 ml elution volume to 13.4 kDa at 18.3 ml. Therefore, even though the protein elutes in a single peak, it is not monodisperse—and not suitable as standard protein.

5. How do detergents and lipids behave in SEC-LS analysis?

Before showing examples of molecular mass determinations of membrane proteins we will first illustrate the behavior of detergent micelles and lipid/detergent micelles in SEC-LS experiments.

To keep membrane proteins in solution all buffers must contain detergent at a concentration slightly above the Critical Micellar Concentration (CMC). Detergent micelles are large molecular assemblies that scatter considerable amounts of light. Therefore, the baseline of the LS detector in a SEC-LS experiment using a buffer containing detergent will be higher than in an experiment using buffers without detergents. Of course, the presence of detergent will not affect the calculations once the baseline has been set correctly, but it is important to realize that negative LS values (below the baseline) can be measured if detergent is depleted.

Fig. 2A shows how micelles of the detergent *n*-dodecyl- β -D-maltopyranoside (DDM) behave on a SEC column. The column was equilibrated with a buffer containing DDM at a concentration slightly above the CMC (0.03%). A solution with a higher concentration of detergent (0.5%) was injected onto the column and the LS, UV and RI signals were recorded. The excess DDM micelles of the

Fig. 2. SEC-LS analysis of detergent micelles and lipids. (A) The column was equilibrated with 20 mM HEPES/KOH, 150 mM NaCl and 0.03% DDM (pH 7.4). A sample of column equilibration buffer with 0.5% DDM was injected. The LS, RI and UV readings are shown in red, green and blue respectively (LS voltage scale on the right-hand axis). The calculated molecular mass of the DDM micelles throughout the peak is indicated by the thick blue line (Molecular mass scale on the left-hand axis). (B) As in panel A but this time the sample injected onto the column did not contain detergent. (C) Four different SEC-LS runs as in panel A of samples containing 0.3% TDM, 0.5% DDM, 0.7% UDM and 0.8% DM injected onto the column equilibrated with 0.01% TDM, 0.03% DDM, 0.1% UM or 0.125% DM, respectively. The traces of the LS detector are shown for TDM, DDM, UDM and DM in red, blue, black and green, respectively. The thick lines indicate the calculated molecular masses of the micelles throughout the peaks (scale on right-hand axis). (D) A sample of column equilibration buffer (as specified for panel A) supplemented with 0.1 mg/ml *E. coli* polar lipids was injected. The traces of the LS (red) and RI (green) detectors (scales on left and right-hand axis, respectively) are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

injected sample eluted from the column at a volume of ~ 15.5 ml as shown by the peaks of the refractive index and the LS signals. The UV detector did not detect the peak because DDM does not absorb at 280 nm. If the dn/dc value of the detergent were known then the two-detector method of Eq. (5a) could be used to calculate the molecular mass of the eluting micelles. Literature values of $(dn/dc)_{\text{DDM}}$ vary between 0.133 and 0.143 depending on the buffer composition. Taking a value of 0.143 the molecular mass of the DDM micelle was calculated to be 66 kDa. dn/dc values of detergents may also be determined experimentally using the differential refractometer off-line [15]. If accurate determination of the micelle size is required it is advisable to do so because the (dn/dc) values are dependent on the exact buffer conditions.

Fig. 2B shows a similar chromatogram, but this time a sample of buffer without detergent was injected onto the column which had been equilibrated in buffer containing 0.03% DDM. At an elution

volume of ~ 15.5 ml the LS and RI detectors now showed negative signals relative to the baseline. Clearly, the depleted micelles eluted at the same volume as excess micelles shown in Fig. 2A. The volume at which excess detergent micelles or depleted micelles elute from the column is important because of possible overlap with the elution volume of the standard proteins. If overlap occurs and the sample of standard protein injected onto the column contains a different concentration of micelles than the column buffer an inaccurate value of K (Eq. (4)) will be obtained, and the calculated protein molecular masses may be far off. In our experience there is always a detectable difference in the detergent concentration between standard protein solutions and the column equilibration buffer, regardless of how carefully the solution was prepared. Micelles of different detergents elute at different volumes from the column (Fig. 2C) and could interfere with different standard protein. Therefore, the standard protein/deter-

gent combination should be chosen so that their peaks are resolved on the column.

If lipids are present in the sample injected onto the column, mixed lipid/detergent micelles will form. Fig. 2D shows the chromatogram of a SEC-LS analysis in which a sample was injected containing column equilibration buffer supplemented with a small amount of lipids (0.1 mg/ml *Escherichia coli* polar lipids). The chromatogram of the RI and LS detectors showed a peak at 15.3 ml immediately followed by a negative peak. Again the UV detector did not detect the peaks. Lipid/detergent micelles are slightly larger assemblies than micelles of detergent only, and therefore they elute earlier (peak at 15.3 ml) from the column. The formation of the lipid/detergent micelles in the injected sample led to a consumption of the empty detergent micelles that were present in the buffer, resulting in a negative peak at 15.6 ml.

6. How do membrane proteins behave in the SEC-LS analysis?

Examples of SEC-LS for three membrane proteins will be given: the glutamate transporter GltT [16], the lactose transporter LacS

[17] and the riboflavin transporter RibU [18]. Other examples can be found in references [2,3,7,19–22]. A monomer of Histidine-tagged GltT from the bacterium *Bacillus stearothermophilus* has a molecular mass of 46.9 kDa calculated from the amino acid sequence. The protein was purified in the detergent DDM in two steps (Ni-sepharose and size exclusion chromatography). Subsequently, the protein was concentrated in a spin-concentrator (cut-off 50 kDa) and the protein was analyzed by SEC-LS. The chromatogram (Fig. 3A) showed a main protein peak (detected by all three detectors) eluting at ~11.5 ml. The molecular mass of the protein ($M_{W,protein}$) in the main peak was calculated using the three-detector method (Eq. (16)) as well as using the ASTRA analysis. Both analyses gave the same result. The protein molecular mass was 154 kDa, confirming that the GltT forms homotrimers [3]. The mass of detergent micelle attached to the protein was determined by calculating δ (from Eqs. (14) and (15), using $(dn/dc)_{protein}$ and $(dn/dc)_{DDM}$ values of 0.182 and 0.143 ml/mg respectively). δ was found to be 0.81 corresponding to an attached detergent micelle of 125 kDa and a total molecular mass of the protein/detergent micelle of 279 kDa. The LS and RI detectors (but not the

Fig. 3. SEC-LS analysis of membrane proteins GltT (A), LacS (B) and RibU (C). The chromatograms show the readings of the LS, RI and UV detectors in red, green and blue, respectively. The scaling for the LS detector readings are shown on the left-hand axes. The thick blue lines indicate the calculated molecular masses of the complete protein/detergent complexes, as well as the contributions of the protein and detergent components throughout the peaks (scales in A and B on the right-hand axes). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

UV detector) showed an additional peak at an elution volume of ~ 15.5 ml. This peak was caused by excess detergent micelles in the injected sample. Empty DDM micelles have a molecular mass of 66 kDa (Fig. 2A) and consequently they were concentrated along with the protein/detergent micelles in the spin concentrator (with a 50 kD cut-off) that was used prior to the SEC-LS analysis. The excess DDM micelles in the injected sample eluted at the expected volume of 15.5 ml (cf. Fig. 2A).

Fig. 3B shows a similar analysis for the Histidine-tagged lactose transporter LacS from *Streptococcus thermophilus*, which has a monomer molecular mass of 72 kDa calculated from the amino acid sequence. Again, the protein was purified in two steps in the detergent DDM. A spin concentrator with a 100 kDa cut-off filter was used to concentrate the protein prior to the SEC-LS analysis. The chromatogram showed a protein peak eluting at ~ 13 ml. The molecular mass of the protein was calculated to be 78 kDa, indicating that the protein was monomeric. Compared to GlT the relative amount of detergent associated with the LacS was much higher (δ was 1.4 corresponding to an associated detergent micelle of 106 kDa), consistent with the notion the amounts of detergent associated with different proteins are highly variable (cf. Table 1). The chromatogram also showed a peak at an elution volume of around 15.5 ml followed by a negative peak, reminiscent of the chromatogram obtained when a lipid solution was injected (Fig. 2D). Apparently, lipid molecules which had remained associated with LacS during the purification procedure leaked off into the buffer of the injected sample, where mixed lipid/detergent micelles formed at the expense of empty detergent micelles. Because the lipid/detergent micelles elute earlier than the empty detergent micelles the chromatogram shows a peak, followed by a negative peak. Delipidation during SEC has been followed in detail by SEC-LS for the metal transporter YiiP [2].

The examples of GlT and LacS show that very little (if any) information about the oligomeric state of a membrane protein is provided by the elution volumes in a SEC experiment. Both proteins elute earlier than catalase (Fig. 1B) but have much lower protein molecular masses. These examples again illustrate that the elution position of a membrane from a size exclusion chromatography column should never be used to determine the subunit stoichiometry or oligomeric state.

The final example is the Riboflavin transporter RibU (calculated molecular mass with a Histidine-tag 24.3 kDa). This small membrane protein was also purified in the detergent DDM in two steps (Ni-Sepharose and size exclusion chromatography) and subsequently analyzed by SEC-LS without prior concentration on a spin concentrator (Fig. 3C). Riboflavin was present in the buffer at a concentration of $1 \mu\text{M}$, which is more than 1000-fold higher than the dissociation constant, thus ensuring saturated binding by RibU and allowing the substrate's absorption at 445 nm to be used for the protein concentration determination [18]. The protein eluted in a single peak at ~ 14 ml, but the protein peak overlapped with the peak of the detergent/lipid micelles (~ 15 ml). Clearly, the resolution of the size exclusion column was not sufficient to separate the protein from the empty detergent and lipid/detergent micelles, thus precluding calculation of the oligomeric state.

7. How accurate is the technique?

The accuracy of SEC-LS for molecular mass determinations has been studied for a large number of soluble proteins by Foltá-Stogniew [6]. The error appeared to be less than 5%, which makes the technique suitable for determination of the oligomeric state homo-oligomers of up to 20 subunits. The error in the determination of the protein molecular mass ($M_{W,\text{protein}}$) of membrane proteins in a protein/lipid/detergent micelle may be slightly larger because three

instead of two detectors are used and the extinction coefficients must be calculated, but membrane protein complexes of up to 10 subunits have been studied [19]. The determination of the attached amounts of detergent are less accurate than the $M_{W,\text{protein}}$ determinations because both $(dn/dc)_{\text{protein}}$ and $(dn/dc)_{\text{detergent}}$ have to be known and their determinations introduce an extra source of error. In addition, there may be other molecules attached to the membrane protein (e.g. lipids or carbohydrates [9]). $(dn/dc)_{\text{lipid}}$ is similar to $(dn/dc)_{\text{detergent}}$ for many phospholipids and detergents and often a single value is taken to calculate the total mass of the attached lipid/detergent micelle [2,3,7]. It is important to note that the determination of the protein molecular mass $M_{W,\text{protein}}$ is not affected by inaccuracies in the determination of the attached mass of detergent/lipid/carbohydrate if the three-detector method is used.

8. How does the technique compare to sedimentation equilibrium centrifugation?

Finally, it is illustrative to make a brief comparison with sedimentation equilibrium centrifugation, the only other well-established technique to determine the absolute molecular mass of proteins in detergent micelles. The accuracy of $M_{W,\text{protein}}$ determinations by both methods is comparable, and both methods determine weight-averaged molecular masses. For sedimentation equilibrium centrifugation, an expression analogous to Eq. (13) may be derived [23]:

$$M_{W,\text{protein}} = \frac{d \ln c_{\text{protein}}}{dr^2} \frac{2RT}{\omega^2 \left(\frac{\partial \rho}{\partial c_{\text{protein}}} \right)_{\mu}} \quad (19)$$

where $M_{W,\text{protein}}$ is the molecular mass (weight averaged) of the protein fraction of the protein/detergent micelle, c_{protein} is the concentration of the protein component (in mg/ml, which can be measured according to Eq. (10)), r is the radial position in the centrifuge tube where the protein concentration c_{protein} is measured, R is the gas constant, T is the temperature and ω is the angular velocity. $(\partial \rho / \partial c_{\text{protein}})_{\mu}$ is the apparent density increment, which describes how the density of the solution changes with the concentration of the protein with all other components in dialysis equilibrium.

In analogy to the apparent refractive index increment $(dn/dc)_{\text{apparent}}$ of Eq. (13) the apparent density increment is the crucial quantity that needs to be determined. Whereas the $(dn/dc)_{\text{apparent}}$ values in a SEC-LS analysis are conveniently determined on-line, the apparent density increment can be measured off-line only, and requires large amounts of protein and extensive dialysis [23]. Therefore SEC-LS is a quicker and more convenient method than sedimentation equilibrium ultracentrifugation. In addition, the coupling of Light Scattering measurements to size exclusion chromatography provides immediate information on the monodispersity of the proteins, which may be used for routine quality control purposes. On the other hand sedimentation equilibrium centrifugation is a true equilibrium technique, whereas size exclusion chromatography is not. Therefore, for proteins undergoing dynamic association/dissociation a full description of the equilibrium constants may be obtained by equilibrium centrifugation only.

9. Conclusion

Size exclusion chromatography coupled to light scattering, absorbance and differential refractive index detectors (SEC-LS) is a technique that allows for the determination of the absolute molecular mass of membrane proteins in protein/detergent/lipid micelles. The technique does not rely on assumptions about the shape of the proteins, nor does it not require prior knowledge of the amount of detergent associated with the protein. The size

exclusion column is used to physically separate aggregates/empty micelles from the protein of interest, and to ensure that the protein is dissolved in the correct buffer. The elution volume is not included in the calculations. The technique provides very similar information as sedimentation equilibrium centrifugation, with similar accuracy of the determined molecular masses. The method is quick (a SEC run takes approximately one hour) and is suitable for routine quality control test of purified membrane proteins.

10. Detailed protocol

10.1. Equipment

Below is a description of the equipment used in our lab, and some alternatives are indicated. For a more comprehensive list of alternatives we refer to Refs. [6,11].

1. HPLC pump. We use an Agilent 1200 series isocratic pump (Agilent Technologies). A pump with low pressure pulsation is required, which can deliver flow rates between 0.2 and 0.7 ml/min during SEC. We have also used the pump of an ÄKTA purifier (GE Healthcare) which worked equally well, but the ÄKTA system is over-specified because the gradient options are not required and a simple isocratic pump without gradient options is sufficient.
2. Vacuum degasser (Biotech model 2003). A vacuum degasser is needed because air bubbles scatter light very effectively, resulting in spikes in the chromatogram. In addition the pump function may be impaired by air bubbles trapped in the pump head.
3. In-line filter. A 0.02 µm pore size Anodisc filter (Millipore) with 2.5 cm diameter in a PEEK filterholder (Wyatt technologies) is present between the pump and the injector. This filter removes any particles that are shed from the pump.
4. A manual injection valve (Rheodyne 9725i (PEEK)) with a 200 µl injection loop. The injector of the ÄKTA purifier works equally well and autosamplers may also be used. The volume of the injected sample should be adjusted to the column size. For good separation a maximum injection volume of about 1% of the column volume is advisable.
5. The column. We routinely use a Superdex 200 10/300 column (GE Healthcare), which has a total volume of 24 ml. An overview of other possible columns and their performances is given in reference [6].
6. Tubing (PEEK). All the post-injection tubing should be of 0.25 mm inner diameter to reduce peak broadening.
7. Absorbance detector (Variable Wavelength Detector (Agilent 1200 series)). We use a semi-micro flowcell (volume 5 µl) to reduce peak broadening. Alternatively, we have used the ÄKTA Purifier UV cell.
8. Light Scattering detector. We use a detector which measures simultaneously at three different angles (minidawn TREOS, Wyatt technologies); the company also produces a similar detector which measures at 18 angles. In the analysis described here there is no angular dependency of the light scattering because the proteins are small compared to the wavelength of the light and measuring at a single angle would be sufficient, but measurements at multiple angles may increase the accuracy. The commercial ASTRA software (Wyatt Technologies) for data analysis requires measurements at more than one angle.
9. A differential refractive index detector. This detector must be the last in line, because it cannot withstand high pressure. We use an Optilab Rex with LED light of 690 nm (Wyatt Technologies).

10. All detectors should be calibrated according to the manufacturer's instructions.

10.2. Reagents

The maltoside detergents *n*-decyl-β-D-maltopyranoside (DM), *n*-undecyl-β-D-maltopyranoside (UM) *n*-dodecyl-β-D-maltopyranoside (DDM) and *n*-tridecyl-β-D-maltopyranoside (TDM) were purchased from Anatrace. *E. coli* polar lipid extract was obtained from Avanti. The standard proteins RNaseA, Catalase and Ovalbumin were from GE Healthcare/Amersham (Gel Filtration calibration kit). All other chemicals as well as the standard proteins BSA and rabbit muscle Aldolase were purchased from Sigma-Aldrich.

10.3. Protein preparation

Histidine tagged LacS ($A_{280} = 1.01 \text{ ml mg}^{-1} \text{ cm}^{-1}$), GltT ($A_{280} = 0.62 \text{ ml mg}^{-1} \text{ cm}^{-1}$) and RibU ($A_{445} = 0.45 \text{ ml mg}^{-1} \text{ cm}^{-1}$) were purified as described using Ni-affinity chromatography [16–18]. The proteins were further purified using size exclusion chromatography in SEC buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.04% DDM, filtered through 0.1 µm pore size VVLP filters (Millipore)) as described for RibU [18]. Spin concentrators (Vivaspin with PES membrane) were used to concentrate the proteins when indicated.

The standard proteins that were used for the molecular mass calculations of the membrane proteins (BSA (monomeric, 66.4 kDa, $A_{280} = 0.65 \text{ ml mg}^{-1} \text{ cm}^{-1}$) and aldolase (tetrameric, 157 kDa $A_{280} = 0.88 \text{ ml mg}^{-1} \text{ cm}^{-1}$)) were dissolved in SEC buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.04% DDM, filtered through 0.1 µm pore size VVLP filters (Millipore)) at a concentration of 4–5 mg/ml. 0.5 ml of the standard protein solution was subjected to a preparative size exclusion chromatography run on a Superdex 200 10/300 column (flow rate 0.5 ml/min). We always do a preparative SEC step before the SEC-LS analysis to isolate the monodisperse peaks of the standard proteins (monomeric BSA, tetrameric aldolase). It is important to choose standard proteins that elute at a volume that is well separated from the elution volume of the detergent micelles (see Figs. 1 and 2).

10.4. The SEC-LS run

The Superdex 200 column used for the SEC-LS analysis was equilibrated with SEC buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.04% DDM, filtered through 0.1 µm pore size VVLP filters (Millipore)) and subsequently the buffer was recirculated through the system for 16 h at 0.5 ml/min. This allowed the buffer to pass several times through the degasser and the pre-injection filter, thereby thoroughly removing air and particles. It also allowed to judge whether the baselines of detectors were stable. During the buffer recirculation the differential refractometer was in the 'purge' position to allow both the reference and the analysis cell to be rinsed extensively. Buffers with detergents produce higher baseline fluctuations than buffers without detergent, both in the RI and in the LS detector. For the RI detector and the SEC buffer specified above we aim to keep the fluctuations below $\Delta RI = 5 \times 10^{-7}$ (ΔRI is the absolute value of the difference in refractive index between the reference and the analysis cell). For the LS detectors (at each of the three angles) we aim to keep the fluctuations not higher than 10-fold the noise when filtered water is used. Purging of the refractometer was switched off, when the baselines were stable and the detector reading was set to zero.

Two hundred micro liters of protein solution (0.2–0.5 mg/ml) was injected and the data from the three detectors were imported

by the ASTRA software package (Wyatt Technologies). The experiments were done at room temperature, but the differential refractometer was precisely thermostatted (at 30 °C) because refractive index measurements are extremely sensitive to temperature fluctuations. It would be even better to temperature control the entire setup (column and all the detectors). Unfortunately, our light scattering and absorption detectors cannot be thermostatted. Tagaki and coworkers have built their own instruments with complete temperature control [7,8].

10.5. Data analysis

We use the ASTRA software package version 5.3.2.10 (Wyatt Technologies) to import the signals from the three detectors. The software also allows for convenient setting of the baselines of the detectors, and automatically corrects for the inter-detector delay volumes (determined from the monodisperse elution peaks of the standard proteins). Then there are two ways to proceed, depending on whether the three-detector method of Eq. (16) or the ASTRA method (Eqs. (17) and (5a)) is used.

For the three-detector methods we export the data to a spreadsheet program. We determine the value of K from the chromatograms of the standard proteins of known molecular mass (from Eq. (16)) using the detector readings at the peak maxima. In case measurements at multiple angles were made the procedure may be repeated for each angle resulting in a more accurate value of K . The value of K is subsequently used to determine the molecular masses of the membrane proteins at their elution peaks, again according to Eq. (16). This calculation requires the extinction coefficients to be known (see below). Finally, the value of δ and the amount of attached detergent are calculated according to Eqs. (14) and (15). For this the dn/dc values of protein and detergent are needed. Although $(dn/dc)_{\text{protein}}$ can be calculated from Eq. (3) using the standard protein peaks we normally use the ASTRA software to do so (see below). $(dn/dc)_{\text{detergent}}$ may be taken from literature or determined off-line [15].

For the molecular mass determination by the ASTRA method we use the 'protein conjugate' module of the ASTRA software package. After defining the peaks and entering the values for $(dn/dc)_{\text{protein}}$, $(dn/dc)_{\text{detergent}}$, $A_{280,\text{protein}}$ and $A_{280,\text{detergent}}$ the program calculates the $M_{W,\text{complex}}$, $M_{W,\text{protein}}$ and $M_{W,\text{detergent}}$ at thin sections throughout the peak. The program also provides information on the monodispersity of the peak. We also use the ASTRA software to determine $(dn/dc)_{\text{protein}}$ from the standard proteins according to Eq. (5a), by adjusting the value until the expected molecular mass is calculated.

10.6. How to determine extinction coefficients?

For homo-oligomeric complexes, the extinction coefficient A_{280} (in $\text{ml mg}^{-1} \text{cm}^{-1}$) may be calculated directly from the amino acid sequence, by first calculating the molar extinction coefficient and then dividing by the molecular mass of a monomer [24]:

$$A_{280} = \frac{5500n_W + 1490n_Y + 125n_{\text{Cysteine}}}{M_{\text{monomer}}} \quad (20)$$

where n_W , n_Y and n_{Cysteine} are the numbers of tryptophans, tyrosines and cystines, respectively, in the amino acid sequence of the monomer, and M_{monomer} is the molecular mass of the monomer calculated from the amino acid sequence.

The ProtParam tool on the ExPASy server (expasy.org) directly calculates A_{280} from the amino acid sequence. It is also possible to determine the extinction coefficient experimentally using quan-

titative amino acid analysis. We have found (for RibU and LacS) that the calculated extinction coefficients are sufficiently accurate and we avoid routine quantitative amino acid analysis.

For hetero-oligomeric complexes of unknown stoichiometry the situation is more complicated. To determine the extinction coefficient $A_{280,\text{heterooligomer}}$, it is necessary to know the subunit stoichiometry beforehand:

$$A_{280,\text{heterooligomer}} = \frac{\sum_i n_i A_{280,i,\text{monomer}} M_{i,\text{monomer}}}{\sum_i n_i M_{i,\text{monomer}}} \quad (21)$$

where $M_{i,\text{monomer}}$ is the molecular mass of a monomer of protein i in the heterooligomeric complex as calculated from the amino acid sequence and $A_{280,i,\text{monomer}}$ is the extinction coefficient of protein i (calculated from Eq. (20)). n_i is the number of proteins i in the complex (the stoichiometric coefficient). Obviously, to determine $A_{280,\text{heterooligomer}}$ we need to know the stoichiometric coefficients, but these are the unknowns that we would like to determine using SEC-LS. The way around this vicious circle is to use a self-consistent method developed by Wen et al. [9]. Various stoichiometries are assumed and the extinction coefficient for each of them is calculated according to Eq. (21). Subsequently, the molecular mass is determined by SEC-LS using each of the calculated extinction coefficients. Finally, the experimentally determined molecular masses are compared with the theoretical molecular masses as calculated from the assumed stoichiometries and the known amino acid sequences. The stoichiometry that gives the best match between the experimental and theoretical molecular mass is taken as the correct one. An example is given by Albright [19].

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