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Article *in* Current protocols in protein science / editorial board, John E. Coligan ... [et al.] · April 2012

DOI: 10.1002/0471140864.ps2905s68 · Source: PubMed

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HPLC-SEC Characterization of Membrane Protein-Detergent Complexes

Alla Korepanova[1] and Edmund D. Matayoshi[2]

[1]Department of Biochemistry, Advanced Technologies, Abbott Laboratories, Abbott Park, Illinois
[2]Department of Structural Biology, Advanced Technologies, Abbott Laboratories, Abbott Park, Illinois

ABSTRACT

Determination of the oligomeric state of integral membrane proteins in detergent solutions is a challenging task because the amount of detergent associated with the protein is typically unknown and unpredictable. Methods that estimate the molecular weight of proteins from their hydrodynamic properties in solution are not suitable for detergent-solubilized membrane proteins. However, size-exclusion chromatography (SEC) performed in combination with analyses of static light scattering (SLS), ultraviolet absorbance (UV), and refractive index (RI) provides a universal method for determination of the molar masses of biopolymers and protein-detergent complexes. The light scattered by a protein is directly proportional to its molecular mass, irrespective of shape, and any additional contributions due to bound detergent molecules can be quantitatively accounted for by the additional combined analysis of ultraviolet absorbance and refractive index information. The primary intention of this unit is to describe how to apply the combination of high-performance liquid chromatography SEC and SLS-UV-RI to evaluate molecular mass and the physicochemical heterogeneity of purified membrane protein-detergent complexes. *Curr. Protoc. Protein Sci.* 68:29.5.1-29.5.12.

Keywords: membrane protein • protein-detergent complexes • static light • scattering • size-exclusion chromatography • HPLC-SEC

BASIC PROTOCOL

Determination of the oligomeric state of integral membrane proteins in detergent solutions is a challenging task because the amount of detergent associated with the protein is unknown. The mass ratio of protein to detergent in a protein-detergent complex (PDC) can vary significantly depending on the protein and the nature of the detergent. Thus, methods that estimate the molecular weight (MW) of proteins from their hydrodynamic properties in solution are not suitable for detergent-solubilized membrane proteins. For example, size-exclusion chromatography (SEC) is used frequently for MW determinations of proteins and assessment of their oligomeric state. Under ideal conditions the elution volume of a protein from SEC column is defined by the protein's hydrodynamic size. The classical SEC method involves calibrating the column with "standard" proteins of known MW values and Stokes radii (globular soluble proteins), which establishes a MW scale used to estimate the MW of an unknown protein from its elution volume.

This method has significant limitations due to the effects of protein shape on the elution volume, as well as from nonspecific interactions between a given protein and the sieving matrix itself. Figure 29.5.1 illustrates a case where protein elution times were poorly correlated with MWs. In this example, a protein with MW of 217 kDa eluted much later than proteins with smaller MWs. Also shown is a reversed order of elution for proteins of MW 143.8 and 148 kDa.

Current Protocols in Protein Science 29.5.1-29.5.12, April 2012
Published online April 2012 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471140864.ps2905s68

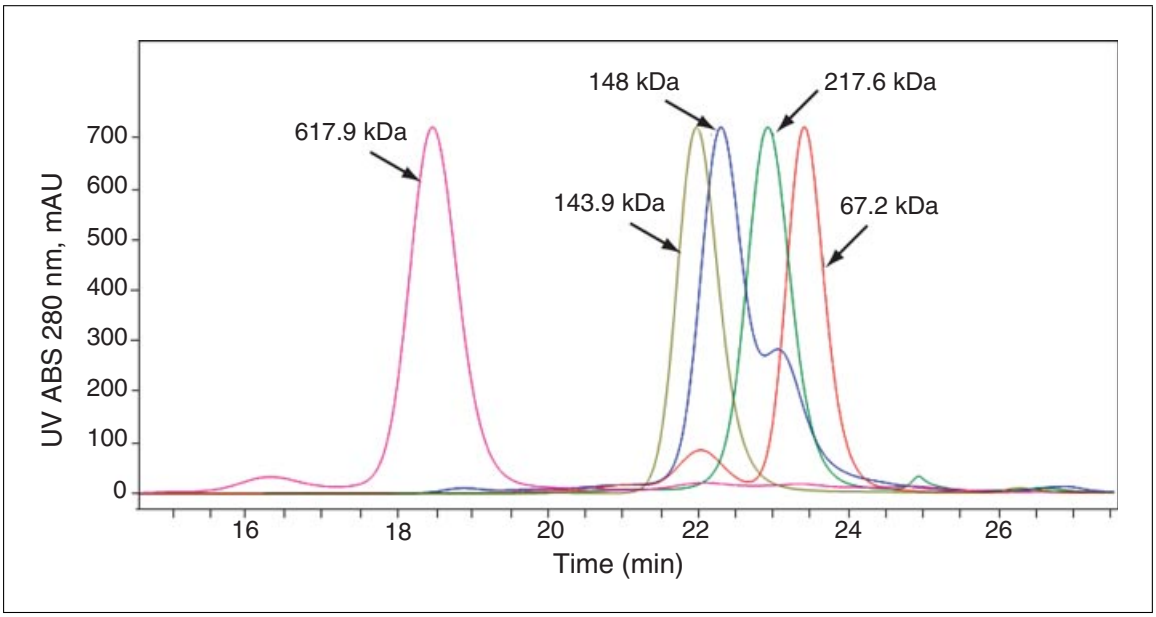

Figure 29.5.1 Size-exclusion chromatography fractionation of several standard proteins. Individual separations were done with a TOSOH G4000SWxl column, 7.8 × 300 mm, run in 0.1 M NaPhosphate, pH 6.6, 0.1 M Na_2SO_4, 0.05 % (w/v) NaN_3, with a flow rate of 0.5 ml/min. Individual chromatograms (normalized, UV absorbance at 280 nm) are superimposed for presentation.

When the standard SEC method is applied to membrane proteins, additional problems are created by the variable amounts of detergent bound to the protein comprising the protein-detergent complex (Moller and le Maire, 1993; Kunji et al., 2008). The elution volume of a membrane protein from an SEC column should never be used to determine its oligomeric state because the amount of bound detergent is a priori unknown and unpredictable.

However, if SEC is performed in conjunction with analyses by static light scattering, ultraviolet absorbance, and refractive index (SEC-SLS-UV-RI), it provides a universal method for determination of the molar masses of biopolymers and PDCs. It is an absolute method because the light scattered by a protein is directly proportional to its molecular mass, irrespective of shape, and any additional contributions due to bound detergent molecules can be quantitatively accounted for by the additional combined analysis of UV and refractive index information. High-performance liquid chromatography (HPLC) is a superior technique for separation of monomeric and oligomeric forms of polypeptides, including membrane proteins.

This unit describes how to combine HPLC-SEC and SLS-UV-RI to evaluate molecular mass and the physicochemical heterogeneity of purified membrane protein-detergent complexes. The general theory and application of SEC-SLS-UV-RI for biopolymers has been reviewed previously (Hayashi et al., 1989; Wyatt, 1991, 1993; Wen et al., 1996; Folta-Stogniew and Williams, 1999; Folta-Stogniew, 2006; Kunji et al., 2008; Slotboom et al., 2008; also see *UNIT 20.6*) and will receive only minimal discussion here. The Basic Protocol describes (1) instrumentation requirements and set up of the system; (2) column and buffer selection; (3) sample preparation, data collection and data processing; and (4) determination of the molar mass of the PDC and its protein and detergent components.

Instruments

High-performance liquid chromatography (HPLC) system: The system should be equipped with a pump providing a stable flow rate of 0.1 to 1 ml/min, and a UV absorbance detector (a diode array detector is not mandatory but advantageous for its

diagnostic utility). An in-line degasser system is highly recommended for minimizing SLS signal noise caused by air bubbles.

Light scattering detector: Although a detector employing a single scattering angle (e.g., 90°) will suffice for the analysis of PDCs with molecular weight up to $\sim 10^6$ Da (wavelength dependent), a multiangle scattering detector is advantageous for optimizing signal-to-noise, versatility in acquisition and interpretation, and applicability to all types of samples. The DAWN HELEOS 18-angle light scattering detector (Wyatt Technology) is recommended while the same manufacturer's TREOS 3-angle light scattering detector represents a less expensive alternative.

Differential refractive index detector: Many commercial units are adequate, but the OPTILAB rEX (Wyatt Technology) is recommended for its sensitivity and unique autoscaling capability.

The data for the examples shown in the current protocol were collected using an SEC-SLS-UV-RI system consisting of an Agilent Model 1100 HPLC system equipped with autosampler and photodiode array UV/VIS detector, and a multiangle SLS detector ("DAWN HELEOS") and differential refractive index detector ("Optilab rEX") from Wyatt Technology.

Software

Software is necessary for instrument control and data acquisition from all three detectors simultaneously. The examples presented here were obtained with two software packages: Chemstation for LC (Agilent) for control of the HPLC system (including absorbance diode array detector), and ASTRA software (Wyatt Technology) for control of SLS and RI detectors and MW analysis based on the three types of collected data. Data processing can be accomplished using a generic spreadsheet; however, specialized software such as ASTRA is superior in its versatility and effectiveness, and is highly recommended.

The example in Figure 29.5.2 shows the data collection window of the ASTRA software with elution profiles of BSA (bovine serum albumin) monitored by SLS (the trace shown corresponds to 90^o scattering), absorbance at 280 nm, and differential refractive index. The ASTRA software can utilize scattering signals collected at multiple angles, and calculates molecular weight at each time slice across any peak or region of the chromatogram where adequate signal from each detector is present. This capability enables a detailed evaluation of mono- or polydispersity of the detergent-solubilized protein with respect to MW.

HPLC-SEC column

Size-exclusion HPLC column: Column selection guidelines are discussed in Critical Parameters and Troubleshooting. An appropriate guard column installed upstream of the SEC column is recommended, and an in-line 1-μm filter installed at the column outlet can be helpful in many instances to counter the effects of column particle shedding (primarily impacting the quality of the SLS data).

Reagents

SEC running buffer: an ionic strength of 0.1 to 0.2 M generally minimizes nonspecific interactions of the protein with the column matrix. The running buffer must contain a detergent known (i.e., determined in advance) to maintain the solubilization of the membrane protein of interest. The HPLC reservoir buffer should be filtered through a 0.22-μm filter. The authors have successfully used SEC-SLS-UV-RI analysis with detergents such as DM (*n*-decyl-β-D-maltopyranoside), DDM (*n*-dodecyl-β-D-maltopyranoside), CYMAL 7 (7-cyclohexyl-1-pentyl-β-D-maltopyranoside), and FOS12, (*n*-dodecylphosphocholine) (all detergents purchased from Anatrace).

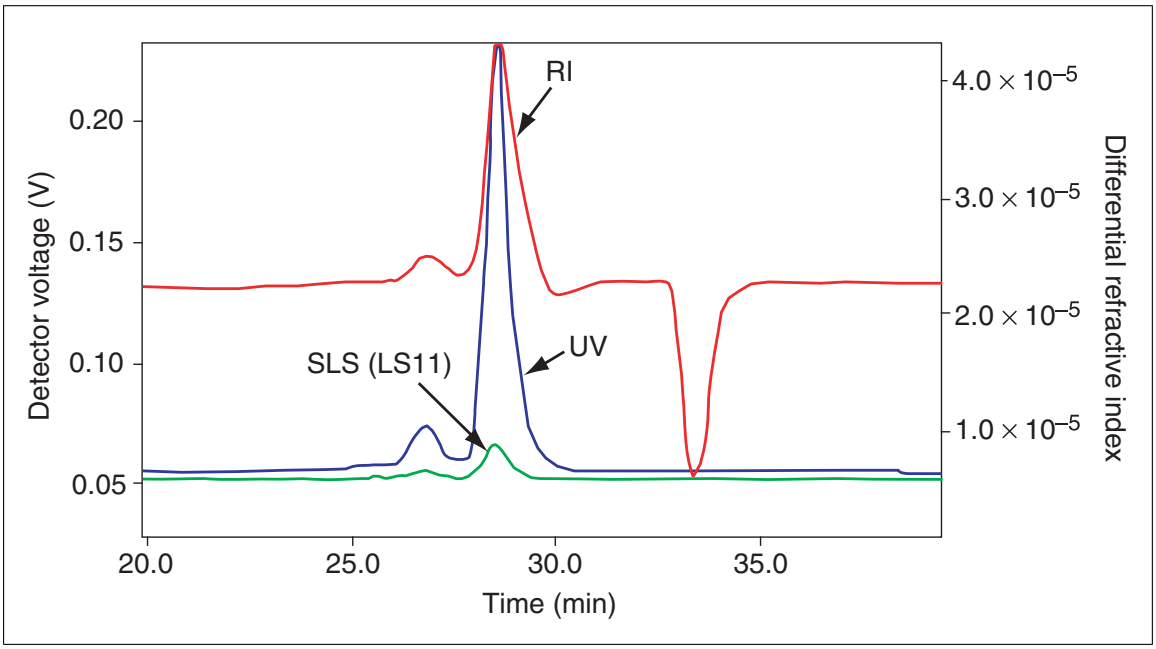

Figure 29.5.2 Data collection window for BSA fractionated in 0.5% (w/v) DDM on a TOSOH G40000SWxl column, 7.8 × 300 mm at 0.4 ml/min. The overlaid real time signals from the SLS ("LS11"-90° detector), UV, and RI detectors are shown.

Protein sample: 20 to 500 µg of protein in a volume corresponding to less than 1% to 2 % (v/v) of the total column volume.

Protein control: bovine serum albumin, BSA, at 1 to 2 mg/ml.

For materials, see above.

Perform chromatography

1. Wash the column for several hours with the detergent-containing mobile phase without connecting it to the detectors (however, see the discussion regarding potential benefit from column pretreatment with buffers containing higher detergent concentration in Critical Parameters and Troubleshooting).

2. Equilibrate the entire system (column in-line with detectors) with the buffer of choice using at least five column volumes, or as dictated by monitoring baseline signal stability at each detector. During the equilibration set the RI detector in the "purge" position. Equilibrate the column at the flow rate that will be used later in protein fractionation, to establish stable baselines in SLS and RI detectors. Monitor the system pressure so as to not exceed maximum pressure specifications for the column or flow cells.

3. Turn on the UV, SLS, and RI detectors and allow sufficient warm-up time for each detector (this should be empirically determined by monitoring the stability of the baselines). If a multiangle SLS detector is employed, the low-angle SLS signals will be more sensitive to the quality of the mobile phase. Monitor the quality of the baselines for at least 30 min to ensure stable, drift-free RI and SLS baselines.

   Making the effort to minimize and stabilize experimental noise in the baselines will enhance the accuracy of the analysis, particularly when injecting small amounts of sample or small proteins that scatter weakly. The ASTRA software allows the user the option of applying post-run filtering of random noise in the SLS data, and selection of a subset of the collected SLS chromatograms (i.e., corresponding to different scattering angles) of sufficient signal-to-noise quality to include in the analysis.

4. Set up the experimental HPLC system run parameters (volume of injection, run duration, spectral parameters for monitoring the elution, etc.). Set up the acquisition parameters for the SLS and RI within the ASTRA software. Select the "trigger upon inject" option to enable parallel recording of UV, SLS and RI data into a single file upon injection of the protein sample into the column.

5. Switch the RI detector from the "purge" mode, and manually "zero" the detector [performed automatically by newer versions of ASTRA (5.5.4) software upon sample injection].

6. Start data collection with the ASTRA software followed by injection of the protein sample.

   As discussed in Critical Parameters and Troubleshooting, it is recommended to run a protein control such as BSA with every new column or run condition.

   An example of the appearance of the BSA control run in the collection window of ASTRA software is shown in Figure 29.5.2.

7. Save the control run data for future diagnostic purposes.

8. Switch the RI detector back to the "purge" mode, and continue running buffer through the system to re-establish stability of all baselines.

9. Repeat steps 5 through 7 with the protein sample.

   Note that the protein of interest should be fractionated in the same buffer and with the same flow rate as the control protein.

Analyze data to determine molecular mass of protein or protein complex with detergent

Start with the analysis of the control protein data. If using ASTRA, a "protein conjugate method" template is provided to help get started. The instruction that follows applies to analysis with the ASTRA software.

10. First, use "baseline" option of the software to optimize the baselines for UV and RI signals, and (in the case of a multi-angle SLS detector) every SLS chromatogram used in the analysis.

11. Apply "despiking" options, if necessary, to manage signal noise.

12. Use "alignment" options of the ASTRA software to generate aligned UV, SLS, and RI chromatograms and determine inter-detector delay volumes.

    The latter will be used when analyzing the protein of interest.

13. Use the "line broadening" protocol in ASTRA software to enable compensating for the different detector flow cell volumes. Two parameters determined during this step, "instrumental term" and "mixing term" will be constants essential to subsequent analyses of protein samples of interest.

14. Select the SLS data sets to be included in the analysis.

15. Select the peak(s) in the chromatogram where protein elutes, and enter the known *dn/dc* values for protein and detergent.

16. Generate a MW ("molar mass") distribution plot.

    Figure 29.5.4 shows an example of a MW distribution plot generated for a BSA control run. For the control protein, the MW across the selected peak (half-maximum region) should not vary by more than ∼5%. The quality of the results obtained with the control protein serves as a check on the validity of the various parameters used in the analysis.

Analyze the protein of interest

17. Optimize the baselines as was done for standard protein (see steps 10 and 11). Do not perform alignment and line broadening steps.

18. Select the SLS detector datasets with adequate signal to noise.

19. Select the chromatographic regions of interest ("peaks") for PDC analysis. Enter the *dn/dc* and values and extinction coefficients for protein and detergent into the table for the selected regions.

20. Enter the various setup parameters obtained from the analysis of the control protein run ("instrumental term," "broadening term," UV-SLS, and SLS-RI inter-detector delay volumes). Run the analysis, and generate a molar mass distribution plot.

COMMENTARY

Background Information

The SEC-SLS-UV-RI method utilizes three detectors placed in line with the chromatography system: detectors of ultraviolet absorption (UV), static light scattering (SLS), and differential refractive index (RI). In this methodology, the function of the selected SEC column is to achieve sufficient spatial and temporal separation of PDCs differing in mass such that small regions of chromatographic slices (i.e., defined ultimately by the detection signal integration interval) correspond to physically monodisperse protein species. Hence, it is not necessary to obtain individual "elution peaks" corresponding to each species present in a physically heterogeneous sample. The SEC column also performs practical tasks, such as efficient exchange of buffer between the mobile phase and sample (essential for accurate extraction of the PDC-specific signal from the solvent background), and filtering particulates that would otherwise render the light scattering measurements unusable.

The SLS analysis calculates weight-averaged molecular masses of species at each point along the SEC chromatogram, without reference to molecular mass standards, and without assumptions about molecular shape. For these calculations, the UV and RI data provide the information required for deconvolving protein and detergent contributions and concentrations. To determine the oligomeric state, the amino acid sequence of the protein is used to define the monomer MW.

The theoretical background for utilizing SLS, UV, and RI data in the analysis of proteins fractionated by SEC has been detailed in previous publications and reviews (Wyatt, 1991, 1993; Folta-Stogniew and Williams, 1999; Kunji et al., 2008) and will not be repeated here. The application of this approach to detergent-solubilized membrane proteins was described initially by Hayashi and coauthors (Hayashi et al., 1989) and subsequently applied to the analysis of both PDC and glycoproteins (Wen et al., 1996; Yernool et al., 2003; Folta-Stogniew, 2006; Slotboom et al., 2008). Briefly, the weight-averaged molecular mass, M_w, is proportional to the light scattering intensity, ΔLS, from the protein or PDC (i.e., in excess over the solvent) by $M_w \propto \Delta LS/[c*(dn/dc)^2]$, where c (g/ml) is the concentration and *dn/dc* (ml/g) is the refractive index increment (instrument constants omitted for clarity). In addition to the SLS detector, key to the PDC analysis is the information provided by two independent concentration sensitive detectors. UV absorbance at 280 nm (A_{280}) and differential refractive index (ΔRI) at the wavelength used for SLS are ideally suited since protein and detergent components contribute additively to the observed signals at each of these detectors:

$$(dn/dc)_{PDC} = f_P * (dn/dc)_P + (1 - f_P) * (dn/dc)_D$$

Equation 29.5.1

$$\varepsilon_{PDC} = f_P * \varepsilon_P + (1 - f_P) * \varepsilon_D$$

Equation 29.5.2

where the subscripts PDC, P, and D refer to PDC, protein, and detergent, respectively, f_P is the mass fraction of the protein in the PDC species, and ε [ml/(g · cm)], the respective extinction coefficient; the corresponding concentrations are simply $c = A_{280}/\varepsilon$ and $c = \Delta RI/(dn/dc)$. Detergents often lack absorbance at the ~280-nm maximum of proteins, and for these cases, $\varepsilon_D = 0$ and the UV signal directly yields the protein concentration alone, further enhancing the accuracy of the analysis.

In the case of nonglycosylated proteins, a value of $(dn/dc)_P$ ~0.185 has been found

by numerous laboratories to be an excellent assumption whenever direct determination is precluded by insufficient availability of purified protein. For membrane glycoproteins, however, this value will be altered (dependent on the degree of glycosylation) and requires modification for the PDC analysis. (Note: Even if sufficient amounts of purified membrane glycoprotein were available, direct experimental determination would usually be impractical because of poor solubility in the absence of detergent.) Since glycoprotein carbohydrates are generally located at the protein surface and exposed to the aqueous solvent, it is reasonable to approximate the modified value, $(dn/dc)_{GP}$, as a linear sum of the mass-weighted contributions from the polypeptide and carbohydrate components:

$$(dn/dc)_{GP} = f_C * (dn/dc)_C + (1 - f_C) * 0.185$$

Equation 29.5.3

where the subscript GP refers to the glycoprotein, f_C is the mass fraction of the glycoprotein due to carbohydrates, and $(dn/dc)_C$ the average refractive index increment for the carbohydrate component. For analysis of detergent-solubilized membrane glycoproteins, $(dn/dc)_{GP}$ will replace $(dn/dc)_P$ in Equation 29.5.1.

The determination of f_C can be accomplished by a combination of several procedures, e.g., by MW determination of the glycoprotein by mass spectrometry (MS) after solubilization of the purified glycoprotein down to a monomeric species with SDS, followed by removal and exchange of the latter with non-interfering agents such as urea (Wisniewski et al., 2009). The difference between the MS-based MW of the glycoprotein and the MW based on the amino acid composition for the nonglycosylated polypeptide thus enables determination of f_C. It should be noted that the glycoprotein MW is generally an ensemble average value representing a distribution of MWs, owing to glycosylation heterogeneity. Likewise, $(dn/dc)_C$ is inherently an average value due to mass and chemical heterogeneity. Standard analytical methods based on glycoprotein digestion and HPLC with or without MS can be used to determine the carbohydrate composition, and an average $(dn/dc)_C$ experimentally measured offline after preparing a mixture of sugars simulating this composition in the buffer used for glycoprotein chromatography (see comments on detergent *dn/dc* determination below in Critical Parameters and Troubleshooting).

Since glycoprotein carbohydrates do not absorb at 280 nm, the molar extinction coefficient is unchanged, but the mass-referenced extinction coefficient ε_P used in Equation 29.5.2 for the pure protein case must be replaced by that for the glycoprotein (ε_{GP}) in accordance with its increased MW:

$$\varepsilon_{GP} = \varepsilon_P * (M_P/M_{GP})$$

Equation 29.5.4

where ε_P is the extinction coefficient [ml/(g · cm] for the pure polypeptide, and M_P and M_{GP} are the molecular weights of the pure polypeptide component and glycoprotein, respectively.

Critical Parameters and Troubleshooting

Instruments

The user should become thoroughly familiar with operation procedures for the system detector components. The SLS and RI detectors should be calibrated according to the protocol provided by manufacturer.

When idle, the system is best maintained by continuously circulating high-purity water containing a bacteriostatic agent such as 0.05% (w/v) sodium azide or 20% (v/v) ethanol through the pumps and detectors.

A series configuration of the detectors is most straightforward and is optimally connected in the following order: UV, SLS, and RI. The order is dictated primarily by the constraints presented by the high pressure tolerances and volumes of the respective flow cells, which differ between detectors. Length and inner diameter of tubing connecting system components should be minimized (within system pressure limitations) to decrease the delay volumes between detectors and experimental band-broadening of the UV, SLS, and RI signals.

Columns

There are many HPLC-SEC columns suitable for SLS analyses and with a broad separation range. Several characteristics that should be considered when selecting the column include column capacity and resolution, protein recovery, potential nonspecific interactions of analyzed protein with column material, column stability in different buffers, and very low shedding of fine particles from the column. For use with detergent-solubilized membrane proteins, it is important to remember that the PDC MW on the column may be significantly higher than the pure protein MW, as a result of the bound detergent.

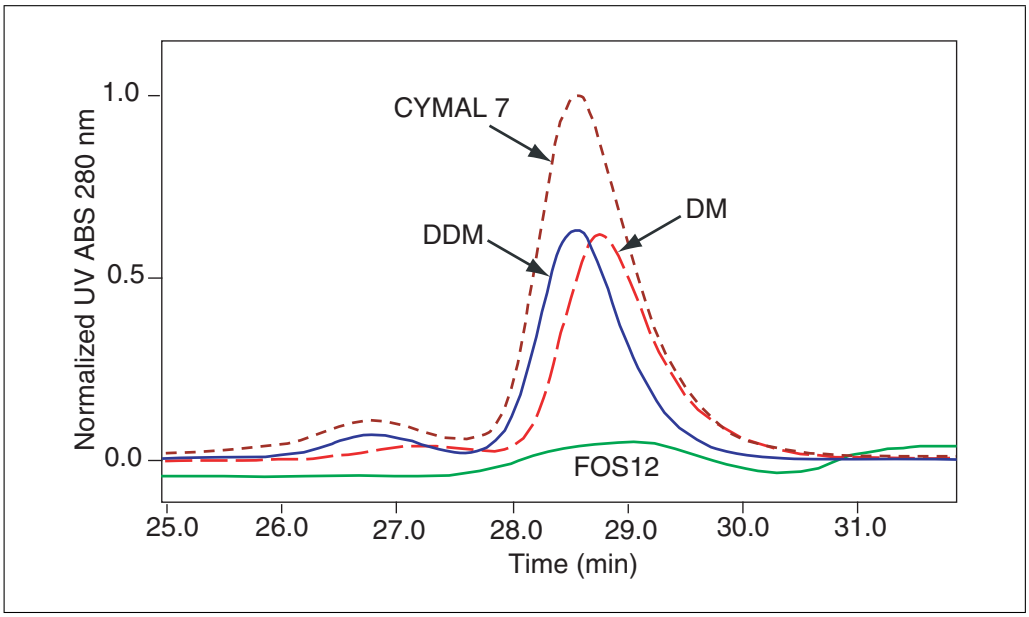

Figure 29.5.3 Optimization of HPLC-SEC: elution profiles of BSA fractionated in buffers with different detergents. A TOSOH G4000SWxl column, 7.8 × 300 mm, was run in buffers with DM (n-decyl-β-D-maltopyranoside), DDM (n-dodecyl-β-D-maltopyranoside), CYMAL 7 (7-cyclohexyl-1-pentyl-β-D-maltopyranoside), and FOS12 (n-dodecylphosphocholine) at 0.4 ml/min. The elution chromatograms are shown as monitored by UV absorbance at 280 nm.

The chemical nature of the column support materials is also important to consider. It has been shown that both polymer- and silica-based columns can perform well for membrane protein fractionation, but no single type of column is universally optimal or adequate for all protein and detergent combinations. Superdex and Superose HR 10/300 series columns have been utilized for many membrane protein fractionations (Folta-Stogniew and Williams, 1999; Yernool et al., 2003). Silica-based columns such as TOSOH TSK gel series that typically provide much higher resolution for SEC in the absence of detergent have also been used successfully in fractionation of several membrane proteins (Winstone et al., 2005; Ravaud et al., 2006). It is recommended to test the performance of the selected column using standard protein in the buffer appropriate for the membrane protein of interest. It is essential to verify for each protein/detergent combination that protein is not retained on the column by measuring the area under the peak(s) in UV ABS 280 chromatogram. As an example, we tested a TOSOH G4000SWxl 7.8 × 300–mm column and several Sepax SEC columns (Sepax Technologies) of similar dimensions, such as SRT-500 and Nanofilm SEC-500, in buffers supporting membrane proteins in solution. We found that the TOSOH column worked well with buffers containing sugar-based detergents such as DM (*n*-decyl-β-D-maltopyranoside) and DDM (*n*-dodecyl-β-D-maltopyranoside), but did not perform well with zwitterionic detergents such as FOS12 (n-dodecylphosphocholine). Figure 29.5.3 shows BSA fractionation on a TOSOH G4000SWxl 7.8 × 300–mm column in buffers containing DM, DDM, and CYMAL 7 (7-Cyclohexyl-1-pentyl-β-D-maltopyranoside). BSA monomer and dimer eluted as well-resolved peaks when fractionation was done in buffers containing DM, DDM, and CYMAL 7 detergents. No separation of BSA monomer and dimer was observed in buffer containing FOS12. In the latter case, BSA recovery was only 10% of the loaded protein, indicating that protein was retained on the column.

In contrast, Sepax SRT and Nanofilm series SEC HPLC columns in our experience proved to be a good choice for FOS12-containing mobile phases. Figure 29.5.4 shows a fractionation of BSA PDCs on a Sepax SEC-500 7.8 × 300–mm column with FOS12, and the quantitative dissection of protein and detergent components. The PDC with a BSA monomer is reasonably resolved from complexes of FOS12 with BSA dimers and higher order oligomers.

The column should be equilibrated with at least two column volumes of the buffer containing detergent. Choice of the column and requirements of the protein will define the amount of the detergent used in the running buffer. It has been shown that detergents like C12E8 (octaethyleneglycol

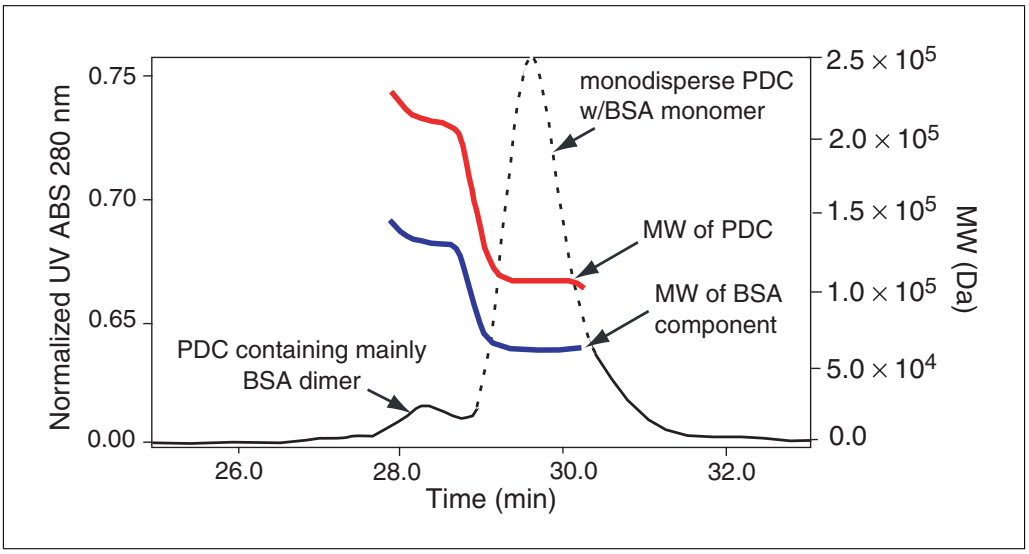

Figure 29.5.4 Analysis of PDCs. The UV 280-nm absorbance chromatogram of BSA in 0.34% (w/v) FOS12 (0.05 M Tris·Cl, pH 8.0, 0.1 M NaCl) is shown. A Sepax Nanofilm SEC-500 column, 7.8 × 300 mm, was used with a flow rate of 0.4 ml/min. The molecular masses of the PDC and its protein component, as obtained from the PDC analysis, are shown as lines superimposed across the eluting peaks.

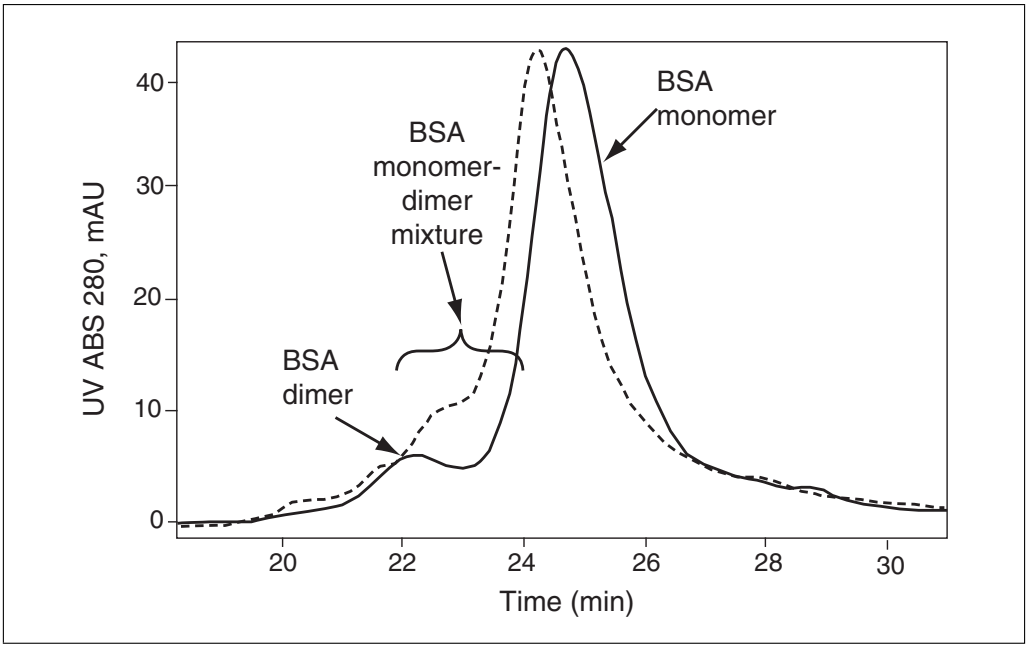

Figure 29.5.5 Optimization of HPLC-SEC: impact of column pre-equilibration with a buffer containing high detergent concentration. A Sepax SRT-500 column, 7.8 × 300 mm, was used for fractionation. Dashed line: column equilibration was done with a buffer containing 0.34% (w/v) FOS12. Solid line: column equilibration was done with a buffer containing 2% (w/v) FOS12. In each case final fractionation was done in 0.34% (w/v) FOS12 containing buffer.

monododecylether), Triton X-100, and DDM bind reversibly to silica columns (Moller and le Maire, 1993). To minimize the likelihood of protein precipitation on the column, the amount of the detergent in the buffer may exceed 2 to 5 times the cmc (critical micelle concentration) value of the detergent normally required to keep the protein in solution (Moller and le Maire, 1993; Winstone et al., 2005). Pre-equilibration of the column with buffer containing higher detergent concentration may help to improve the resolution (Winstone et al., 2005); an example is shown in Figure 29.5.5, where separation of PDCs with BSA monomer and dimer was improved when the column was pre-equilibrated with 2% (w/v) FOS12,

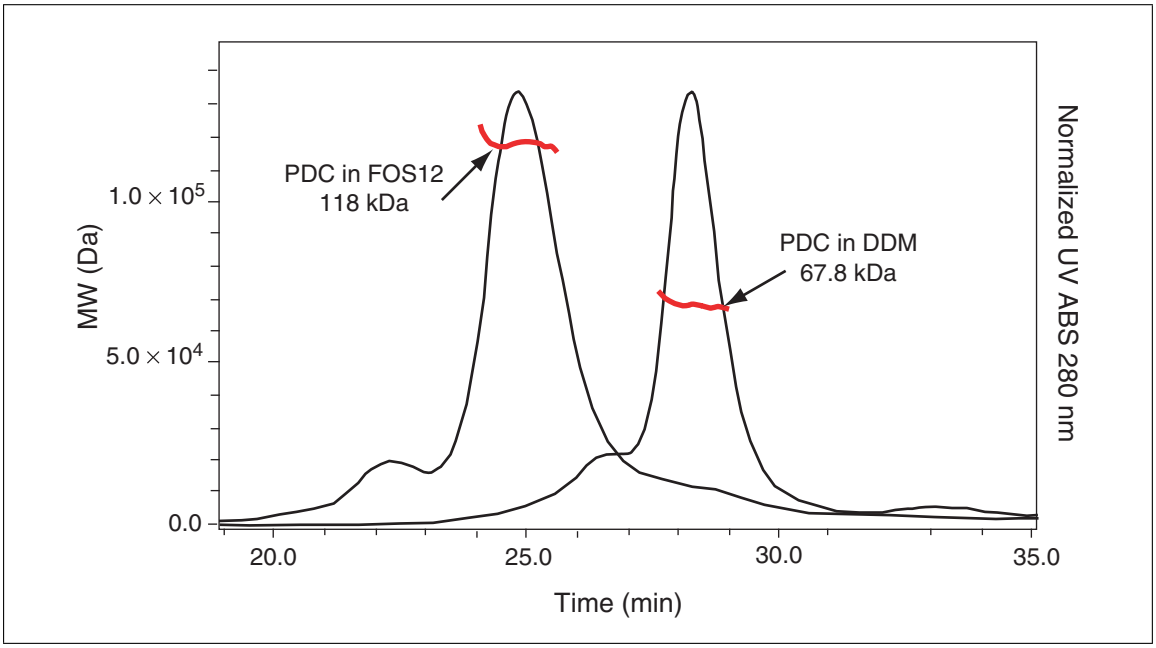

Figure 29.5.6 Optimization of HPLC-SEC: comparative effect of two detergents on the elution profile of a protein. The UV 280-nm absorbance chromatograms of BSA in buffer with 0.5% (w/v) DDM versus 0.34% (w/v) FOS12 are shown, with the corresponding MW distributions of the PDCs across their respective elution peaks superimposed.

followed by equilibration and fractionation in 0.34% (w/v) FOS12. Guard columns should be included in the pre-equilibration protocol.

An additional factor in column selection for detergent applications is the quality of the SLS baseline. Columns may shed particles that create unacceptable levels of noise in the SLS baseline. Baselines should improve after flushing the column with ten to twenty column volumes of buffer. An in-line filter placed after the column can be further helpful in preventing particles from entering the detector flow cell.

Protein standards

In principle, SEC based on an SLS analysis is an "absolute" technique without the need for MW standards. While this is essentially true in practice, we find it useful to regularly run a single protein "control" as a diagnostic aid for monitoring overall system performance and for verifying parameters such as inter-detector volumes and detector calibration constants, which are critical to the accuracy of the PDC analysis. Such a protein control can be selected from the group of protein standards used for traditional SEC calibration without detergents. However, their performance in the presence of different detergents bears careful evaluation (often unpredictable, as noted above). BSA at 1 to 2 mg/ml serves as a good control and diagnostic of column behavior for most column/detergent combinations. The classical separation of its dominant monomer peak (66.7 kD) from the dimer and higher oligomers should be preserved in the presence of bound detergent.

In our studies, BSA has been exploited for PDC method development because of its well-characterized and stable solution properties, its oligomeric forms, and its amphiphilic ligand-binding promiscuity. The latter property in particular makes it an ideal model system for developing a quantitative analysis of detergent binding to proteins, and for the testing of both chromatographic and analytical procedures with a wide variety of detergents.

Protein samples

The maximum injection volume of a protein sample is defined by the column specifications. The amount of the protein also depends on its molecular weight: more sample is required for small proteins than for large proteins because of the large reduction in scattered light intensity. As a general rule, injection of 50 to 100 μg of a protein with MW of 40 to 200 kDa should result in good SLS signal. Proteins with smaller MW would require injection of twice as much protein.

The extinction coefficient of the analyzed protein expressed in terms of weight concentration is required for the analysis by ASTRA software. It can be calculated based on its primary amino acid sequence using ExPASY

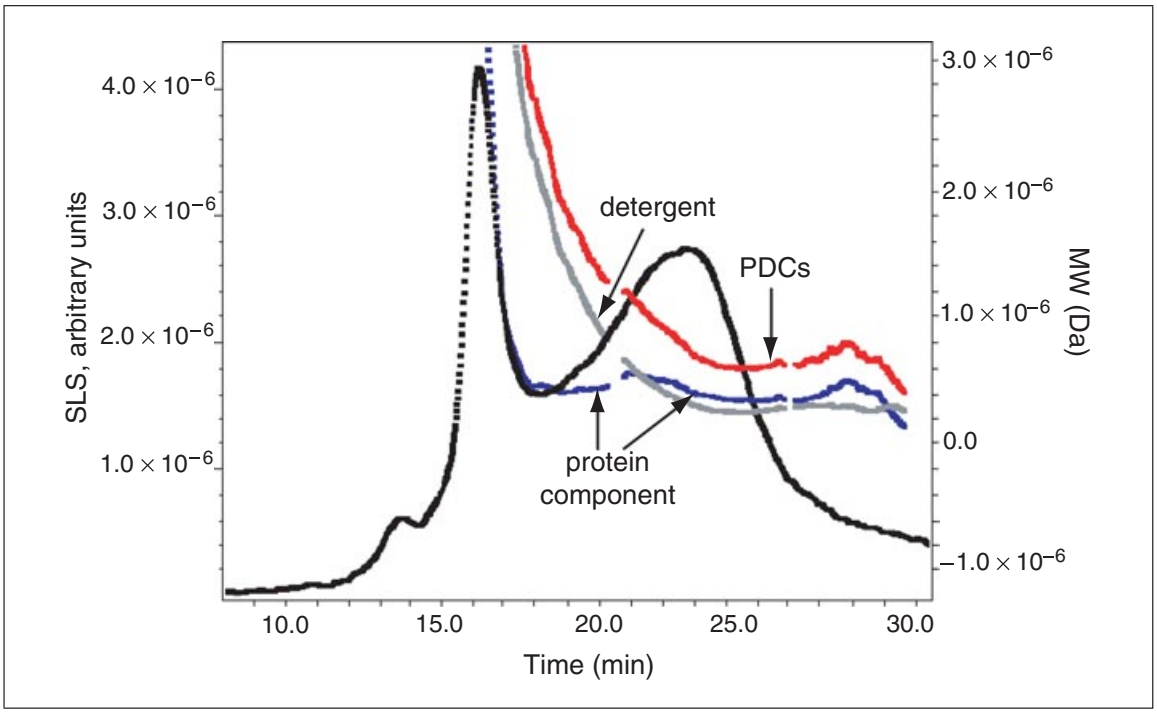

Figure 29.5.7 Example of a (proprietary) membrane protein with a complex MW distribution of PDCs. The elution chromatogram of the protein from Sepax SRT-500 (10-nm beads) column, 7.8 × 300 mm, as monitored by SLS (90° scattering), is shown. MW distributions across the broadly eluting material are shown for PDC, protein, and bound detergent.

Server (*http://ca.expasy.org/tools/protparam.html*) or any other available method (Pace et al., 1995).

The dn/dc value of the protein and detergent

As discussed above, the measured differential refractive index signal from a PDC contains contributions from both protein and bound detergent moieties. The analysis of MW and fractional contributions requires accurate values for both $(dn/dc)_P$ and $(dn/dc)_D$. As noted, a value of 0.185 ml/g for nonglycosylated proteins has been validated for many protein standards. The value for a given glycosylated protein (Kendrick et al., 2001) will be dependent on the composition and relative mass of the carbohydrate component, and can be determined as discussed above.

A broadly cited literature value of 0.134 ml/g for $(dn/dc)_D$ was determined solely for the C12E8 detergent (Hayashi et al. 1989); however, $(dn/dc)_D$ can be expected to vary significantly for different detergents. It can be simply and accurately determined offline (Strop and Brunger, 2005) by flow injection into the differential refractive index detector with a stepped concentration gradient of detergent in the mobile phase buffer. A linear plot of Δn versus concentration yields $(dn/dc)_D$ from the slope.

Analysis of protein-detergent complexes

The ASTRA software contains a fitting protocol with general applicability, which can dissect the potentially varying stoichiometry within complexes (covalent or noncovalent) formed between proteins and other molecules such as detergents, lipids, or sugars, as they elute from an SEC column. Figure 29.5.4 shows an analysis of the PDCs of BSA and the detergent FOS12. The elution profile appears qualitatively similar to that for BSA in the absence of detergent, and the PDC analysis verifies that the dominant peak is indeed monomeric, monodisperse BSA, while the FOS12 bound to the monomer adds ~49 kD to the complex. The PDC analysis also demonstrates quantitatively that the secondary peak eluting in front of the monomer PDC consists of a BSA dimer and bound detergent associated at a similar mass ratio.

This analytical approach is invaluable for a comparative investigation of the interaction between a protein and different detergents. Figure 29.5.6 shows not only that BSA elutes at very different times in buffers containing DDM versus FOS12, but that the dominant species in each case contains the 68-kDa BSA monomer. The PDC analysis furthermore leads to the finding that whereas FOS12 forms a 118-kD complex with the BSA monomer,

DDM does not bind appreciably to BSA under these conditions.

It is expected that detergent-solubilized membrane proteins will frequently consist of a complex heterogeneous distribution of PDCs of varying MW and stoichiometry. It is important to have tools on hand that permit systematic optimization of the choice of detergent and solubilizing conditions. The ASTRA software facilitates this process by enabling rapid analysis of the oligomeric state of PDCs at individual slices across the elution chromatogram. Figure 29.5.7 presents an example of a membrane protein preparation with a complex MW distribution of PDCs, which vary continuously across a broad elution profile. It leads to the conclusion that FOS12 solubilization of this membrane protein produces substantial physical heterogeneity, where protein exists in varying oligomeric/aggregated states with varying mass ratios of bound detergent to protein.

Anticipated Results

Figure 29.5.4 demonstrates a nearly ideal fractionation of a detergent-solubilized protein sample and the application of the UV-SLS-RI analysis in characterizing the resultant PDC species. Figure 29.5.7 further shows the power of the method in assessing a "real" membrane protein sample of great complexity, which is comprised of a broad distribution of PDCs.

Time Considerations

The Basic Protocol can typically be completed in one day with at least two HPLC-SEC runs including the standard protein and the protein of interest. The initial column equilibration can be performed overnight. The time required for detailed data analysis using ASTRA software will depend greatly on sample complexity, but with user experience will not be a major factor in the total time for carrying out experiments. A high-throughput format can also be developed that employs automated injections of multiple protein samples and well-established SEC protocols.

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