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Starting with an Integral Membrane Protein Project for Structural Biology: Production, Purification, Detergent Quantification, and Buffer Optimization—Case Study of the Exporter CntI from *Pseudomonas aeruginosa*

Maxime Mégret-Cavalier, Alexandre Pozza, Quentin Cece, Françoise Bonneté, Isabelle Broutin, and Gilles Phan

Abstract

Production, extraction, purification, and stabilization of integral membrane proteins are key steps for successful structural biology studies, in particular for X-ray crystallography or single particle microscopy. Here, we present the purification protocol of CntI from *Pseudomonas aeruginosa*, a new metallophore exporter of the Drug Metabolite Transporter (DMT) family involved in pseudopaline secretion. Subsequent to CntI purification, we optimized the buffer pH, salts, and additives by differential scanning fluorimetry (DSF), also known as ThermoFluor Assay (TFA) or fluorescent thermal stability assay (FTSA), with the use of dye 1-AnilinoNaphthalene-8-Sulfonic acid (ANS), a fluorescent molecule compatible with detergents. After the buffer optimization, the purified CntI was analyzed by Size Exclusion Chromatography coupled with Multi-Angle Laser Light Scattering (SEC-MALLS), UV absorbance, and Refractive Index detectors, in order to determine the absolute molar mass of the protein–detergent complex, the detergent amount bound to the protein and the amount of protein-free detergent micelles. Altogether, these biophysical techniques give preliminary and mandatory information about the suitability of the purified membrane protein for further biophysical or structural investigations.

Key words Membrane protein, Detergent, Purification, Ultrafiltration, Size exclusion chromatography, Multi-angle light scattering, Differential scanning fluorimetry, Melting temperature, Refractive index

1 Introduction

Membrane proteins are essential in many cellular functions, such as secretion, metabolite transport, ion diffusion, cellular communication, and signaling. In pharmaceutical industry, membrane proteins represent more than 65% of the drug targets [1, 2]. Nevertheless, the number of membrane protein structures in the Protein Data Bank (PDB) is still low (3.8% according to the PDB statistics,

<https://www.rcsb.org/stats>) and grows slowly because of technical difficulties in producing and purifying milligrams of functional and stable membrane proteins for structural studies. Typical reasons are protein overexpression into already crowded biological membrane of the host-cell and stability of the protein extracted from physiological membrane with the help of non-biological surfactants (mainly detergents). Stabilization of the membrane protein in detergent for structural biology is highly empirical [3, 4]. Fortunately, the hindsight of many decades of membrane protein purification and crystallization [5] teaches us to carefully look at the biochemical and structural characterization of the closest protein homologue to start with, in particular for buffer and detergent choices during the first purification attempt. In this chapter, we present the production and purification protocol of the integral membrane protein CntI, the exporter of pseudopaline, a large-spectrum metallophore discovered recently in the pathogen *Pseudomonas aeruginosa* [6]. CntI is a polytopic integral membrane protein of the Drug Metabolite Transporter (DMT) family, made of 284 amino acids (MW = 30.696 Da, calculated pI = 9.45) and predicted with ten transmembrane α -helices. The closest homologue of known 3D-structure is YddG from *Starkeya novella* with 26.7% amino acids identity (MW = 29.452 Da, calculated pI = 9.59), which was successfully extracted and purified in Dodecyl β -Maltoside (DDM) detergent [7]. Because of protein similarities in terms of size, amino acid composition, and calculated charge repartition with YddG (calculated charges at physiological pH are +5.9 and + 5.2 for YddG and CntI, respectively, <https://www.protpi.ch/Calculator/ProteinTool>), we first decided to purify CntI in a similar way. Subsequently, buffer, salts, and additives were optimized by Differential Scanning Fluorimetry (DSF), also known as Thermofluor Assay (TFA) or fluorescent thermal stability assay (FTSA), in order to identify the best buffer compositions that would promote thermal stability of CntI, and likely crystallization. In our case, we used the hydrophobic fluorescent dye 1-anilinonaphthalene-8-sulfonic acid (ANS), which seems to favor interaction with denatured over folded membrane protein–detergent complexes [8]. Usually, excess of ANS is added to the protein at μ M concentration, with protein–dye molar ratio of 1:50 to 1:400. The fluorescence signal of the dye is then monitored (excitation wavelength at 350 nm/emission wavelength at 480 nm) while a temperature gradient is applied to the mixture, usually from 20 °C to 90 °C. With increasing temperature, the protein unfolds and provides hydrophobic patches for dye interaction, which increases the fluorescence signal. The interaction of the dye with the denaturing protein leads to a sigmoidal curve over temperature increase, and the inflexion point of the slope is defined as an apparent melting temperature of the protein (T_m), quantifying the relative thermal stability of the protein.

DSF approach allows us to screen stability effect of different buffer variations such as pH, concentration of salt, type of detergent, or other additives, like glycerol or imidazole used as eluant in the specific case of metal affinity column chromatography purification.

Biochemical analysis of membrane protein molecular weight (MW) could be tricky. Indeed, typical Size Exclusion Chromatography (SEC) analysis, once calibrated with appropriate MW standards, gives an estimation of the protein–detergent complex MW, which varies depending on the class and the amount of associated detergent molecules. Besides, analysis of MW from Sodium Dodecyl Sulfate–Poly Acrylamide Gel Electrophoresis (SDS-PAGE) often shows unexpected migration distance compared to the MW standards, with around 10–30% differences from expected weight [9]. This is generally due to partial unfolding of the membrane protein with the Laemmli sample buffer. Consequently, determining the absolute molecular weight of the protein by triple-detection SEC-Multi Angle Laser Light Scattering (MALLS) analysis is a suitable biophysical approach since it does not rely on the shape of the protein, or the amount of associated detergent. SEC-MALLS analysis consists in separation of protein–detergent complex and protein-free detergent micelles by SEC coupled to triple detection by simultaneous measurements of ultraviolet absorbance at 280 nm (UV_{280}) mainly of the protein, static light scattering at 658 nm at three different angles $\Delta(LS(\theta))$, and refractive index (ΔRI) of both protein and detergent [10]. The light scattering difference between the sample and the buffer is converted into Rayleigh ratio by the Astra software (from Wyatt Technology) using the following equation:

$$R(\theta) = \Delta(LS(\theta)) \cdot \left(\frac{n_0}{n_t}\right)^2 \cdot \left(\frac{R_t}{I_t}\right)$$

in which n_0 and n_t are the refractive index of the buffer and the toluene, respectively, R_t and I_t the Rayleigh ratio and the scattering light intensity of toluene at $\theta = 90^\circ$.

The Astra software allows then to determine the detergent/protein mass ratio (δ) in the protein–detergent complex using the values of protein and detergent refractive index increments, $((dn/dc)_{\text{protein}}; (dn/dc)_{\text{detergent}})$, together with the extinction coefficient of protein and detergent ($A_{280 \text{ protein}}; A_{280 \text{ detergent}}$) with the following formula:

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

The refractive index increment for the protein can be obtained from the SedFit software and for the detergent directly from batch measurements using the refractometer.

In this formula, the left-hand side term corresponds to the protein–detergent complex refractive index increment:

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

while the right-hand side term corresponds to the protein–detergent complex refractive index increment, using the experimental values of UV_{280} , ΔRI , and both protein and detergent extinction coefficients:

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

The calculated Rayleigh ratio $R(\theta)$ and the other parameters ($(dn/dc)_{\text{complex}}$ and c_{complex}) are therefore used to determine the absolute molar mass of the protein–detergent complex following the formula:

$$MW_{\text{complex}} = \frac{R(\theta)}{k \cdot \left(\frac{dn}{dc}\right)_{\text{complex}}^2 \cdot c_{\text{complex}}}$$

where c_{complex} is the concentration of the protein–detergent complex and k an apparatus constant. Finally, the absolute protein molar mass is determined with the formula:

$$MW_{\text{protein}} = \frac{MW_{\text{complex}}}{(1+\delta)}$$

Finally, protein concentration, usually around 10–20 mg/mL, is necessary for structural biology, in particular for crystallization. Ultrafiltration by medium speed centrifugation is commonly used to concentrate the protein. Nevertheless, depending on the type of membrane filter materials (cellulose triacetate or polyether-sulfone), molecular weight cut-off, geometry (dead-end versus cross flow filtration) and centrifugation speed (500–5000 g), protein-free detergent micelles could dramatically accumulate and possibly influence the crystallization process, or also destabilize and aggregate the protein [11, 12]. An accurate quantification of detergent micelles is essential for further structural studies, but depends on specific state-of-the-art technologies, such as mass spectrometry [13]. Here, we took advantage of SEC-MALLS analysis to quantify protein-free detergent micelles after linear correlation of the integrated refractive index (ΔRI) and the quantity of DDM [14].

2 Materials

2.1 Bacterial Culture and Cntl Production

1. *E. coli* strain C43 (DE3) deleted from the *acrb* gene ($\Delta acrb$) transformed with pET-TEV expression plasmid, kanamycin resistant, with T7 inducible promoter of recombinant Cntl (with eight His N-terminal tag followed by a TEV protease cleavage site) (*see Note 1*).
2. Six flasks of 2.5 L volume size containing 1 L of sterilized Terrific Broth (TB) media: 12 g/L tryptone, 24 g/L yeast extract, 5 g/L glycerol, and 89 mM phosphate buffer (17 mM KH_2PO_4 and 72 mM K_2HPO_4).
3. 1 M isopropyl β -D-1-thiogalactopyranoside (IPTG) and 50 mg/mL kanamycin.
4. Orbital incubator shaker with capacity for at least six flasks of 1 L.

2.2 Bacterial Cell Lysis and Membrane Isolation

1. 100 mL of bacterial lysis buffer: 20 mM HEPES, pH 7.5, 200 mM NaCl, EDTA-free protease inhibitor cocktail tablet, and 250 U/ μL benzonase.
2. Centrifuge with rotor suitable for 50 mL conical plastic tubes.
3. Ultracentrifuge with rotor Ti 45 (Beckman).
4. Two polycarbonate bottles of 70 mL (Beckman).
5. Four 50 mL conical bottom tubes.
6. Cell disruptor (CellD, Constant Systems Ltd. or equivalent).
7. Protein assay BiCinchoninic Acid (BCA) kit for total protein quantification (e.g., QuantiPro™ BCA Assay Kit, Sigma-Aldrich).

2.3 Solubilization of Cntl with Detergent

1. 1 L of solubilization buffer: 20 mM HEPES, pH 8, 200 mM NaCl, 1% (w/v) n-Dodecyl- β -D-Maltopyranoside (DDM), 10 mM imidazole, pH 8, EDTA-free protease inhibitor cocktail (to be added extemporaneously), 10% glycerol (*see Note 2*).
2. Cell homogenizer Potter type of 50 mL.
3. Ultracentrifuge with rotor Ti 45 (Beckman).
4. Six polycarbonate bottles of 70 mL (Beckman).
5. Stirrer and 4 cm magnetic bar.

2.4 Purification of Cntl Solubilized in Detergent

2.4.1 Immobilized Affinity Chromatography (IMAC)

1. Automated protein purification system (ÄKTA Pure or Purifier, Cytiva, or equivalent) equipped with 96 deep well fraction collector and a loading sample pump.
2. HisTrap Ni-NTA pre-packed column of 5 mL (Cytiva) (or equivalent).

3. 500 mL of equilibration buffer A: 20 mM HEPES, pH 8, 200 mM NaCl, 10 mM imidazole, 0.03% (w/v) DDM
4. 250 mL of elution buffer B: 20 mM HEPES, pH 8, 200 mM NaCl, 500 mM Imidazole, 0.03% (w/v) DDM.

2.4.2 Size Exclusion Chromatography (SEC)

1. Automated protein purification system (ÄKTA Pure or Purifier, Cytiva, or equivalent) equipped with 96 deep well fraction collector.
2. 1 L SEC running buffer: 20 mM MES, pH 6, 150 mM NaCl, 0.03% (w/v) DDM (*see Note 3*)
3. Superdex 200 SEC column with a total volume of 124 mL (e.g., HiLoad 16/600, Cytiva) (*see Note 4*).
4. Vivaspin® Ultra centrifugal filtration device for protein concentration, with membrane molecular weight cut-off (MWCO) of 50,000 Da (Sartorius, or equivalent) (*see Note 5*).
5. 96 deep wells plate adapted to AKTA pure fraction collector.

2.5 ThermoFluor Assay (or Differential Scanning Fluorescence)

1. qPCR thermocycler instrument (e.g., CFX384 Touch Real-Time PCR System, Biorad).
2. qPCR microplate (e.g., Hard-Shell-PCR plates, 384 wells, thin wall, white well, Biorad) (*see Note 6*).
3. Plastic film for plate sealing (e.g., MicroAmp™ optical adhesive film, ThermoFischer).
4. Fluorescent dye 1-Anilinonaphthalene-8-Sulfonic Acid (1,8-ANS), >98% purity (Invitrogen).
5. 2× ready to use screening buffer of different conditions.
6. Dimethylsulfoxide (DMSO).
7. n-Dodecyl-β-D-Maltopyranoside (DDM).
8. Centrifuge with rotor adapted to 96 wells plate.

2.6 SEC-MALLS (Size Exclusion Chromatography—Multi Angle Laser Light Scattering)

1. High Pressure Liquid Chromatography (HPLC) pump with low-pressure pulsation (e.g., Solvent-delivery system LC-20 AD, Shimadzu Technology).
2. Vacuum degasser (e.g., DGU-20A, Shimadzu Technology).
3. On-line filter (e.g., 0.1 μm pore size).
4. Hamilton syringe for microvolume injection (e.g., 25 μL).
5. A manual injection valve with injection loop of adapted volume (e.g., 20 μL).
6. 1 M NaOH and/or 1% SDS; Washing solution A: 0.5 M NaCl, 0.5 M NaOH; washing solution B: 1% (w/v) SDS, 10 mM EDTA, 100 mM NaCl
7. Deionized and degassed water for washing or equilibrating HPLC system.

8. SEC mobile phase: 20 mM MES, pH 6.0, 150 mM NaCl, 0.03% (w/v) DDM.
9. An analytical SEC column (e.g., Superdex 200 increase 10/300, Cytiva).
10. UV-Vis Absorbance detector (e.g., SPD-20A, Shimadzu Technology).
11. Multi-Angle Laser Light Scattering (MALLS) and Refractive Index (RI) detectors (miniDAWN TREOS and Optilab T-rEX respectively, from Wyatt Technology).
12. Astra software (Wyatt Technology).
13. Bovine Serum Albumine (BSA) at 5 mg/mL in the mobile phase (filtered and centrifuged).
14. Amicon® and Vivaspin® commercial ultra-centrifugal filters of different types, shape, and molecular weight cut-off (MWCO) (e.g., polyether sulfone or cellulose; conic or flat; classical or inversed gravity flow; 30, 50, or 100 kDa MWCO) for sample preparation.

3 Methods

3.1 Cell Culture and CntI Expression

Here, we describe an optimized protocol to produce CntI using 2 L flask culture.

1. Inoculate 100 mL of freshly prepared TB media with *E. coli* C43 (DE3) transformed with the expression plasmid pET-TEV-CntI from LB agar plate (or frozen glycerol stock). Grow the cell primary culture overnight at 37 °C, with shaking at 180 rpm (rotation per minute).
2. The day after, inoculate 6 × 1 L of TB media supplemented with kanamycin at 50 µg/mL final (plasmid resistance), with around 10 mL per flask to start with OD_{600nm} of 0.05.
3. Grow the cells at 37 °C, 180 rpm until OD_{600nm} of 0.6–0.9 (*see Note 7*).
4. Induce the T7 promoter expression plasmid using 1 mM IPTG and grow overnight (~16 h) at 18 °C, with shaking at 180 rpm.
5. Harvest the cells by centrifugation for 20 min at 6000 g.
6. Discard supernatant and resuspend the cell pellet in lysis buffer.
7. Homogenize the cells with the Potter. Refrigerate the pellet on ice for immediate lysis or store at –80 °C for several weeks.

3.2 Bacterial Cell Lysis and Membrane Preparation

1. Thaw pellet and add lysozyme at 1 mg/mL final, benzonase at 50 U/100 mL, and EDTA-free protease inhibitors cocktail.
2. Lyse cells using a cell disruptor refrigerated at 4 °C, by three passages at 2.4 kbar.

3. Pellet unbroken cell and insoluble debris by centrifugation (12,500 g, 25 min, 4 °C) (*see Note 8*).
4. Discard the pellet and pour the supernatant into chilled ultra-centrifuge tubes and centrifuge at 150,000 g for 1 h (*see Note 9*).
5. Resuspend the membrane pellet in lysis buffer with the Potter homogenizer. The sample will be called “crude extract” of CntI.
6. Measure the total concentration of protein by colorimetric assay compatible with detergent such as BCA assay, using Bovine Serum Albumin (BSA) as standard protein, following commercial recommendations.
7. Freeze with liquid nitrogen and store for several weeks at –80 °C, or pursue to the Subheading 3.3.

3.3 Extraction and Solubilization of CntI

1. Prepare 500 mL of crude extract diluted at 1–2 mg/mL of total protein with the solubilization buffer supplemented with 1% (w/v) DDM (*see Note 10*). Incubate at 4 °C under agitation overnight.
2. Centrifuge at 150,000 g during 1 h, at 4 °C to pellet the insoluble material.

3.4 Purification of Solubilized CntI

3.4.1 Immobilized Affinity Chromatography (IMAC)

1. Load supernatant of solubilized protein extract onto 5 mL HisTrap Ni-NTA column equilibrated beforehand in buffer A with peristaltic pump of the AKTA system, at 1 mL/min (*see Note 11*).
2. Wash with 10 Column Volume (CV) of 50 mM then 100 mM of imidazole until UV absorbance at 280 nm falls back to the baseline. Make sure to keep all the proteins eluted from the washing steps, in case of binding issue with the protein of interest CntI.
3. Eluate CntI with buffer B at a flow rate of 2 mL/min, collect 10 mL per fraction.

3.4.2 Size Exclusion Chromatography (SEC)

1. Equilibrate the preparative Superdex 200 SEC column with two CV (250 mL approximately) of SEC buffer.
2. Concentrate the IMAC elution fractions, around 40 mL, with 50 kDa MWCO ultra-centrifugal filter at 3500 g, 4 °C, by steps of 5–10 min in order to avoid protein aggregation by over-concentrating, to obtain a final volume of 1–2 mL.
3. Estimate the protein concentration by UV absorbance and theoretical extinction coefficient $50,880 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (<https://web.expasy.org/protparam/>).

4. Introduce the concentrated IMAC elution in the injection loop of the AKTA system. Make sure the injection loop size is two times higher than the sample volume to inject (e.g., for 2 mL loop, inject 1 mL of sample).
5. Perform purification with SEC buffer at a flow rate of 0.35 mL/min. Collect 1 mL fractions in 96 deep wells plate.
6. SEC elution fractions are analyzed by SDS-PAGE. The sample is denaturated in Laemmli buffer without boiling. Indeed, CntI is an integral α -helical membrane protein that aggregated at high temperature, i.e., 95 °C.
7. Either pursue with Subheading 3.6.2 or concentrate with 50 kDa MWCO ultra-centrifugal filter of adapted volume by 3500 g, 5 min cycle of centrifugation.

3.5 Stability Condition Screening by Fluorescent Thermal Stability Assay (FTSA)

FTSA can readily be used to compare relative protein stability under different conditions, therefore facilitating the optimization of protein preparations for further studies.

The fluorophore must be protected from light when stored and moved from plate to qPCR thermocycler to avoid photobleaching.

3.5.1 Determination of Appropriate Concentration of Fluorophore and Protein

1. Dissolve and aliquot ANS stock solution at 10 mM in DMSO from the manufacture powder bottle.
2. In a qPCR plate (96- to 384-wells format), screen varying concentrations of ANS from 0.05 to 1.5 mM (e.g., 0.05, 0.10, 0.25, 0.50, 0.75, 1.0, and 1.5 mM) and protein from 0.5 to 10 μ M (e.g., 0.5, 1.0, 2.0, 4.0, and 10 μ M) in 20 μ L of total volume per well: in 10 μ L final volume, first, add water; second, purified protein CntI; third, fluorophore ANS; and finally, add 10 μ L of protein buffer with detergent at 2 \times CMC. In our specific case, 10 μ M of CntI and 50–100 μ M of ANS gave the best results (*see Note 12*) (Fig. 1).
3. Prepare in the same way the controls without protein, i.e., with ANS, DDM, or ANS + DDM.
4. Centrifuge the plate at 5000 g (*see Note 13*).
5. Selection of the wavelength filters compatible with ANS fluorescence: in our case, we used the same parameters as SYBR Safe dye, i.e., $\lambda_{\text{excitation}}$ 450–490 nm and $\lambda_{\text{emission}}$ 515–530 nm.
6. Apply a temperature range from 20 to 90 °C (increasing speed of 0.75 °C/min) with 0.5 °C reading step.

3.5.2 Screen of Buffer Solutions and Additives

1. Prepare screening buffer solutions 2 \times with commercial or home-made kit, including various pH (4.2–10.2), buffering agents (phosphate, cacodylate, acetate, citrate, HEPES, MES, TRIS, BIS-TRIS, and bicine), salt concentration (NaCl), and additives (e.g., imidazole or glycerol in our specific case).

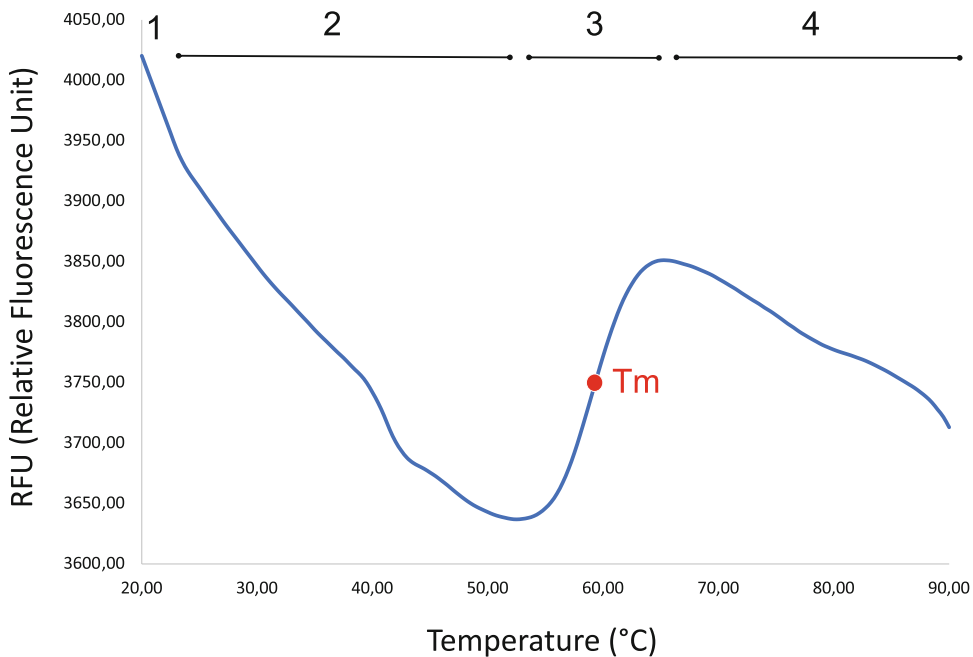


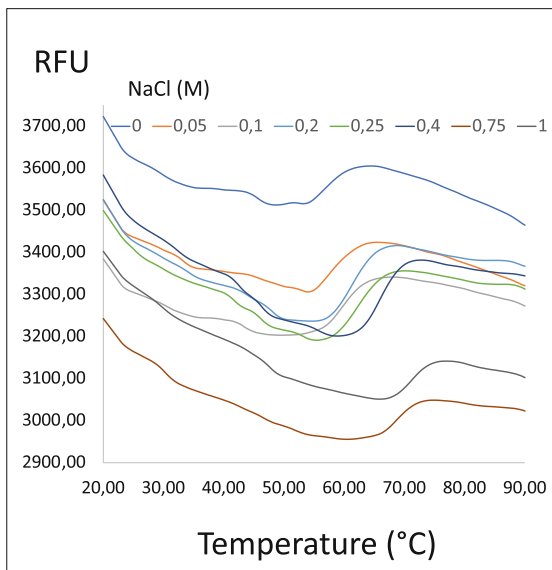
Fig. 1 Thermofluor assay of Cntl (membrane protein) with the fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS). The melting curve of Cntl is tracked thanks to its interaction with ANS. There are four phases during temperature increase: (1) binding of ANS with Cntl at room temperature shows initial fluorescence; (2) from 20 °C to 55 °C, fluorescence quenching is probably due to ANS dissociation; (3) from 55 °C to 65 °C, the interaction of ANS with denatured Cntl enhances the fluorescence. We observe a sigmoidal curve where the inflection point corresponds to the melting temperature (T_m) of the protein; (4) from 65 °C to 90 °C, ANS dissociates from aggregated Cntl, resulting in fluorescence quenching

2. Prepare pre-mixed solution of protein, fluorophore and detergent optimized previously (Subheading 3.5.1), concentrated 2 \times .
3. Repeat **step 2** without Cntl protein as control.
4. In each well, deposit 10 μ L of screen buffer solution 2 \times , then 10 μ L of pre-mixed protein 2 \times or control solution 2 \times .
5. Proceed to **steps 4–6** of Subheading 3.5.1.
6. Identification of stabilizing buffers from primary screening should be refined by additional screening, as long as the protein quantity is enough. For instance, exchange Cntl buffer with the best one identified from the first screen, then go back to **step 1** for another round of screening in order to improve the protein stability condition.

3.5.3 Data Analysis

1. Export data of Relative Fluorescence Unit (RFU) and first derivative of RFU (dF/dT) versus temperature in spreadsheet file format with the qPCR software.

2A



2B

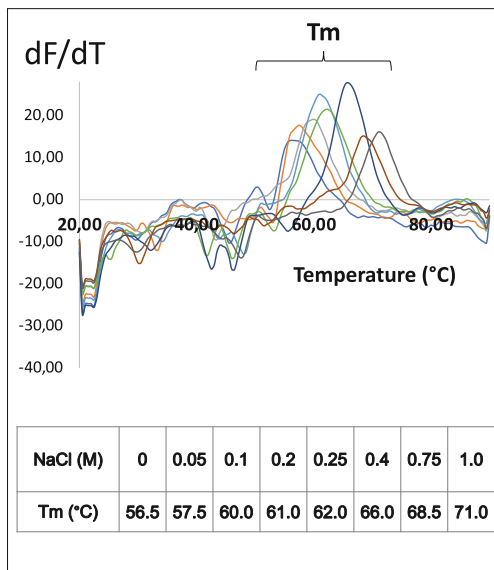


Fig. 2 Effect of ionic strength on Cntl stabilization by thermofluor assay. Cntl is initially in the buffer 20 mM MES, pH 6.0, 150 mM NaCl, DDM 0.12 mM. We tested the increasing concentration of additional NaCl (0.05–1 M) on Cntl stability. 2A, melting curves of Cntl (RFU, Relative Fluorescence Unit) with ANS dye and different concentration of additional NaCl (0.05–1 M). 2B, first derivative of melting curves (dF/dT) highlights the T_m (melting temperature) as the maximum dF/dT . T_m of the starting buffer is 56.5 °C. Increment of NaCl tends to increase T_m , i.e., adding 1 M of NaCl results in T_m of 71 °C. The ionic strength clearly stabilizes Cntl

2. Determine the melting temperature (T_m) with first derivative numerical analysis of the melt curves using data visualization software. Protein stability with highest T_m is desired (*see Note 14*) (Fig. 2).

3.6 Analysis of Protein Detergent Complexes (PDC) by SEC-MALLS

Separated peaks of protein detergent complex (PDC) and detergent micelles are required to determine molar masses unambiguously.

3.6.1 Preparation of the SEC-MALLS System

1. Proceed to an optimal wash of the SEC column with two CV of 1 M NaOH and/or 1% SDS (*see Note 15*). Finish by abundant washing with deionized water (at least three CV).
2. Equilibrate the system and the column with appropriate solvent (or buffer) for enough time until the light scattering and refractometer baselines are stable with minimum background noise (e.g., overnight) and signal drift.
3. For a membrane protein, select the “Protein Conjugate” mode that includes the analysis with the three detectors (laser light scattering, UV absorbance, and refractive index). This mode

allows to obtain the absolute molecular weight of the membrane protein–detergent complex, and both protein and detergent mass fractions. It also determines the peak molecular composition.

4. In order to establish the experiment template, a run with a monodisperse protein standard (e.g., BSA) dissolved in the running buffer is done. This step is mandatory to calibrate the SEC-MALLS system. The calibration of the SEC-MALLS system is done in three steps:
 - (a) The normalization of each light scattering (LS) detectors with the 90° LS detector.
 - (b) The alignment of UV, LS, and RI peaks of the BSA monomer. This step allows to determine the inter-detector volumes.
 - (c) The band broadening correction to take the sample diffusion through the detectors into account.

3.6.2 Preparation and Analysis of PDC (Protein–Detergent Complex)

1. Pool the monodisperse elution fractions of the protein from the last purification step (Subheading 3.4.2). The total volume is 15–20 mL.
2. Estimate the concentration of the purified protein in the total homogenized elution by UV absorbance at 280 nm, using the extinction coefficient.
3. Split the total volume of elution into the appropriate number of identical fractions, i.e., 1.5–2 mL, for a desired number of protein concentration tests to be analyzed by SEC-MALLS.
4. Concentrate by ultrafiltration the fractions under the different conditions to be compared, e.g., speed (500 or 3500 g), MWCO of the filter (10, 30, 50, or 100 KDa), type, and shape of centrifuge filter unit (Vivaspin®, Amicon® or Centriprep®).
5. Flash cool the samples in liquid nitrogen if the SEC-MALLS experiments cannot be performed the same day.
6. Determine the % of protein recovery after ultrafiltration by measuring the UV absorbance at 280 nm and using the extinction coefficient of 50,880 M⁻¹.cm⁻¹ (see Note 16).
7. Concentrate under the same condition the buffer without protein for control analysis.
8. For each sample, inject into the SEC-MALLS system 20 µL of CntI concentrated at 5–6 mg/mL (see Note 17).
9. Fractionate and eluate at 0.3 mL/min, at 25 °C (see Note 18).
10. Save the results and analyze the chromatograms with Astra software (Wyatt Technology) (Fig. 3).

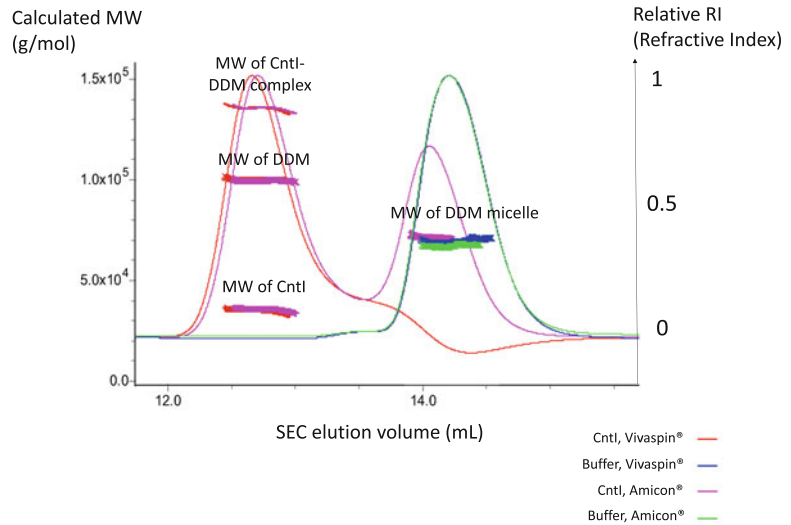
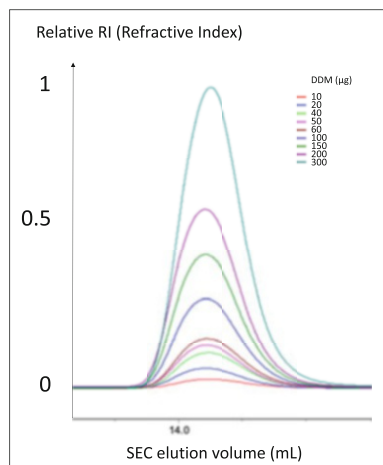


Fig. 3 Effect of ultrafiltration unit type on detergent micelle concentration analyzed by SEC-MALLS. After concentration of Cntl with Vivaspin® or Amicon® ultrafiltration unit with a membrane MWCO of 30 kDa, Cntl is analyzed by SEC-MALLS (SEC column S200 increase 10/300, Cytiva). Cntl and detergent micelle elution peaks are observed by refractive index (RI) detection. The chromatography plot shows Cntl elution as red and pink lines, after concentration with Vivaspin® and Amicon® respectively. The expected elution volume of DDM micelles is shown as blue and green lines after concentration of the buffer (20 mM MES, pH 6; 150 mM NaCl, 0.03% DDM) with Vivaspin® and Amicon® respectively. Calculated molecular weights (MWs) are indicated for Cntl-DDM complex, DDM, and Cntl. In the same concentration condition, we observed that Amicon® unit concentrates DDM micelle, unlike Vivaspin®

3.6.3 Evaluation of Detergent Micelles Accumulated During Protein Concentration

1. Inject into the SEC-MALLS system, for calibration, accurate amounts of DDM from 10 to 300 μg prepared in the same condition as protein analysis in Subheading 3.6.2.
2. Calculate with any suitable software (Origin, Excel, etc.) the peak area of refractive index signal subtracted from the baseline (ΔRI).
3. Plot integrated ΔRI against the corresponding detergent quantity. If the curve is linear, use the linear fit equation as a standard to calculate the quantity of detergent micelles of the concentrated samples (Fig. 4).
4. Establish the optimal filter and centrifugation speed for protein concentration step by choosing the condition where protein recovery is the highest and detergent micelle concentration is the lowest (*see Note 19*).

4A



4B

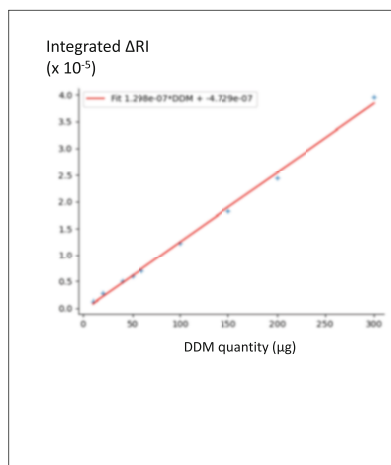


Fig. 4 Quantification of DDM micelles by SEC-MALLS. (a) different quantities of DDM (from 10 to 300 µg) are injected on a SEC column (S200 increase 10/300, Cytiva). (b) linear correlation of the integrated area under the peak of RI (Refractive Index) with the quantity of DDM is used as standard

4 Notes

1. It is usual to include a tag removal strategy for crystallization of the recombinant protein. The TEV cleavage site is made of eight amino acids, and the specific proteolysis takes place at carboxy side of the sixth residue. That is why the His tag with TEV site must be added at the N-terminus to remove most of the extra amino acids from the cleavage site. The plasmid we used was designed and made by the team of Dr. Pascal Arnoux (CEA, Cité des Energies, France).
2. Glycerol is a common, but not mandatory, protein stabilizer additive in particular for membrane protein.
3. If the protein-free detergent micelle concentration is too high at the end of the purification, reduce the detergent concentration of the SEC running buffer down to $1.1 \times \text{CMC}$.
4. The choice of the SEC column is based on the hypothetical size of the molecules to fractionate, i.e., CntI as protein detergent complex (PDC) and protein-free detergent micelle. Often, PDC molar mass is higher than the membrane protein by itself, and detergent micelles could unexpectedly show the same observed size as PDC.
5. In order to reduce the quantity of protein-free micelles concentration, the choice of the membrane molecular weight cut-off (MWCO) should be the closest to the PDC size.

6. Beware: The qPCR plate we used shows background fluorescence in our testing conditions.
7. Bacterial culture reaches OD_{600nm} of 8 with baffled flask (oxygenation enhancer) and OD_{600nm} of 3.2 with standard flasks.
8. A supplemented centrifugation step can be made after lysis if there are excessive foam and turbidity (e.g., 5 min at 12,500 g).
9. It is possible to check by Western-blot with an anti-His tag antibody if CntI remains in the supernatant. In that case, increase centrifugation duration by two.
10. From 6 L of cell culture, we usually obtain 100 mL of membrane crude extract at 20–50 mg/mL of total membrane protein. Solubilizing the whole crude extract at once would end up with many liters to deal with. Solubilizing 500 mL volume of crude extract is easier to manipulate during the next steps of ultracentrifugation and affinity chromatography purification. The remains of the non-solubilized crude extract could be saved in –80 °C for later preparation.
11. Supernatant loading at low flow increases the protein fixation on the affinity column. It is also possible to load the supernatant in a closed circuit overnight at 4 °C.
12. Since ANS causes background fluorescence via detergent-fluorophore interaction, we recommend to use minimum of detergent concentration, i.e., at 1× CMC, or even 0.5× CMC since an excess of detergent is already in the buffer of the protein.
13. Foam formation can easily happen when pipetting micro-volume of detergent and protein. When there are bubbles, try to centrifuge the plate at 5000 g for 15–30 min. Note that centrifugation might not be enough to remove all the foam.
14. Compare data with and without protein (control) to spot the fluorescence artifact. The expected melting temperatures (T_m) are usually around 60–80 °C.
15. The washing step of the column is crucial because of the high sensibility of MALLS detector. Especially with SEC columns, washing with only NaOH might not be enough. Add a supplementary wash with high concentration of SDS to solubilize hydrophobic contaminant that could cause strong background noise to the light scattering detection. Wash with 1% SDS at room temperature to avoid SDS crystallization at low temperature.
16. A gradient of protein concentration is formed during ultrafiltration. Make sure to mix the concentrated sample before measuring the absorbance.
17. In order to compare the quantity of free detergent micelle retained by the filter membrane, it is important to inject the same amount of protein after concentration.

18. For better resolution of SEC peak separation, the flow can be reduced at 0.2–0.1 mL/min. Nevertheless, the same speed has to be used for all the samples for proper comparison.
19. During protein purification steps, concentration by ultrafiltration is already applied before the SEC. Note that free micelle could already accumulate at this step. Besides, in our case, SEC elution volume of CntI and free detergent micelle tend to overlap. It is thus difficult to separate completely free detergent micelle.

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