High-Throughput Screening of Protein-Detergent Complexes Using Fluorescence Polarization Spectroscopy

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This article provides detailed protocols for a high-throughput fluorescence polarization (FP) spectroscopy approach to disentangle the interactions of membrane proteins with solubilizing detergents. Existing techniques for examining the membrane protein-detergent complex (PDC) interactions are low throughput and require high amounts of proteins. Here, we describe a 96-well analytical approach, which facilitates a scalable analysis of the PDC interactions at low-nanomolar concentrations of membrane proteins in native solutions. At detergent concentrations much greater than the equilibrium dissociation constant of the PDC, K_d, the FP anisotropy reaches a saturated value, so it is independent of the detergent concentration. On the contrary, at detergent concentrations comparable with or lower than the K_d, the FP anisotropy readout undergoes a time-dependent decrease, exhibiting a sensitive and specific detergent-dissociation signature. Our approach can also be used for determining the kinetic rate constants of association and dissociation. With further development, these protocols might be used in various arenas of membrane protein research that pertain to extraction, solubilization, and stabilization.

Keywords: fluorescent labeling • kinetics • membrane protein engineering • protein folding • protein-detergent interactions • proteomicelles

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INTRODUCTION

This article provides a general procedure for the development of fluorescence polarization (FP) anisotropy assays that can monitor the interactions of membrane proteins with solubilizing amphiphilic molecules in a high-throughput fashion. Steady-state FP spectroscopy enables the examination of changes in the rotational mobility of a fluorescently labeled protein (Kwok & Cheung, 2010; Rossi & Taylor, 2011; Stoddart, White, Nguyen, Hill, & Pfleger, 2016; Turman, Nathanson, Stockbridge, Street, & Miller, 2015). This approach can be conducted by exciting a chemically attached fluorophore with plane-polarized light (Moerke, 2009; Swonger & Robinson, 2018). Let us assume that the

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labeled membrane protein binds to detergent monomers, leading to the formation of a protein-detergent complex (PDC), also called a proteomicelle. This process is accompanied by a slowed rotational diffusion of the protein-detergent complex (PDC) with respect to unbound protein, leading to a decreased emission in the plane perpendicular to the polarized light and an increased emission in the plane parallel to the polarized light. Therefore, the ratio between the numbers of free and bound proteins in solution can be monitored using this emission change (Rossi & Taylor, 2011). Specifically, our approach relies on changes in steady-state FP anisotropy, r, at detergent concentrations below and above the equilibrium dissociation constant, K_d, of the proteomicelles. Here, the K_d of the proteomicelle is the detergent concentration at which only half of the membrane proteins are solubilized in solution. This parameter pertains to any membrane protein that forms a complex with a detergent micelle. The FP anisotropy is calculated using the parallel, $I_p(t)$, and orthogonal, $I_o(t)$, time-dependent components of the emission intensity (Gradinaru, Marushchak, Samim, & Krull, 2010; Jameson & Ross, 2010):

$$r(t) = \frac{I_p(t) - G I_o(t)}{I_p(t) + 2G I_o(t)}$$

Equation 1

Here, G denotes a correction factor for the detection modes when emission polarizers are oriented vertically and horizontally. This sensitivity correction factor is given by the following equation:

$$G = \frac{I_{HV}}{I_{HH}}$$

Equation 2

I_{HV} indicates the intensity with the excitation and emission polarizers in a horizontal and vertical orientation, respectively. On the contrary, I_{HH} shows the intensity with both the excitation and emission polarizers in a horizontal orientation.

If the detergent concentration is brought to a value lower than the K_d, then a gradual dissociation of detergent monomers from membrane proteins occurs. Here, both the K_d and detergent concentration are provided in molarity units. This alteration in the overall mass of the proteomicelles is observed through a time-dependent decrease in the FP anisotropy. Because detergent dissociation from membrane proteins leads to protein aggregation, this process is not reversible. In contrast, at detergent concentrations much greater than the K_d, no net dissociation of detergent monomers from membrane proteins is expected, indicating no change in the FP anisotropy. In Figure 1, an example of such time-dependent changes in the FP anisotropy is provided for screening various concentrations of the detergent 4-cyclohexyl-1-butyl-β-D-maltoside (CYMAL-4) used for solubilizing outer membrane protein G (OmpG), a monomeric β-barrel of *E. coli* (Subbarao & van den Berg, 2006; Yildiz, Vinothkumar, Goswami, & Kuhlbrandt, 2006). This figure also shows that the FP anisotropy undergoes changes between an initial maximum anisotropy value (r_{max}), when the membrane protein is fully detergent solvated and a minimum anisotropy value (r_{min}), when the protein is detergent desolvated. Therefore, this time-dependent change in the FP anisotropy is faster at lower detergent concentrations in the sample well.

It is important to mention that for complete solubilization of a membrane protein in a detergent micelle, the K_d should be of the same order of magnitude as the critical micelle concentration (CMC) of the detergent. The CMC is the detergent concentration at which the detergent monomers and detergent micelles are at thermodynamic equilibrium. Its value depends on the physicochemical conditions of the aqueous solution (salt concentration, solution viscosity, temperature) and the detergent properties

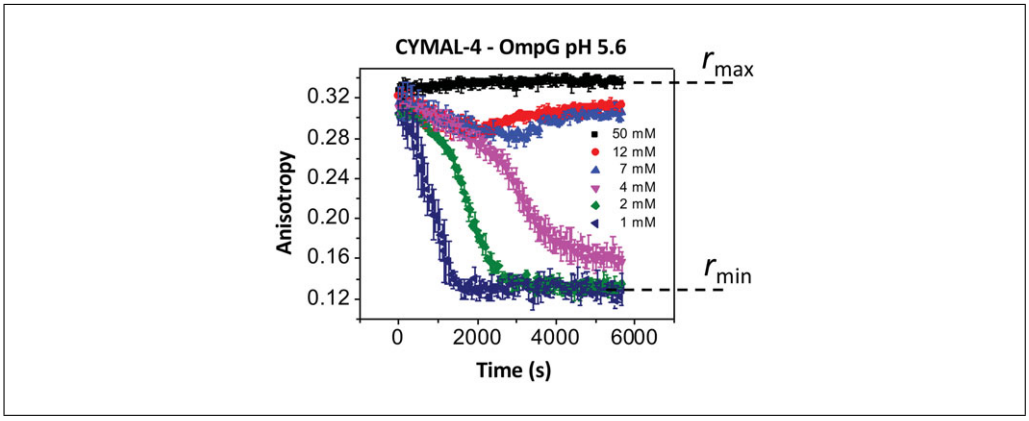

Figure 1 Representative example of the time-dependent FP anisotropy change as a result of detergent desolvation of a membrane protein. This data was acquired for outer membrane protein G, a monomeric outer β-barrel membrane protein of *E. coli* (Subbarao & van den Berg, 2006; Yildiz et al., 2006). This protein was refolded in 4-cyclohexyl-1-butyl-β-D-maltoside (CYMAL-4). The starting detergent concentration was 50 mM. The figure also indicates the absolute maximum (r_{max}) and minimum (r_{min}) FP anisotropy. The figure legend shows the final detergent concentrations in the protein sample; the final protein concentration was kept constant at 28 nM. The buffer solution contained 200 mM NaCl and 50 mM sodium acetate, pH 5.6. All FP anisotropy data were derived at room temperature as mean ± s.d. of at least three independent acquisitions. Reprinted with permission from reference Wolfe, Gugel, Chen, and Movileanu (2018b) Copyright 2017, American Chemical Society.

(e.g., polar head group, length of the hydrophobic tail). As a test case, we provide examples of the PDC interactions of monomeric β-barrel membrane proteins, such as OmpG of *E. coli* (Subbarao & van den Berg, 2006; Yildiz et al., 2006) and three truncation derivatives of ferric hydroxamate uptake component A (FhuA) of *E. coli* (Ferguson, Hofmann, Coulton, Diederichs, & Welte, 1998; Mohammad et al., 2012). These protocols can be employed to examine the interactions of numerous detergents with membrane proteins within the same FP anisotropy recording. This approach facilitates the determination of the apparent dissociation constants of the PDC, K_d, over a range of several orders of magnitude (Wolfe, Hsueh et al., 2017). We want to highlight that the detergent desolvation of a membrane protein at concentrations below K_d is mechanistically related to its unfolding, owing to lack of solubilization. This path of protein unfolding pertains to protein aggregation because of the hydrophobic effect. Although this article provides some detergent examples, our approach can be extended to other amphiphiles, including native and synthetic lipids. The reduction of the concentration of detergent in the sample well of the assay plate determines a two-state transition of membrane proteins between a detergent-solubilized state (e.g., folded state) and detergent-desolvated state (e.g., unfolded state). The nature of this transition depends on the interfacial PDC interactions, including electrostatic and hydrophobic interactions of the membrane proteins with the polar headgroups and alkyl chains of the detergent monomers, respectively. The results of these measurements can be used to determine the model-dependent rate constants of association (k_{on}) and dissociation (k_{off}) of the proteomicelles. Finally, we show that these assays can also be employed in the case of α-helical membrane proteins. Because these FP anisotropy recordings are executed in a high-throughput format, a broad range of physicochemical conditions, membrane proteins, and detergent species can be screened over a duration of several hours.

STRATEGIC PLANNING

FP Anisotropy Measurements

These FP anisotropy measurements can be conducted using a standard plate reader (e.g., SpectraMax I3 plate reader, Molecular Devices) that is equipped with a detection

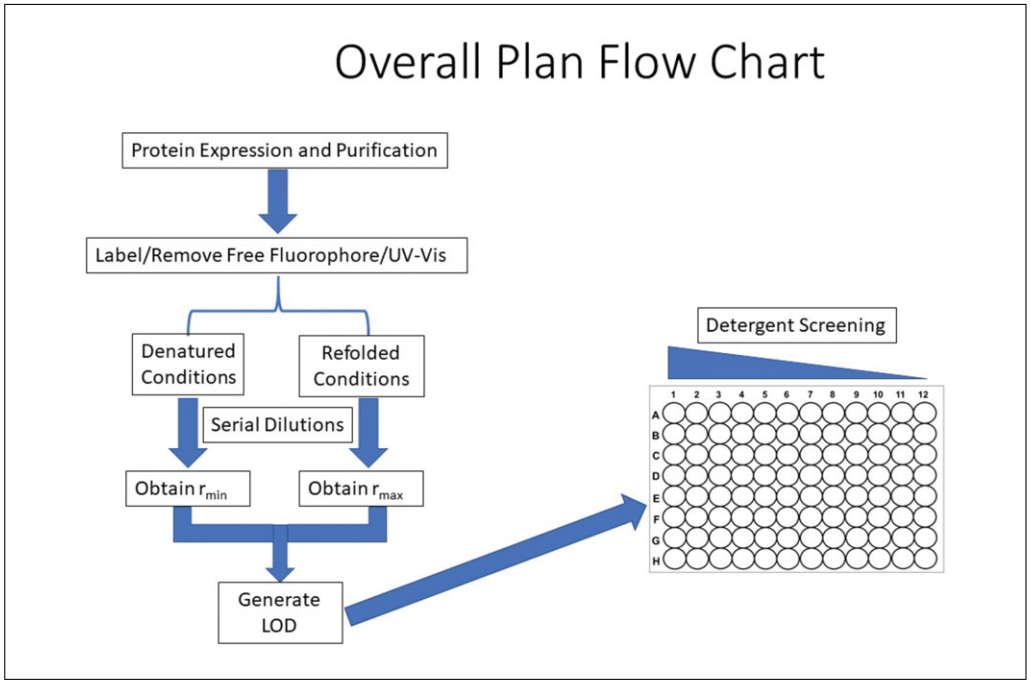

Figure 2 Flow chart representation for establishing a detergent screen. This figure chronologically displays the steps for establishing an effective detergent screen. Several critical steps are required prior to executing the detergent screen. The initial step involves purifying the protein of interest and bioconjugation of a fluorophore to the targeted membrane protein. Removal of free fluorophore is commonly performed after bioconjugation reactions. Several detailed examples of protein preparation are provided. The fluorescently labeled protein will then be subjected to both denaturing and refolding conditions. The data acquired from this step is ancillary to determining the limit of detection (LOD). Instrument sensitivity parameters (LOD) are absolutely required prior to screening for various detergents and their dilution factors.

cartridge for a targeted fluorophore. For example, if a rhodamine derivative will be used, then such a cartridge is required for rhodamine FP spectroscopy. We have only optimized this experiment for use on the SpectraMax I3 plate reader, where there is no ability to modulate the slit width or filter bandwidths on the FP cartridges. If dyes are used outside of the rhodamine or fluorescein wavelengths for which the SpectraMax I3 FP cartridges are optimized, then extra care will have to be taken in performing the optimization of the signal for limit-of-detection (LOD) experiments (see below). Certainly, we suggest that you pick a more suitable dye (e.g., a fluorophore with a longer fluorescence lifetime for a slower-rotational diffusion protein), if at all possible.

To acquire an enhanced signal sensitivity, these experiments necessitate the use of an optically intense and stable fluorophore under a broad range of physicochemical conditions. A fluorophore that features such physical properties is Texas Red (Titus, Haugland, Sharrow, & Segal, 1982). Indeed, because Texas Red is a bright fluorophore (Gradinaru et al., 2010), this FP protocol can be executed using a tiny amount of membrane protein in the low-nanomolar concentration range (Wolfe, Hsueh et al., 2017). A fundamental requirement of this protocol is the availability of a single cysteine sulfhydryl or multiple cysteine sulfhydryls, which can be reacted with a Texas Red fluorophore. In an ideal case, a single cysteine sulfhydryl in a membrane protein covalently modified with a Texas Red fluorophore would facilitate kinetic measurements of the PDC interactions. Because of its hydrophilic nature (Titus et al., 1982), Texas Red can be covalently attached on the aqueous phase–exposed domain of the targeted membrane protein.

The overall strategy of this protocol is to probe the isothermal desorption of detergent from a refolded membrane protein (Fig. 2). These protocols can be applied for neutral,

ionic, and zwitterionic detergents, including both low- and high-CMC detergents (Wolfe, Gugel, Chen, & Movileanu, 2018a, 2018b, Wolfe, Hsueh et al., 2017; Wolfe, Si et al., 2017). These measurements can be conducted by diluting the refolded membrane protein sample within individual wells of an assay plate with buffer of varying detergent concentrations. It is very important to mention that individual wells contain gradually decreasing detergent concentrations, while the final protein concentration is kept unchanged at a low-nanomolar value. The time-dependent FP anisotropy measurements are determined for time periods in the range of 20 to 100 min. For equilibrium determinations and model-dependent dissociation constants, the protein samples are covered and placed at a cold temperature (e.g., 4°C). Then, the FP anisotropy endpoints are collected 24 hr after the beginning of the desorption reaction. Finally, the FP anisotropy traces will be processed as mean ± SD over a at least three independent data recordings.

Selection of Fluorophore for Bioconjugation

Smaller proteins, for example those with a molecular mass less than 50 kDa, can be properly labeled with many commercially available fluorophores (i.e., maleimide-directed Texas Red). If a protein is larger than 50 kDa, it may be necessary to utilize a fluorophore that features a longer fluorescence lifetime, τ_F (Zhang, Wu, & Berezin, 2015). This is because a larger protein shows a significantly slower rotational diffusion (Roos et al., 2016; Yusko et al., 2017), so a longer lifetime of the fluorophore is needed for statistically significant recordings. It is imperative to select a dye that complements the functional groups on the protein. For example, for cystine sulfhydryl–containing proteins, maleimide-based bioconjugates can be produced. On the other hand, for lysine-containing proteins, N-hydroxysuccinimide ester-driven bioconjugates can be made. It is also essential that the fluorophore excitation and emission properties be complementary to the plate reader cartridge. Traditionally, fluorophore labeling is conducted at 10-fold to 50-fold molar excess of fluorophore to protein (Hermanson, 2013). In our bioconjugation reactions, we have routinely used a 20-fold molar excess of fluorophore to protein. It should be noted that this ratio is protein specific and should be optimized for a given bioconjugation reaction.

Selection of Plate

The FP anisotropy recordings will be accomplished using black flat-bottom 96-well Costar assay plates (Corning, Inc.). The only absolute criterion for selecting a plate in FP anisotropy–based assays is that the plate be black-opaque. However, once a plate has been selected for an assay, it is highly recommended that the type of plate not be changed. The differences between different types of black-opaque plates may seem negligible, but they will alter the results of the FP assay. If a change in plate type must be made after assay development, there is a need to re-perform the necessary quality-control experiments. This includes read-height calibration, LOD, and limit of quantification.

Preparation of Detergent Dilutions of the Refolded Protein Samples at a Constant Protein Concentration

Steady-state FP anisotropy recordings are executed with diluted refolded proteins in individual plate wells while maintaining the protein concentration of each sample constant. This can be achieved by diluting the refolded protein sample with buffer solutions containing detergents at different concentrations. The final detergent concentration for the FP anisotropy measurements is obtained using the equation:

$$C_f V = C_s V_s + C_d V_d = (C_s f_s + C_d f_d) V$$

Equation 3

Here, V and C_f are the well volume and the final detergent concentration of the protein sample, respectively. V_s and f_s show the volume and fractional volume (V_s/V), respectively, of the refolded protein sample at a starting detergent concentration. V_d and f_d denote the volume and fractional volume (V_d/V), respectively, of the diluting buffer containing detergents at various concentrations. C_s and C_d denote the detergent concentrations of the refolded protein (starting concentrations) and diluting buffer, respectively. The pre-incubation time of the solubilized protein in low-detergent-concentration wells has to be minimal, in order to start time-dependent FP anisotropy readings at an initial maximum anisotropy value (r_{max}) (Fig. 1).

Determination of the Equilibrium Dissociation Constants of the Proteomicelles

Detergent dilutions are followed by time-dependent FP anisotropy reads at the beginning of the detergent desolvation reactions. The end points of the detergent desolvation reactions are collected 24 hr after the initiation of the detergent desolvation. These end points are employed to achieve the detergent dissociation isotherms. One asset of this approach is the high quality of the FP readout despite protein aggregation in the sample well over time upon drastic detergent depletion. The Hill-Langmuir isothermal dissociation curves were fitted by two-state logistic functions, as follows (Prinz, 2010):

$$r(c) = \frac{r_{min} + r_{max}\left(\frac{c}{K_d}\right)^p}{1 + \left(\frac{c}{K_d}\right)^p}$$

Equation 4

r_{min} and r_{max} indicate the minimum and maximum FP anisotropy values, respectively (Textor & Keller, 2015). p and K_d are the Hill coefficient and the apparent dissociation constant of the proteomicelles, respectively. The above equation can be used for the isothermal detergent desorption curves by assuming that the protein surface shows individual binding sites for detergent monomers. This fit approach can also provide the opportunity for evaluation the steepness of the two-state detergent desolvation transition (q), which occurs at half detergent saturation:

$$q = \frac{p\,(r_{max} - r_{min})}{4K_d}$$

Equation 5

In Figure 3, we show that this approach is highly sensitive to both the nature of the detergent monomers and protein electrostatics. Specifically, two-state detergent desorption transitions are presented for the interactions of two basic (FhuA ΔC/Δ5L_25N and FhuA ΔC/Δ7L_30N) and two acidic (OmpG and FhuA ΔC/Δ5L) β barrels that are solubilized with either 1-lauroyl-2-hydroxy-*sn*-glycero-3-phosphcholine (LysoFos) or *n*-octyl-β-D-glucopyranoside (OG) (Fig. 3A; Wolfe, Hsueh et al., 2017). In Figures 3B and 3C, we illustrate the dose-response FP anisotropy caused by LysoFos and OG depletion in the sample well, respectively. In these examples, the proteins were polyhistidine-tagged and purified using affinity chromatography. Alternatively, β barrels can be purified by tag-free anion-exchange chromatography (Thakur, Larimi, Gooden, & Movileanu, 2017; Thakur & Movileanu, 2019; Wolfe, Mohammad, Thakur, & Movileanu, 2016). We think that the polyhistidine tag does not significantly affect the detergent solubilization properties of a β barrel protein.

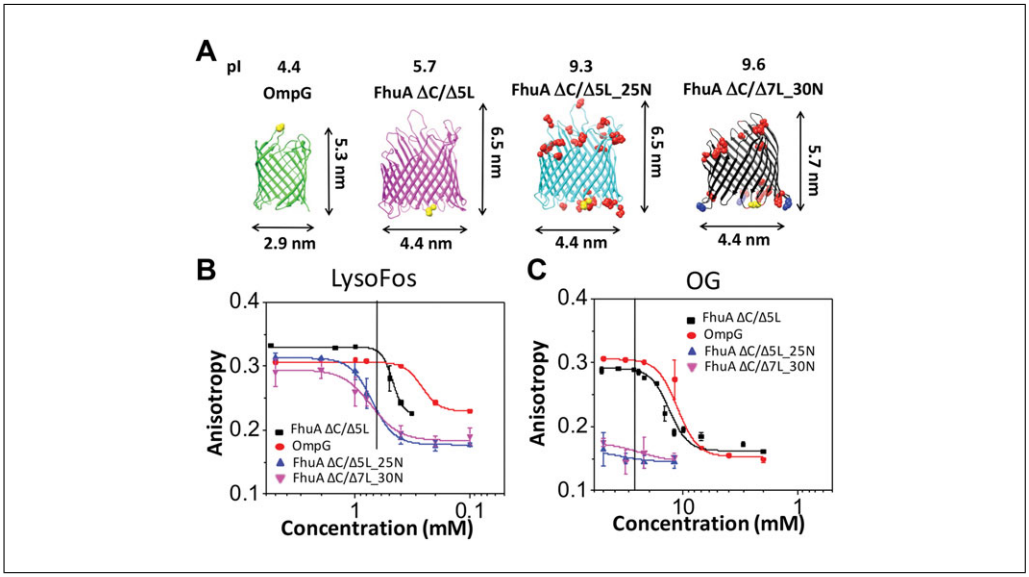

Figure 3 Representative examples of two-state isothermal desorption transition of detergents from outer membrane proteins of varying isoelectric point. (**A**) This panel shows the side views of OmpG and three truncation FhuA mutants. The isoelectric points of all proteins are displayed at the top of the panel. Texas Red (marked in yellow) was covalently attached to an engineered cysteine sulfhydryl. The alterations in the isoelectric point, which are marked in red (FhuA ΔC/Δ5L_25N and FhuA ΔC/Δ7L_30N), were achieved via negative charge neutralizations with respect to FhuA ΔC/Δ5L. FhuA ΔC/Δ7L_30N features three lysine mutations, which are marked in blue. Two of these mutations are negative-to-positive charge reversals. (**B**) Two-state desorption transitions of these proteins in 1-lauroyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LysoFos), a zwitterionic detergent. (**C**) Two-state desorption transitions of these proteins in n-octyl-β-D-glucopyranoside (OG), a neutral detergent. The CMC values of these detergents are shown by vertical bars. The horizontal axis illustrates the final detergent concentrations above and below the CMC; the final protein concentration was kept constant at 28 nM. The vertical axis shows the equilibrium FP anisotropy values collected 24 hr after the beginning of the detergent desorption reaction. All the other experimental conditions were the same as in Figure 1. Reprinted with permission from Wolfe et al. (2017). Copyright 2017, American Chemical Society.

PROTEIN EXPRESSION AND PURIFCATION: EXAMPLE FOR A ROBUST β-BARREL

BASIC PROTOCOL 1

Here, we provide detailed experimental steps for a monomeric β-barrel outer protein. FhuA ΔC/Δ5L forms inclusion bodies during recombinant overexpression (Wolfe et al., 2016). This protocol describes a process to purify FhuA ΔC/Δ5L from inclusion bodies via polyhistidine tag–based affinity chromatography.

Materials

BL21 (DE3) *E. coli* cells (New England BioLabs, C2571)
Plasmid vector compatible with selected strain of *E. coli*
Antibiotic compatible with the resistance gene in selected vector
Lysogeny broth (LB) medium (IBI Scientific, IB49040)
Isopropyl β-D-1-thiogalactopyranoside (IPTG; Gold Bio, I2481)
Resuspension buffer (see recipe)
Inclusion body cleaning buffer (see recipe)
Denaturing buffer (see recipe)
Nickel nitrilotriacetic acid (Ni-NTA) equilibration buffer (see recipe)
Ni-NTA wash buffer (see recipe)
Ni-NTA elution buffer (see recipe)

Growth vessel (shaker flask/bioreactor/incubator)
Spectrophotometer with ability to read the optical density (OD) at 600 nm

Glass or plastic cuvette
Refrigerated centrifuge, which must have capacity to spin at at least 16,000 × *g*
Microfluidizer (Microfluidics, Model 110 L)
Potter-Elvehjem homogenizer
0.2-µm filter (Thomas Scientific, 1222B32)
Ni-NTA column and resin (gravity or FPLC; see Current Protocols article: Petty, 1996)
Dialysis tubing (with appropriate molecular weight cutoff for the protein of interest)
Lyophilizer (FreeZone, 710201000)

Additional reagents and equipment for SDS-PAGE (see Current Protocols article: Gallagher, 2012)

1. Transform plasmids into BL21 (DE3) *E. coli* cells according to manufacturer's instructions.
2. Grow the bacterial culture in LB containing selection antibiotic for the resistance gene in the plasmid vector at 37°C until the OD at 600 nm is 0.4 to 0.6.

   In the detergent screening, a final protein concentration of 28 nM was used. To fill the entirety of a 96-well plate, 9.6 ml of solution is required. In the case of FhuA ΔC/Δ5L, roughly 0.25 mg of protein is required to fill an entire 96-well plate.
3. Add IPTG stock to growth vessel so that the final concentration is 1 mM.

   IPTG concentration optimization should be performed for each different expressed recombinant protein.
4. Grow the cells over a duration of 3 to 4 hr after addition of IPTG.
5. Harvest the cells by centrifugation for 20 min at 3700 × *g*, 4°C. Then, decant the supernatant and keep the pelleted cells.
6. Resuspend the cell pellet with resuspension buffer.
7. Lyse the cells using an ice-chilled microfluidizer. Pass the lysate through the microfluidizer approximately 15 times.

   If solution is viscous, add DNase. Lysis can also be performed by enzymatic digestion or by sonication.
8. Centrifuge the homogenate 20 min at 16,000 × *g*, 4°C, then decant and discard the supernatant.
9. Resuspend the pelleted material with inclusion body cleaning buffer. Keep the solution on ice.
10. Homogenize the resuspended pellet using a Potter-Elvehjem homogenizer on ice.
11. Centrifuge the sample 20 min at 16,000 × *g*, 4°C, then decant the supernatant.
12. Repeat steps 9 to 11 a total of three times.
13. Resuspend the pelleted material in denaturing buffer and centrifuge the sample 20 min at 16,000 × *g*, 4°C. Do not discard the supernatant.

    The addition of denaturing agents allows for removing the protein from the inclusion bodies. Centrifugation removes insoluble materials. At this step, the desired soluble protein resides within the supernatant. The supernatant is kept for subsequent steps.
14. Filter the supernatant with a 0.2-µm filter and load it onto a Ni-NTA column equilibrated in Ni-NTA equilibration buffer.

15. Wash the column with 5 column volumes of Ni-NTA wash buffer. Then, elute fractions with 10 column volumes of Ni-NTA elution buffer, and collect the fractions.
16. Analyze the purity of fractions via SDS-PAGE (see Current Protocols article: Gallagher, 2012) for pool-pure fractions.
17. Dialyze the pure protein from step 16 against deionized water at 4°C.

    The length of dialysis will depend on the size of the vessel used, as well as the size of dialysis tubing. Dialyzing the protein overnight in a 4-L vessel has been successful in a variety of examples.

18. Aliquot the sample from step 17 and lyophilize. Store the sample at −80°C once complete lyophilization has occurred.

DETERGENT-EXTRACTED PREPARATION OF MEMBRANE PROTEINS: EXPRESSION AND PURIFICATION OF AN α-HELICAL PROTEIN

ALTERNATE PROTOCOL 1

Proteins vary in their biophysical characteristics. Depending on these characteristics, different purification techniques will be utilized. Basic Protocol 1 describes a procedure for extracting a recombinant β-barrel protein from inclusion bodies. This alternate protocol describes the purification of SELENOK U92C, a transmembrane α-helical protein by membrane extraction (Wolfe, Si et al., 2017).

Materials

E. coli cells and appropriate medium
Plasmid vector compatible with selected strain of *E. coli*
Antibiotic compatible with the resistance gene in selected vector
Isopropyl β-D-1-thiogalactopyranoside (IPTG; Gold Bio, I2481)
Amylose buffer (see recipe)
Liquid N_2
Exchange buffer (see recipe)
Amylose elution buffer (see recipe)
StrepTrap elution buffer (see recipe)
Poly-histidine-tagged tobacco etch virus (TEV) protease (MilliporeSigma, T4455)

Spectrophotometer
Glass cuvette
Probe sonicator
Amylose column (see Current Protocols article: Kimple, Brill, & Pasker, 2018)
Immobilized metal affinity chromatography (IMAC) column (see Current Protocols article: Petty, 1996)
GE Healthcare StrepTrap HP column (or commercial equivalent)

Additional reagents and equipment for SDS-PAGE (see Current Protocols article: Gallagher, 2012)

1. Transform *E. coli* cells according to manufacturer's instructions.
2. Grow *E. coli* cells in appropriate medium (incorporate missing nutrients, correct antibiotic) at 37°C until OD at 600 nm reaches 1.0.
3. Lower temperature to 18°C and add IPTG at a final concentration of 0.5 mM.
4. Harvest the cells 14 to 16 hr after the IPTG induction by centrifugation for 10 min at 4000 × *g*, 4°C. Decant medium and resuspend cell pellet with amylose buffer.
5. Flash freeze cells in liquid nitrogen and store them in aliquots at −80°C.

6. Thaw the cells and sonicate them in amylose buffer using a probe-type sonicator.

   Dilute the cell pellet in 1 volumetric equivalent of amylose buffer, and cool the sample on ice for 10 min. While on ice, sonicate the sample 10 intervals of 10 s. Allow a 30-s cooling period after 10 sonication intervals. Repeat this strategy as needed. Ensure foaming does not occur at any point during this process. If foaming occurs, then lower the power settings.

7. Centrifuge solution 1 hr at 20,000 × *g*, 4°C.
8. Equilibrate the amylose column with 5 column volumes of amylose buffer
9. Load the supernatant from step 7 onto the amylose column.
10. Wash the amylose column with 5 column volumes of amylose buffer.
11. Wash the amylose column with 5 column volumes of exchange buffer.
12. Elute the sample by adding 5 column volumes of amylose elution buffer.
13. Remove the solubility tag by incubating with TEV protease overnight at 4°C.

    This example utilized a 1:10 molar ratio of TEV to SELENOK U92C in dialysis tubing. A different molar ratio may be optimal for optimal cleavage of different fusion constructs.

14. Remove the poly-histidine tagged maltose-binding protein (MBP) and TEV from the sample by utilizing IMAC.

    The MBP and TEV both have affinity tags that will bind to IMAC resin (i.e., Ni-NTA). A standard IMAC method can be utilized here. Follow steps 14 to 16 of Basic Protocol 1. The difference here is that the desired material will not bind to the resin and will elute from the column during the load and wash steps. Also ensure that the IMAC column for this procedure is equilibrated with amylose elution buffer.

15. Load the flow-through sample collected from step 14 onto a StrepTrap HP equilibrated with exchange buffer.
16. Elute the protein from the StrepTrap column with the StrepTrap elution buffer.
17. Analyze the purity of fractions via SDS-PAGE (see Current Protocols article: Gallagher, 2012) and pool relevant fractions. Store samples at 4°C.

BASIC PROTOCOL 2

BIOCONJUGATION OF FLUOROPHORE TO MEMBRANE PROTEIN AND REMOVAL OF FREE FLUOROPHORE: EXAMPLE OF A β-BARREL PROTEIN

Prior to initiating the labeling reaction, ensure that the sample does not contain agents that will interfere with the labeling chemistry. For example, reducing agents with the thiol functional groups β-mercaptoethanol (BME) or dithiothreitol (DTT) will compete for coupling sites and hinder the labeling efficiency of maleimide bioconjugation reactions. Specific reaction of the maleimide group with reduced sulfhydryl groups occurs when the pH of the reaction buffer is in the range 6.5 to 7.5. Under more alkaline conditions, with a pH $>$ 8.5, the reactivity of amines becomes a potential issue. Maleimides do not normally react with histidines, methionines, and tyrosines. We have successfully conducted fluorophore conjugation reactions at pH 7.5.

If the purified protein sample contains a reagent that will hinder labeling, desalt or dialyze into an appropriate buffer. Tris(2-carboxyethyl)phosphine (TCEP) is an appropriate substitute for BME or DTT. Free fluorophore can interfere with the readout of this fluorescence anisotropy assay. When measuring the labeling efficiency, ensure the dye/protein ratio does not exceed 1. If the dye/protein ratio does exceed 1, subsequent desalting is necessary.

Materials

10 μM purified protein
Fluorophore: Texas Red C2 maleimide (store several times more concentrated than 200 μM)
Reaction buffer for Basic Protocol 2 (see recipe)
Ni-NTA Elution Buffer A (see recipe)
Ni-NTA Elution Buffer B (see recipe)

Aluminum foil
Agitation apparatus (stir plate, rocker, or orbital shaker)
Ni-NTA column (see Current Protocols article: Petty, 1996)
Quartz cuvette
UV-VIS spectrophotometer

1. Resuspend the lyophilized protein from Basic Protocol 1, step 18, in reaction buffer so that the protein concentration is 10 μM.

   The final volume needed for the 96-well plate depends on the FP anisotropy signal that is generated by the protein-fluorophore conjugate, which is generated in this protocol. The stronger the FP anisotropy signal, the less concentration will be needed in the well and the lower the total necessary volume will be. This will have to be calculated for each experiment.

2. Add the fluorophore to the reaction vessel so that the final concentration of dye is 200 μM.

3. Immediately cover the reaction vessel with aluminum foil to shield it from light. Then, gently agitate overnight at room temperature.

4. Load the reaction mixture onto an Ni-NTA column equilibrated with reaction buffer.

5. Wash column with 5 column volumes of reaction buffer.

6. Elute the protein off the column with a 10 to 200 mM stepwise imidazole elution by applying 5 column volumes of elution buffer A, followed by 5 elution volumes of elution buffer B.

7. Get UV-VIS spectra of the Ni-NTA elution steps, then calculate the dye/protein molar ratio.

   The protein will have a maximum absorbance at 280 nm, while the dye will have a different maximum absorbance. Both absorbance values must be divided by their respective extinction coefficients to get the concentrations. It is possible for dyes to also absorb at 280 nm, so multiply this value by the correction factor to account for this factor.

BIOCONJUGATION OF FLUOROPHORE TO PROTEIN AND REMOVAL OF UNREACTED FLUOROPHORE: EXAMPLE OF AN α-HELICAL PROTEIN

ALTERNATE PROTOCOL 2

Basic Protocol 2 utilizes an Ni-NTA column to remove non-conjugated fluorophore from solution. Another common method of removing non-conjugated fluorophore is utilizing a desalting column (i.e., PD-10 column). If a desalting column is not available, dialysis may be used instead.

As mentioned in Basic Protocol 2, thiol-containing reducing agents will interfere with the labeling chemistry and should be removed by desalting or dialysis.

Materials

10 μM purified protein
Reaction buffer for Alternate Protocol 2 (see recipe)

Fluorophore: Texas Red C2 maleimide (store several times more concentrated than 200 μM)

PD-10 desalting column (see Current Protocols article: Hagel, 1998)
Aluminum foil
Agitation apparatus (stir plate, rocker, etc.)
PD-10 desalting column (see Current Protocols article: Hagel, 1998)
Quartz cuvette
UV-vis spectrophotometer

Additional reagents and equipment for dialysis (*optional*; see Current Protocols article: Zumstein, 1995)

1. Exchange protein into reaction buffer utilizing a desalting column. Ensure that the final protein concentration is 10 μM.

   The final volume needed for the 96-well plate depends on the FP anisotropy signal that is generated by the protein-fluorophore conjugate, which is generated in this protocol. The stronger the FP anisotropy signal, the less concentration will be needed in the well and the lower the total necessary volume will be. This will have to be calculated for each experiment.

2. Add the fluorophore to the reaction vessel so that the final concentration of dye is 200 μM.

3. Immediately cover the reaction vessel with aluminum foil to shield from light. Then, gently agitate at room temperature for 1 hr.

   Because the SELENOK U92C protein is α-helical, its thermodynamic stability is much lower than that of the readily refolded β-barrel protein that was labeled in Basic Protocol 2. Therefore, care is taken to reduce protein-handling time in this protocol. It should be noted that the 1-hr incubation could also work for the reaction described in Basic Protocol 2, but its efficiency would be lower.

4. Remove free fluorophore by desalting with a PD-10 desalting cartridge (see Current Protocols article: Hagel, 1998) or dialyzing in the dark overnight at 4°C.

5. Obtain UV-VIS spectra of the desalted/dialyzed material. Use absorbance values to calculate the dye/protein molar ratio.

   The protein will have a maximum absorbance at 280 nm, while the dye will have a different maximum absorbance. Both values must be divided by their respective extinction coefficients to get the respective concentrations. It is possible for dyes to also absorb at 280 nm. To account for this, multiply the recorded A_{280} by the dye's correction factor.

SUPPORT PROTOCOL 1

REFOLDING OF DENATURED PROTEIN: EXAMPLE OF A β-BARREL PROTEIN

Inclusion body preparation, as well as subsequent chemical denaturing and detergent-mediated refolding, is necessary for effective purification of FhuA ΔC/Δ5L. In order to effectively refold a chemically-denatured membrane protein, many techniques have been described, and each protein requires its own conditions. Here, we will describe a rapid-dilution refolding protocol that has been shown to effectively refold FhuA ΔC/Δ5L (Wolfe et al., 2016). One should note that all detergent-containing buffers should be freshly prepared to avoid hydrolysis and oxidation. This example uses *n*-decyl-β-D-maltoside (DDM) which has a manufacturer-provided CMC value of 1.8 mM. This protocol utilizes a final detergent concentration of 3× CMC in the dilution buffer. The concentrations and volumes for this dilution protocol are specific examples and will need to be modified for each specific experiment. Specific values are provided for the

purpose of revealing commonly encountered procedural oversights. In short, be mindful to account for the change in detergent concentration upon combination with the protein sample.

Materials

Detergent: *n*-decyl-β-D-maltoside (DDM; Anatrace, D310)
Dilution buffer (see recipe)
Fluorescently labeled protein

Agitation apparatus (stir plate, rocker, or orbital shaker)
Refrigerated centrifuge with the ability to spin at 16,000 × *g*

1. Make a stock solution of 5.5 mM DDM in dilution buffer.

   This is one example of detergent that can be used in this protocol.

2. Dilute the protein 50-fold by combining 50 µl of protein sample with 2450 µl of dilution buffer.

   This brings the final concentration of DDM to 5.4 mM, which is 3× the CMC.

3. Allow the sample to gently mix overnight at 4°C. This can be done by rocking on a tilted plate.

   Excessive agitation can generate non-productive micelles and disrupt the refolding process.

4. Spin the sample 15 min at 16,000 × *g*, 4°C. Retain the supernatant without disturbing the solution and discard any particulate that was noticed. Then, re-calculate the concentration of labeled protein as described in Basic Protocol 2.

   This step serves the purpose of removing misfolded or aggregated proteins that may be generated during this step. The protein aggregation must be removed to properly calculate the soluble protein concentration. This is accomplished by the previous centrifugation step, removing the soluble protein that is left in the supernatant.

5. Protein solutions can be aliquotted and stored at −80°C until needed for new experiments.

DATA ACQUISITION FOR THE LIMIT OF DETECTION

BASIC PROTOCOL 3

Purification of membrane proteins is a time-consuming process. It is desirable to use the least amount of protein possible for any given experiment. Determining a range of protein concentrations that can be reliably measured is essential before executing experiments. The limit of detection (LOD) is commonly described as the lowest quantity of analyte that can be distinguished from instrument noise. It is desirable to reach the highest signal-to-noise ratio, but three times the signal-to-noise ratio should be the lowest accepted value. In this case, the relative fluorescent units (RFUs) of the labeled protein sample should be three times greater than the signal measured from a blank sample. Each protein-fluorophore combination will have a unique labeling efficiency, and therefore will generate a different signal. Due to the difference in photophysical properties of each protein-fluorophore combination, the LOD is unique to each protein-fluorophore combination. The denaturing condition described in this protocol and subsequent protocols is a buffer compatible with the protein, but the buffer also contains a denaturing agent at a high concentration (see "denaturing assay buffer" recipe in Reagents and Solutions). Figure 4 shows a linear dilution scheme to obtain the LOD. To screen a wider range of concentrations, a log scale dilution can be used. If the plate reader is moved or a novel experiment is being executed, it is recommended to perform a read-height calibration.

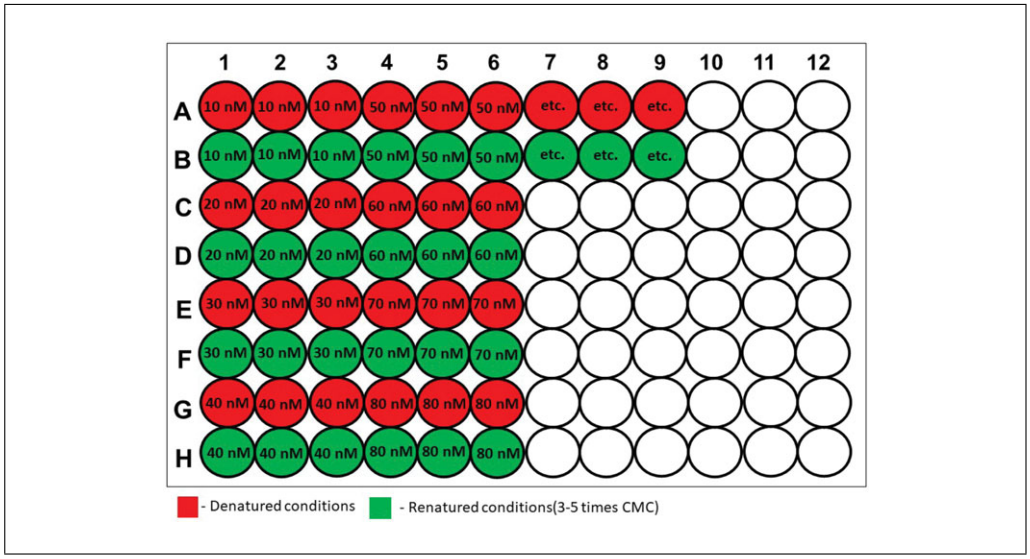

Figure 4 Linear dilution scheme for LOD. This figure shows a linear dilution scheme of protein concentrations in denatured and renatured conditions. Denatured and renatured conditions correspond to denaturing assay buffer and renaturing assay buffer, respectively. The displayed concentrations are suggestions and do not need to be followed exactly. However, any dilution pattern chosen must be subjected to denatured and renatured conditions. It is important to have the CMC near the center of the dilution scheme, as this will allow for the creation of curves that are easily fit. Yet, it is sometimes difficult to get many usable points below the CMC due to the loss of signal and deterioration of the signal-to-noise ratio.

NOTE: This is a standard procedure implemented in the experimental settings on the SpectraMax I3 instrument.

Materials

Assay buffer (not defined because it varies based on protein and condition being screened)
Renaturing assay buffer (same as assay buffer, but with detergent concentration three to five times the CMC)
Denaturing assay buffer (same as assay buffer but with high concentration of denaturing agent)

96-well plate, black-opaque (Corning Costar)
Plate reader (equipped with the appropriate FP cartridge)

1. Determine the desired concentration range to scan. For each desired concentration, calculate the volumes of protein stock and assay buffer to obtain that concentration.

   This ensures that accurate volumes are pipetted, because temperature can affect volume, especially when working in the microliter range.

2. Allow all reagents used in the experiment to equilibrate to room temperature for 10 min prior to addition to plate.

   This ensures that accurate volumes are pipetted, because temperature can affect volume, especially when working in the microliter range.

3. First, add an appropriate amount of assay buffer to the wells. Then, add the labeled protein stock, so that the final volume is that of the working volume of the plate and the protein is at the desired concentration (Fig. 4).

   Note that the denatured and renatured conditions correspond to denaturing assay buffer and renaturing assay buffer, respectively.

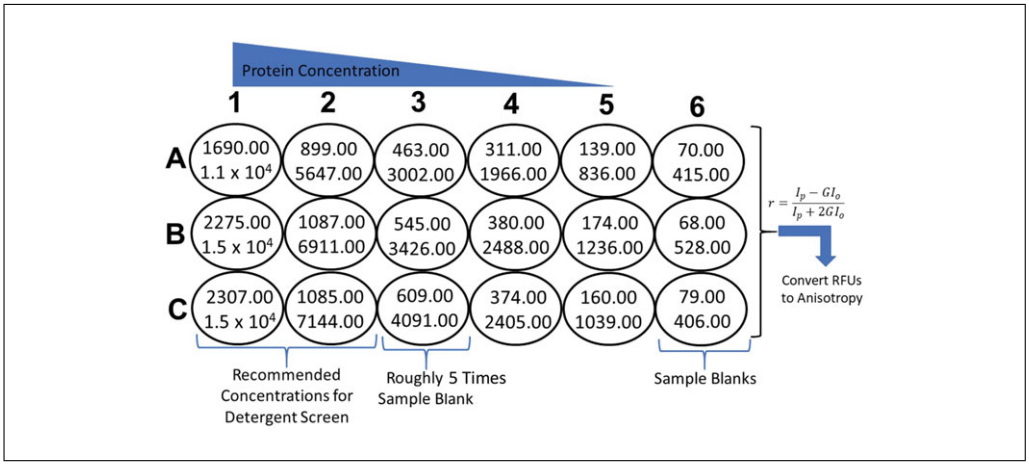

Figure 5 Example of raw data generated while determining the limit of detection. Displayed above are RFUs generated by a serially diluted labeled protein. Rows A, B, and C are triplicate repeats of each other. Columns 1 to 5 contain a serial dilution of labeled protein, while column 6 contains buffer only (sample blank). The top number corresponds to orthogonal emission intensity (I_o) and the bottom number corresponds to parallel emission intensity (I_p). The average relative fluorescence unit (RFU) values in column 3 are just above five times the value generated by average sample blanks. The RFUs in columns 4 and 5 would not generate reliable anisotropy, *r*, values, because they are not five times greater than those of sample blanks. The protein concentrations that generated the RFUs observed in columns 1 and 2 should be used in subsequent assays. Protein aggregates are likely to occur at low detergent concentrations, which lower the signal intensity. The raw values of RFUs can be converted to anisotropy values, *r*, by utilizing the displayed formula [equations. (1) and (2)]. The LOD can be obtained by applying this strategy to the labeled protein in the refolded and denatured state.

4. Ensure that there are at least three wells within the plate that contain assay buffer only. These will act as blanks.

   Note that the blanks are not shown in Figure 4. Within the plate, select a group of wells that can be used for blanks.

5. If the plate reader has been recently moved, or this is a novel protein-fluorophore combination, perform a read-height calibration.

6. Set the acquisition settings to complement the excitation and emission properties of the bioconjugate being used, and acquire the data.

7. Raw data will populate as RFUs (Fig. 5).

DATA ASSESSMENT FOR DETERMINING THE LIMIT OF DETECTION

SUPPORT PROTOCOL 2

This section utilizes the data acquired from Basic Protocol 3 for calculating limit of detection (LOD). The LOD calculation will provide the researcher with the lowest limit of signal needed to generate reliable anisotropy values. It is this check that will provide the researcher with the confidence that the FP anisotropy values are real. Relative fluorescence is directly related to the concentration of labeled protein within the sample. If the RFU values observed while executing experiments drop below the LOD, the FP anisotropy data cannot be considered genuine. Therefore, it is important to remember that we will be measuring proteins in conditions where aggregation will be present, and that the effective protein concentration will be decreasing due to the time-dependent loss of solubility. For this reason, it is important to work with protein concentrations much higher than the LOD and check that FP anisotropy data generated from conditions below the CMC of a detergent-protein complex still produce a satisfactory signal. The CMC values can be found in different sources of literature (le Maire, Champeil, & Moller, 2000; Prive, 2007, 2009). RFU values should be at least three to five times that of the sample

blank. The only variable being assessed in this protocol is the protein concentration. Be sure to keep the concentrations of denaturing agent and detergent constant in each well.

Materials

Denaturing agent(s) and detergent(s) of interest
Raw data acquired from Basic Protocol 3

1. Leave data in units of RFU for calculating the LOD.
2. Average the triplicate values of the RFU values at each protein concentration.
3. Average the values for the blanks (buffer-only wells).
4. The lowest protein concentration that generates an RFU signal at least three times the blank signal is the LOD.

   If no values three times blank signal (or greater) are generated, execute these steps again using higher protein concentrations. A concentration range of 10 to 250 nM is typically sufficient.

5. Using the FP anisotropy formula [equation (1)] and G factor [equation (2)] convert all RFU values to FP anisotropy values.
6. The FP anisotropy values gathered from denatured conditions (as described in the introduction of Basic Protocol 3) correspond to $r_{\min.}$ In this case, there is no detergent present, but a chemical denaturant [e.g., guanidinium hydrochloride (Gdm-HCl)] to show the minimum FP anisotropy value corresponding to the detergent-free protein.
7. The FP anisotropy values gathered from three to five times CMC values correspond to $r_{\max}$.
8. Compare the $r_{\min}$ and $r_{\max}$ values at different protein concentrations.

   Since the FP anisotropy value is concentration independent, it should remain true that at various concentrations of proteins, r_{min} and r_{max} will remain the same even though various protein concentrations will result in different RFU signals. This control of various concentrations while checking r_{min} and r_{max} should be run on all protein-fluorophore conjugate pairs. It is important to remember that because the protein used in these experiments starts in detergent, the final concentration of detergent at the various concentrations of detergents is held steady.

BASIC PROTOCOL 4

DETERGENT SCREENING

To generate a useful detergent dilution curve, several FP anisotropy values above the CMC and below the CMC should be present in the dilution. If this is not done, it can result in no observable FP anisotropy change. The samples that contain detergent concentrations below the CMC are prone to protein aggregation. Aggregated proteins will lower the concentration of fluorophore, resulting in a reduction of the RFU value. Keep in mind that RFUs are utilized to calculate the FP anisotropy values. If any RFUs fall below the previously determined LOD, the resulting FP anisotropy values cannot be considered genuine. The protein solutions will have detergents present prior to their addition to the plate. It is crucial to account for the detergent concentrations in the protein sample prior to making calculations for the detergent screen. Protein samples that contain excess detergent may need to be extensively diluted. Therefore, ensure that the protein concentration does not fall below the LOD during an extensive dilution. It is also important to ensure that the protein concentration is kept constant and the final volume is the working volume of the plate [see equation (3)].

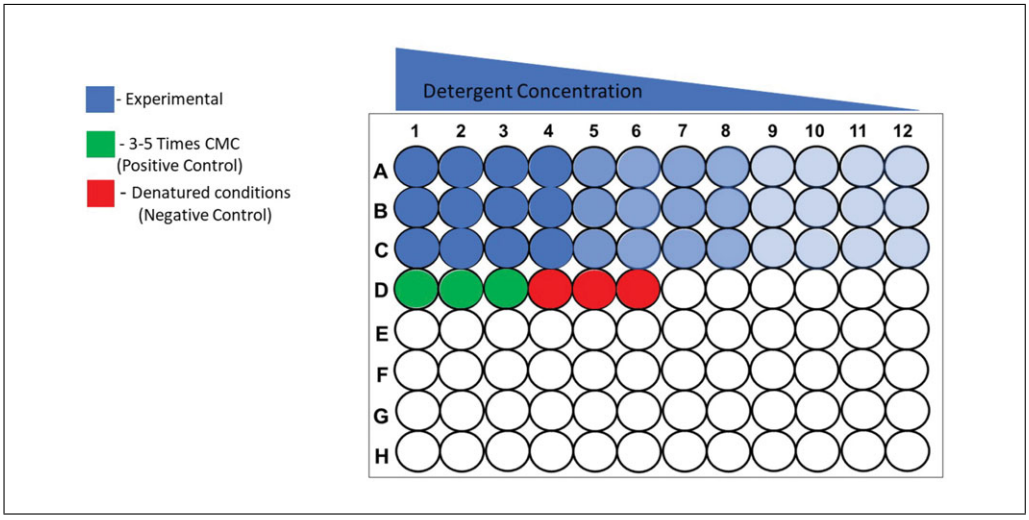

Figure 6 Plate layout for detergent screening. This figure is a representation of a 96-well plate arrangement that can be used for detergent screening. The blue wells represent experimental protein samples of varying detergent concentration. The green wells represent the labeled protein samples, which are refolded in a detergent that is different from the detergent being screened. The red wells represent the protein samples in denaturing conditions. The plate layout includes samples in the denatured and refolded states, which act as internal controls. If there is no observable change in anisotropy between the positive and negative control, an error has occurred. It is recommended to have internal control samples on each plate being analyzed. This diagram shows each sample being replicated in triplicate. Of course, this can be changed as desired.

Materials

Stock solution of the detergent being screened
Assay buffer (not defined because it varies based on protein and condition being screened)
Labeled-protein stock solution (the stock protein concentration should be several times more concentrated than the concentration of protein that generated the LOD in Support Protocol 2)
Positive-control detergent (the same as Basic Protocol 3)
Denaturing buffer (the same as Basic Protocol 3)

96-well plate, black-opaque (Corning Costar)
Plate reader equipped with appropriate cartridge

1. Add the detergent being screened (“experimental”) to wells at positions A1, B1, and C1 on the 96-well plate. The starting concentration should be five times the CMC (Fig. 6).
2. In rows A, B, and C, columns 2 to 12, add assay buffer with a final volume half of that which was added at positions A1, B1, and C1.
3. Perform a serial two-fold dilution by drawing up half of the volume from the wells at positions A1, B1, and C1 and dispensing into the respective positions A2, B2, and C2. Pipette up and down three times in positions A2, B2, and C2, ending by fully dispensing into column 2. Repeat this procedure across columns 2 to 11, leaving column 12 as a true blank.
4. Using the detergent system from Basic Protocol 3, subject the protein to these conditions in triplicate. This will act as an internal positive control.
5. Subject the protein to denaturing conditions, as in Basic Protocol 3, but in triplicate. This will act as the internal negative control.

6. Using equation (3), add enough protein stock to bring the final volume to the working volume of the plate. The final concentration of protein should also be at or above the previously determined minimum RFU value.

7. Set the acquisition settings as previously performed in Basic Protocol 3 (step 6).

8. Allow the plate to continuously read for 30 to 90 min, while gently shaking (kinetic read). A steady decrease in anisotropy should be observed, eventually flatlining between the theoretical r_{min} and r_{max} (Fig. 1).

   Once a steady flatline in the FP anisotropy value is observed, it can be assumed that the PDC has reached equilibrium. We have performed these experiments with incubation steps at 4°C and up to 48 hr and have seen no change. We suggest overnight incubations. If no observable change in the FP anisotropy value is present during the kinetic read, the desolvation may have gone to completion.

9. Once a flatline in the FP anisotropy is observed, allow the plate to incubate at 4°C overnight. Re-read the plate as an endpoint assay.

   Ensure that the FP anisotropy value observed after the overnight incubation has not decreased toward the theoretical minimum.

REAGENTS AND SOLUTIONS

Amylose buffer (Alternate Protocol 1)

50 mM sodium phosphate buffer, pH 7.5 (Current Protocols, 1998)
200 mM NaCl
% (v/v) Triton X-100
Store up to 4 weeks at 4°C

Amylose elution buffer (Alternate Protocol 1)

50 mM sodium phosphate buffer, pH 7.5 (Current Protocols, 1998)
200 mM NaCl
1 mM EDTA
0.067% (w/v) *n*-dodecyl-β-D-maltopyranoside (DDM; Anatrace, D310)
20 mM maltose
Prepare fresh buffer for each use

Denaturing buffer (Basic Protocol 1)

50 mM Tris·Cl, pH 8 (Current Protocols, 1998)
8 M urea
Prepare fresh with each use

Dilution buffer (Support Protocol 1)

200 mM NaCl
50 mM HEPES, pH 7.4
Store up to 4 weeks at 4°C

Exchange buffer (Alternate Protocol 1)

50 mM sodium phosphate buffer, pH 7.5 (Current Protocols, 1998)
200 mM NaCl
1 mM EDTA
0.067% (w/v) *n*-dodecyl-β-D-maltopyranoside (DDM
Prepare fresh with each use

Inclusion body cleaning buffer (Basic Protocol 1)

50 mM Tris·Cl, pH 8 (Current Protocols, 1998)

0.1% (v/v) Triton X-100, pH 8
Store up to 4 weeks at 4°C

Ni-NTA elution buffer (Basic Protocol 1)

500 mM NaCl
50 mM Tris·Cl, pH 8 (Current Protocols, 1998)
8 M urea
350 mM imidazole
Prepare fresh with each use

Ni-NTA elution buffer A (Basic Protocol 2)

200 mM NaCl
50 mM Tris·Cl, pH 7.5 (Current Protocols, 1998)
1 mM TCEP (MilliporeSigma, C4706)
6 M guanidinium hydrochloride (Gdm-HCl)
10 mM imidazole
Prepare fresh with each use

Ni-NTA elution buffer B (Basic Protocol 2)

200 mM NaCl
50 mM Tris·Cl, pH 7.5 (Current Protocols, 1998)
1 mM TCEP (MilliporeSigma, C4706)
6 M guanidinium hydrochloride (Gdm-HCl)
200 mM imidazole
Prepare fresh with each use

Ni-NTA equilibration buffer (Basic Protocol 1)

500 mM NaCl
50 mM Tris·Cl, pH 8 (Current Protocols, 1998)
8 M urea
Prepare fresh with each use

Ni-NTA wash buffer (Basic Protocol 1)

500 mM NaCl
50 mM Tris·Cl, pH 8 (Current Protocols, 1998)
8 M urea
5 mM imidazole
Prepare fresh with each use

Reaction buffer (Alternate Protocol 2)

50 mM sodium phosphate buffer, pH 7.5 (Current Protocols, 1998)
200 mM NaCl
0.067% (w/v) *n*-dodecyl-β-D-maltopyranoside (DDM; Anatrace, D310)
1 mM EDTA
Prepare fresh with each use

Reaction buffer (Basic Protocol 2)

200 mM NaCl
50 mM Tris·Cl, pH 7.5 (Current Protocols, 1998)
1 mM TCEP (MilliporeSigma, C4706)
6 M guanidinium hydrochloride (Gdm-HCl)

Prepare fresh with each use

Resuspension buffer (Basic Protocol 1)

100 mM NaCl
50 mM Tris·Cl, pH 8 (Current Protocols, 1998)
10 mM $MgCl_2$
Store up to 4 weeks at 4°C

StrepTrap elution buffer (Alternate Protocol 1)

50 mM sodium phosphate buffer, pH 7.5 (Current Protocols, 1998)
200 mM NaCl
1 mM EDTA
0.067% (w/v) *n*-dodecyl-β-D-maltopyranoside (DDM; Anatrace, D310)
2.5 mM desthiobiotin (MilliporeSigma, D1411)
Prepare fresh with each use

COMMENTARY

Background Information

Determination of the rotational diffusion coefficient of the proteomicelles

The time-dependent FP anisotropy recordings can be used to infer the hydrodynamic radius of the proteomicelles under detergent solvation and desolvation conditions. Specifically, we can use Perrin's equation, which provides a relationship between the recorded steady-state FP anisotropy, r, and rotational diffusion coefficient, D_r (Gradinaru et al., 2010):

$$\frac{r_0}{r} = 1 + 6D_r\tau_F$$

Equation 6

This equation also includes the fluorescence lifetime of the fluorophore, τ_F, and the fundamental maximum anisotropy value, r_0. For example, for Texas Red, τ_F is 4.2 ns (Lakowicz, 2006) and r_0 is 0.4 (Prazeres, Fedorov, Barbosa, Martinho, & Berberan-Santos, 2008). The rotational correlation time, θ, depends on the apparent hydrodynamic volume of the labeled proteomicelle, V_h, as follows (Lakowicz, 2006):

$$\theta = \frac{1}{6D_r}$$

Equation 7

$$V_h = \frac{\theta k_B T}{\eta} = \frac{k_B T}{6\eta D_r}$$

Equation 8

k_B and T indicate the Boltzmann constant and absolute temperature, respectively. Viscosity of the buffer solution can be inferred using viscosity tables for the known salt concentration. For example, the viscosity of solutions containing 200 mM NaCl, η, is 1.028 mPa s (Lide, 2008).

In practice, if the viscosity is too high, the rotational diffusion of the fluorophore will be too slow to see an FP anisotropy change at any detergent concentration.

Using equations (6) and (7), we obtain the following equation:

$$\frac{r_0}{r} = 1 + \frac{\tau_F}{\theta}$$

Equation 9

The rotational diffusion coefficients can be determined for the fully solvated proteins (proteomicelles, D_r^{slow}) and detergent-desolvated proteins (denatured proteins, D_r^{fast}).

Determining the kinetics of the predesolvation and desolvation phases

At detergent concentrations lower than K_d, a predesolvation phase precedes the desolvation phase (Fig. 7). Because the predesolvation phase follows a linear time-dependent FP anisotropy change, its observable rate constant, k_{obs}^{pre}, is determined using a linear fit of the time-dependent FP anisotropy, $r(t)$:

$$r(t) = -k_{obs}^{pre}t + r_{max}$$

Equation 10

Here, t and r_{max} denote the recording time during the predesolvation phase and the maximum FP anisotropy (recorded at time $t = 0$), respectively. Because the predesolvation phase follows an exponential-decay FP anisotropy change, its observable rate constant, k_{obs}^{des}, is inferred at various detergent concentrations lower than or comparable with the CMC using a single-exponential fit, as follows:

$$r(t) = r_d e^{-\frac{t}{\tau}} + r_{min}$$

Equation 11

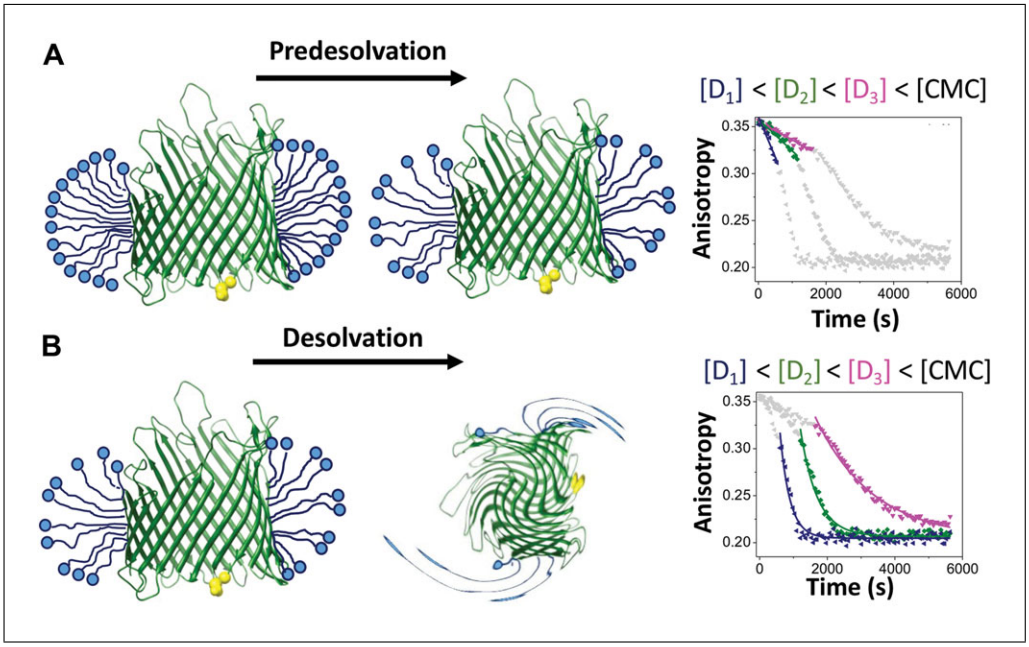

Figure 7 Representative two phases of the isothermal detergent desorption of membrane proteins. (**A**) The initial phase is detergent predesolvation. (**B**) The follow-up, time-dependent phase is detergent desolvation. Predesolvation results from the dissociation of a relatively small number of detergent monomers from membrane proteins. This process is accompanied by a relatively small and slow change in the FP anisotropy with respect to the initial value, r_{max}. The second phase depicts a large and fast alteration in the FP anisotropy, as it is representative of the loss of many detergent monomers from membrane proteins. This major change in the average molecular mass of proteomicelles is reflected in a drastic alteration of the FP anisotropy, and occurs in a single-exponential fashion (right-hand panels). The kinetic rates of both predesolvation and desolvation phases are dependent on the final detergent concentration (Wolfe et al., 2018a). For example, the kinetic rates are greater for lower detergent concentrations in the protein sample. Reprinted with permission from reference Wolfe et al. (2018b) Copyright 2018, American Chemical Society.

Here, t shows the recorded time during the desolvation phase, including the total time of predesolvation, T^{pre}. r_{min} is the minimum FP anisotropy, which is recorded at time infinity of the desolvation phase. Therefore, k_{obs}^{des} is $1/\tau$, where τ is the desolvation time constant. k_{obs}^{des} can also be used as the apparent first-order rate constant (Movileanu, Cheley, Howorka, Braha, & Bayley, 2001) for the desolvation reaction of the proteomicelles.

This rate constant includes the kinetic rate constants of association (k_{on}) and dissociation (k_{off}) of the proteomicelles (Fig. 8) (Stoddart et al., 2016), as follows:

$$k_{obs}^{des} = -k_{on}[D] + k_{off}$$

Equation 12

The initial FP anisotropy value during the desolvation phase, r_{in}, is given by the following equation:

$$r_{in} = r\left(T^{pre}\right) = r_d e^{-\frac{T^{pre}}{\tau}} + r_{min}$$

Equation 13

which provides r_d, a parameter reaction of the desolvation phase:

$$r_d = \frac{r_{in} - r_{min}}{e^{-\frac{T^{pre}}{\tau}}}$$

Equation 14

Using equations (10) to (14), one obtains the FP anisotropy value for the detergent desolvation phase:

$$r(t) = (r_{in} - r_{min})\, e^{-\frac{t - T^{pre}}{\tau}} + r_{min}$$

Equation 15

The time-dependent protein concentration, $[P(t)]$, is given by the following equation:

$$[P(t)] = [P_t]\left(\frac{r(t) - r_{min}}{r_{in} - r_{min}}\right)$$

Equation 16

Here, $[P_t]$ denotes the total protein concentration at the beginning of the desolvation process.

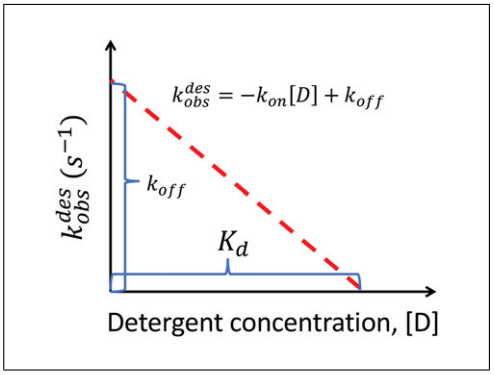

Figure 8 Kinetic analysis of the time-dependent FP anisotropy data. It is expected that the detergent desolvation is faster for a lower detergent concentration due to an accelerated dissociation process of detergent monomers from the membrane protein. This figure shows the linear relationship of the observable rate of desolvation, k_{obs}^{des}, on the final detergent concentration of the protein sample. The intercept of the linear fit of kinetic rate data acquired for various detergent dilutions with the vertical axis is the dissociation rate constant, k_{off}. On the other hand, the slope of the linear fit is the association rate constant, k_{on}. Therefore, the intercept of the linear fit with the horizontal axis is the model-independent dissociation constant of the proteomicelles, K_d.

Therefore, the observable desolvation rate is:

$$R^{\mathrm{des}}(t) = \left|\frac{d[P(t)]}{dt}\right| = [P_t]\frac{1}{\tau e^{\frac{t-T^{\mathrm{pre}}}{\tau}}}$$

Equation 17

The initial observable desolvation rate, R_{in}^{des}, is the rate calculated at the initial time, $t = T^{\mathrm{pre}}$:

$$R_{\mathrm{in}}^{\mathrm{des}} = \frac{[P_t]}{\tau} = [P_t]k_{\mathrm{obs}}^{\mathrm{des}}$$

Equation 18

Critical Parameters

Tests for self-quenching of the fluorophore

It should be verified that quenching of the fluorophore does not impact the FP anisotropy of the proteomicelles. Therefore, there is a need for control FP anisotropy experiments, as follows: (i) at the beginning of the measurements for detergent concentrations much greater than the CMC; (ii) 24 hr after the detergent desolvation reaction. In either case, no time-dependent alterations of the FP anisotropy should be found.

Contributions of light scattering effects

It should be noted that these FP anisotropy measurements can be affected by light-scattering factors. First, a Spectramax i3 plate reader (Molecular Devices) is equipped with emission filters for rhodamine derivatives (Texas Red is one of them). These filters are designed for the excitation and emission wavelengths of 535 nm and 595 nm, respectively. A large separation between excitation and emission always ensures that scattering is minimal. Second, a large wavelength of emission should be used to avoid Raman and Rayleigh light scattering effects. Both Raman and Rayleigh scattering factors are proportional to λ^{-4}, where λ is the emission wavelength (Splinter & Hooper, 2007). Third, the protein concentration under native conditions needs to reach a value at which the FP anisotropy readout is independent of protein concentration (Zhang et al., 2015). Fourth, additional control experiments need to be conducted with proteins of closely similar molecular weight, but that have varying detergent solubilization properties under identical detergent conditions. For example, in Figure 3C, we show that the basic proteins FhuA ΔC/Δ5L_25N (pI 9.3) and FhuA ΔC/Δ7L_30N (pI 9.6) cannot be solubilized by OG, so that they exhibit FP anisotropy values closely similar to the basal value of 0.16, which corresponds to the denatured FhuA variants (e.g., in the presence of high concentrations of urea or Gdm-HCl). In contrast, acidic proteins OmpG (pI 4.4) and FhuA ΔC/Δ5 L (pI 4.7) are well solubilized by OG at detergent concentrations greater than the CMC.

Troubleshooting

Protein aggregation may and most likely will occur while screening for detergents, especially at detergent concentrations well below the CMC. Aggregation of labeled protein will result in a lower RFU signal. It is possible that significant protein aggregation occurs, so that the RFU value falls below the LOD. If this is the case, starting with a higher protein concentration can overcome this issue. If the protein concentration is increased, be sure to account for the increased addition of detergent. If no change in the FP anisotropy value is observed while performing a kinetic read, it is possible that the desolvation reaction has gone to completion and the kinetic desolvation was missed. To mitigate this, attempt to lessen the dead time between the addition of reagents to the plate and the time at which the plate is read. This may also happen at time scales where it is not possible to detect the change without

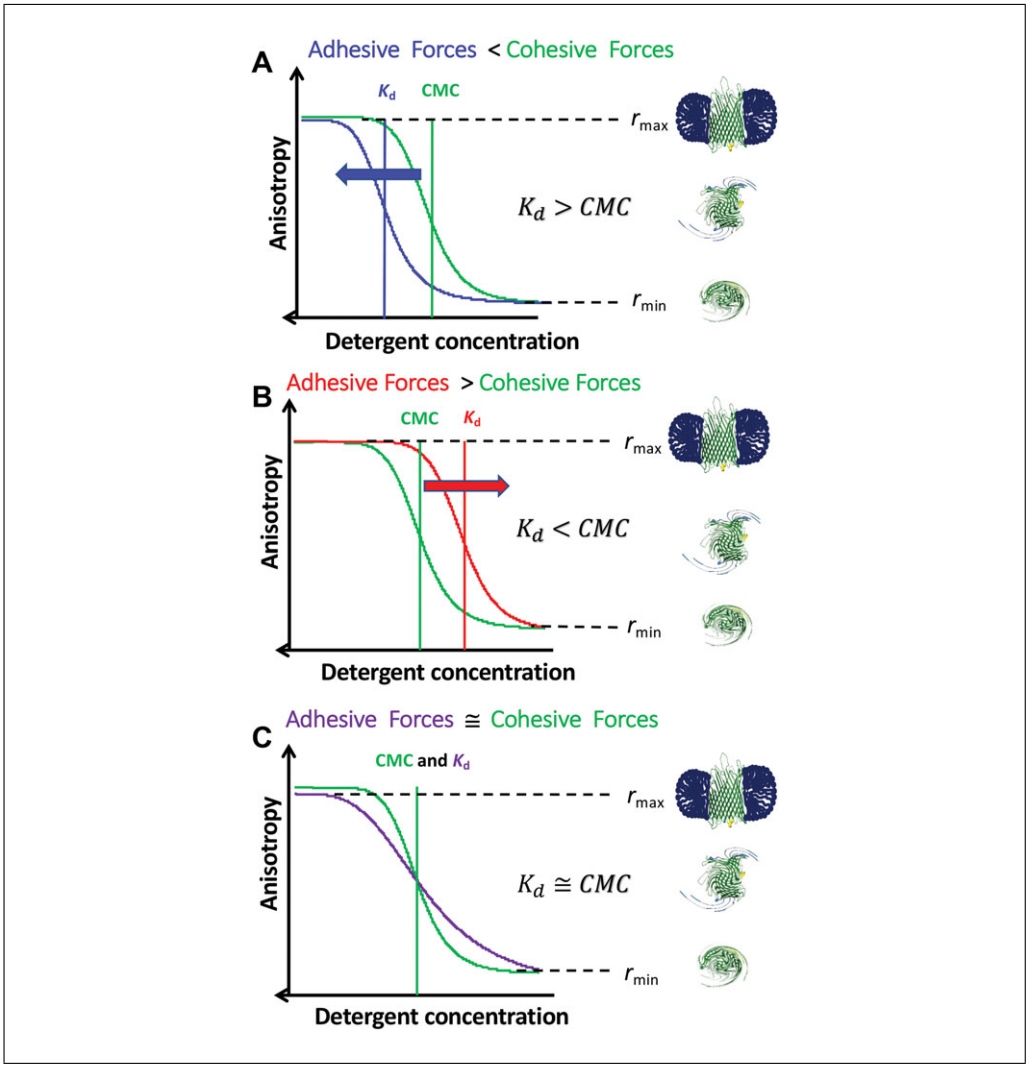

Figure 9 Hypothetical cartoons that illustrate three possible outcomes of the two-state isothermal desorption transition with respect to the CMC value. (**A**) The adhesive interactions between detergents and external surface of the membrane proteins are weaker than the cohesive interactions among the detergent monomers. (**B**) The adhesive interactions are stronger than the cohesive interactions. (**C**) The adhesive and cohesive interactions are closely similar. Reprinted with permission from Wolfe et al. (2017). Copyright 2017, American Chemical Society.

a more sophisticated stopped-flow instrument. Protein solutions containing detergents with exceptionally low CMC values will have high detergent concentrations. It is possible for the detergent concentration to be so high that it cannot be diluted to a value below the CMC when performing these detergent dilutions.

Understanding Results

For a satisfactorily solubilizing detergent, at detergent concentrations much greater than the K_d, the FP anisotropy reaches a value r_{max} that is independent of detergent concentration. In contrast, at detergent concentrations comparable with or below the K_d, the FP anisotropy decreases to a concentration-dependent value, $r(c) < r_{max}$. Yet, at detergent concentrations much lower than the K_d, the FP anisotropy decreases to an absolute minimum value, r_{min}. OmpG (Grosse et al., 2014) and FhuA (Mohammad, Howard, & Movileanu, 2011; Wolfe, Hsueh et al., 2017) proteins show anti-parallel β-sheet structure in solution under detergent-refolding conditions. For each case, the center of the isothermal desorption transition, K_d, should be compared with the CMC value. If $K_d >$ CMC, then the cohesive interactions between detergent monomers are greater than the adhesive interactions between the detergent monomers and membrane protein (Fig. 9A). The opposite is true if $K_d <$ CMC (Fig. 9B). Finally, if the adhesive and cohesive interactions are closely similar, then $K_d \cong$ CMC

(Fig. 9C). This approach can be used to obtain a high-throughput screening of the stability of PDCs in various contexts. For instance, these measurements may be extended to acquire mechanistic information regarding the electrostatic and hydrophobic interactions at the PDC interface for a variety of synthetic detergents, including amphipols (Kleinschmidt & Popot, 2014) and lipopeptides (Nazari, Kurdi, & Heerklotz, 2012; Prive, 2009). The outcomes of these studies pertain to general handling of membrane proteins in extraction, solubilization, and refolding, as well as in stabilization and crystallization.

Time Considerations

Transformation of *E. coli,* then subsequent growth and overexpression of recombinant proteins, can be completed in 3 days. However, if the protein of interest is a novel recombinant construct, growth optimization may need to be performed. Purification of recombinant proteins via affinity chromatography will take several hours for each purification performed. Fluorescent labeling of the protein and removal of the free fluorophore can be completed in little as 1.5 hr. This depends on whether the reaction is completed at room temperature or under refrigerated conditions. Removal of the free fluorophore can be completed in seconds if a desalting cartridge is available. If dialysis is utilized to remove free fluorophore, this should be done overnight. This assay can be executed with extremely small quantities of protein (e.g., tens of nanograms/trial). Our detergent-screening protocol can be conducted in a microplate format, allowing for parallel assessment of hundreds to thousands of conditions in minutes to hours.

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