Chapter 15

High-Throughput Methods for Electron Crystallography

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Abstract

Membrane proteins play a tremendously important role in cell physiology and serve as a target for an increasing number of drugs. Structural information is key to understanding their function and for developing new strategies for combating disease. However, the complex physical chemistry associated with membrane proteins has made them more difficult to study than their soluble cousins. Electron crystallography has historically been a successful method for solving membrane protein structures and has the advantage of providing a native lipid environment for these proteins. Specifically, when membrane proteins form two-dimensional arrays within a lipid bilayer, electron microscopy can be used to collect images and diffraction and the corresponding data can be combined to produce a three-dimensional reconstruction, which under favorable conditions can extend to atomic resolution. Like X-ray crystallography, the quality of the structures are very much dependent on the order and size of the crystals. However, unlike X-ray crystallography, high-throughput methods for screening crystallization trials for electron crystallography are not in general use. In this chapter, we describe two alternative methods for high-throughput screening of membrane protein crystallization within the lipid bilayer. The first method relies on the conventional use of dialysis for removing detergent and thus reconstituting the bilayer; an array of dialysis wells in the standard 96-well format allows the use of a liquid-handling robot and greatly increases throughput. The second method relies on titration of cyclodextrin as a chelating agent for detergent; a specialized pipetting robot has been designed not only to add cyclodextrin in a systematic way, but to use light scattering to monitor the reconstitution process. In addition, the use of liquid-handling robots for making negatively stained grids and methods for automatically imaging samples in the electron microscope are described.

Key words: Electron crystallography, Electron microscopy, Membrane proteins, Protein structure, High-throughput, Crystallization, Dialysis, Cyclodextrin, Negative stain

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1. Introduction

Electron microscopy (EM) has made a significant contribution to our understanding of membrane protein structure through the application of electron crystallography (1–3). As with X-ray crystallography, the formation of suitable crystals is the first and often biggest hurdle to overcome (4). Crystallographic methods cannot be applied without crystals and their quality is primarily responsible for the resolution of the final structure. The number of structures solved by X-ray crystallography has experienced exponential growth in the last two decades. Although membrane proteins have lagged behind their soluble counterparts, recent successes show a marked acceleration not only in numbers of structures, but also in their biological impact (5). Much of this success is attributable to automation, which allows X-ray crystallographers to implement high-throughput approaches at various stages of the crystallization pipeline. Specifically, automation is employed for screening genetic constructs for expression, screening of detergents for protein stability and, of course, screening tens of thousands of conditions for producing well-ordered crystals (6, 7). With regard to EM, methods for automation are routinely employed for collecting image tilt series for tomographic reconstruction (8–12) and for collecting images to include in single particle reconstructions (13–16). In addition, prototypical robotic systems have been reported for exchanging samples in the electron microscope (17–19). However, relatively little attention has been paid to automating the process of forming two-dimensional (2D) arrays of membrane proteins within the lipid bilayer, so-called 2D crystals. Such regular assemblies are amenable to atomic scale resolution assessment by electron crystallography and they yield the structure of membrane proteins in their native environment and thus in a functional state. High-throughput automation of 2D crystallization is critical to more widespread application of electron crystallography. In this review, we will describe recent developments in high-throughput 2D crystallization employing both detergent dialysis and detergent complexation with cyclodextrin. In addition, we describe facilities necessary for imaging these large-scale 2D crystallization screens in the electron microscope.

2. Materials

2.1. Sample Characterization

1. Purified, detergent-solubilized protein sample with concentration 1–2 mg/ml. Unlike X-ray crystallography, it is not necessary to have a highly concentrated protein solution and

fractions collected from the last step in the purification (e.g., size-exclusion column) may be sufficient to use directly for crystallization trials (see Note 1).

2. SDS polyacrylamide gel electrophoresis system, including glass plates, combs, tank, casting frame, power supply (e.g., Mini-Protean Electrophoresis System, BioRad Laboratories, Hercules, CA).
3. Bovine serum albumin (BSA) stock solution of 1 mg/ml in pH 7 buffer (Sigma-Aldrich, St. Louis, MO).
4. Densitometer for acrylamide gels (e.g., Molecular Imager from BioRad Laboratories or an inexpensive document scanner).
5. Device for measuring contact angles from small drops of detergent solutions. A self-contained drop-box as described by Kaufmann et al. (20) can be built according to plans available at http://temimps.nysbc.org/ or can be purchased commercially. A homemade device can be built by mounting a digital camera on a tripod and by using an optical rail to support an *x*–*y*–*z* translation stage (Fig. 1). Software specifically developed for the drop analysis can be downloaded at http://temimps.nysbc.org/.
6. Parafilm M, laboratory sealing film (Pechiney Plastic Packaging Company, Chicago, IL).
7. Bench-top centrifuge (e.g., Eppendorf).
8. Thin layer chromatography (TLC) setup for measuring lipid concentration. This consists of 20 × 20 cm TLC plates with silica gel $60F_{254}$ coating (Merck & Co., Whitehouse Station, NJ), a Glass chromatography tank (25 cm × 27 cm × 10 cm), a Kontes Chromatography TLC Reagent Sprayer with Standard Ground Joint (Fisher Scientific, Pittsburgh PA).
9. Chloroform, methanol, aqueous ammonia, and 8-anilino-1-naphthalene sulfonic acid (ANSA) (Sigma, St. Louis, MO) solubilized in water at 0.1% (w/w).
10. UV lamp and digital camera or UV imaging densitometer.
11. Spectrophotometer (e.g., NanoDrop 2000c, Thermo Scientific, Wilmington DE).

2.2. Preparation of Lipid

1. Selection of detergents that are compatible with the protein(s) of interest (Anatrace, Inc. Maumee OH). Commonly used long-chain detergents are *n*-dodecyl-β-D-maltopyranoside (DDM), *n*-decyl-β-D-maltopyranoside (DM), dodecyl octaethylene glycol ether ($C_{12}E_8$) Triton X-100 (TX-100), all of which have a very low critical micelle concentration (cmc, see Note 2) in the range of 0.1–1 mM. Commonly used short-chain detergents are *n*-octyl-β-D-glucopyranoside (OG), *n*-octyl-β-D-thioglucopyranoside (OTG), pentaethylene glycol

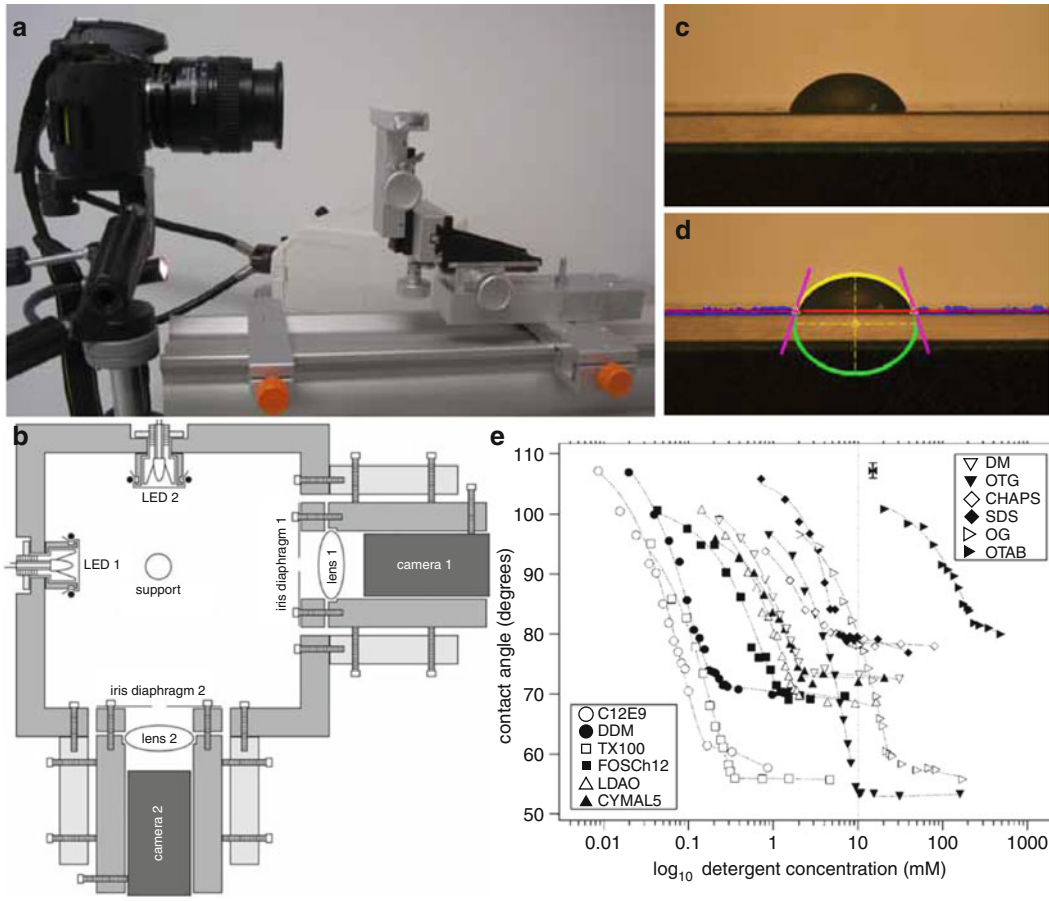


Fig. 1. Using drop shape as a measurement of detergent concentration. (**a**) Simple apparatus for recording an image of a drop. A digital camera is mounted on a tripod and drops are placed on a horizontal plate (*arrow*). The use of optical components to support the plate allows convenient translation of the drops along *x*–*y*–*z* axes. Diffuse backlighting of the drops provides a suitable image for analysis. (**b**) Diagram of the "drop box" described by Kaufmann et al. (20) (more detailed planes available at http://temimps.nysbc.org). In this device, the drop is imaged from two orthogonal angles by CCD cameras connected to a frame grabber. (**c**) Typical image of a drop containing 15 mM OG. (**d**) Fitting of the drop shape by the drop box program. *Yellow line* indicates the points used for fitting an ellipse to the drop. *Red line* corresponds to the surface of the susbstrate and the *pink lines* are the tangents to the drop at this surface, thus defining the contact angle. (**e**) Calibration curves for several detergents; adapted from Kaufmann et al. (20).

monooctyl ether (C_8E_5), which have relatively high cmc's in the range of 10–30 mM. Aqueous stock solutions should be made at 10 mg/ml.

2. Selection of lipids that are compatible with the protein(s) of interest (Avanti Polar Lipids, Alabaster, AL). These should include different chain lengths with differing degrees of saturation as well as head groups with different sizes, shapes, and charge distribution. A good initial selection includes 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-Dioleoyl-sn-

glycero-3-phosphocholine (DOPC), 1,2-Dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG), 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphatidic acid (POPA) (see Note 3).

3. Glass test tubes (7 cm × 1 cm) or 25 ml round-bottom flasks.
4. Hamilton syringes.
5. Lyophilizer or vacuum desiccator.
6. Dry N_2 gas supply.

2.3. Crystallization by Dialysis

1. 96-Well dialysis block capable of dialyzing 96 unique protein samples against 96 unique dialysis buffers. One design is described by Vink et al. (21), another is available commercially (XZ-HT-96-8k, GN Biosystems, Santa Clara CA).
2. 8–12 Channel micropipettor (e.g., Hamilton Co., Reno, NV) or liquid handling robot (e.g., Biomek FX or Biomek NXP, Beckman Coulter, Fullerton, CA).
3. Dialysis membrane in sheets with 12 kDa MW cutoff (Spectrum Laboratories, Inc. Rancho Dominguez, CA).
4. Small crystallization incubator.
5. Stock solutions of buffers for the crystallization matrix. Buffers with p*K*a of 6, 7, 8 are appropriate for initial screens, e.g., 2-(*N*-morpholino)ethanesulfonic acid (MES), *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), and 3-[4-(2-hydroxyethyl)-1-piperazinyl] propanesulfonic acid (EPPS). Stock solutions should be 0.5–1 M with their pH adjusted to the desired value.
6. Stock solutions of NaCl and $MgCl_2$ with a concentration of 0.5 or 1 M.
7. Dialysis buttons with volumes of 5, 10, 20, and 50 µl for optimization of crystal conditions (Hampton Research, Aliso Viejo, CA).

2.4. Crystallization with Cyclodextrin

1. Methyl-β-cyclodextrin and/or 2-hydroxylpropyl-β-cyclodextrin (Aldrich). Stock solutions should be made in deionized water at 50 mg/ml and filtered through a 0.2 µm filter.
2. 96-Well microtiter plate.
3. Cyclodextrin dispensing robot as described by Iacovache et al. (22) (Seyonic PCNC-0034-00-V00 pipettor) or 8–12 channel micropipettor (e.g., Hamilton Co., Reno, NV) or liquid handling robot (e.g., Biomek FX or Biomek NXP, Beckman Coulter, Fullerton, CA).
4. Stock solutions of buffers for the crystallization matrix as specified in Subheading 2.3.
5. Stock solutions of NaCl and $MgCl_2$ with a concentration of 0.5 or 1 M.

2.5. Negative Staining

1. Uranyl acetate (Electron Microscopy Science, Hatfield, PA) dissolved in water at 2 mg/ml.
2. 300 Mesh Ni electron microscopy grids (Electron Microscopy Sciences).
3. 1% Solution of collodion (parlodion) dissolved in amyl acetate (Electron Microscopy Sciences, Hatfield, PA).
4. Crystallizing dish (10 cm diameter).
5. Vacuum evaporator equipped with carbon rod or carbon thread (e.g., Auto 306, BOC Edwards, Crawley, UK).
6. Glow discharge apparatus, typically an accessory for the vacuum evaporator. Because a large number of grids are prepared simultaneously, the small chamber offered by plasma cleaners is not convenient.
7. 96-Position magnetic grid support platform (SPRI plate 384 Post Magnet Plate, Agencourt, Beverly MA) or magnetic grid tray described by Coudray et al. (19).

2.6. Electron Microscopy

1. Electron microscope suitable for screening negatively stained grids (e.g., Morgagni M268 or Tecnai T12, FEI Corp., Hillsboro OR, or JEM-1400 JEOL Ltd. Tokyo).
2. Digital camera for electron microscope (e.g., ES500W Erlangshen, Gatan Inc, Pleasanton CA).
3. Software for automated imaging of samples (where available).

3. Methods

As with 3D crystallization of detergent-solubilized protein for X-ray crystallography, 2D crystallization within the lipid bilayer is favored by a homogeneous starting solution, in which the protein adopts a single oligomeric state and the mixed micelles of protein, detergent, and lipid are monodisperse. Detergents for promoting monodispersity and stability can be screened using an FPLC system equipped with a size exclusion chromatography column. A protein solution that is well behaved will form a single Gaussian-shaped peak on the elution profile. The presence of peaks in the void volume, or the presence of multiple or asymmetric peaks in the elution volume indicates that the protein micelles are not monodisperse, either due to the presence of multiple oligomeric states or aggregation. After using chromatography to optimize the biochemical parameters of a preparation, it is also useful to examine the sample by negative stain EM to further verify its monodispersity and stability over time.

Prior to screening a membrane protein for crystallization, it is essential to characterize the relative amounts of protein, detergent,

and lipid in the starting ternary mixture. This information allows one to accurately and reproducibly control important parameters such as the lipid-to-protein ratio and the rate of detergent removal. Crystallization itself relies on the controlled removal of the detergent in the presence of a defined mixture of lipids. We present two alternative approaches for removing the detergent, namely by dialysis and by complexation with cyclodextrin. Both of these techniques are amenable to parallelization, though some special facilities are required. Specifically, a 96-well dialysis chamber has been devised, which is compatible with the use of a liquid-handling robot or multichannel pipettor. For cyclodextrins, a pipetting robot has been designed and built to systematically add nanoliter quantities of cyclodextrin stock solutions to commercially available 96-well microtiter plates. This so-called 2DX robot is also equipped with a level sensor, to allow compensation for evaporation, a shaker to ensure mixing, a temperature controller, and a laser for measuring light scattering to follow the reconstitution process. All of these parameters are displayed in real time by the control software. Alternate approaches for crystallization are dilution of the detergent below its cmc (23) or addition of polystyrene beads (BioBeads) to adsorb the detergent (24). Although the former is suitable for parallelization, it's disadvantage is the inevitable dilution of the protein as well. Whereas good results have been obtained with BioBeads, this method is not amenable to automation, given the difficulty in handling the beads.

3.1. Sample Characterization

3.1.1. Protein

In this section, we present methods for determining protein concentration using SDS-PAGE, detergent concentration using contact angle measurements, and lipid concentration using TLC. Although there are easier, faster ways to determine protein concentration (i.e., absorbance at 280 nm, Lowry assay, Bradford Assay), these are often inaccurate and are sometimes influenced by the detergent present in the solution. We therefore recommend SDS-PAGE to compare the staining of the purified protein relative to known amounts of BSA. This method removes the variability due to detergent, requires only minimal amounts of material and also verifies the purity of the sample. Furthermore, the Coomassie-stained protein band can be excised from the gel and sent to a protein sequencing facility to confirm identity, which is especially useful if the sample is provided by another laboratory. It should be noted that the Coomassie stain used for SDS-PAGE does not stain all proteins equally, so the comparison of staining intensity relative to BSA cannot be assumed to represent an exact measure of concentration. Moreover, SDS-PAGE is time consuming and may not be the best choice for unstable proteins. In these cases it may be necessary to use one of the aforementioned spectroscopic techniques, perhaps after

calibrating them relative to SDS-PAGE. The NanoDrop 2000c spectrophotometer is an excellent alternative given its extremely small sample size.

1. Prepare acrylamide gel with an appropriate concentration (e.g., 8%) to resolve your protein of interest as well as BSA (66 kDa).
2. Load a series of lanes with 0.25, 0.5, 1.0, and 1.5 μg of BSA as well as the protein of interest.
3. Run gel according to standard protocols and stain with Coomassie blue.
4. Scan gel with a densitometer and use either associated software or ImageJ to calibrate the integrated intensities of each protein sample. Plot a standard curve for the BSA samples and use this to determine the concentration of the protein of interest.

3.1.2. Detergent

Purified membrane proteins may have a poorly defined detergent concentration due to the use of a concentrator, which often retains detergent and increases its concentration in an unpredictable way. Although a concentrator with a molecular weight cutoff of 50 kDa will remove short- and medium-chain detergents (e.g., OG and DM) together with the filtrate, long-chain detergents are generally retained with the protein with molecular weight cutoffs up to 100 kDa, which may be larger than the expected micelle size. In this case, detergent concentration can be determined from the shape of droplets. This shape is governed by surface tension. Detergent molecules that line the air/water interface decrease the surface tension and cause the drop to spread. The contact angle between the drop and the supporting surface decreases monotonically with increasing detergent concentration up to the cmc. This behavior is characteristic for all detergents and needs to be calibrated (20). Above the cmc, the concentration of free detergent molecules remains unchanged, so there are no further changes in drop shape. Thus, solutions with higher detergent concentrations, such as those used to purify proteins, must be diluted to bring the detergent below the cmc. This may cause the protein to precipitate, but this precipitate can be removed by centrifugation.

1. Cover the substrate (e.g., glass slide) with a fresh piece of parafilm or Teflon tape (see Note 4).
2. Collect data for a standard curve. Prepare a series of detergent solutions with concentrations ranging from 0 to twice the cmc (e.g., 0, 2, 4, 8, 16, 24, 30, 36, 48 mM OG). Place two drops on the substrate for each concentration. Equilibrate for 30 s before recording an image.
3. Collect data for unknown sample. Dilute protein solution based on estimated detergent concentration to achieve half the cmc.

Centrifuge this solution for 30 s using a benchtop centrifuge. Place two drops on the substrate and, after 30 s incubation, record images.

4. The substrate may be washed and reused several times, but should be changed after a maximum of five drops. Thus, using a very long plate with the ability to translate the plate along its length can save a lot of time.
5. Use a computer program (e.g., ImageJ, xdroptrace, dropbox) to measure contact angles (see Note 5) and to plot a standard curve from which the concentration of detergent in the protein sample can be determined.

3.1.3. Lipids

It has frequently been observed that lipids co-purify with membrane proteins. Also, addition of exogenous lipid is a common way to optimize membrane protein stability during purification. TLC is a well-established method both to quantify the total amount of lipid and to identify different lipid species. Specifically, if the appropriate standard lipids are run together with the protein sample, it is possible to fully characterize the lipid composition in the solution.

1. Combine 65 ml chloroform, 25 ml methanol and 5 ml 25% aqueous ammonia in a glass graduated cylinder. Add this solution to a chromatography tank lined with filter paper and allow it to equilibrate for 10 min.
2. Cut TLC plates into 5 × 10 cm pieces using a glass cutter. Draw a light pencil line across the plates ~1.5 cm from the bottom. Use gloves to handle the plates to prevent contamination.
3. Prepare standard samples of lipid with a concentration of 1 mg/ml. These standards can be either in water or in chloroform/methanol.
4. Spot 2–4 μl of sample at 5 mm intervals along the pencil line. Include 1–5 μg of the standard lipids (see Note 6).
5. Dry TLC plates under a stream of N_2 gas.
6. Place plates into glass tank such that samples lay above the level of the solvent. Cover the tank and allow solvent front to ascend to the top of the plate (30–45 min).
7. Remove plates and dry under a stream of N_2 gas in a chemical fume hood.
8. Spray plates with 0.1% ANSA solution.
9. Image plates with either transmitted or reflected UV illumination using a digital camera or a specialized imaging device.
10. Quantify strength of the bands using ImageJ or similar software and determine the lipid composition and concentration by comparison with the standard solutions (Fig. 2).

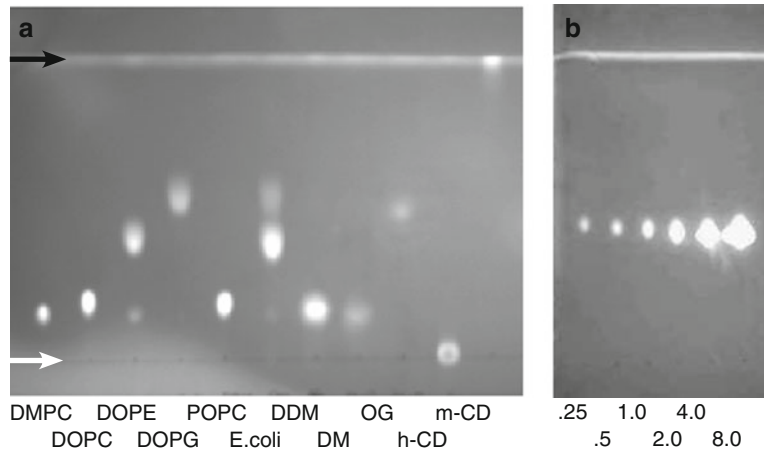


Fig. 2. Use of thin layer chromatography to characterize lipid. (**a**) Various species migrate differently on the TLC plate and can thus be distinguished. DMPC, DOPC, DOPE, DOPG, POPG, and *E. coli* correspond to the lipids mentioned in the text. DDM, DM, and OG are detergents mentioned in the text. h-CD and m-CD are 2-hydroxypropyl-β-cyclodextrin and methyl-β-cyclodextrin, respectively. The *white arrow* indicates the origin, where samples were all initially applied, and the *black arrow* indicates the solvent front at the end of the run. (**b**) Ladder of lipid concentrations show how TLC can be used to determine lipid concentration as well as separate different species. This amount of lipid added to each lane ranges from 0.25 to 8 μg, as indicated along the bottom.

3.2. Preparation of Lipid Solutions for 2D Crystallization Screens

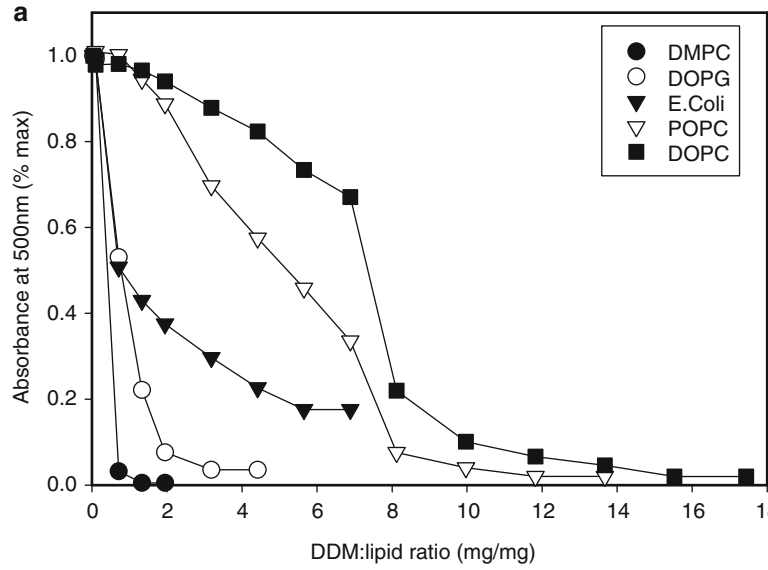
1. Prepare stock solution of lipids in chloroform at 25–50 mg/ml. Lipid is susceptible to oxidation and care must be taken during storage. For long-term storage, it is safest to keep lipid in the powdered form at −80°C. When storing chloroform stock solutions at −80°C, vials with Teflon seals should be used and should be purged with N_2 gas before sealing. Chloroform solutions are also susceptible to evaporation, which will alter the lipid concentration, so care should be taken to keep these solutions cold (e.g., on ice) and capped when not in use.
2. Make a thin film of dried lipid. Use a Hamilton syringe to pipette the desired amount of lipid (e.g., 500 μl) into a glass test tube or 25 ml round-bottom flask (see Note 7). Swirl the test tube while allowing the chloroform to evaporate in a chemical fume hood. A thin stream of N_2 gas will accelerate this process, though the rate of evaporation should be relatively slow in order to obtain a thin, even film of lipid around the walls of the test tube. This film should appear homogeneous and white. If thick, clear areas are present, the film should be redissolved with a small amount of chloroform and the process repeated.
3. If making multiple lipid stock solutions, purge the test tube with N_2 gas and store capped on ice until the next step. This

thin film is particularly susceptible to oxidation given its high surface area.

4. Remove traces of chloroform by placing lipid film in a lyophilizer or vacuum desiccator. A cold trap is highly recommended in order to prevent contamination of lipid samples with back-streaming oil vapor from the vacuum pump. Pump for at least 1 h.
5. Prepare detergent stock solutions at 100 mg/ml. All of the detergents should dissolve readily in water. These can be aliquoted and stored briefly at 4 °C, but should be frozen at –20 or –80 °C for longer periods.
6. Prepare aqueous lipid stock solutions by adding detergent solutions to the dried lipid films (see Note 8). The amount of detergent required for solubilization depends both on the lipid species and on the detergent (Fig. 3). Generally speaking, the choice of both detergent and lipid will be governed by the preference of the protein. For particularly difficult lipid/detergent combinations, one should consider using a different detergent for solubilization and thus attempting crystallization from a solution with a mixture of detergents. After adding detergent to the lipid film, the solution should be stirred on ice for 1 h to ensure a clear, homogeneous solution of solubilized lipid.

3.3. Crystallization by Dialysis

1. Define conditions for the crystallization screen. General parameters that influence crystallization are pH, lipid composition, and lipid-to-protein ratio (LPR) of the bilayer, and ionic composition of the aqueous phase. Therefore, a basic screen for a novel protein with unknown behavior, includes pH 6, 7, and 8, a range of lipids such as DMPC, DOPC, POPC, DOPG, DOPE, or *Escherichia coli* lipid extract for bacterial proteins, LPR's from 0.25 to 1.5, presence of either 100 mM NaCl or 5–10 mM $MgCl_2$. By using a 96-well dialysis block, it is possible to obtain a linear sampling for all of these parameters: e.g., a matrix of conditions with three values of pH, five species of lipid, three LPR's, in the presence of either NaCl or $MgCl_2$.
2. Information about the protein of interest may guide the selection of parameters used for the screen. Specific ligands should be tested as an additive, either in native state (e.g., transported ions) or as an enzymatically inactive state (e.g., non-hydrolyzable nucleotides or transition state analogues). Mammalian proteins call for testing of cholesterol or sphingolipids as a component of the bilayer, or a species-specific lipid extract may be used (e.g., from egg-yolk, brain, wheat germ, or *E. coli*).
3. Prepare the protein/detergent/lipid solutions in 96-well microtiter plates and incubate at 20 °C for an hour to ensure complete mixing of these amphipathic components. The goal is to start with a completely homogeneous preparation of



b Solubilization of lipids by detergent*

	DMPC	DOPC	DOPG	POPC	E. coli
OG	4.4	6.8	2.5	6.8	5.6
OTG	5.0	5.0	2.5	3.7	3.1
C8E5	2.5	5.0	1.9	3.7	3.7
DM	1.0	15.7	n.d.	15.7	2.0
DDM	1.3	10.0	1.9	10.0	4.4
C12E8	1.0	1.4	1.0	1.4	1.0
TX100	1.0	1.9	1.3	1.9	1.9

* values correspond to the weight ratio of detergent required to reduce Abs^{500} to baseline for each lipid species at a concentration of 1.5 mg/ml

Fig. 3. Solubilization of lipid by detergent as followed by light scattering. (**a**) Light scattering (absorbance at 500 nm) is plotted against the weight ratio of DDM relative to a variety of lipids as indicated on the upper right. Solubilization is complete once the light scattering signal reaches a baseline close to zero. Note that some lipids are considerably easier to solubilize than others, and this effect depends on the detergent. (**b**) Concentrations of detergent required to solubilize 1.5 mg/ml solution of various lipids. This table summarizes the results of plots like that shown in panel (**a**). These results will depend on the state of lipids and will be most reproducible if starting with unilamellar vesicles. For these data, dried lipid films were resuspended in small amounts of each detergent by vortexing. Additional aliquots of detergent were then added, the solution stirred and Abs^{500} was measured.

mixed micelles. Shaking the plates during this incubation helps with this mixing. Both the protein and the lipid solutions should contain sufficient detergent for solubilization, so no additional detergent is required at this point. However, it may be desirable to adjust the detergent concentrations to be equal in all of the crystallization trials, thus ensuring a consistent rate

of detergent removal across all the conditions. This strategy requires addition of detergent to conditions with lower LPR. For long-chain detergents, this will unnecessarily prolong an already long crystallization period (1–2 weeks) and it may be better to consider the variable reconstitution rates as another parameter in the crystallization.

4. Prepare dialysis solutions in a way that is compatible with the liquid handling device. The geometry of the 96-well format restricts the volume of the dialysis buffer relative to the protein solution, but this restriction can be overcome by frequently changing the dialysis buffer over the course of the crystallization. Typically, this buffer is changed twice per day and a programmable liquid-handling robot is therefore extremely useful. To facilitate this process, the dialysis buffers should be arrayed in a 96-well format (e.g., deep-well plates). Dialysis buffers should contain any soluble components that distinguish the crystallization trial, e.g., pH, salts, nucleotides, and other soluble ligands. Membrane bound additives are likely to stay inside the dialysis chamber in association with either the micelles or the bilayer.
5. Assemble the dialysis block (Fig. 4) in a way that minimizes bubbles in the individual dialysis chamber. Specifically, excess protein solution should be added to each well (e.g., 55 μl to a 50 μl well) to ensure complete whetting of the dialysis membrane prior to sealing the device. Bubbles will potentially block the dialysis membrane and slow the rate of detergent removal. To minimize this problem, the dialysis block can be stored on its side so that any bubbles will rise to the side of the well rather than block the dialysis membrane.
6. Place the dialysis block in a temperature-controlled environment at the selected temperature. Typical temperatures to test are 4, 20, and 37 °C and the initial choice should depend on the stability of the protein sample, as assayed by size-exclusion chromatography. Temperature cycling has also been effective in promoting crystal formation, which involves shuttling samples between high and low temperature environments. Higher temperatures promote bilayer fluidity, mixing of hydrophobic components, and perhaps formation of crystal seeds, whereas the lower temperatures promote annealing of larger crystalline domains. Prolonged periods at high temperature may also lead to denaturation of protein, especially in the presence of high amounts of detergent. Therefore, a useful strategy might be to remove detergent initially at low temperature and then to switch to a period of thermal cycling as the solution transitions from the micellar phase to the bilayer phase.
7. The time required to complete the dialysis should be studied prior to a crystallization screen and can be estimated by visual

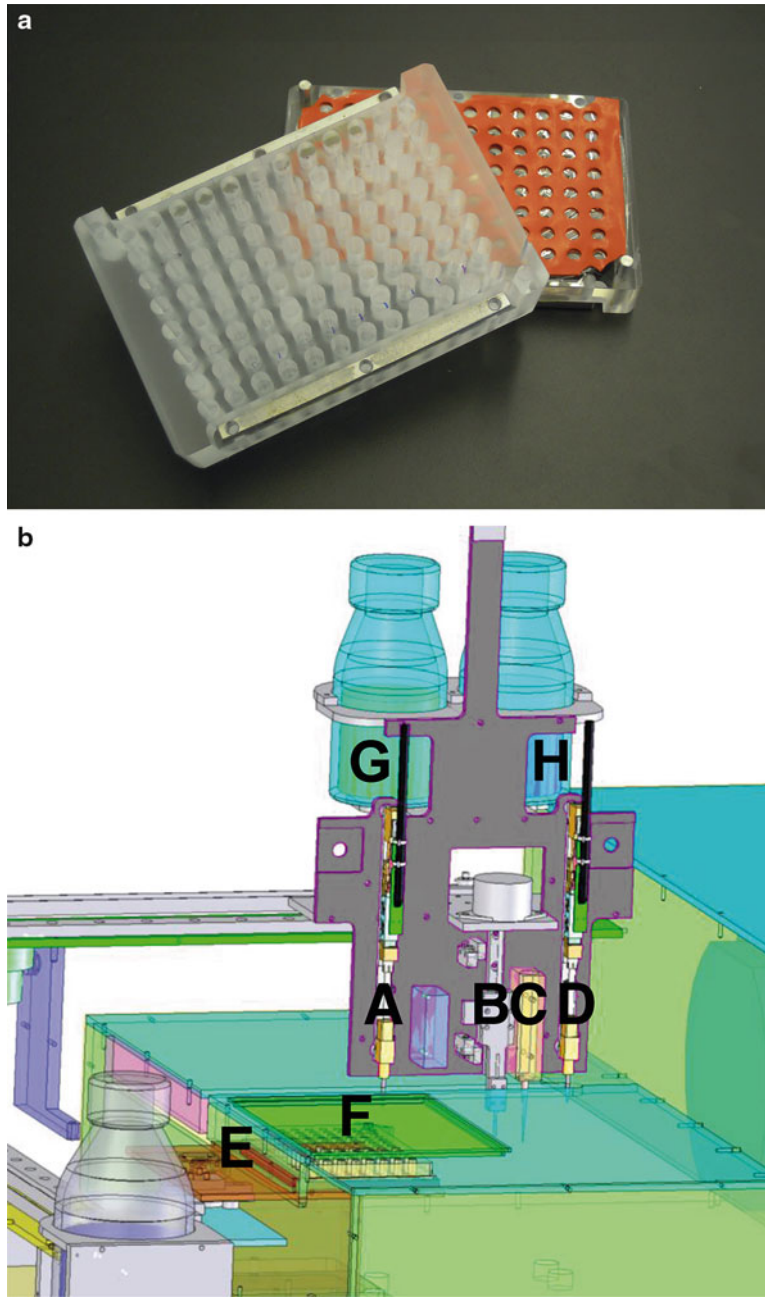


Fig. 4. Devices for high-throughput crystallization trials. (**a**) Dialysis block accommodating 96 independent samples, each with a unique dialysis buffer, as described by Vink, et al. (21). The samples containing protein and lipid are placed in the lower chamber, fitted with a sheet of dialysis membrane and a gasket (orange), and secured to the upper chamber, which holds the dialysis buffers. The wells are arrayed with standard 96-well geometry such that loading and dialysis buffer exchanges can be done with a commercial liquid-handling robot. (**b**) Cyclodextrin crystallization robot described by Iacovache et al. (22) with plans available at http://temimps.nysbc.org. This robot uses nanoliter scale pipettors (Seyonic) to add either a cyclodexrin stock solution (A/G) or water (D/H) to each of 96 well in a commercial microtiter plate. It is equipped with a volume sensor (B) and additions of water are used to compensate for evaporation during the period of crystallization. In addition, light scattering is measured (C) and the plates are shaken to ensure mixing of the solutions (F).

inspection of the turbidity of the samples during crystallization. Certain additives, such as glycerol, have a tendency to slow dialysis significantly, whereas elevated temperatures may speed dialysis; such parameters need to be taken into consideration. Therefore, a mock crystallization trial should be set up in advance, omitting the protein from the sample but otherwise maintaining concentrations of detergent and lipid. After each exchange of dialysis buffer, the detergent concentration of these buffers can be analyzed, using either drop shape measurements or TLC, and the removal of detergent can thus be plotted as a function of time.

8. The time required to reach the transition point can be inferred from the rate of detergent removal and knowledge of the starting concentrations of lipid and detergent. A good rule of thumb is that the transition to lipid bilayers will occur when the detergent-to-lipid ratio is 1:1. In reality, this transition varies depending on the specific lipid and detergent species and can be studied by measuring light scattering of detergent–lipid solutions as the detergent concentration is increased (Fig. 3).

3.4. Crystallization Using Cyclodextrin

1. Define conditions of the screen as above.
2. Prepare the individual detergent/lipid/protein samples in a 96-well microtiter plate as above, but include all cofactors to be tested, because unlike with dialysis, these cofactors cannot be introduced through the dialysis buffer. This fact means that a more concentrated protein solution is desirable for the cyclodextrin method, which can be diluted into a buffer with defined salt composition and pH. Alternatively, the protein solution may be dialyzed prior to crystallization setup.
3. Set up the 2DX robot (Fig. 4). The rate of adding the cyclodextrin solution (µl/h) depends on the detergent concentration in the well and the specific detergent adsorption capacity of the cyclodextrin. One should plan to reach the cmc within 6–12 h. For example, if the ternary mixture containing protein, lipids, and detergent contains 5 mM DDM, a total of 10 mM cyclodextrin will need to be added, because it takes 2 cyclodextrin molecules to chelate 1 molecule of DDM (25). Given a well volume of 20 µl, 0.286 mg of cyclodextrin will be required for full complexation of the detergent, which corresponds to 5.72 µl of a 5% stock solution of cyclodextrin. In this case, a rate of 0.5–1 µl/h would be suitable for adding the cyclodextrin, typically at intervals of one drop every 15 min (~200 nl/drop).
4. Set the initial temperature. Ideally, the initial temperature should be as low as possible during early stages of detergent removal, while remaining above the phase transition temperature of the

lipid. In particular, the transition temperature is 25 °C for DMPC and <0 °C for the lipids with unsaturated acyl chains.

5. Monitor the reconstitution/crystallization process by light scattering. The light scattering signal will increase as the detergent concentration reaches the cmc, indicating the aggregation of membrane proteins and lipids, hopefully forming bilayers.
6. Increase the temperature over the next 6–12 h, typically to 37 °C. Cyclodextrin may be added over another 12 h to ensure complete complexation of the detergent.

3.5. Negative Staining

1. Prepare a solid plastic film by pipetting 25 μl of collodion solution onto the surface of water-filled crystallizing dish. Prior to adding the collodion, the surface of the water should be swept free of dust with a glass rod or pipette. After allowing the film to dry briefly, EM grids are placed on its surface in a defined orientation (e.g., polished side contacting the film). Plastic-coated EM grids are picked up from the water surface using a flat piece of lint-free, unprinted newspaper (see Note 9). Allow the grids and newspaper to dry in a clean environment.
2. Coat the grids with carbon using a vacuum evaporator (see Note 10).
3. Make the grids hydrophilic by glow discharge (see Note 11).
4. Place the grids onto the magnetic posts of 96-position magnetic grid support platform or tray (Fig. 5).
5. Use liquid handling robot or multichannel pipette to carry out pipetting steps for negative staining. The sequence of steps depends on the particular application. Specifically, Kim et al. (26) added 2 μl individual crystallization trials to individual EM grids and allowed them to incubate for 30 s. Thereafter, three sequential drops containing 8 μl of 0.25% uranyl acetate were added, using the pipette to remove each drop prior to adding the next. Alternatively, a home built staining robot pipettes 4 μl of each protein sample on the grids, incubates for 60 s, and then washes grids three times with water to remove cyclodextrin. After removing the final water wash, 3 μl of 2% uranyl acetate are added and removed after 15 s by aspiration (19).
6. Remove final drop of stain either by aspiration with the pipettor or by blotting the grids with filter paper. For the latter, a strip of filter paper can be used to blot an entire row of EM grids simultaneously (see Note 12).

3.6. Electron Microscopy

1. Insert negatively stained samples into an electron microscope. This can be done manually by following the manufacturers procedures, or in an automated fashion using a robotic sample changer. The latter is preferable for screening large numbers of samples and a number of alternative systems have been

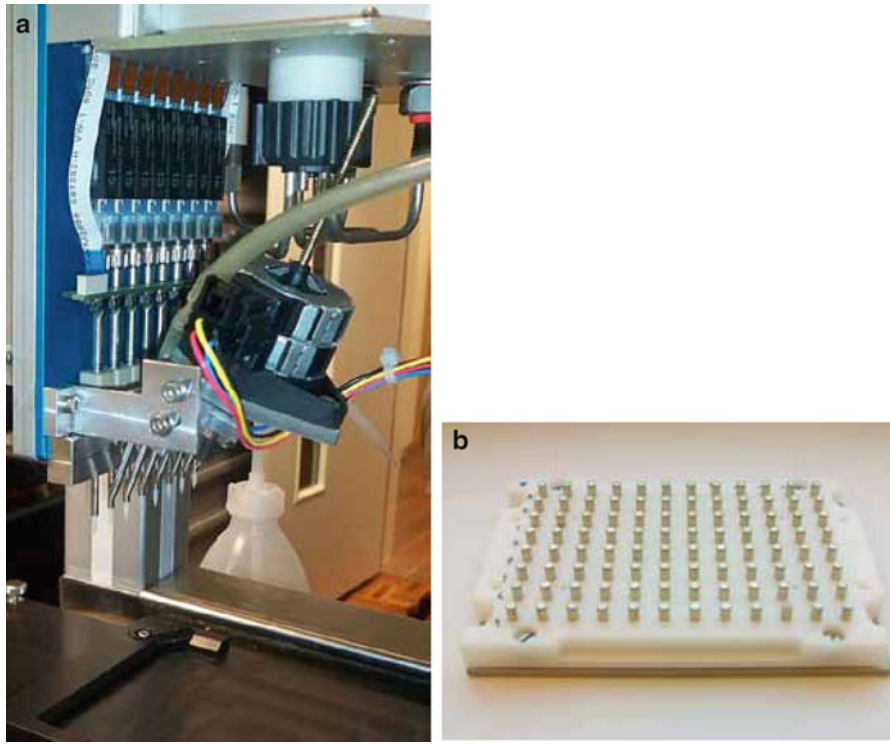


Fig. 5. Apparatus for high-throughput negative staining. (**a**) A negative staining robot described by Coudray et al. (19) for applying negative stain to grids placed on a magnetic platform. This device stains four grids at a time and is capable of working through a set of 96 grids in ~2 h. A computer control interface has been developed that allows the user to specify all the relevant parameters of staining, such as sample incubation time, number of washes, number of stain cycles. (**b**) A commercial magnetic platform that can be used for negative staining. The posts contain rare earth magnets and firmly hold Ni grids, such that they are not lifted by capillary action during the multiple pipetting steps. The posts are arrayed according to standard 96-well dimensions and can be accessed either with commercial liquid-handling systems or with the negative staining robot in panel (**a**).

developed. Most recently, Coudray et al. (19) have described the retrofit of a Tecnai T12 electron microscope with the "Autoloader" from FEI Corp coupled with a carousel for holding up to 96 samples. For this system, EM grids are loaded into 12-grid Autoloader cassettes and 8 of these cassettes are mounted on the carousel within the vacuum of the microscope. A similar strategy was used for the Gatling Gun designed by Lefman et al. (27), in collaboration with Gatan Inc. This device consisted of a spiral drum that accommodates 100 grid cartridges from the FEI Polara microscope, also within the vacuum of the microscope. An alternative approach for loading samples was originally reported by Cheng et al. (17) and more recently elaborated by Hu et al. (18). Both of these groups built free-standing robots to load grids from a tray onto the standard EM sample holder and then to transfer the holder through the air-lock of the microscope. The latter

systems take slightly longer for loading each grids and require considerable care in maintaining the mechanical alignment between the robot and the microscope (specifically the axis of sample insertion). However, they require minimal modifications to the electron microscope and grids do not have to be individually mounted into specialized cartridges.

2. Record a series of representative images from each sample. A number of different software packages have been developed for automated imaging, though they have varying abilities to target suitable objects on the grid. Both of the external robots described in the previous section are controlled by the Leginon software program, which also includes applications for automatically collecting images from each grid after loading. The basic steps, described in detail by both Hu et al. (18) and Cheng et al. (17), involve identifying suitable grid squares from a montage of images at very low magnification, identifying suitable areas within these grid squares at low magnification and recording images at medium magnification for later evaluation. Although this software is able to identify simple objects in the intermediate images, like regular holes in a perforated carbon support, it currently lacks the ability to identify objects of interest for crystallization screens, though this is an area of active development. Similarly, the Gatling Gun included a script for directing Gatan's Digital Micrograph software to record a series of images at designated magnifications, but no effective algorithms for targeting (27). Commercial software called PASys from JEOL has been developed in collaboration with the Fujiyoshi labortory that does include a shape analysis of objects in images, but this software appears to only be compatible with JEOL microscopes. A more satisfactory solution is provided by the AnimatedEM program that was developed in conjunction with the Autoloader retrofit of the Tecnai T12 microscope (19). This program collects the usual series of images at increasing magnification, but performs a sophisticated shape analysis of intermediate images to select uniform, isolated sheets that might contain 2D arrays. Higher magnification images are then recorded from these areas and Fourier transforms are inspected for the presence of Bragg reflections, which would reflect not only the presence of 2D arrays, but also their relative order.
3. Store images of crystallization trials in a database for evaluation and cross-referencing. Although a laboratory notebook is the conventional way of recording crystallization results, as the number of conditions and the number of protein targets increase, accessibility of images and correlation with their respective crystallization conditions becomes extremely important. A laboratory information management system (LIMS)

such as those used by high-throughput X-ray crystallography centers is ideal, given their existing facility for storing information about sequence, expression, purification, crystallization. In particular, the Sesame LIMS (28, 29) has been modified to import images and associate them with their respective crystallization conditions (18). This import can be done with any generic set of images, or it can be done automatically from the Leginon database using a shared database identifier. The Sesame user interface then allows users to scan through images and assign corresponding scores reflecting the success of the crystallization trial.

3.7. Conclusion

The dialysis and cyclodextrin methods represent two complimentary approaches to crystallization. On the one hand, the ability to dictate the rate of cyclodextrin addition can be used to precisely control the rate of detergent removal and consequent reconstitution. It is reasonable to assume that the transition between the micellar phase and the bilayer phase is critical to the crystallization process, and manipulation of cyclodextrin addition offers the opportunity to explore this transition. On the other hand, dialysis has a longer track record in reconstitution and membrane protein crystallization. Although the level of control is somewhat limited, the resulting solutions do not have high levels of cyclodextrin present, simplifying the preparation of samples for electron microscopy. With either approach, differences in the physical chemistry of detergent removal can be expected to produce different results and it is impossible to know a priori which will yield the best crystals for a given protein target. In the spirit of assessing the effect of all possible parameters, it may be useful to attempt both methods of crystallization. This is analogous to empirical testing of different 3D crystallization methods for X-ray crystallography (e.g., hanging drop, sitting drop, batch, lipidic cubic phase) in order to obtain the best possible result.

A combination of these two methods may also be a productive avenue to explore. In particular, the addition of cyclodextrin to the dialysis buffer has the potential to accelerate and to provide finer control over dialysis rates. Dialysis rates are fundamentally limited by the relatively low concentration of monomeric detergent molecules, which are the only species that can equilibrate across the dialysis membrane. Detergent micelles typically contain ~100 molecules and are therefore too large to move across this membrane. The limitation is particularly acute for long-chain detergents, which have a very low cmc. However, both cyclodextrin and the cyclodextrin-detergent complexes are able to migrate across the dialysis membrane, thus effectively increasing the pool of monomeric detergent molecules that can be equilibrated and thus removed by dialysis. Preliminary analysis has shown cyclodextrin to accelerate the removal of DDM greatly, achieving complete detergent removal

in only 4 days, compared with the typical 2 weeks in the absence of cyclodextrin. The overall cyclodextrin concentration remains relatively low throughout the process and can be completely eliminated by simply omitting cyclodextrin in the last change of dialysis buffer. Because this buffer is frequently changed throughout the procedure, one can maintain a certain control over the rate of detergent removal, for example, pausing when the protein solution reaches the transition from micellar to bilayer phases. Also, a bolus of cyclodextran can be added to the starting protein solution to establish a starting condition that is relatively close to the transition, thus minimizing the unproductive period that a protein spends in the micellar state. This may be particularly important for unstable membrane proteins.

4. Notes

1. Ideally, protein is used immediately after purification without freezing, storage, or concentration. By freezing or otherwise storing the protein, the possibility of denaturation or aggregation increases. Concentrators typically increase the detergent concentration in an unpredictable way, making it difficult to precisely define the starting point for crystallization.
2. The cmc is defined as the concentration at which micelles start to form. Below this concentration, all detergent molecules are monomeric in solution. Above this concentration, there is a mixture of monomeric detergent molecules and micelles, which are in dynamic equilibrium. Above the cmc, the concentration of monomers remains constant at the cmc. The cmc is characteristic for each detergent, depending on its chemical composition, and is affected by physical/chemical parameters such as the presence of solutes and temperature.
3. This selection of lipids has hydrocarbon chain lengths of 14 (myristol), 16 (palmitoyl) and 18 (oleoyl). The latter has one unsaturated bond, whereas the former are both fully saturated. The head groups range from the zwitterionic phosphatidylcholine and phosphatidyl ethanolamine, to the negatively charged phosphatidylglycerol and phosphatidic acid. Although this is a good starting set of lipids, in many cases it is also worth using other membrane components, such as cardiolipin, cholesterol, and sphingolipids, or using lipid extracts from the relevant organism or tissue, such as liver, heart, brain *E. coli*, or yeast.
4. Parafilm is the standard substrate reported by Kaufmann et al. (20) that generally works well. Teflon tape (e.g., for pipe fittings) is an alternate choice of substrate and may be suitable for short-chain detergents. Parafilm has the advantage of

maximizing changes in the contact angle as a function of concentration. Teflon is more hydrophobic and thus limits the extreme flatness of drops produced by short-chain detergents (e.g., OG) as their concentration approaches the cmc. With some software, it is difficult to determine the contact angle for very flat drops and measurements may therefore be more reproducible using Teflon tape.

5. Although contact angle is the conventional and most physically rigorous measure of surface tension, the ratio of drop width and height also produces reliable results, at least for small drops (e.g., 20 μl where the shape is well approximated by an ellipse). The programs Xdroptrace and DropBox both fit the drop shape with an ellipse and calculate either the width/height ratio (based on the major and minor axes) or the contact angle (based on the intersection with the planar substrate). The measurement of contact angle is very sensitive to the precise position of the substrate and the axial ratio may therefore be more robust in practice. Both Xdroptrace can be downloaded from http://temimps.nysbc.org.
6. If the concentration of lipid in the protein solution is low, then the lipids may be readily extracted from the solution with chloroform. After separating the organic phase from the aqueous phase, the chloroform can be evaporated and the resulting lipid film resolubilized in a smaller amount of chloroform prior to running the TLC. Extraction into an organic phase is also a method for separating the lipid from the detergent, which will be abundant and perhaps interfere with the signal from the lipid. Although nonionic detergents partition between the aqueous and organic phases, multiple washings of the organic phase with water will greatly reduce the amount of detergent present; lipid has a negligible solubility in water and will remain almost completely in the organic phase.
7. It is judicious to include a small amount of butylated hydroxytoluene (BHT) as a scavenger of free radicals and therefore as a mechanism to minimize lipid oxidation. Prepare a stock solution of 20% BHT in ethanol and add a sufficient amount to obtain 0.2% BHT in the final aqueous stock solution of lipid.
8. It is possible to bring lipid into the aqueous phase in the absence of detergent, but the lipid will form very heterogeneous multivesicular structures that may prove difficult to solubilize at a later stage. A more homogeneous, pure lipid solution can be obtained by sonicating the solution using a probe sonicator (e.g., Branson Sonifier S-250 fitted with a microtip). The effectiveness at producing unilamellar vesicles depends on the nature of the lipid; lipids with a net charge more readily form unilamellar structures, whereas neutral lipids are more difficult to disperse. Sonication puts a significant

amount of energy into the solution and can cause chemical changes (oxidation, cleavage of headgroups, creation of lysolipids); the solution should therefore be kept cold (on ice and in a cold room) and sonication periods should be minimal (1–3 minutes with 50% duty cycle and with breaks to allow the solution to remain cold). Since a detergent-solubilized, homogeneous solution is required to start crystallization, it therefore makes sense to directly solubilize the lipid film with detergent and avoid these potential problems, but the ability of a given detergent to solubilize the lipid also depends on the physical state of the lipid.

9. To remove the EM grids from the water surface, layer the newspaper on top of the EM grids, allow the paper to absorb a bit of water and then peel the paper off of the surface, thus removing the EM grids as a sandwich between the newspaper and the plastic film. Place the sandwich into a petri dish with the plastic facing upwards and allow to air dry (30).
10. Depending on the apparatus, either a carbon rod (graphite or amorphous carbon) or a carbon thread can be used for evaporation. For high resolution work, the flatness and conductivity of the carbon support is critical (31). For evaluating negatively stained samples, however, one simply requires a uniform carbon film that provides a reasonable proportion of unbroken grid squares (the broken squares can be identified by automated imaging programs and easily avoided). The underlying plastic film is helpful in this regard. It is also important to control the current during carbon evaporation in order to avoid sparking, which produces inhomogeneities in the resulting film. Also, many investigators evaporate several thin layers of carbon in order to improve strength.
11. An optional step in EM grid preparation is to remove the plastic film prior to glow discharge. This improves the clarity of high magnification images and also reduces residual stickiness that results from the ragged edges of the plastic film at the periphery of the grid. However, this step is not easily automated and represents an interruption in the workflow. To remove the plastic, place several pieces of filter paper into a glass petri dish. Saturate the filter paper with amyl amine. Place EM grids onto the filter paper with carbon side facing upwards. Cover the petri dish and allow grids to incubate for 15–30 min in a chemical fume hood. Remove the grids and place on a fresh piece of filter paper for glow discharge. An alternative method for producing carbon film is evaporate carbon onto a freshly cleaved mica surface and then to float this carbon onto a water surface. This method, also less amenable to high-throughput methods due to fragility in the resulting film, is described by Stokes and Ubarretxena (30).

12. The choice between blotting and aspiration will depend on the quality of the samples as assessed by electron microscopy. The goal is to obtain an even, thin layer of solution across the entire EM grid, which after drying encases all of the samples in an even layer of negative stain.

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