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Liposomes on a streptavidin crystal: a system to study membrane proteins by cryo-EM

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Abstract

In this chapter we describe the preparation of cryo-EM specimens for random spherically-constrained (RSC) single-particle reconstruction of membrane proteins. The specimen consists of liposomes into which the purified membrane protein is reconstituted at low density. The substrate is a 2D streptavidin crystal, which serves as an affinity surface that tethers the liposomes, which are doped with biotinylated lipids; the crystal also can serve as an image-quality and image-calibration reference. After subtraction of the crystal and lipid membrane contributions to the image, the remaining particle images can be used for 3D reconstruction.

1. Introduction

A long-sought goal in structural biology has been the imaging of membrane proteins in their lipid environments. This goal has been achieved in several cases where the proteins can form two-dimensional crystalline arrays in planar membranes (Walz, this volume) or in helical membrane tubes (Kubalek, Young chapters in this volume). As an alternative approach, we have sought to extend the generality and power of the single-particle reconstruction technique to membrane proteins. Tilley et al. (Tilley et al., 2005) were the first to obtain single-particle reconstructions of membrane proteins in lipid membranes from hundreds of “side views” (within 20 degrees of the equator) of the proteins in liposomes of variable size. We have recently described a more general method, in which membrane protein particles are imaged in spherical liposomes. The spherical shape allows the lipid membrane contribution to each image to be computationally removed, regardless of the liposome size, and also aids in the determination of the orientation of each protein particle. This method has been successfully employed to study the structure of the BK “big K⁺” calcium-activated potassium channel (Wang and Sigworth, 2009).

In this chapter we describe the steps in assembling the particular type of cryo-EM specimen that we have used for this method (Fig. 1). Membrane proteins are reconstituted at low density into liposomes, and the resulting proteoliposomes are applied to a nano-structured substrate. The substrate is a two-dimensional streptavidin crystal, which spans the holes in a perforated carbon film. The streptavidin serves as an affinity surface, tethering liposomes at a uniform density. The ordered lattice of this crystal serves as a built-in image calibration and evaluation reference. The periodic lattice also allows efficient subtraction of the signal from the streptavidin layer, yielding less background noise than traditional ultra-thin carbon films.

A typical image of this specimen is shown in Fig 1C. In order to determine the structure of the membrane protein, the contributions to the image of the 2D crystal and the lipid

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membrane are first removed, and then the protein particles are picked from the resulting images (Fig. 1D). From the position of each imaged particle with respect to the center of the liposome, two of the particle's three Euler angles can be estimated. This greatly reduces the computational cost of determining the orientation of each particle, and increases the reliability of the orientation assignment. Given orientations of the particles, the 3D structure of the protein can then be determined using conventional 3D reconstruction methods.

2. Methods

2.1 Streptavidin crystals

Streptavidin is a 15 kDa protein of 159 residues (Sano et al., 1995). It is proteolyzed naturally at both ends, and the most stable and well-studied form of this protein (called core-streptavidin) contains 125 to 127 residues. The atomic structure has been determined by X-ray diffraction (Hendrickson et al., 1989; Weber et al., 1989). Other structures (full length, biotin-bound complex and mutants) have also been determined (Freitag et al., 1999; Freitag et al., 1997; Izrailev et al., 1997). Streptavidin forms a two-dimensional crystal on a monolayer of biotinylated lipid; this assembly has been studied by electron crystallography (Avila-SakarTT and Chiu, 1996; Darst et al., 1991; Kubalek et al., 1991; Le Trong et al., 2006; Wang et al., 1999). The crystal structures show that the protein is a homotetramer of identical subunits. Each subunit contains a β -barrel with the biotin-binding site located at one end (Fig. 2A).

Two-dimensional crystals grown on a lipid monolayer also show a tetrameric arrangement of subunits (Fig. 2A). The 2D crystal has a unit cell with $a=b=8.23$ nm, $\gamma=90^\circ$ (Avila-Sakar and Chiu, 1996). Due to the mirror symmetry in the projection map, the reflection spots with indices $h+k = 2n+1$ are absent; thus in projection the crystal appears to have a square lattice with $a=b=5.82$ nm and $\gamma=90^\circ$. We use these parameters in analyzing our cryoEM images.

The 2D streptavidin crystal has been shown to be readily picked up by an EM grid coated with a perforated carbon film (Avila-Sakar and Chiu, 1996; Crucifix et al., 2004; Kubalek et al., 1991), and crystal sizes up to a few μm^2 have been observed. Crystals fast-frozen in vitreous ice show electron diffraction to atomic resolution (Avila-Sakar and Chiu, 1996), and the 2D crystals have been shown to be useful as tethering surfaces, e.g. tethering biotinylated DNA molecules (Crucifix et al., 2004).

2.2 Growth of the streptavidin crystal

The 2D streptavidin crystal is grown on a lipid monolayer containing biotinylated lipid (Darst et al., 1991) as shown in Fig 2B. Streptavidin solution is loaded into a well, onto whose surface a lipid monolayer containing biotinylated lipids has been formed. As a first step in the formation of the 2D crystal, two of the four binding sites on the streptavidin tetramer bind sequentially to biotin lipid headgroups. Then, by diffusing in the plane of the monolayer, the tetramers interact to form two-dimensional arrays. In the resulting crystal, each streptavidin tetramer has two vacant biotin binding sites that are available to bind to other molecules.

Streptavidin was purchased from Sigma-Aldrich (St Louis, MO). Biotinylated dipalmitoyl-phosphatidylethanolamine (biotin-DPPE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (biotin-cap-DPPE) and dioleoyl-phosphatidylcholine (DOPC) (Avanti, Alabaster, AL) were used as received. Two-dimensional streptavidin crystals are grown at room temperature using a procedure similar to that described by Kubalek et al. (Kubalek et al., 1991). 67 μl of Tris buffer (150 mM NaCl, 50 mM Tris, pH 7.0) containing 0.05-0.40 mg/ml of streptavidin is deposited in a micro-well formed by the

polypropylene cap of a 0.2 ml PCR tube. Then 0.5–1.5 μ l of lipid solution is spread on the surface; the lipid solution is chloroform/hexane (1:1) containing 0.1 mg/ml biotin-DPPE and 0.4 mg/ml DOPC. The PCR caps are placed in a humid chamber and kept at room temperature for 2–20 hr.

Several crystal growth conditions were investigated. We found that when the growth time was short (e.g. 2 hr), the average crystal domain size decreased as the streptavidin concentration in the subphase under the biotinylated lipid monolayer was increased from 0.05 to 0.2 mg/ml (Figs. 2 C and D). However, the average domain size increased with the protein concentration when the growth time was 6 hr, as shown in Fig. 2E. With an overnight growth at a protein concentration of 0.2 mg/ml, individual crystal domains were usually large enough to cover entire 2 μ m-diameter holes. Furthermore, the 2D crystal layer covered more than 90% of the holes in an EM grid, and the crystal over holes remained intact even in negative stain.

2.3 Crystal transfer and liposome tethering

The streptavidin crystal is picked up by a perforated carbon film on an EM grid. We prefer to use “aged” perforated carbon film, at least 4–7 days old, since freshly-deposited carbon film is hydrophilic and cannot be used to pick up the hydrophobic lipid monolayer where the 2D crystal is grown. We have used standard 400-mesh EM grids coated with a perforated carbon film made by the stamping method (Chester et al., 2007), or alternatively C-flat™ Holey Carbon Grids (Electron Microscopy Sciences, Hatfield, PA). A grid is washed with hexane and dried in air for an hour, and then is placed for one minute on the surface of the well where the crystal has been grown. Then the grid is lifted vertically away from the surface by tweezers having a bent tip, and placed onto a drop (25 μ l on a sheet of Parafilm) of Tris buffer to wash away free streptavidin molecules, as shown in Fig. 3A. In an early version of this procedure we continued to use tweezers to transfer the grid from one drop to another, but we have found that the large surface-tension effects of lifting the grid from a drop (Fig. 3B) breaks the crystal into small domains, smaller than 0.5 μ m² (see also Walz, this volume).

To minimize the disruption we now use a “loop-fishing” method for transferring the grid from one solution to another. The 2D crystal is removed from the crystallization well as described above but is then transferred onto a well of the wash buffer to remove free streptavidin molecules. This well is the cap of a 1.5 ml Eppendorf tube (Fig. 3C). Starting from this point, the handling of the grid is by a metal loop instead of a pair of tweezers. The loop is about 3.5 mm in diameter, slightly larger than the EM grid; it can be made by wrapping a piece of metal wire around a metal rod. The important point is that the loop should be as circular and planar as possible. Such a loop may also be bought from Ernest F. Fullam, Inc (Latham, NY). The loop is inserted into the buffer at an angle perpendicular to the surface, rotated to be parallel to the grid and moved under the grid, and then withdrawn straight up to pick up the grid. In this process, the loop receives the surface-tension forces during the removal of the grid from the well; the grid itself floats in the water layer trapped in the loop, as shown in Fig. 3D. The grid is transferred to two additional wash-wells in the same manner. Note that the water surface in the wells should be flat. A concave or convex surface causes the grid to jump during its landing from the loop to the surface.

After the free streptavidin is washed away, the grid, floating on the water layer in the loop, is placed onto a drop of the sample solution—here the proteoliposome suspension—on a sheet of parafilm (Fig. 3E). The sample volume is 6–10 μ l, chosen small to allow a gentle merging of the loop buffer with the drop. The side with the streptavidin crystal faces down and makes contact with the liposome suspension in the 3x diluted running buffer (composition is given below). The liposomes are doped with biotin-cap-DPPE at 1:3600

molar ratio with POPC to yield about three copies per 30 nm liposome. The low density of biotinylated lipid is chosen for two reasons. First, we seek to avoid the flattening of liposomes which can occur when multiple tethers attach to the streptavidin crystal. We do not know to what extent the liposome is distorted when a few tethers are present, but we do not observe any distortion effects in our projection images and assume the distortion to be small. Second, we find that at higher biotinylated lipid concentrations considerable direct cross-linking of liposomes occurs through residual free streptavidin in the solution.

The specific binding between biotin and streptavidin allows the desired density of liposomes on the EM grid to be achieved despite the relatively low concentration of liposomes in the sample solution. By controlling the tethering time, the density of tethered liposomes can be tuned as shown in Fig. 2F. This is especially critical for membrane proteins that are hard to express. It turns out that the biotin-DPPE inferior to the biotin-cap-DPPE for doping the liposomes because it binds preferentially to disordered regions, but poorly to ordered crystalline regions of the streptavidin surface. This presumably arises from the position of the binding pocket at one end of the β -barrel (Fig. 2A) and the fact that in biotin-cap-DPPE the longer linker gives more mobility to the biotin group. It can be imagined that biotin binding might cause a local distortion of the crystal, but we have not investigated this possibility.

After the desired time of incubation, typically 5–20 minutes in a humid atmosphere, a 25 μ l drop of dilution buffer, having the same composition as the sample solution but 3x diluted with water, is put onto the parafilm and dragged to the loop via a 200 μ l pipette tip, as shown in Fig. 3F. This dilution represents the first of several osmotic shocks that are used to swell the liposomes, as will be described below. The dragging process should be slow to prevent abrupt merging of the droplet with the loop, again to protect the integrity of the 2D crystal. The grid is then picked up by tweezers, and either stained or fast-frozen for negative-stain or cryo imaging, respectively. In preparing cryo-samples, 6 μ l of 5x diluted buffer is added right before freezing to ensure that the external osmotic pressure is lower than the internal osmotic pressure even if water evaporates during the freezing process.

Plunge-freezing is carried out by standard techniques (McDowell, this volume). We blot grids manually, from one side. The frozen ice layer is flat, because the crystal presents its hydrophilic surface on the side opposite the carbon support film. We seek to have an ice thickness of 100–150 nm, considerably greater than the liposome diameter, to avoid flattening of the liposomes.

2.4 Analysis and removal of crystal information from micrographs

A simple method was employed by Crucifix et al. to remove the periodic signal from images (Crucifix et al., 2004). Basically, the image is Fourier transformed, the data at the positions of the reciprocal-lattice peaks are set to zero, and then the inverse Fourier transform is performed. With this simple method the authors were able to obtain, without artifacts from the crystal, a 3D reconstruction of RNA polymerase to a resolution of 3.0 nm. From simulations of a liposome on the 2D crystal we found that a slightly better strategy is to scale the pixels in the neighborhood of the lattice peaks to the locally averaged amplitude, instead of zero. This improved method was used to remove the crystal information from our cryo-EM images (Wang et al., 2008). Firstly, a cryo-EM micrograph ($0.51 \times 0.51 \mu\text{m}$ of specimen area) is Fourier transformed. Then a mask having the same reciprocal lattice as the 2D crystal, with a 3-pixelradius disc at each lattice point, is applied to the Fourier transform. An annulus (inner and outer radii 3 and 4 pixels) defines the region over which the local background value is computed. From theory one expects optimum removal of the crystal information would occur when the mean of the complex values in the annulus is applied to each of the masked points. We find however that slightly better results are obtained by

scaling the masked Fourier pixel values so that their magnitude is equal to the rms magnitude in the annulus, while the phase of each masked pixels is left unchanged. There is no visible crystal residue left in the resultant liposome images, as illustrated in Fig. 1D.

The signal from the 2D crystal can be used as a built-in reference to calibrate the image formation process, as described previously (Wang et al., 2008). We found that Fourier components up to fourth order (index 4,1) are readily visible in the power spectrum of a micrograph. There is often distortion in the crystal lattice, undoubtedly due to mechanical strain of the unsupported crystal during transfer and blotting. If however we perform image-warping to unbend the lattice (Henderson et al., 1986) components up to fifth order are significant, providing information to about 1 nm resolution from a single micrograph. For quantitative evaluation of the crystal image we constructed a model image of a hydrated streptavidin tetramer, which we take to represent a unit cell of the crystal. By comparing the data with the model in Fourier space, the ratios of the intensities at different spatial frequencies can be used to define the envelope function (Wang et al., 2008). The magnitude and phases of the Fourier components can also be used to determine the CTF parameters. In addition, the signal-to-noise ratio can be estimated by comparing the intensities of the reflection spots to the surrounding background.

2.5 Proteoliposomes

As most membrane proteins are isolated and purified in detergent solution, the most successful and frequently used strategy for reconstitution of membrane proteins is detergent removal. The detergent-solubilized membrane proteins are mixed with detergent-solubilized lipid; then the detergent is removed and liposomes with incorporated proteins (proteoliposomes) form spontaneously. The molecular mechanism for the formation of proteoliposomes is still not well understood. The simplest theory is that the proteins participate in the membrane formation process (Rigaud et al., 1995). Presumably the membrane-formation process is the reverse of the liposome solubilization process (Fig. 4), in which case there are three stages of liposome formation: micelles corresponding to stage III in the solubilization scheme, transformation of micelles into detergent-saturated liposomes corresponding to stage II, and transformation of detergent-saturated liposomes to detergent-free liposomes corresponding to stage I.

The main approaches to remove detergents are dialysis, gel chromatography, and adsorption on hydrophobic resins. The rate of detergent removal depends largely on the critical micelle concentration (cmc), which is defined as the concentration at which the detergent monomers begin to form micellar aggregates. The higher the cmc, the more easily can the detergent be removed. Detergents with a low cmc are not readily removed by gel chromatography, and even less by dialysis, but can be efficiently removed through adsorption on hydrophobic resins such as the polystyrene Bio-Beads (Bio-Rad Laboratories).

Membrane proteins are extracted from cellular membranes using a detergent that is selected to provide high extraction efficiency and to stabilize the protein in solution. We use dodecylmaltoside (DDM) to extract the BK potassium channel (Wang and Sigworth, 2009), but DDM has an extremely low cmc (0.2 mM). Although it can be removed by Bio-Beads, we prefer to use gel chromatography. The biggest concern is the amount of detergent to be removed. Since only a few tens of micrograms of the BK channel is used for each reconstitution, only about 1 μ mol of DDM needs to be removed, which requires only one or two Bio-Beads. It is impractical to control the rate of detergent removal in this case because of the large size variation of the Bio-Beads, and because the removal of detergent is highly localized around the hydrophobic beads. Therefore we prefer to replace the DDM with decylmaltoside (DM), which has a much higher cmc ($\sim$ 2 mM) and can be removed easily by dialysis or gel chromatography.

2.6 Purification and reconstitution of BK channels

The BK channel alpha-subunit is stably expressed in an HEK293 cell line as the full-length KCNMA1 (gi:507922) gene product, carrying an N-terminal Flag tag. Cells are disrupted and the BK protein is subsequently bound to FLAG-affinity beads (Sigma-Aldrich, St Louis, MO) in the presence of 8 mM DDM in a buffer containing 200 mM KCl, 50 mM Tris, pH=7.4. The beads are collected into a column, which is first washed with 10–20 volumes of the same buffer containing 8 mM DDM followed by 10 volumes of buffer containing 4 mM DM. Finally the BK protein is eluted with five sequential applications, at intervals of 20 min, of 0.4 ml DM buffer containing 0.1–0.2 mg/ml FLAG peptide. The eluate is concentrated to 0.2–0.3 ml using a concentrator with a cutoff of 100 kDa (Sartorius North America Inc., Bohemia, NY). A detergent-lipid mixture is then added to the detergent-solubilized membrane protein solution. For the BK channel, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was used since the thickness of the POPC bilayer matches the transmembrane region of another potassium channel, KcsA (Doyle et al., 1998). The detergent-to-lipid ratio is chosen to be larger than R_{sol} , which is usually about 1:1. To ensure a well solubilized lipid solution, the molar ratio was chosen to be DM:POPC = 3:1.

If there are many copies of the membrane protein in a proteoliposome, the protein particles will overlap in the cryo-EM projection images, rendering the images uninterpretable. Thus it is important to control the protein-to-lipid ratio for reconstitution. A final protein-to-lipid molar ratio of 1:5,000 is used in the reconstitution of the BK channel, which yields about two copies of the BK channel in a 30 nm liposome. To ensure complete mixing between the detergent-solubilized membrane protein and the lipid-detergent micelles, the solution is shaken gently for an hour or overnight at 4 °C before detergent removal.

The detergent is removed using gel chromatography at room temperature. A column (1 cm² × 25 cm) is packed with well-soaked Sephadex® G-50 resin (Sigma-Aldrich Corp. St. Louis, MO), and equilibrated with running buffer (135 mM KCl, 5 mM NaCl, 1 mM EDTA, 10 mM HEPES, pH=7.4). About 250 µl of the protein-lipid-detergent (~0.2 nM protein, 1 mM POPC, ~3 mM DM, 135 mM KCl, 5 mM NaCl, 1 mM EDTA, 10 mM HEPES, pH=7.4) is loaded onto the top of the column to initiate slow detergent removal by diffusion. After an hour, running buffer is added to the column at 0.2 ml/min, and the eluate is fractionated. The fractions are analyzed using dynamic light scattering. Proteoliposomes elute at 30% to 45% of the packed volume, and the detergent elutes at about 100% of the packed volume. During the detergent-removal process, the flow rate is controlled by the height difference between the buffer container and the end of the eluate tubing. If the flow rate is faster than 0.5 ml/min, the detergent will not be removed completely. Slower detergent removal is expected to yield larger liposomes. Although our target size was a diameter of 40–50 nm, even with the attempts to reduce the detergent removal rate most of the resulting liposomes were smaller than 40 nm.

After detergent removal the proteoliposomes, empty liposomes and free protein aggregates coexist in the solution. To separate them, the sample is incorporated into the 20% layer of a discontinuous Nycodenz gradient (Fig. 5A). All the layers have the same osmotic pressure (~300 mOsm) to ensure no swelling or shrinkage of liposomes. After ultracentrifugation (238,000 × g for 18 hr) the protein-free liposomes float up to the 3% Nycodenz band, while proteoliposomes appear at the 5%-15% boundary (Figs. 5B and C). Lipid concentrations are determined by measuring phosphate using a colorimeter; protein is determined with the BCA (bicinchoninic acid) assay (Pierce), and the relative fraction of BK in each layer is determined by densitometry of a Western blot.

In the density gradient the position of the liposomes depends on the liposome size (smaller liposomes are more dense than larger ones) as well as on the presence of incorporated

protein. The clear segregation of empty liposomes and proteoliposomes in the 3% and the 5–15% boundary, respectively, is consistent with an effective separation due to the added density from protein. The reconstitution efficiency (reconstituted slo / total slo protein) is about 70%, and about 80% of liposomes are proteoliposomes (fraction of lipid in proteoliposomes / total lipid). The layers containing both liposomes and proteins are collected and the Nycodenz is removed by the concentration-dilution method using a Vivaspın ultrafiltration spin column (Sartorius North America Inc., Bohemia, NY) with a cutoff of 100 kDa. The solution was concentrated at around $1000 \times g$, and diluted with running buffer for 3–4 cycles to reach 10,000 times dilution in total.

In the RSC method, it is essential that the liposomes have a spherical shape. To make them highly spherical, we swell them by repeated osmotic shocks, adding water to the liposome suspension (11%, 14%, 18%, 24% and 33% of the original volume) at one-hour intervals at room temperature. We found that when we used a single swelling step, many of the liposomes were not spherical. We speculate that the reason is the following: to change from an ellipsoid to a sphere, some of the lipid molecules in the inner leaflet of the liposome membrane have to move to the outer leaflet. The spontaneous flip-flop rate is very slow, but with an osmotic shock the transient formation of a membrane defect allows flip-flop to occur. The multiple osmotic shocks allow equilibration of the two leaflets to be achieved to form highly-spherical liposomes.

2.7 Flux assay to test the function of reconstituted proteins

The function of reconstituted BK channels is assayed using the cationic voltage-sensitive dye, 5P,5P,6,6P-Tetrachloro-1,1,3,3P-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Invitrogen), to monitor K^+ -induced changes in membrane potential (Chanda and Mathew, 1999; Reers, 1991). BK proteoliposomes loaded with 135 mM KCl are incubated with channel blockers if desired, and diluted into 5 mM KCl, 135 mM NaCl solution containing 1.6 μ M of JC-1, giving a total lipid concentration of $\sim 6 \mu$ M. The fluorescence signal of the J-aggregates ($\lambda_{ex}=480$ nm, $\lambda_{em}=590$ nm) is monitored as the external K^+ concentration is increased by the addition of small volumes of 2M KCl solution. The formation of aggregates appears to be a non-equilibrium process, so for reproducibility all steps following the addition of JC-1 are held to a fixed schedule, with the KCl additions made at intervals of 20 to 40 minutes.

The large permeability of BK channels ($\sim 10^8$ ions/s) and the small liposome size means that the membrane potential of a liposome will be established very quickly once a channel opens, as the movement of only ~ 100 ions is required. The membrane potential in turn drives an influx of the cationic JC-1 dye, so that an exchange of an unknown number of JC-1 molecules for K^+ ions occurs. We perform the flux assays in nanomolar free Ca^{2+} , and the potassium-dependent membrane potentials (~ 80 mV maximum) are such that the inside-out channels are expected to have an open probability up to 10^{-2} while right-side-out channels have an open probability of 10^{-6} to 10^{-5} . Even so, a functional right-side-out channel is highly likely to open during a 100 s interval, so that a single channel may provide a maximal fluorescence signal in a given liposome. The time scale of charging is so much faster than the time scale of redistribution of JC-1 across the membrane (tens of seconds) that the block of BK channels must be very complete if a reduction in the fluorescence response to K^+ gradients is to be observed. Thus we applied channel blockers at about $1000 \times K_d$ in order to reduce the net permeability to control levels.

2.8 Computational removal of the lipid membrane contribution

In the RSC method, entire proteoliposomes are imaged. It is however not practical to perform a 3D reconstruction of entire proteoliposomes because of the large variation in their

diameters. Instead, we subtract the membrane contribution from the proteoliposome images. To do this we need an accurate, scalable model of a liposome image, which in turn requires an accurate profile of electron scattering by the phospholipid bilayer. Although a model for the electron scattering can be derived from molecular dynamics simulations and computations of electrostatic potentials, we prefer to obtain the membrane profile empirically (Wang et al., 2006).

The model is constructed in the following way. Having identified 10–20 liposomes in a representative micrograph, we apply an inverse filter to compensate for the low-frequency variations in the CTF, and compute the circularly averaged intensity of each liposome image. The Fourier transform of this one-dimensional function then yields experimental structure factors. From these the scattering profile of a slice through the spherical vesicle is obtained (Wang et al., 2006) by the Hankel transform (Bracewell, 2000). Finally, the averaged membrane profile is obtained after shifting each profile to align the membrane center, as in Fig. 4A of (Wang et al., 2006). This profile is used in computing the image of a liposome of arbitrary radius.

For each crystal-subtracted micrograph the CTF parameters are first determined, and then the CTF-modified liposome model is fitted, by nonlinear least-squares, to each liposome in the micrograph. The center and radius of each fitted liposome model is recorded, and the model is subtracted from the micrograph. The subtraction is imperfect, because a "pure" liposome model is fitted to images of liposomes containing protein particles, but the protein contribution is so weak that the fits are little affected by the extra density. In the case of the BK channel where no initial 3D model was available we picked the particles manually from the liposome-subtracted micrographs. Picking was done with reference to the corresponding unsubtracted micrographs so that particles in liposomes could be distinguished from artifacts in the images.

2.9 Using the liposome image to aid the orientation determination of each particle

The most difficult and time-consuming computational step in the single-particle reconstruction process is the determination of the orientation of each protein particle that is imaged (Ludtke, this volume). Usually this is done by comparing each particle image with reprojections of the particle model in all possible orientations. One has to search over the three Euler angles ϕ , θ , and ψ which define the orientation of the protein. With the use of liposomes, two of the three Euler angles (θ and ψ) can be determined from the location of the particle in the liposome image, relative to the liposome center (Fig. 6B) (Jiang et al., 2001; Wang and Sigworth, 2009). The angles can be estimated up to a four-fold ambiguity: the protein can be either on the top or on the bottom half of the liposome and can be inserted either inside-out or right-side-out. This four-fold ambiguity can be resolved while searching over the Euler angle ϕ by considering the two angles θ and $\pi - \theta$ that are consistent with the particle location, and also considering inside-out and right-side-out orientations. Nevertheless the use of the spherical geometry as a constraint greatly eases the determination of particle orientations. In the case of the BK channel, we attempted to make a 3D reconstruction from images of the detergent-solubilized protein, but this reconstruction was of poor quality, presumably because of the difficulty of determining all three Euler angles from particles of a roughly spherical shape in the absence of lipid membranes, a difficulty also encountered by other researchers (Alabi et al., 2007; Jiang et al., 2003).

The determination of the two angles allows a cylindrically-symmetric initial model to be built directly. For the BK channel we constructed such a model directly from the estimated θ and ψ values by applying a 32-fold symmetry around the self-rotation axis. We then forced a reduction to the assumed C4 symmetry by subtracting density in four wedges normal to the rotation axis. This initial model was refined to the final density map using the FREALIGN

algorithm (Grigorieff, 2007) with the search over ϕ varying from 0 to $\pi/2$, but the search over the other variables (θ , ψ , x , y) being tightly constrained. All of the image processing and reconstruction steps were carried out using homemade computer programs in the Matlab programming environment.

The resulting map has an artificially low density in the transmembrane region, because a continuous membrane density was subtracted from each liposome image. The interpretation of this map can be approached in two ways. First, the density that is subtracted shows little variation near the center of the membrane plane (say within ± 1 nm of the center) so that the interpretation of transmembrane density variations in that region is straightforward. Second, it is possible to computationally restore the membrane density. Given the estimated position and θ and ψ angles of the protein particle, the modeled image of a circular membrane patch surrounding the particle can be restored to each particle image and the final 3D reconstruction step re-run. The result is a map of the protein with the membrane density restored, which can be interpreted in the same way as a cryo-EM map from a 2D crystal of a membrane protein embedded in a membrane.

3. Conclusion

The use of a streptavidin crystal as a substrate allows the tethering of liposomes at a uniform density over the entire EM grid. The streptavidin crystal also serves as a built-in reference for image quality control and the calibration of the image formation process. Meanwhile, the reconstitution of membrane proteins into liposomes has the considerable advantage that the integrity and activity of the proteins is preserved, while functional assays as well as structural studies can be carried out on the same sample. The spherical geometry of the liposome also eases the task of determining the orientation of each particle image and increases the accuracy of particle alignment and thus the resolution of the determined structure. This new system has been successfully applied to study the structure of a potassium channel, the BK channel (Wang and Sigworth, 2009), and can be applied to study other membrane proteins and their complexes.

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Figure 1.

Liposomes on a streptavidin crystal. (A) Scheme of the tethering of liposomes in a vitreous ice layer (light gray) on a 2D crystal substrate (gray) spanning a hole in the carbon support film (black). (B) Scale drawing of the tethering system. The 2D crystal layer (5.0 nm thick) is bound to a lipid monolayer (2.5 nm) which spans a hole in the perforated carbon film (15–30 nm in thickness). (C) Portion of a cryo-EM micrograph showing proteoliposomes and the streptavidin-crystal background. (D) The same image, after the contribution of lipid membrane and 2D crystals is removed. Manually-picked particles are marked by white boxes.

Figure 2.

The streptavidin crystal as a tethering substrate. (A) Side view of the streptavidin tetramer. One bound biotin is shown as a space-filling model, and the other three binding sites are marked with asterisks. (B) 2D crystals are formed by attaching streptavidin molecules to a lipid monolayer via specific biotin-avidin binding. Biotinylated lipid molecules are indicated by triangles. (C-D) Negative-stain EM images show crystals grown from 0.2 mg/ml and 0.05 mg/ml streptavidin solutions for 2 hours at room temperature. The black lines outline the domain boundaries, the arrow shows the crystal orientation in each region, and the insets are 3× close-ups. (E) Dependence of the average domain size on the protein concentration and growth time. The crystal growth time was varied from 2 hr (dashed line) to 6 hr (solid

line) and overnight (data not shown, but the crystal covered entire 2 μm -diameter holes for 0.2 mg/ml of protein concentration). Error bars are the standard deviation of crystal domain sizes with $n = 20$. (F) The dependence of the average liposome density on the incubation time. The lines represent the liposome densities on 2D crystal (solid line) and on a continuous carbon support film (dashed line), respectively.

Figure 3.

Crystal washing and transfer techniques. (A) an EM grid with the 2D crystal is placed onto a droplet of wash buffer. (B) When the EM grid is picked up by tweezers, strong surface-tension effects deform the crystal. (C) an EM grid with the 2D crystal is placed onto a well filled with wash buffer. (D) The grid shown in C is picked up by a wire loop. The side view shows the grid floating on the water layer trapped in the loop. (E) The grid is placed onto a droplet of proteoliposome suspension. (F) A droplet of dilution buffer is placed near the grid and then dragged to the grid with a pipette tip.

Figure 4.

Schematic representation of the liposome solubilization process. Optical density (as a reporter of light scattering due to particle size) is plotted as a function of the detergent concentration. The main lipid phase is the lipid bilayer (liposomes) in stage I. Bilayers and micelles coexist in stage II, and stage III represents complete solubilization of lipid in mixed micelles. R_{sat} and R_{sol} are the critical detergent-to-lipid ratios where the lipid phase changes from stages I to II, and II to III.

Figure 5. Separation of BK proteoliposomes. (A) Density gradient flotation of proteoliposomes; the weight percent Nycodenz is given for each layer. (B) Distribution of protein (gray bars) and phospholipid (black bars) in the gradient layers after flotation (18 hours at $238,000 \times g$). Empty liposomes are seen to float to layer 3. (C) A coomassie-stained SDS-PAGE gel shows protein extracted from the BK proteoliposomes as compared to the purified, solubilized BK protein before reconstitution.

Figure 6. The spherical geometry of a liposome allows estimation of the two Euler angles θ and ψ . (A) A 3D model of a proteoliposome and its projection image. (B) The geometry used to determine the two Euler angles.