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Reconstitution of Membrane Proteins in Phospholipid Bilayer Nanodiscs

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Abstract

Self-assembled phospholipid bilayer Nanodiscs have become an important and versatile tool among model membrane systems to functionally reconstitute membrane proteins. Nanodiscs consist of lipid domains encased within an engineered derivative of apolipoprotein A-I scaffold proteins, which can be tailored to yield homogeneous preparations of disks with different diameters, and with epitope tags for exploitation in various purification strategies. A critical aspect of the self-assembly of target membranes into Nanodiscs lies in the optimization of the lipid:protein ratio. Here we describe strategies for performing this optimization and provide examples for reconstituting bacteriorhodopsin as a trimer, rhodopsin, and functionally active P-glycoprotein. Together these demonstrate the versatility of Nanodisc technology for preparing monodisperse samples of membrane proteins of wide-ranging structure.

1. Introduction

As this volume highlights, model membrane systems are essential for ongoing research aimed at understanding lipid dynamics in complex biological membranes, membrane protein function and molecular recognition between lipids and proteins or small molecules. In addition, several lipid membrane-based systems have been developed for drug delivery or other applications. Over the course of the past several decades the study of membrane proteins has been accelerated by membrane models including detergent micelles, mixed detergent/lipid micelles, bicelles, and liposomes, facilitating structural determination and functional studies. Although each of these established systems has distinct advantages, none are perfect for all applications and, in fact, each has significant limitations. Therefore, when considering methods for reconstituting membrane proteins, or designing lipid-based nanodevices, a recently established tool based on self-assembling lipid bilayer Nanodiscs is an important consideration (Bayburt et al., 2002; Bayburt et al., 2006; Bayburt et al., 2007; Bayburt and Sligar, 2002; Bayburt and Sligar, 2003; Chouguet et al., 2007; Denisov et al., 2004; Marin et al., 2007; Morrissey et al., 2008; Nath et al., 2007a; Sligar, 2003). Nanodisc

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technology provides many advantages for the controlling physical parameters of protein-lipid particles, and they are likely to have utility as components to be incorporated into more complex nanodevices (Das, 2009; Goluch et al., 2008; Nath et al., 2008; Zhao et al., 2008). Here we aim to describe the methods used for self-assembly of Nanodiscs and their application for reconstituting various membrane proteins into soluble nanoscale lipid bilayers with controlled composition and stoichiometry.

2. Overview of Nanodisc Technology

Phospholipid bilayer Nanodiscs are similar in structure to nascent discoidal high-density lipoprotein particles. They consist of a circular fragment of the phospholipid bilayer encapsulated by two copies of a membrane scaffold protein (MSP), which has been derived from apolipoprotein A-1 (Bayburt et al., 2002; Denisov et al., 2004), as illustrated in Figure 1. A detailed review of the structural and biological aspects of apolipoprotein A-1 and its modification to yield MSPs has been presented (Nath et al., 2007a). Currently available MSP constructs are represented in Table I. They consist of an N-terminal hexa-histidine tag, a linker containing a protease site enabling the tag to be removed, and the main MSP sequences. Incorporation of membrane proteins into Nanodiscs with the histidine tag removed after purification of MSP enables the separation of empty disks from those containing histidine-tagged target proteins. The main MSP sequence can be varied by changing the number of amphipathic helices punctuated by prolines and glycines, to allow for disks of varying sizes. As summarized in Table I, these scaffold proteins provide a collective set of tools to generate Nanodiscs ranging in outer diameter from 9.8 to 17 nm, which can accommodate a range of membrane proteins.

2.1 Structure and Properties of Nanodiscs

Optimization of the lipid:protein stoichiometry during the self-assembly process allows production of Nanodiscs of uniform size. The effect of scaffold protein length was examined by determining the concentration of radiolabeled lipid and scaffold protein in the Nanodisc-containing size exclusion peak (Denisov et al., 2004). These results, summarized in Figure 2, illustrate an interesting trend. Insertion of extra helices in the central portion of the scaffold protein (MSP1E1, MSP1E2, and MSP1E3) results in Nanodiscs of increasing size, while deletions of the affinity tag and the first 22 amino acids of the N-terminus do not significantly decrease the size of the disk formed, implying that the first 22 amino acids are marginally, if at all, involved in the self-assembly process and resultant stabilization of the discoidal nanoparticle. Truncation past the first 22 amino acids leads to a gradual decrease in lipid:protein ratio accompanied by a decrease in the major monodisperse Nanodisc component and an increase in aggregated fractions.

Systematic studies of the lipid:protein ratio in Nanodiscs made from different MSP constructs has shown that the number of lipids per Nanodisc, N_L , and the number of amino acids in the scaffold protein, M , can be described by the following simple relationship (Eq. 1, modified equation (2) from (Denisov et al., 2004)):

$$N_L \bullet S = (0.423 \bullet M - 9.75)^2 \quad (\text{Eq. 1})$$

Where S represents the mean surface area per lipid used to form the Nanodisc, measured in $\text{\AA}^2$. The quadratic relationship between the number of lipid molecules per Nanodisc and the length of the scaffold protein confirms the flat two-dimensional morphology of Nanodisc particles, illustrated in Figure 1. The size similarity of Nanodiscs formed using the same scaffold protein but different lipids clearly indicates that the length of the protein's amphipathic helix is the sole determinant of Nanodisc diameter, while different lipid:protein stoichiometries are due to the different surface area per lipid. For example, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) is in gel state below 314 K, with the area per lipid in the range of 52 – 57 $\text{\AA}^2$, while 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) is in liquid crystalline state above 278 K, with the area per lipid approximately 70 $\text{\AA}^2$.

2.2 MSP expression

MSPs are expressed using the pET expression system (Novagen) with the BL21-Gold (DE3) strain (Stratagene) as a host. The expression is very efficient, and a large amount of protein is produced in just a few hours after induction with IPTG. However, MSPs are noticeably susceptible to proteolysis, and prolonged post-induction growth results in significant decrease of the MSP yield. Different modifications of the N- and C-termini of the MSP can affect stability *in vivo*, and for some MSPs (e.g. fusion constructs or epitope tagged MSPs), shortening of the post-induction time and/or lowering the temperature during the growth in comparison with the standard protocol improves yield. The highest yield is achieved with a rich medium such as terrific broth (TB), however, minimal medium was used successfully for production of the isotope labeled MSP (Li et al., 2006). Relatively high oxygenation level, which is essential for good yields, can be easily maintained in a fermenter, such as Bio-Flow III. However, satisfactory yields can also be achieved in flasks by using relatively small culture volume (e.g. 500 mL in a two liter Fernbach flask). The detailed method is outlined below:

1. Prepare a starting culture as follows: inoculate 30 mL of Luria Broth (LB) medium containing kanamycin (30 mg/L) with a single colony from freshly streaked plate. Incubate at 37°C with shaking at 250 rpm until OD_{600} is approximately 0.4–0.6 (usually 5–6 hours). At this point the culture can be used immediately or stored overnight at 4°C.
2. Prepare and sterilize 2.5 L TB medium and set the fermenter parameters (37°C, 500 rpm, and air – 3 L/min). When the temperature reaches 37°C, add 25 mg kanamycin, a few drops of antifoam and inoculate the fermenter with the starting culture.
3. Check OD_{600} every hour. When the OD reaches 2.5–3.0 (usually in 3–4 hours), induce the culture with 1 mM IPTG. Stop the fermentation 3 hours after induction. Typically OD_{600} reaches 10–15 by the end of fermentation.
4. Harvest the cells by centrifugation at 8000×g for 10 min. The weight of the wet pellet collected from 2.5 L of culture grown on TB medium usually is between 50 and 60 g. Store the cell pellet at –80°C.

2.3 MSP purification

MSPs are purified using Chelating Sepharose FF (GE Healthcare), charged with Ni²⁺, following the general protocol for purification of polyhistidine-tagged proteins with additional washing steps using detergent-containing buffers to disrupt interaction of MSP with other proteins:

1. Charge the metal-chelating column (3.4×6 cm) by passing through one bed volume (50 mL) of 0.1 M NiSO₄, followed by 100 mL of water. Equilibrate the column with 250 mL of 40 mM phosphate buffer, pH 7.4.
2. Resuspend cell pellet collected from 2.5 L fermentation (40–60 g) in 200 mL of 20 mM phosphate buffer pH 7.4. Add stock solution of phenylmethylsulfonyl fluoride (PMSF) in ethanol to make 1 mM. After the cells are completely resuspended, add stock solution of 10% Triton X-100 to a final concentration of 1%. Add approximately 5 mg of deoxyribonuclease I (Sigma, DN-25). Lyse the cells by sonication (three one-minute rounds). Clarify the lysate by centrifugation at 30,000×g for 30 min.
3. Load the lysate on the column. Make sure the flow rate does not exceed 10 mL/min (about 1 mL/min•cm²). Wash the column with 250 mL of each of the following:
 - 40 mM Tris/HCl, 0.3 M NaCl, 1% Triton X-100, pH 8.0
 - 40 mM Tris/HCl, 0.3 M NaCl, 50 mM Na-cholate, 20 mM imidazole, pH 8.0
 - 40 mM Tris/HCl, 0.3 M NaCl, 50 mM imidazole, pH 8.0
4. Elute MSP with 40 mM Tris/HCl, 0.3 M NaCl, 0.4 M imidazole. Collect 10–14 mL fractions, check for protein with Coomassie G-250 reagent (Pierce). Pool the fractions containing MSP and dialyze the sample against buffer 1 (20 mM Tris/HCl, 0.1 M NaCl, 0.5 mM EDTA, pH 7.4) at 4°C. Filter the protein sample using 0.22 µm syringe filter, and add 0.01% NaN₃ for storage.
5. Analyze the sample: check protein purity by running SDS-PAGE and performing electro-spray mass spectrometry (see Table I for molecular masses). Measure absorbance at 280 nm using 1 mm path length quartz cuvette against standard buffer, and calculate protein concentration. If necessary, concentrate to 4–10 mg/mL. MSP can be stored for several days at 4°C. For long-term storage, freeze the sample or lyophilize it, and store at –20°C or below.
6. After purification, regenerate the column with 50 mM EDTA, wash with water, and equilibrate with 20% ethanol. Regenerate the column after every purification round.

3. Reconstitution Considerations

As of the end of 2008, the list of membrane protein reconstituted into Nanodiscs for functional studies include the cytochromes P450 (Baas et al., 2004; Bayburt and Sligar, 2002; Civjan et al., 2003; Das et al., 2007; Das, 2009; Denisov et al., 2006; Denisov et al., 2007; Duan et al., 2004; Grinkova et al., 2008; Kijac et al., 2007; Nath et al., 2007b)

bacteriorhodopsin as a monomer and trimer (Bayburt et al., 2006; Bayburt and Sligar, 2003), G-protein coupled receptors as monomers and dimers (Bayburt et al., 2007; Leitz et al., 2006; Marin et al., 2007), other receptors (Boldog et al., 2006; Boldog et al., 2007; Mi et al., 2008), toxins (Borch et al., 2008), blood coagulation protein tissue factor (Morrissey et al., 2008; Shaw et al., 2007), protein complexes of the translocon (Alami et al., 2007; Dalal et al., 2009), and monoamine oxidase (Cruz and Edmondson, 2007). The potential of Nanodiscs is exemplified by their utility in diverse biochemical and biophysical methodologies, including solid state NMR (Kijac et al., 2007; Li et al., 2006), single molecule fluorescence experiments (Nath et al., 2008), and solubilizing functional receptors (Bayburt et al., 2007; Boldog et al., 2007; Leitz et al., 2006; Mi et al., 2008). Importantly, these methods may be modified to accommodate other membrane proteins.

As an example, we describe the methods of reconstitution of bacteriorhodopsin (bR) trimer and rhodopsin monomer. Assembly of membrane proteins into Nanodiscs follows the rules for empty Nanodiscs. Cholate-solubilized phospholipids (see section 3.1) are mixed with MSP and detergent-solubilized membrane protein. Following detergent removal with adsorbant beads (Bio-beads SM-2, Biorad or amberlite XAD-2, Sigma Aldrich), the assembly is analyzed and purified by size exclusion chromatography. Additional parameters to consider are choice of detergent to initially solubilize the protein from its membrane, choice of Nanodisc size, and the lipid to MSP to membrane protein ratios.

Incorporation of a membrane protein into Nanodiscs requires the protein to be initially solubilized by treatment with a detergent. For a practical guide to membrane protein solubilization, see (Hjelmeland and Chrambach, 1984). The crude solubilized protein can be put directly into Nanodiscs or purified beforehand. A distinct advantage of using the crude solubilized membrane is that membrane proteins tend to be labile in detergent, and affinity purification can be done after the target is in the Nanodiscs. The use of protein purified in detergent has the advantage that the native lipid is mostly removed, thus simplifying determination of the correct MSP to phospholipid ratio. However, when using purified protein, the presence of relatively high glycerol concentrations can interfere with the assembly process, so the final concentration in the reconstitution mixture should be kept below 4%.

As with empty disks, the phospholipid:MSP ratio must be satisfied (see Table II). Stated more precisely, the surface area of the target plus phospholipid bilayer needs to be matched to the size of Nanodisc being assembled. It should be recognized that target protein, along with any associated native lipid, will displace exogenously added phospholipid from the Nanodisc structure. The mean surface area per lipid in Nanodiscs is $52 \text{ \AA}^2$ for DPPC, $57 \text{ \AA}^2$ for 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), and $69 \text{ \AA}^2$ for POPC (Bayburt et al., 2002; Bayburt et al., 2006; Denisov et al., 2004). These numbers can be used as a starting point for determining the necessary amount of phospholipid, shown in Table II for empty Nanodiscs. If the structure of the target is known, an estimate of displaced lipid can be made based on cross-sectional area of the membrane domain. If the structure is not known, an estimate can be made using an area of $140 \text{ \AA}^2$ per transmembrane helix. The Swiss-Prot database (www.expasy.org) annotates potential transmembrane helices for proteins in its database and ExPASy provides links to topology prediction tools for unknown

proteins. One then simply subtracts the number of phospholipids displaced by the target protein, and any native lipid present, from the amount of lipid that would be used to form empty Nanodiscs of the same size and phospholipid type. Bacteriorhodopsin was found to displace ~37 DMPC molecules and rhodopsin displaced ~50 POPC molecules based on chemical and spectral analysis of purified Nanodiscs (Bayburt et al., 2006; Bayburt et al., 2007). The experimentally determined numbers are consistent with the cross-sectional areas of bR trimer corresponding to ~40 DMPC and rhodopsin corresponding to ~43 POPC estimated from the crystal structures. These results indicate that a simple subtraction of phospholipid to account for the surface area of protein is a valid approximation.

Endogenous lipid must also be accounted for when reconstituting from whole solubilized membrane. A crude approximation is that the weight of lipid is equal to the weight of total protein in a membrane. We estimate the concentration of lipid using the molecular weight of POPC (MW 760). It is often convenient to use a large excess of MSP and synthetic phospholipid compared to native membrane lipid so that the contribution of native lipid and membrane protein can be neglected. Once a reconstitution has been performed and analyzed by size exclusion chromatography, the lipid:MSP ratio can be adjusted to optimize the formation of Nanodiscs. Another obvious consideration is the choice of the Nanodisc size. The bilayer area for several disk sizes is given in Table II. Importantly, a critical number of phospholipids associated with the Nanodisc-protein complex may be necessary for native-like structure. Theoretically, three bR can fit into MSP1 Nanodiscs, but the trimer only forms in the larger Nanodiscs, which suggests that sufficient phospholipid must be present to allow unperturbed oligomer formation.

A final consideration is the target protein to disk ratio in the assembly mixture. Single monomeric membrane proteins will assemble into Nanodiscs as long as the ratio of Nanodisc to target is high (i.e. the number per Nanodisc follows the Poisson distribution for non-interacting target). If an oligomeric membrane protein is desired then one must consider the strength of interaction, as increasing the phospholipid component can dissociate oligomers by a surface dilution effect. For weakly interacting proteins, such as the bR homotrimer, the choice of Nanodisc to target ratio is critical (Bayburt et al., 2006). Experimentally, the ratio of bR to Nanodisc was varied to find the optimal ratio. A similar approach was used for the Tar receptor (Boldog et al., 2006). Bacteriorhodopsin trimer exhibits exciton formation that was used as a convenient assay for trimer formation. In the case of Tar, a functional assay suggested that a trimer of dimers formed at a specific reconstitution ratio.

A few simple tests for assembly of a target protein with Nanodiscs can be performed to ensure efficient reconstitution. Separation of the reconstituted sample using a calibrated Superdex 200 column will allow determination of size and homogeneity of the Nanodiscs. If excess empty disks are present, column fractions can be analyzed for the presence of target by techniques such as SDS-PAGE or activity assays. Upon reinjection, the peak target fraction should elute at the same position without degradation or aggregation; size changes in the peak fraction indicate improper Nanodisc formation. The amount of phospholipid can be measured and should correspond to the expected value, as described above. However, for

the measurement to be meaningful, the target-containing Nanodiscs must first be isolated from any empty Nanodiscs.

3.1. Preparing the Reconstitution Mixture

Prepare lipid stocks in chloroform at 25–100 mM and store at –20°C in glass vials with Teflon-lined screw caps. Determine the concentration of the stock solution by phosphate analysis (Chen, 1956). The desired amount of chloroform lipid stock is dispensed into a disposable glass culture tube and dried using a gentle stream of nitrogen gas in a fume hood; a thin film on the lower walls of the tube can be obtained by rotating the tube while holding it at an angle. To remove residual solvent, place the tube in a vacuum dessicator under high vacuum overnight. Add buffer containing sodium cholate to the dried lipid film. Typically cholate is added to twice the desired concentration of lipid, for example, if 200 µl of 100 mM lipid stock was used, add 200 µl of 200 mM cholate or 400 µl of 100 mM cholate. Vortex the tube, heat it under hot tap water (about 60°C), and sonicate in an ultrasonic bath until the solution is completely clear, and no lipid remains on the walls of the tube. Scaffold protein is added to cholate-solubilized phospholipid to yield desired lipid:protein ratio, ensuring the final cholate concentration in the reconstitution mixture is between 12 – 40 mM, supplementing with standard buffer or cholate stock solution if necessary. Incubate the mixture at the appropriate incubation temperature, which is dependent on the lipid used, for 15 minutes or longer. The temperature of the self-assembly should be near the T_m of the lipid being used. Assembly with POPC is done on ice or at 4°C, DMPC at room temperature, and DPPC at 37°C. Prepared disk reconstitution mixtures can be used immediately to make Nanodiscs or incorporate membrane proteins, or lyophilized for prolonged storage. Specific examples below demonstrate these steps with different proteins.

3.2 Reconstitution of bR trimer

Purple membrane is isolated from *H. salinarum* JW-3 cultures and solubilized with 4% w/v Triton X-100 as described (Dencher and Heyn, 1978; Oesterhelt and Stoekenius, 1974). MSP1E3 stock solutions (~200 µM) and a DMPC/cholate mixture (200 mM/400 mM in buffer 1, prepared as described above) are added to bR (~200 µM) in a microfuge tube to give MSP1E3:bR:DMPC ratio of 2:3:160. Protease inhibitors can be included in the assembly. The final concentration of DMPC should be above 7 mM, below which poor disk formation occurs (Bayburt et al., 2006). If low phospholipid concentrations are necessary, Nanodisc formation can be aided by using sodium cholate at a final concentration of 14 mM. After one hour incubation at room temperature, detergent is removed by treatment for 3–4 hours at room temperature with ~500 mg wet Bio-beads SM-2 per mL of solution, with gentle agitation to keep the beads suspended. Bio-beads SM-2 or Amberlite XAD-2 are prepared by suspending in methanol, washing with several volumes of methanol in a sintered glass funnel, and rinsing with large amounts of Milli-Q treated water (Millipore) to remove traces of methanol. Amberlite XAD-2 additionally requires removal of fine particles by decantation. Prepared beads are stored in water containing 0.01% w/v NaN_3 as preservative. Incubation temperature and amount of beads are factors in the rate and completeness of detergent removal (Rigaud, 1998). We generally use an equal volume of beads to sample and an overnight incubation to remove detergents at 4°C. Room temperature or 37°C assemblies require several hours. A table of adsorption capacities for

various detergents has been compiled (Rigaud, 1998). If it is critical that the amount of residual detergent is known, the assembly should be tested using radiolabeled detergent.

Bio-beads are removed by punching a hole in the bottom of the microfuge tube with a needle, placing the tube snugly through a hole made in the cap of a 15 mL Falcon tube (Corning), and punching a vent hole in the cap of the microfuge tube. The assembly is centrifuged briefly using the Falcon tube to collect the sample. The sample is filtered using a 0.22 μ m filter and injected onto the gel filtration column run at 0.5 mL/minute while monitoring A₂₈₀ and A₅₆₀. A typical elution profile after assembly of trimer is shown in Figure 3, panel A. The reconstitution was made using optimal amount of phospholipid, yet the Nanodisc peak is still accompanied by larger aggregates that also contain bR. One possible explanation for the presence of aggregates is that multiple bR interactions promote an aggregation pathway as opposed to formation of Nanodiscs of fixed size. Fractions containing the bR Nanodiscs are pooled and the presence of trimer is assessed by measuring the visible circular dichroism spectrum which shows a positive and negative peak, due to exciton splitting (Bayburt et al., 2006).

3.3 Assembly of Monomeric Rhodopsin Nanodiscs

The assembly described herein uses whole membrane and added synthetic phospholipid to generate rhodopsin monomer Nanodiscs. Rhodopsin is handled in a darkroom under dim red light (Kodak #1 filter, 7.5 W bulb). Rod outer segments (Papermaster, 1982) are solubilized in 135 mM nonyl glucoside to give 143 μ M solubilized rhodopsin. Rod outer segments contain on the order of 100 native phospholipids per rhodopsin. MSP1E3D1 (183 μ M) and POPC (0.1 M in buffer 1 containing 0.2 M cholate) were mixed with solubilized membranes at ratios of 1:168:0.05 (MSP:POPC:rho) on ice followed by overnight removal of detergent with Bio-beads at 4°C with gentle agitation. The sample was filtered and run on a Superdex 200 HR 10/30 column run at 0.5 mL/min. The elution profile monitored at 500 nm is given in Figure 3, panel B. The elution profile shows a sharp Gaussian peak, though there are small amounts of larger aggregates. The aggregates indicate that the amount of POPC in the reconstitution could be lowered somewhat to optimize assembly of Nanodiscs.

4. Optimizing the Reconstitution for P-glycoprotein (P-gp)

When embarking on the incorporation of a new target into Nanodiscs, one must not only consider the requirements of the Nanodisc system, but also any unique requirements of the target of interest. Herein we describe the tailoring of the reconstitution to an important mammalian protein, P-glycoprotein (P-gp). P-gp is a member of the ATP-binding cassette (ABC) transporter family which has been implicated in the phenomenon of multidrug resistance in tumor cells (Higgins, 2007), as well as the absorption and disposition of many pharmaceutical compounds (Zhou, 2008), yet there is still a great deal about the mechanism and interaction with substrates that is unknown. In fact, structure-function studies of P-gp have been seriously hampered by the difficulty of obtaining large quantities of stable P-gp. Presumably, this difficulty results from the structural complexity of P-gp which comprises a 1280 amino acid protein with 12 transmembrane helices punctuated by two cytoplasmic nucleotide-binding domains (NBDs) (Higgins et al., 1997). A recent crystal structure of

mouse P-gp (Abcb1a, 87% homology with human P-gp) is shown in Figure 4, to illustrate the domain architecture (Aller et al., 2009).

P-gp is known to be sensitive to both the lipid environment (Orlowski et al., 2006) and the detergent used during the purification process (Bucher et al., 2007). Disruption of the lipid-protein interface has been shown to result in almost complete inactivation of the protein (Callaghan et al., 1997); in fact, a common practice in the purification of P-gp is to add external lipid to maintain this crucial interface (Ambudkar et al., 1998; Taylor et al., 2001). Many detergents commonly used to solubilize membrane proteins disrupt the protein-lipid interaction, and are thus detrimental for use with P-gp (Naito and Tsuruo, 1995). N-Dodecyl- β -D-maltoside (DDM) is a mild, non-ionic detergent which is commonly used in the solubilization and reconstitution of P-gp (Kimura et al., 2007; McDevitt et al., 2008), and which has also previously been used in the formation of Nanodiscs (Alami et al., 2007; Boldog et al., 2006; Dalal et al., 2009). It was, therefore, chosen to use in the incorporation of P-gp into Nanodiscs. The standard lipid used during the purification and liposomal reconstitution of P-gp is an *E. coli* total lipid extract (Kim et al., 2006; Taylor et al., 2001), which is a mixture of phosphatidylethanolamine (57.5%), phosphatidylglycerol (15.1%), cardiolipin (9.8%) and 'other' lipids (17.6%). This mixture seems to satisfy the requirement P-gp has for the lipid content, as exemplified by high levels of drug-stimulated ATPase activity in reconstituted proteoliposomes (Ambudkar et al., 1998; Taylor et al., 2001) and has also been used, with DDM, in the formation of Nanodiscs (Alami et al., 2007; Dalal et al., 2009).

4.1. P-gp as a target for incorporation

There are currently four *in vitro* systems routinely utilized to study P-gp: whole cells overexpressing P-gp (Adachi et al., 2001; Polli et al., 2001; Takano et al., 1998; Wang et al., 2002), membrane fractions from those cells (Loo et al., 2003; Loo and Clarke, 2005; Zolnerchik et al., 2007), purified protein that has been solubilized in detergent (Liu et al., 2000; Qu et al., 2003; Rosenberg et al., 2005), and purified protein that has been reconstituted into proteoliposomes (Kim et al., 2006; Lu et al., 2001; Taylor et al., 2001). Each system has strengths and weaknesses; in the whole cell and membrane fraction systems the protein is in the most native form but there is the obvious concern about the complexity of the system. Human P-gp that has been detergent-solubilized shows no ATPase activity, whereas protein that has been reconstituted into proteoliposomes has ATPase activity (Ambudkar et al., 1998), but is not particularly stable. In fact, at room temperature P-gp-proteoliposomes have a half life of less than one day (Heikal et al., 2009). Nanodiscs afford an attractive system to study P-gp because they allow for a relatively simple, controlled system in which P-gp is solubilized, yet in an active form.

4.2. Reconstitution of P-gp

Baculovirus encoding dodeca-histidine-tagged-P-gp was a generous gift from Dr. Kenneth Linton (Imperial College, London). Production of P-gp containing insect cell membranes and protein purification was as previously described (Taylor et al., 2001), with modifications. Briefly, insect cell membrane fractions were solubilized in solubilization buffer (20 mM Tris, 150 mM NaCl, 1.5 mM MgCl₂, 20% glycerol, 0.4% lipid (80:20 *E. coli*

total lipid:cholesterol) and 2% DDM, pH 6.8) with repeated extrusion through a 25 Ga needle. Insoluble protein was separated by centrifugation at 100,000×g for 40 minutes. The resulting solubilized protein was incubated with ProBond Nickel-Chelating Resin (Invitrogen) for 1 hour at 4°C with constant agitation, with the addition of 20 mM imidazole to reduce non-specific binding. The resin was washed with 20 bed volumes of wash buffer (20 mM Tris, 150 mM NaCl, 1.5 mM MgCl₂, 20% glycerol, 0.1% DDM, pH 8) with increasing concentrations of imidazole (80–150 mM). P-gp containing fractions were eluted with 500 mM imidazole in elution buffer (same as wash buffer, pH 6.8) and stored at –80°C until used.

1. Prepare a lipid film of 12 µmol *E. coli* total lipid (molar concentration determined as described above) and vacuum desiccate overnight.
2. Resuspend the lipid film in 17 µmol DDM and 1 mL buffer 1 (20 mM Tris, 100 mM NaCl, pH 7.4). Sonicate and vortex until the solution is clear and free of lumps of lipid.
3. Add 500 µL of purified P-gp in elution buffer, protease inhibitors (20 µM leupeptin, 1 µM benzamidine, and 1 µM pepstatin), 100 nmol MSP1E3D1, and enough buffer 1 to make a total volume of 2.5 mL, ensuring the final glycerol concentration is less than 4%. Incubate at room temperature with constant agitation for 1 hour.
4. To initiate self-assembly, add 0.6 g/mL washed Bio-beads SM-2 and incubate at room temperature for 2 hours with constant agitation.
5. Remove reconstituted Nanodiscs from Bio-beads with a 25 Ga needle and store at 4°C until used.
6. Empty Nanodiscs can be made in parallel, adding 500 µL of elution buffer in place of purified P-gp.

4.3. Functional activity of P-gp in liposomes vs. Nanodiscs

Functional characterization of a transporter protein in Nanodiscs has unique challenges. A disadvantage of using Nanodiscs to study transporters, such as P-gp, is the inability to study true vectorial transport, per se, because there is no internal or external compartment. Fortunately, a majority of the substrates transported by P-gp stimulate ATPase activity, which can be used as a surrogate for many of the conformational and chemical processes functionally coupled to transport (Polli et al., 2001). As mentioned previously, human P-gp has no detectable ATPase activity when solubilized in DDM, but regains activity when reconstituted. The amount of lipid is stringently controlled during the reconstitution process to prevent the concurrent formation of liposomes. Thus, the activity that is determined after reconstitution can be attributed to P-gp in Nanodiscs. For an initial characterization, the activity of P-gp reconstituted in Nanodiscs was determined by measuring the basal and drug-stimulated ATPase activity in MSP1E3D1 disks and in proteoliposomes, the standard reconstitution system for P-gp.

Proteoliposomes were formed as previously described, with modifications (Taylor et al., 2001). Briefly, a mixture of *E. coli* lipid and cholesterol (80:20, w:w) was dried to a lipid

film, before rehydration in elution buffer without DDM. The solution was sonicated and vortexed to make unilamellar liposomes. DDM was added to completely solubilize the lipid, and the solution was incubated at room temperature for 1 hour to equilibrate. Equal volumes of the solubilized lipid and purified P-gp were incubated with protease inhibitors for 30 minutes at room temperature with constant agitation. Detergent was selectively removed by addition of 0.3 g/mL of Bio-beads SM-2 for 2 hours at room temperature with constant agitation. Proteoliposomes were recovered with a 25 Ga needle and stored on ice until used.

Basal and drug-stimulated ATPase activity was determined by phosphate release using a colorimetric assay, as previously described (Chifflet et al., 1988), at 50 μ M nicardipine, with varying concentrations of ATP (Taylor et al., 2001). Empty disks or liposomes made in parallel were used as a control. Figure 5 shows the comparison of basal and nicardipine-stimulated activity of P-gp in MSP1E3D1 Nanodiscs and liposomes. A two-fold increase in the maximum drug-stimulated ATPase activity in Nanodiscs, compared to liposomes, is seen, while the K_m values are comparable. This could be due to the uniform orientation of P-gp in Nanodiscs, whereas in liposomes there are two possible orientations: right-side out (NBDs on the interior of the liposomes, and therefore inaccessible to ATP) and inside-out (NBDs on the exterior of the liposomes, and therefore accessible to ATP). This scrambled orientation in liposomes is consistent with incorporation of the protein using completely solubilized lipid (Rigaud, 2002). An increase in basal activity is also seen in disks as compared to liposomes, where the basal activity is almost undetectable.

These data not only show that P-gp is functionally active when reconstituted into Nanodiscs, but that it exhibits higher specific activity than the current standard reconstitution system. P-gp is a complex, integral membrane protein containing 12 transmembrane helices that was incorporated into Nanodiscs in a fairly straightforward manner, after small modifications to the standard procedure. This will facilitate a more detailed study into the mechanism of P-gp and its interaction with substrates and serves to exemplify the utility of Nanodiscs in the study of membrane proteins.

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References

- Adachi Y, Suzuki H, Sugiyama Y. Comparative studies on in vitro methods for evaluating in vivo function of MDR1 P-glycoprotein. *Pharm Res.* 2001; 18:1660–8. [PubMed: 11785684]
- Alami M, Dalal K, Lelj-Garolla B, Sligar SG, Duong F. Nanodiscs unravel the interaction between the SecYEG channel and its cytosolic partner SecA. *EMBO J.* 2007; 26:1995–2004. [PubMed: 17396152]
- Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, Chang G. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science.* 2009; 323:1718–22. [PubMed: 19325113]
- Ambudkar SV, Lelong IH, Zhang J, Cardarelli C. Purification and reconstitution of human P-glycoprotein. *Methods Enzymol.* 1998; 292:492–504. [PubMed: 9711577]
- Baas BJ, Denisov IG, Sligar SG. Homotropic cooperativity of monomeric cytochrome P450 3A4 in a nanoscale native bilayer environment. *Arch Biochem Biophys.* 2004; 430:218–228. [PubMed: 15369821]

- Bayburt TH, Grinkova YV, Sligar SG. Self-assembly of discoidal phospholipid bilayer nanoparticles with membrane scaffold proteins. *Nano Lett.* 2002; 2:853–856.
- Bayburt TH, Grinkova YV, Sligar SG. Assembly of single bacteriorhodopsin trimers in bilayer nanodiscs. *Arch Biochem Biophys.* 2006; 450:215–222. [PubMed: 16620766]
- Bayburt TH, Leitz AJ, Xie G, Oprian DD, Sligar SG. Transducin Activation by Nanoscale Lipid Bilayers Containing One and Two Rhodopsins. *J Biol Chem.* 2007; 282:14875–14881. [PubMed: 17395586]
- Bayburt TH, Sligar SG. Single-molecule height measurements on microsomal cytochrome P450 in nanometer-scale phospholipid bilayer disks. *Proc Natl Acad Sci U S A.* 2002; 99:6725–6730. [PubMed: 11997441]
- Bayburt TH, Sligar SG. Self-assembly of single integral membrane proteins into soluble nanoscale phospholipid bilayers. *Protein Sci.* 2003; 12:2476–2481. [PubMed: 14573860]
- Boldog T, Grimme S, Li M, Sligar SG, Hazelbauer GL. Nanodiscs separate chemoreceptor oligomeric states and reveal their signaling properties. *Proc Natl Acad Sci U S A.* 2006; 103:11509–11514. [PubMed: 16864771]
- Boldog T, Li M, Hazelbauer GL. Using Nanodiscs to create water-soluble transmembrane chemoreceptors inserted in lipid bilayers. *Methods Enzymol.* 2007; 423:317–35. [PubMed: 17609138]
- Borch J, Torta F, Sligar SG, Roepstorff P. Nanodiscs for Immobilization of Lipid Bilayers and Membrane Receptors: Kinetic Analysis of Cholera Toxin Binding to a Glycolipid Receptor. *Anal Chem.* 2008; 80:6245–6252. [PubMed: 18616345]
- Bucher K, Belli S, Wunderli-Allenspach H, Kramer SD. P-glycoprotein in proteoliposomes with low residual detergent: the effects of cholesterol. *Pharm Res.* 2007; 24:1993–2004. [PubMed: 17497080]
- Callaghan R, Berridge G, Ferry DR, Higgins CF. The functional purification of P-glycoprotein is dependent on maintenance of a lipid-protein interface. *Biochim Biophys Acta.* 1997; 1328:109–24. [PubMed: 9315609]
- Chen PS, Toribara TY, Warner H. Microdetermination of Phosphorus. *Analytical Chemistry.* 1956; 28:1756–1759.
- Chifflet S, Torriglia A, Chiesa R, Tolosa S. A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: application to lens ATPases. *Anal Biochem.* 1988; 168:1–4. [PubMed: 2834977]
- Choungnet A, Grinkova Y, Ricard D, Sligar S, Woggon W-D. Fluorescent probes for rapid screening of potential drug-drug interactions at the CYP3A4 level. *Chem Med Chem.* 2007; 2:717–724. [PubMed: 17357170]
- Civjan NR, Bayburt TH, Schuler MA, Sligar SG. Direct solubilization of heterologously expressed membrane proteins by incorporation into nanoscale lipid bilayers. *BioTechniques.* 2003; 35:556–558. 560, 562–563. [PubMed: 14513561]
- Cruz F, Edmondson DE. Kinetic properties of recombinant MAO-A on incorporation into phospholipid nanodisks. *J Neural Transm.* 2007; 114:699–702. [PubMed: 17393065]
- Dalal K, Nguyen N, Alami M, Tan J, Moraes TF, Lee WC, Maurus R, Sligar SS, Brayer GD, Duong F. Structure, Binding, and Activity of Syd, a SecY-interacting Protein. *J Biol Chem.* 2009; 284:7897–902. [PubMed: 19139097]
- Das A, Grinkova YV, Sligar SG. Redox potential control by drug binding to cytochrome P 450 3A4. *J Am Chem Soc.* 2007; 129:13778–13779. [PubMed: 17948999]
- Das A, Zhao J, Schatz GC, Sligar SG, Van Dyne RP. Screening of Type I and II Drug Binding to Human Cytochrome P450-3A4 in Nanodiscs by Localized Surface Plasmon Resonance Spectroscopy. *Analytical Chemistry.* 2009; 81
- Dencher NA, Heyn MP. Formation and properties of bacteriorhodopsin monomers in the non-ionic detergents octyl-beta -D-glucoside and Triton X-100. *FEBS Lett.* 1978; 96:322–6. [PubMed: 32075]
- Denisov IG, Grinkova YV, Baas BJ, Sligar SG. The Ferrous-Dioxygen Intermediate in Human Cytochrome P450 3A4: Substrate dependence of formation of decay kinetics. *J Biol Chem.* 2006; 281:23313–23318. [PubMed: 16762915]

- Denisov IG, Grinkova YV, Lazarides AA, Sligar SG. Directed Self-Assembly of Monodisperse Phospholipid Bilayer Nanodiscs with Controlled Size. *J Am Chem Soc.* 2004; 126:3477–3487. [PubMed: 15025475]
- Denisov IG, Grinkova YV, McLean MA, Sligar SG. The One-electron Autoxidation of Human Cytochrome P450 3A4. *J Biol Chem.* 2007; 282:26865–26873. [PubMed: 17650504]
- Duan H, Civjan NR, Sligar SG, Schuler MA. Co-incorporation of heterologously expressed Arabidopsis cytochrome P450 and P450 reductase into soluble nanoscale lipid bilayers. *Arch Biochem Biophys.* 2004; 424:141–153. [PubMed: 15047186]
- Goluch ED, Shaw AW, Sligar SG, Liu C. Microfluidic patterning of nanodisc lipid bilayers and multiplexed analysis of protein interaction. *Lab on the Chip.* 2008; 8:1723–1728.
- Grinkova YV, Denisov IG, Waterman MR, Arase M, Kagawa N, Sligar SG. The ferrous-oxy complex of human aromatase. *Biochem Biophys Res Commun.* 2008; 372:379–382. [PubMed: 18482580]
- Heikal A, Box K, Rothnie A, Storm J, Callaghan R, Allen M. The stabilisation of purified, reconstituted P-glycoprotein by freeze drying with disaccharides. *Cryobiology.* 2009; 58:37–44. [PubMed: 18983838]
- Higgins CF. Multiple molecular mechanisms for multidrug resistance transporters. *Nature.* 2007; 446:749–57. [PubMed: 17429392]
- Higgins CF, Callaghan R, Linton KJ, Rosenberg MF, Ford RC. Structure of the multidrug resistance P-glycoprotein. *Semin Cancer Biol.* 1997; 8:135–42. [PubMed: 9441943]
- Hjelmeland LM, Chrambach A. Solubilization of functional membrane proteins. *Methods Enzymol.* 1984; 104:305–18. [PubMed: 6232441]
- Kijac AZ, Li Y, Sligar SG, Rienstra CM. Magic-Angle Spinning Solid-State NMR Spectroscopy of Nanodisc-Embedded Human CYP3A4. *Biochemistry.* 2007; 46:13696–13703. [PubMed: 17985934]
- Kim IW, Peng XH, Sauna ZE, FitzGerald PC, Xia D, Muller M, Nandigama K, Ambudkar SV. The conserved tyrosine residues 401 and 1044 in ATP sites of human P-glycoprotein are critical for ATP binding and hydrolysis: evidence for a conserved subdomain, the A-loop in the ATP-binding cassette. *Biochemistry.* 2006; 45:7605–16. [PubMed: 16768456]
- Kimura Y, Kioka N, Kato H, Matsuo M, Ueda K. Modulation of drug-stimulated ATPase activity of human MDR1/P-glycoprotein by cholesterol. *Biochem J.* 2007; 401:597–605. [PubMed: 17029589]
- Leitz AJ, Bayburt TH, Barnakov AN, Springer BA, Sligar SG. Functional reconstitution of beta 2-adrenergic receptors utilizing self-assembling nanodisc technology. *BioTechniques.* 2006; 40:601–602, 604, 606, 608, 610, 612. [PubMed: 16708760]
- Li Y, Kijac AZ, Sligar SG, Rienstra CM. Structural analysis of nanoscale self-assembled discoidal lipid bilayers by solid-state NMR spectroscopy. *Biophys J.* 2006; 91:3819–3828. [PubMed: 16905610]
- Liu R, Siemiarczuk A, Sharom FJ. Intrinsic fluorescence of the P-glycoprotein multidrug transporter: sensitivity of tryptophan residues to binding of drugs and nucleotides. *Biochemistry.* 2000; 39:14927–38. [PubMed: 11101309]
- Loo TW, Bartlett MC, Clarke DM. Drug binding in human P-glycoprotein causes conformational changes in both nucleotide-binding domains. *J Biol Chem.* 2003; 278:1575–8. [PubMed: 12421806]
- Loo TW, Clarke DM. Do drug substrates enter the common drug-binding pocket of P-glycoprotein through “gates”? *Biochem Biophys Res Commun.* 2005; 329:419–22. [PubMed: 15737603]
- Lu P, Liu R, Sharom FJ. Drug transport by reconstituted P-glycoprotein in proteoliposomes. Effect of substrates and modulators, and dependence on bilayer phase state. *Eur J Biochem.* 2001; 268:1687–97. [PubMed: 11248688]
- Marin VL, Bayburt TH, Sligar SG, Mrksich M. Functional assays of membrane-bound proteins with SAMDI-TOF mass spectrometry. *Angew Chem, Int Ed.* 2007; 46:8796–8798.
- McDevitt CA, Shintre CA, Grossmann JG, Pollock NL, Prince SM, Callaghan R, Ford RC. Structural insights into P-glycoprotein (ABCB1) by small angle X-ray scattering and electron crystallography. *FEBS Lett.* 2008; 582:2950–6. [PubMed: 18657537]

- Mi LZ, Grey MJ, Nishida N, Walz T, Lu C, Springer TA. Functional and structural stability of the epidermal growth factor receptor in detergent micelles and phospholipid nanodiscs. *Biochemistry*. 2008; 47:10314–23. [PubMed: 18771282]
- Morrissey JH, Pureza V, Davis-Harrison RL, Sligar SG, Ohkubo YZ, Tajkhorshid E. Blood clotting reactions on nanoscale phospholipid bilayers. *Thromb Res*. 2008; 122:S23–S26. [PubMed: 18691494]
- Naito M, Tsuruo T. Reconstitution of purified P-glycoprotein into liposomes. *J Cancer Res Clin Oncol*. 1995; 121:582–6. [PubMed: 7559741]
- Nath A, Atkins WM, Sligar SG. Applications of Phospholipid Bilayer Nanodiscs in the Study of Membranes and Membrane Proteins. *Biochemistry*. 2007a; 46:2059–2069. [PubMed: 17263563]
- Nath A, Grinkova YV, Sligar SG, Atkins WM. Ligand Binding to Cytochrome P450 3A4 in Phospholipid Bilayer Nanodiscs: The Effect of Model Membranes. *J Biol Chem*. 2007b; 282:28309–28320. [PubMed: 17573349]
- Nath A, Koo PK, Rhoades E, Atkins WM. Allosteric effects on substrate dissociation from cytochrome P450 3A4 in nanodiscs observed by ensemble and single-molecule fluorescence spectroscopy. *J Am Chem Soc*. 2008; 130:15746–7. [PubMed: 18980315]
- Oosterhelt D, Stoekenius W. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Meth Enzymol*. 1974; 31:667–678. [PubMed: 4418026]
- Orlowski S, Martin S, Escargueil A. P-glycoprotein and 'lipid rafts': some ambiguous mutual relationships (floating on them, building them or meeting them by chance? *Cell Mol Life Sci*. 2006; 63:1038–59. [PubMed: 16721513]
- Papernmaster DS. Preparation of retinal rod outer segments. *Methods Enzymol*. 1982; 81:48–52. [PubMed: 6212746]
- Polli JW, Wring SA, Humphreys JE, Huang L, Morgan JB, Webster LO, Serabjit-Singh CS. Rational use of in vitro P-glycoprotein assays in drug discovery. *J Pharmacol Exp Ther*. 2001; 299:620–8. [PubMed: 11602674]
- Qu Q, Russell PL, Sharom FJ. Stoichiometry and affinity of nucleotide binding to P-glycoprotein during the catalytic cycle. *Biochemistry*. 2003; 42:1170–7. [PubMed: 12549939]
- Rigaud JL. Membrane proteins: functional and structural studies using reconstituted proteoliposomes and 2-D crystals. *Braz J Med Biol Res*. 2002; 35:753–66. [PubMed: 12131914]
- Rigaud JL, Levy D, Mosser G, Lambert O. Detergent removal by non-polar polystyrene beads. Applications to membrane protein reconstitution and two-dimensional crystallization. *European Biophysical Journal*. 1998; 27:305–319.
- Rosenberg MF, Callaghan R, Modok S, Higgins CF, Ford RC. Three-dimensional structure of P-glycoprotein: the transmembrane regions adopt an asymmetric configuration in the nucleotide-bound state. *J Biol Chem*. 2005; 280:2857–62. [PubMed: 15485807]
- Shaw AW, Pureza VS, Sligar SG, Morrissey JH. The Local Phospholipid Environment Modulates the Activation of Blood Clotting. *J Biol Chem*. 2007; 282:6556–6563. [PubMed: 17200119]
- Sligar SG. Finding a single-molecule solution for membrane proteins. *Biochem Biophys Res Commun*. 2003; 312:115–119. [PubMed: 14630028]
- Takano M, Hasegawa R, Fukuda T, Yumoto R, Nagai J, Murakami T. Interaction with P-glycoprotein and transport of erythromycin, midazolam and ketoconazole in Caco-2 cells. *Eur J Pharmacol*. 1998; 358:289–94. [PubMed: 9822896]
- Taylor AM, Storm J, Soceneantu L, Linton KJ, Gabriel M, Martin C, Woodhouse J, Blott E, Higgins CF, Callaghan R. Detailed characterization of cysteine-less P-glycoprotein reveals subtle pharmacological differences in function from wild-type protein. *Br J Pharmacol*. 2001; 134:1609–18. [PubMed: 11739236]
- Wang EJ, Lew K, Casciano CN, Clement RP, Johnson WW. Interaction of common azole antifungals with P glycoprotein. *Antimicrob Agents Chemother*. 2002; 46:160–5. [PubMed: 11751127]
- Zhao J, Das A, Schatz GC, Sligar SG, Van Duyne RP. Resonance Localized Surface Plasmon Spectroscopy: Sensing Substrate and Inhibitor Binding to Cytochrome P450. *J Phys Chem C*. 2008; 112:13084–13088.

- Zhou SF. Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. *Xenobiotica*. 2008; 38:802–32. [PubMed: 18668431]
- Zolnercijs JK, Wooding C, Linton KJ. Evidence for a Sav1866-like architecture for the human multidrug transporter P-glycoprotein. *FASEB J*. 2007; 21:3937–48. [PubMed: 17627029]

Figure 1. Structure of Nanodiscs, modeled with POPC as lipid. Lipid bilayer fragment (white space filling) is encircled by two amphipathic helices of MSP (gray ribbon). The graphic was generating using the PyMOL Molecular Graphics system.

Figure 2. Number of DPPC molecules per Nanodisc experimentally determined using tritiated lipids. Panel A, number of lipids in Nanodiscs formed with extended MSP proteins. Panel B, number of lipids in Nanodiscs formed with truncated MSP proteins. For the description of MSP constructs see Table I.

Figure 3.

Elution profile from Nanodisc reconstitutions. Panel A, elution profile of MSP1E3 bR trimer Nanodiscs after assembly. After detergent removal the sample was injected onto a Superdex 200 prep grade column at a flow rate of 0.5 mL/min. The main peak corresponds to Nanodiscs containing three bR. Panel B, elution profile of MSP1E3 rhodopsin Nanodisc assembly mixture produced from solubilized rod outer segments. The sample was injected onto a Superdex 200 HR 10/30 column run at a flow rate of 0.5 mL/min.

Figure 4. Crystal structure of mouse P-gp (PDB: 3G5U) in the nucleotide-free state, as seen from the plane of the membrane (Aller et al., 2009). The TMDs are embedded in the membrane, while the NBDs protrude into the interior of the cell. The graphic was generated using the PyMOL Molecular Graphics system.

Figure 5.

ATPase activity of P-gp in MSP1E3D1 Nanodiscs as compared to proteoliposomes. Squares represent activity of P-gp in MSP1E3D1 Nanodiscs and circles represent activity in liposomes. Open symbols show basal activity in the absence of drug and filled symbols show activity in the presence of 50 μ M nicardipine.

Table 1

Membrane Scaffold Protein Constructs

Protein	N-terminus	Disk size, nm	MW	ϵ_{280}	Features
MSP1*	FX	9.7 ^a /9.8 ^b	24608	23950	Original MSP1 (deletion 1-43 mutant of human Apo A-1)
MSP1TEV	TEV	9.7 ^a /10 ^b	25947	26930	MSP1 with removable 7-his tag
MSP1D1*	TEV	9.5 ^a /9.7 ^b	24662	21430	Deletion 1-11 mutant of MSP1TEV
MSP1D1 D73C	TEV	9.6 ^a	24650	21430	Cysteine in helix 2, Apo A-1 numbering, mutant of MSP1D1
MSP1D1(-)	TEV	9.6 ^a /9.6 ^b	22044	18450	MSP1D1 with removed 7-His tag
MSP1E1*	FX	10.4 ^a /10.6 ^b	27494	32430	Extended MSP1, helix 4 repeated
MSP1E1D1	TEV	10.5 ^a	27547	29910	Extended MSP1D1, helix 4 repeated
MSP1E2*	FX	11.1 ^a /11.9 ^b	30049	32430	Extended MSP1, helices 4 and 5 repeated
MSP1E2D1	TEV	11.1 ^a	30103	29910	Extended MSP1D1, helices 4 and 5 repeated
MSP1E3*	FX	12.1 ^a /12.9 ^b	32546	32430	Extended MSP1, helices 4, 5 and 6 repeated
MSP1E3D1*	TEV	12.1 ^a	32600	29910	Extended MSP1D1, helices 4, 5 and 6 repeated
MSP1E3D1 D73C	TEV	12.0 ^a	32588	29910	Cysteine in helix 2, Apo A-1 numbering, mutant of MSP1E3D1
MSP1D1-22	TEV	9.4 ^a	23404	21430	Deletion 1-22 mutant of MSP1TEV
MSP1D1-33	TEV	9.0 ^a	22055	15930	Deletion 1-33 mutant of MSP1TEV
MSP1D1-44	TEV	8.6 ^a	20765	15930	Deletion 1-44 mutant of MSP1TEV
MSP2	FX	9.5 ^a	48020	47900	Fusion of two MSP1 with GT-linker
MSP2N2	TEV	15.0 ^a /16.5 ^b	45541	39430	Fusion of MSP1D1-11 and MSP1D1-22 with GT-linker
MSP2N3	TEV	15.2 ^a /17 ^b	46125	39430	Fusion of MSP1D1-11 and MSP1D1-17 with GT-linker
MSP1FC	TEV	9.7 ^a	25714	22400	MSP1D1 with C-terminal FLAG-tag
MSP1FN	TEVF	9.6 ^a	25714	22400	MSP1D1 with N-terminal FLAG-tag

* the plasmid is available through Addgene (www.addgene.org)

FX = GHHHHHHHIEGR; TEV = GHHHHHHHHDYDIPTTENLYFQG; TEVF = GHHHHHHHHDYDIPTTENLYFQGS DYKDDDDKKG

^a Stokes hydrodynamic diameter, determined by size-exclusion chromatography (Denisov et al., 2004);

^b Nanodisc diameter determined by SAXS (Denisov et al., 2004).

Table II

Reconstitution Ratios for Empty Disks

	POPC	DPPC	DMPC	Bilayer area per Nanodisc, Å ²
MSP1D1	65	90	80	4400
MSP1E1D1	85	115	100	5700
MSP1E2D1	105	145	130	7200
MSP1E3D1	130	180	160	8900