CHAPTER ELEVEN

Reconstitution of Membrane Proteins in Phospholipid Bilayer Nanodiscs

T. K. Ritchie,[*] Y. V. Grinkova,[†] T. H. Bayburt,[†] I. G. Denisov,[†]
J. K. Zolnerciks,[*] W. M. Atkins,[*] *and* S. G. Sligar[†]

Contents

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-1 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

[*] Department of Medicinal Chemistry, University of Washington, Seattle, Washington, USA
[†] Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA

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 development (Bayburt and Sligar, 2002, 2003; Bayburt *et al.*, 2002, 2006, 2007; Chougnet *et al.*, 2007; Denisov *et al.*, 2004; Marin *et al.*, 2007; Morrissey *et al.*, 2008; Nath *et al.*, 2007a; Sligar, 2003). Nanodisc technology provides many advantages for controlling the physical parameters of protein–lipid particles, and they are likely to have utility as components to be incorporated into more complex nanodevices (Das *et al.*, 2009; Goluch *et al.*, 2008; Nath *et al.*, 2008; Zhao *et al.*, 2008). Here we 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) derived from apolipoprotein A-1 (Bayburt *et al.*, 2002; Denisov *et al.*, 2004), as illustrated in Fig. 11.1. 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 11.1. They consist of an N-terminal
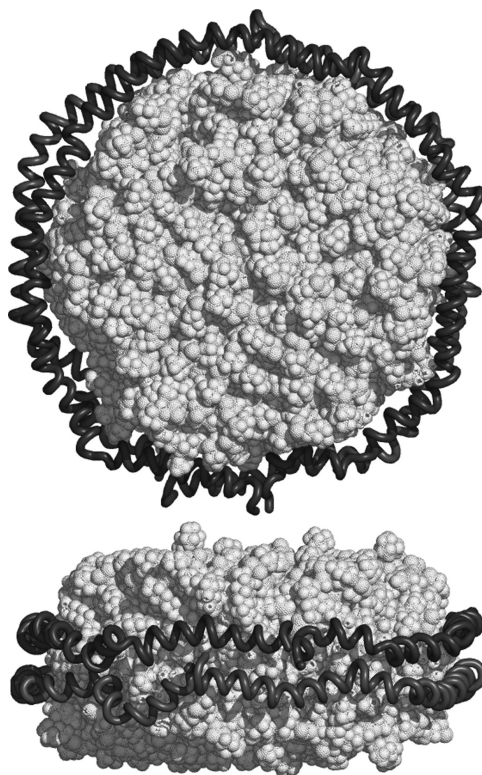
Figure 11.1 Structure of Nanodiscs, modeled with POPC as lipid. Lipid bilayer fragment (white space filling) is encircled by two amphipathic helices of MSP (gray ribbon). The graphic was generated using the PyMOL Molecular Graphics system.

hexahistidine 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 11.1, 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

Table 11.1   Membrane scaffold protein constructs

Protein	N-terminus	Disk size (nm)	MW	ε_{280}	Features
MSP1[a]	FX	$9.7^b/9.8^c$	24,608	23,950	Original MSP1 (deletion 1–43 mutant of human Apo A-1)
MSP1TEV	TEV	$9.7^b/10^c$	25,947	26,930	MSP1 with removable 7-his tag
MSP1D1[a]	TEV	$9.5^b/9.7^c$	24,662	21,430	Deletion 1–11 mutant of MSP1TEV
MSP1D1 D73C	TEV	9.6^b	24,650	21,430	Cysteine in helix 2, Apo A-1 numbering, mutant of MSP1D1
MSP1D1(−)	TEV	$9.6^b/9.6^c$	22,044	18,450	MSP1D1 with removed 7-His tag
MSP1E1[a]	FX	$10.4^b/10.6^c$	27,494	32,430	Extended MSP1, helix 4 repeated
MSP1E1D1	TEV	10.5^b	27,547	29,910	Extended MSP1D1, helix 4 repeated
MSP1E2[a]	FX	$11.1^b/11.9^c$	30,049	32,430	Extended MSP1, helices 4 and 5 repeated
MSP1E2D1	TEV	11.1^b	30,103	29,910	Extended MSP1D1, helices 4 and 5 repeated
MSP1E3[a]	FX	$12.1^b/12.9^c$	32,546	32,430	Extended MSP1, helices 4, 5, and 6 repeated
MSP1E3D1[a]	TEV	12.1^b	32,600	29,910	Extended MSP1D1, helices 4, 5, and 6 repeated
MSP1E3D1 D73C	TEV	12.0^b	32,588	29,910	Cysteine in helix 2, Apo A-1 numbering, mutant of MSP1E3D1
MSP1D1–22	TEV	9.4^b	23,404	21,430	Deletion 1–22 mutant of MSP1TEV
MSP1D1–33	TEV	9.0^b	22,055	15,930	Deletion 1–33 mutant of MSP1TEV
MSP1D1–44	TEV	8.6^b	20,765	15,930	Deletion 1–44 mutant of MSP1TEV
MSP2	FX	9.5^b	48,020	47,900	Fusion of two MSP1 with GT-linker
MSP2N2	TEV	$15.0^b/16.5^c$	45,541	39,430	Fusion of MSP1D1–11 and MSP1D1–22 with GT-linker
MSP2N3	TEV	$15.2^b/17^c$	46,125	39,430	Fusion of MSP1D1–11 and MSP1D1–17 with GT-linker
MSP1FC	TEV	9.7^b	25,714	22,400	MSP1D1 with C-terminal FLAG-tag
MSP1FN	TEVF	9.6^b	25,714	22,400	MSP1D1 with N-terminal FLAG-tag

FX = GHHHHHHIEGR; TEV = GHHHHHHHDYDIPTTENLYFQG; TEVF = GHHHHHHHDYDIPTTENLYFQGSDYKDDDDKG.

[a] The plasmid is available through Addgene (http://www.addgene.org).

[b] Stokes hydrodynamic diameter, determined by size–exclusion chromatography (Denisov et al., 2004).

[c] Nanodisc diameter determined by SAXS (Denisov et al., 2004).

radiolabeled lipid and scaffold protein in the Nanodisc–containing size exclusion peak (Denisov *et al.*, 2004). These results, summarized in Fig. 11.2, 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. (11.1), modified Eq. (11.2) from Denisov *et al.*, 2004):

$$N_L S = (0.423M - 9.75)^2 \qquad (11.1)$$

where S represents the mean surface area per lipid used to form the Nanodisc, measured in Å^2. The quadratic relationship between the number of lipid molecules per Nanodisc and the length of the scaffold protein
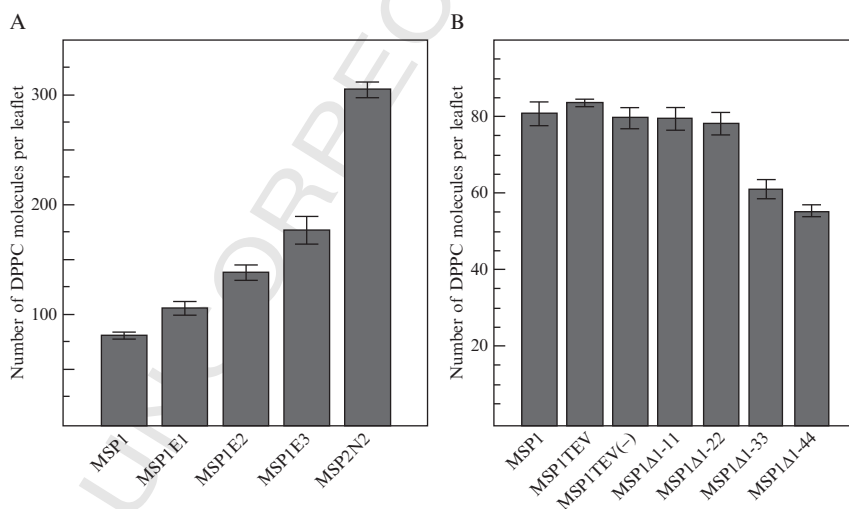
Figure 11.2 Number of DPPC molecules per Nanodisc determined experimentally using tritiated lipids. Panel A: number of lipids in Nanodiscs formed with extended MSP proteins. Panel B: number of lipids in Nanodiscs formed with truncated MSP proteins. For the description of MSP constructs, see Table 11.1.

confirms the flat two-dimensional morphology of Nanodisc particles, illustrated in Fig. 11.1. 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 Å^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 Å^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 postinduction 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 postinduction 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 2-L Fernbach flask). The detailed method is outlined below:

(1) A starting culture is prepared as follows: 30 mL of Luria Broth (LB) medium containing kanamycin (30 mg/L) is inoculated with a single colony from a freshly streaked plate. The suspension is incubated at 37 °C with shaking at 250 rpm until the OD_{600} is approximately 0.4–0.6 (usually 5–6 h). At this point the culture can be used immediately or stored overnight at 4 °C.

(2) 2.5 L TB medium is prepared and sterilized and the fermenter parameters (37 °C, 500 rpm, and air—3 L/min) are set. When the temperature reaches 37 °C, 25 mg kanamycin and a few drops of antifoam are added and the fermenter is inoculated with the starting culture.

(3) OD_{600} is checked every hour. When the OD reaches 2.5–3.0 (usually in 3–4 h), the culture is induced with 1 m*M* IPTG. The fermentation is stopped 3 h after induction. Typically, OD_{600} reaches 10–15 by the end of fermentation.

(4) The cells are harvested by centrifugation at $8000 \times g$ for 10 min. The weight of the wet pellet collected from 2.5 L of culture grown on TB medium is usually between 50 and 60 g. The cell pellet is stored at $-80\ ^{\circ}C$.

2.3. MSP purification

MSPs are purified using Chelating Sepharose FF (GE Healthcare), charged with Ni^{2+}, 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) The metal-chelating column (3.4×6 cm) is charged by passing through 1 bed volume (50 mL) of 0.1 M $NiSO_4$, followed by 100 mL of water. The column is equilibrated with 250 mL of 40 mM phosphate buffer, pH 7.4.

(2) Cell pellet collected from 2.5 L fermentation (40–60 g) is resuspended in 200 mL of 20 mM phosphate buffer, pH 7.4. Stock solution of phenylmethylsulfonyl fluoride (PMSF) is added in ethanol to make 1 mM. After the cells are completely resuspended, stock solution of 10% Triton X-100 is added to a final concentration of 1%. Approximately 5 mg of deoxyribonuclease I (Sigma, DN-25) is added. The cells are lysed by sonication (three 1-min rounds). The lysate is clarified by centrifugation at $30,000 \times g$ for 30 min.

(3) The lysate is loaded on the column. Care should be taken to make sure the flow rate does not exceed 10 mL/min (about 1 mL/min cm²). The column is washed 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) MSP is eluted with 40 mM Tris/HCl, 0.3 M NaCl, 0.4 M imidazole. 10–14 mL fractions are collected, and protein is checked with Coomassie G-250 reagent (Pierce). The fractions containing MSP is pooled and the sample is dialyzed against buffer 1 (20 mM Tris/HCl, 0.1 M NaCl, 0.5 mM EDTA, pH 7.4) at 4 $^{\circ}C$. The protein sample is filtered using 0.22 μm syringe filter, and 0.01% NaN_3 is added for storage.

(5) *Analyze the sample*: protein purity is checked by running SDS–PAGE and performing electrospray mass spectrometry (see Table 11.1 for molecular masses). Absorbance is measured at 280 nm using 1 mm path length quartz cuvette against standard buffer, and protein concentration is calculated. If necessary, it is concentrated to 4–10 mg/mL. MSP can be stored for several days at 4 $^{\circ}C$. For long-term storage, the sample is frozen or lyophilized, and is stored at $-20\ ^{\circ}C$ or below.

Au1

(6) After purification, the column is regenerated with 50 m*M* EDTA, is
washed with water, and is equilibrated with 20% ethanol. The column
is regenerated after every purification round.

3. Reconstitution Considerations

As of 2009, 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, 2009;
Denisov *et al.*, 2006, 2007; Duan *et al.*, 2004; Grinkova *et al.*, 2008; Kijac
et al., 2007; Nath *et al.*, 2007b) bacteriorhodopsin as a monomer and trimer
(Bayburt and Sligar, 2003; Bayburt *et al.*, 2006), 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, 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 bacterio-
rhodopsin (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 deter-
gent-solubilized membrane protein. Following detergent removal with
adsorbent beads (Bio-beads SM-2, Biorad or Amberlite XAD-2; Sigma-
Aldrich), the assembly is analyzed and purified by size-exclusion chroma-
tography. Additional parameters to consider are the 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.

When using purified protein, however, 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 11.2). 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 Å^2 for DPPC, 57 Å^2 for 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and 69 Å^2 for POPC (Bayburt $et\ al.$, 2002, 2006; Denisov $et\ al.$, 2004). These numbers can be used as a starting point for determining the necessary amount of phospholipid, shown in Table 11.2 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 Å^2 per transmembrane helix. The Swiss-Prot database (http://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, 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

Table 11.2 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

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 11.2. 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 noninteracting 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 homo-trimer, 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. For the measurement to be meaningful, however, the target-containing Nanodiscs must be isolated first from any empty Nanodiscs.

3.1. Preparing the reconstitution mixture

Lipid stocks are prepared in chloroform at 25–100 m*M* and stored at −20 °C in glass vials with Teflon-lined screw caps. The concentration of the stock solution is determined by phosphate analysis (Chen *et al.*, 1956; Düzgüneş, 2003). 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, the tube is placed in a vacuum dessicator under high
vacuum overnight. Buffer containing sodium cholate is added 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, 200 μL of
200 mM cholate or 400 μL of 100 mM cholate is added. The tube is
vortexed, heated under hot tap water (about 60 °C), and sonicated 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 and 40 mM,
supplementing with standard buffer or cholate stock solution if necessary.
The mixture is incubated at the appropriate incubation temperature, which
is dependent on the lipid used, for 15 min 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 in the following subsections demon-
strate these steps with different proteins.

3.2. Reconstitution of bR trimer

Purple membrane is isolated from *Halobacterium salinarum* JW-3 cultures and
solubilized with 4% (w/v) Triton X-100 as described (Dencher and Heyn,
1978; Oesterhelt and Stoeckenius, 1974). MSP1E3 stock solutions
($\sim$200 μM) and a DMPC/cholate mixture (200 mM/400 mM in buffer 1,
prepared as described above) are added to bR ($\sim$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 forma-
tion can be aided by using sodium cholate at a final concentration of 14 mM.
After 1 h incubation at room temperature, detergent is removed by treat-
ment for 3–4 h at room temperature with $\sim$500 mg wet Bio-beads SM-
2 per mL of solution, with gentle agitation to keep the beads suspended.
Bio-beads SM-2 or Amberlite XAD-2 are prepared by suspending in
methanol, washing with several volumes of methanol in a sintered glass
funnel, and rinsing with large amounts of Milli-Q treated water (Millipore)
to remove traces of methanol. Amberlite XAD-2 additionally requires
removal of fine particles by decantation. Prepared beads are stored in
water containing 0.01% (w/v) NaN$_3$ as preservative. Incubation tempera-
ture and amount of beads are factors in the rate and completeness of
detergent removal (Rigaud *et al.*, 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 *et al.*, 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-μm filter and injected onto the gel filtration column run at 0.5 mL/min while monitoring A_{280} and A_{560}. A typical elution profile after assembly of trimer is shown in Fig. 11.3, 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).
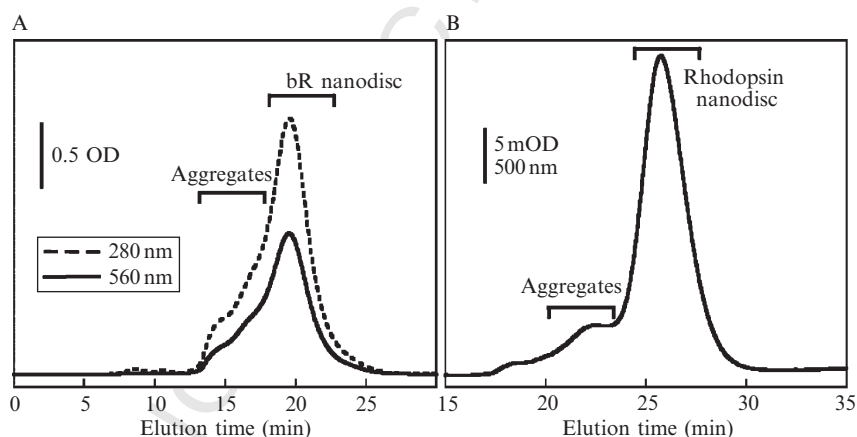
Figure 11.3   Elution profile from Nanodisc reconstitutions. Panel A: elution profile of MSP1E3 bR trimer Nanodiscs after assembly. After detergent removal the sample was injected onto a Superdex 200 prep grade column at a flow rate of 0.5 mL/min. The main peak corresponds to Nanodiscs containing three 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.

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 μM solubilized rhodopsin. Rod outer segments contain on the order of 100 native phospholipids per rhodopsin. MSP1E3D1 (183 μM) and POPC (0.1 M in buffer 1 containing 0.2 M cholate) are 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 is 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 Fig. 11.3, 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

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 Fig. 11.4, 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
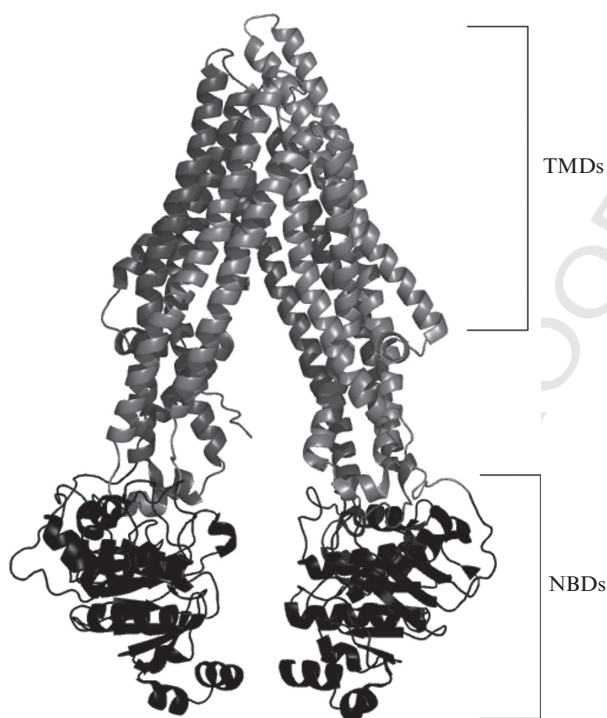
Figure 11.4 Crystal structure of mouse P-gp (PDB: 3G5U) in the nucleotide-free state, as seen from the plane of the membrane (Aller *et al.*, 2009). The TMDs are embedded in the membrane, while the NBDs protrude into the interior of the cell. The graphic was generated using the PyMOL Molecular Graphics system.

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, nonionic detergent that 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 *Escherichia 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 and Clarke, 2005; Loo *et al.*, 2003; Zolnerciks *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 1 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 is performed as previously described (Taylor *et al.*, 2001), with modifications. Briefly, insect cell membrane fractions are solubilized in solubilization buffer (20 mM Tris, 150 mM NaCl, 1.5 mM MgCl$_2$, 20% glycerol, 0.4% lipid (80:20 *E. coli* total lipid:cholesterol), and 2% DDM, pH 6.8) with repeated extrusion through a 25-gauze needle. Insoluble protein is separated by centrifugation at $100,000 \times g$ for 40 min. The resulting solubilized protein is incubated with ProBond Nickel-Chelating Resin (Invitrogen) for 1 h at 4 °C with constant agitation, with the addition of 20 mM imidazole to reduce nonspecific binding. The resin is washed with 20 bed volumes of wash buffer (20 mM Tris, 150 mM NaCl, 1.5 mM MgCl$_2$, 20% glycerol, 0.1% DDM, pH 8) with increasing concentrations of imidazole (80–150 mM). P-gp containing fractions are eluted with 500 mM imidazole in elution buffer (same as wash buffer, pH 6.8) and stored at -80 °C until used.

(1) A lipid film of 12 μmol *E. coli* total lipid (molar concentration deter-
    mined as described above) is prepared and vacuum desiccated
    overnight.
(2) The lipid film is resuspended in 17 μmol DDM and 1 mL buffer 1
    (20 m*M* Tris, 100 m*M* NaCl, pH 7.4). It is sonicated and vortexed until
    the solution is clear and free of lumps of lipid.
(3) 500 μL of purified P-gp is added 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%. It is then
    incubated at room temperature with constant agitation for 1 h.
(4) To initiate self-assembly, 0.6 g/mL washed Bio-beads SM-2 is added
    and incubated at room temperature for 2 h with constant agitation.
(5) Reconstituted Nanodiscs is removed from Bio-beads with a 25-gauge
    needle and is stored 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 versus 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 sub-
strates transported by P-gp stimulate ATPase activity, which can be used as a
surrogate for many of the conformational and chemical processes function-
ally 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 deter-
mined by measuring the basal and drug-stimulated ATPase activity
in MSP1E3D1 disks and in proteoliposomes, the standard reconstitution
system for P-gp.

Proteoliposomes are formed as previously described, with modifications
(Taylor *et al.*, 2001). Briefly, a mixture of *E. coli* lipid and cholesterol (80:20,
w/w) is dried to a lipid film, before rehydration in elution buffer without
DDM. The solution is sonicated and vortexed to make unilamelar lipo-
somes. DDM is added to completely solubilize the lipid, and the solution is
incubated at room temperature for 1 h to equilibrate. Equal volumes of the
solubilized lipid and purified P-gp are incubated with protease inhibitors for
30 min at room temperature with constant agitation. Detergent is selectively

removed by addition of 0.3 g/mL of Bio-beads SM-2 for 2 h at room temperature with constant agitation. Proteoliposomes are recovered with a 25-gauze 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 μM nicardipine, with varying concentrations of ATP (Taylor *et al.*, 2001). Empty disks or liposomes made in parallel were used as a control. Figure 11.5 shows the comparison of basal and nicardipine-stimulated activity of P-gp in MSP1E3D1 Nanodiscs and liposomes. A twofold 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 as well. 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
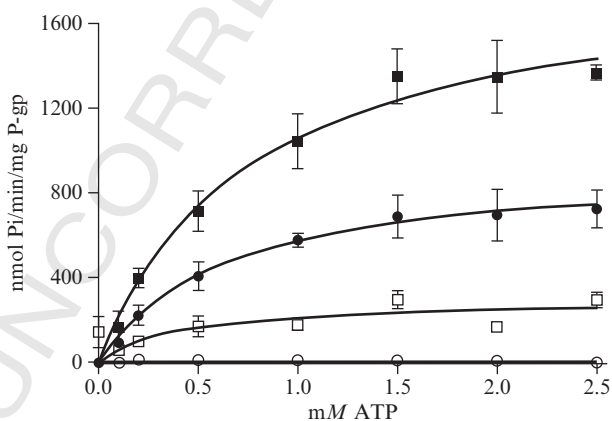
Figure 11.5 ATPase activity of P-gp in MSP1E3D1 Nanodiscs as compared to proteoliposomes. Squares represent the activity of P-gp in MSP1E3D1 Nanodiscs and circles represent activity in liposomes. Open symbols show basal activity in the absence of drug and filled symbols show activity in the presence of 50 μM nicardipine.

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.

REFERENCES

Adachi, Y., Suzuki, H., and Sugiyama, Y. (2001). Comparative studies on *in vitro* methods for evaluating *in vivo* function of MDR1 P-glycoprotein. *Pharm. Res.* **18,** 1660–1668.

Alami, M., Dalal, K., Lelj-Garolla, B., Sligar, S. G., and Duong, F. (2007). Nanodiscs unravel the interaction between the SecYEG channel and its cytosolic partner SecA. *EMBO J.* **26,** 1995–2004.

Aller, S. G., Yu, J., Ward, A., Weng, Y., Chittaboina, S., Zhuo, R., Harrell, P. M., Trinh, Y. T., Zhang, Q., Urbatsch, I. L., and Chang, G. (2009). Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* **323,** 1718–1722.

Ambudkar, S. V., Lelong, I. H., Zhang, J., and Cardarelli, C. (1998). Purification and reconstitution of human P-glycoprotein. *Methods Enzymol.* **292,** 492–504.

Baas, B. J., Denisov, I. G., and Sligar, S. G. (2004). Homotropic cooperativity of monomeric cytochrome P450 3A4 in a nanoscale native bilayer environment. *Arch. Biochem. Biophys.* **430,** 218–228.

Bayburt, T. H., and Sligar, S. G. (2002). Single-molecule height measurements on microsomal cytochrome P450 in nanometer-scale phospholipid bilayer disks. *Proc. Natl. Acad. Sci. USA* **99,** 6725–6730.

Bayburt, T. H., and Sligar, S. G. (2003). Self-assembly of single integral membrane proteins into soluble nanoscale phospholipid bilayers. *Protein Sci.* **12,** 2476–2481.

Bayburt, T. H., Grinkova, Y. V., and Sligar, S. G. (2002). Self-assembly of discoidal phospholipid bilayer nanoparticles with membrane scaffold proteins. *Nano Lett.* **2,** 853–856.

Bayburt, T. H., Grinkova, Y. V., and Sligar, S. G. (2006). Assembly of single bacteriorhodopsin trimers in bilayer nanodiscs. *Arch. Biochem. Biophys.* **450,** 215–222.

Bayburt, T. H., Leitz, A. J., Xie, G., Oprian, D. D., and Sligar, S. G. (2007). Transducin activation by nanoscale lipid bilayers containing one and two rhodopsins. *J. Biol. Chem.* **282,** 14875–14881.

Boldog, T., Grimme, S., Li, M., Sligar, S. G., and Hazelbauer, G. L. (2006). Nanodiscs separate chemoreceptor oligomeric states and reveal their signaling properties. *Proc. Natl. Acad. Sci. USA* **103,** 11509–11514.

Boldog, T., Li, M., and Hazelbauer, G. L. (2007). Using Nanodiscs to create water-soluble transmembrane chemoreceptors inserted in lipid bilayers. *Methods Enzymol.* **423,** 317–335.

Borch, J., Torta, F., Sligar, S. G., and Roepstorff, P. (2008). Nanodiscs for immobilization of lipid bilayers and membrane receptors: Kinetic analysis of cholera toxin binding to a glycolipid receptor. *Anal. Chem.* **80,** 6245–6252.

Bucher, K., Belli, S., Wunderli-Allenspach, H., and Kramer, S. D. (2007). P-glycoprotein in proteoliposomes with low residual detergent: The effects of cholesterol. *Pharm. Res.* **24,** 1993–2004.

Callaghan, R., Berridge, G., Ferry, D. R., and Higgins, C. F. (1997). The functional purification of P-glycoprotein is dependent on maintenance of a lipid-protein interface. *Biochim. Biophys. Acta* **1328,** 109–124.

Chen, P. S., Toribara, T. Y., and Warner, H. (1956). Microdetermination of phosphorus. *Anal. Chem.* **28,** 1756–1759.

Chifflet, S., Torriglia, A., Chiesa, R., and Tolosa, S. (1988). 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.* **168,** 1–4.

Chougnet, A., Grinkova, Y., Ricard, D., Sligar, S., and Woggon, W.-D. (2007). Fluorescent probes for rapid screening of potential drug-drug interactions at the CYP3A4 level. *Chem*M*ed*Chem **2,** 717–724.

Civjan, N. R., Bayburt, T. H., Schuler, M. A., and Sligar, S. G. (2003). Direct solubilization of heterologously expressed membrane proteins by incorporation into nanoscale lipid bilayers. *Biotechniques* **35,** 556–558, 560, 562–563.

Cruz, F., and Edmondson, D. E. (2007). Kinetic properties of recombinant MAO-A on incorporation into phospholipid nanodisks. *J. Neural Transm.* **114,** 699–702.

Dalal, K., Nguyen, N., Alami, M., Tan, J., Moraes, T. F., Lee, W. C., Maurus, R., Sligar, S. S., Brayer, G. D., and Duong, F. (2009). Structure, binding, and activity of Syd, a SecY-interacting protein. *J. Biol. Chem.* **284,** 7897–7902.

Das, A., Grinkova, Y. V., and Sligar, S. G. (2007). Redox potential control by drug binding to cytochrome P 450 3A4. *J. Am. Chem. Soc.* **129,** 13778–13779.

Das, A., Zhao, J., Schatz, G. C., Sligar, S. G., and Van Dyne, R. P. (2009). Screening of type I and II drug binding to human cytochrome P450–3A4 in nanodiscs by localized surface plasmon resonance spectroscopy. *Anal. Chem.* **81,** 3754–3759.

Dencher, N. A., and Heyn, M. P. (1978). Formation and properties of bacteriorhodopsin monomers in the non-ionic detergents octyl-beta-D-glucoside and Triton X-100. *FEBS Lett.* **96,** 322–326.

Denisov, I. G., Grinkova, Y. V., Lazarides, A. A., and Sligar, S. G. (2004). Directed self-assembly of monodisperse phospholipid bilayer nanodiscs with controlled Size. *J. Am. Chem. Soc.* **126,** 3477–3487.

Denisov, I. G., Grinkova, Y. V., Baas, B. J., and Sligar, S. G. (2006). The ferrous-dioxygen intermediate in human cytochrome P450 3A4: Substrate dependence of formation of decay kinetics. *J. Biol. Chem.* **281,** 23313–23318.

Denisov, I. G., Grinkova, Y. V., McLean, M. A., and Sligar, S. G. (2007). The one-electron autoxidation of human cytochrome P450 3A4. *J. Biol. Chem.* **282,** 26865–26873.

Duan, H., Civjan, N. R., Sligar, S. G., and Schuler, M. A. (2004). Co-incorporation of heterologously expressed Arabidopsis cytochrome P450 and P450 reductase into soluble nanoscale lipid bilayers. *Arch. Biochem. Biophys.* **424,** 141–153.

Düzgüneş, N. (2003). Preparation and quantitation of small unilamellar liposomes and large unilamellar reverse-phase evaporation liposomes. *Methods Enzymol.* **367,** 23–27.

Goluch, E. D., Shaw, A. W., Sligar, S. G., and Liu, C. (2008). Microfluidic patterning of nanodisc lipid bilayers and multiplexed analysis of protein interaction. *Lab Chip* **8,** 1723–1728.

Grinkova, Y. V., Denisov, I. G., Waterman, M. R., Arase, M., Kagawa, N., and Sligar, S. G. (2008). The ferrous-oxy complex of human aromatase. *Biochem. Biophys. Res. Commun.* **372,** 379–382.

Heikal, A., Box, K., Rothnie, A., Storm, J., Callaghan, R., and Allen, M. (2009). The stabilisation of purified, reconstituted P-glycoprotein by freeze drying with disaccharides. *Cryobiology* **58,** 37–44.

Higgins, C. F. (2007). Multiple molecular mechanisms for multidrug resistance transporters. *Nature* **446,** 749–757.

Higgins, C. F., Callaghan, R., Linton, K. J., Rosenberg, M. F., and Ford, R. C. (1997). Structure of the multidrug resistance P-glycoprotein. *Semin. Cancer Biol.* **8,** 135–142.

Hjelmeland, L. M., and Chrambach, A. (1984). Solubilization of functional membrane proteins. *Methods Enzymol.* **104,** 305–318.

Kijac, A. Z., Li, Y., Sligar, S. G., and Rienstra, C. M. (2007). Magic-angle spinning solid-state NMR spectroscopy of nanodisc-embedded human CYP3A4. *Biochemistry* **46,** 13696–13703.

Kim, I. W., Peng, X. H., Sauna, Z. E., FitzGerald, P. C., Xia, D., Muller, M., Nandigama, K., and Ambudkar, S. V. (2006). 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* **45,** 7605–7616.

Kimura, Y., Kioka, N., Kato, H., Matsuo, M., and Ueda, K. (2007). Modulation of drug-stimulated ATPase activity of human MDR1/P-glycoprotein by cholesterol. *Biochem. J.* **401,** 597–605.

Leitz, A. J., Bayburt, T. H., Barnakov, A. N., Springer, B. A., and Sligar, S. G. (2006). Functional reconstitution of beta 2-adrenergic receptors utilizing self-assembling nanodisc technology. *Biotechniques* **40,** 601–602, 604, 606, 608, 610, 612.

Li, Y., Kijac, A. Z., Sligar, S. G., and Rienstra, C. M. (2006). Structural analysis of nanoscale self-assembled discoidal lipid bilayers by solid-state NMR spectroscopy. *Biophys. J.* **91,** 3819–3828.

Liu, R., Siemiarczuk, A., and Sharom, F. J. (2000). Intrinsic fluorescence of the P-glyco-protein multidrug transporter: Sensitivity of tryptophan residues to binding of drugs and nucleotides. *Biochemistry* **39,** 14927–14938.

Loo, T. W., and Clarke, D. M. (2005). Do drug substrates enter the common drug-binding pocket of P-glycoprotein through "gates"? *Biochem. Biophys. Res. Commun.* **329,** 419–422.

Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2003). Drug binding in human P-glycoprotein causes conformational changes in both nucleotide-binding domains. *J. Biol. Chem.* **278,** 1575–1578.

Lu, P., Liu, R., and Sharom, F. J. (2001). Drug transport by reconstituted P-glycoprotein in proteoliposomes. Effect of substrates and modulators, and dependence on bilayer phase state. *Eur. J. Biochem.* **268,** 1687–1697.

Marin, V. L., Bayburt, T. H., Sligar, S. G., and Mrksich, M. (2007). Functional assays of membrane-bound proteins with SAMDI-TOF mass spectrometry. *Angew. Chem. Int. Ed.* **46,** 8796–8798.

McDevitt, C. A., Shintre, C. A., Grossmann, J. G., Pollock, N. L., Prince, S. M., Callaghan, R., and Ford, R. C. (2008). Structural insights into P-glycoprotein (ABCB1) by small angle X-ray scattering and electron crystallography. *FEBS Lett.* **582,** 2950–2956.

Mi, L. Z., Grey, M. J., Nishida, N., Walz, T., Lu, C., and Springer, T. A. (2008). Functional and structural stability of the epidermal growth factor receptor in detergent micelles and phospholipid nanodiscs. *Biochemistry* **47,** 10314–10323.

Morrissey, J. H., Pureza, V., Davis-Harrison, R. L., Sligar, S. G., Ohkubo, Y. Z., and Tajkhorshid, E. (2008). Blood clotting reactions on nanoscale phospholipid bilayers. *Thromb. Res.* **122,** S23–S26.

Naito, M., and Tsuruo, T. (1995). Reconstitution of purified P-glycoprotein into liposomes. *J. Cancer Res. Clin. Oncol.* **121,** 582–586.

Nath, A., Atkins, W. M., and Sligar, S. G. (2007a). Applications of phospholipid bilayer nanodiscs in the study of membranes and membrane proteins. *Biochemistry* **46,** 2059–2069.

Nath, A., Grinkova, Y. V., Sligar, S. G., and Atkins, W. M. (2007b). Ligand binding to cytochrome P450 3A4 in phospholipid bilayer nanodiscs: The effect of model membranes. *J. Biol. Chem.* **282,** 28309–28320.

Nath, A., Koo, P. K., Rhoades, E., and Atkins, W. M. (2008). Allosteric effects on substrate dissociation from cytochrome P450 3A4 in nanodiscs observed by ensemble and single-molecule fluorescence spectroscopy. *J. Am. Chem. Soc.* **130,** 15746–15747.

Oesterhelt, D., and Stoeckenius, W. (1974). Isolation of the cell membrane of Halobacterium halobium and its fractionation into red and purple membrane. *Methods Enzymol.* **31,** 667–678.

Orlowski, S., Martin, S., and Escargueil, A. (2006). P-glycoprotein and 'lipid rafts': Some ambiguous mutual relationships (floating on them, building them or meeting them by chance?). *Cell. Mol. Life Sci.* **63,** 1038–1059.

Papermaster, D. S. (1982). Preparation of retinal rod outer segments. *Methods Enzymol.* **81,** 48–52.

Polli, J. W., Wring, S. A., Humphreys, J. E., Huang, L., Morgan, J. B., Webster, L. O., and Serabjit-Singh, C. S. (2001). Rational use of *in vitro* P-glycoprotein assays in drug discovery. *J. Pharmacol. Exp. Ther.* **299,** 620–628.

Qu, Q., Russell, P. L., and Sharom, F. J. (2003). Stoichiometry and affinity of nucleotide binding to P-glycoprotein during the catalytic cycle. *Biochemistry* **42,** 1170–1177.

Rigaud, J. L. (2002). Membrane proteins: Functional and structural studies using reconstituted proteoliposomes and 2-D crystals. *Braz. J. Med. Biol. Res.* **35,** 753–766.

Rigaud, J. L., Levy, D., Mosser, G., and Lambert, O. (1998). Detergent removal by nonpolar polystyrene beads. Applications to membrane protein reconstitution and two-dimensional crystallization. *Eur. Biophys. J.* **27,** 305–319.

Rosenberg, M. F., Callaghan, R., Modok, S., Higgins, C. F., and Ford, R. C. (2005). Three-dimensional structure of P-glycoprotein: The transmembrane regions adopt an asymmetric configuration in the nucleotide-bound state. *J. Biol. Chem.* **280,** 2857–2862.

Shaw, A. W., Pureza, V. S., Sligar, S. G., and Morrissey, J. H. (2007). The local phospholipid environment modulates the activation of blood clotting. *J. Biol. Chem.* **282,** 6556–6563.

Sligar, S. G. (2003). Finding a single-molecule solution for membrane proteins. *Biochem. Biophys. Res. Commun.* **312,** 115–119.

Takano, M., Hasegawa, R., Fukuda, T., Yumoto, R., Nagai, J., and Murakami, T. (1998). Interaction with P-glycoprotein and transport of erythromycin, midazolam and ketoconazole in Caco-2 cells. *Eur. J. Pharmacol.* **358,** 289–294.

Taylor, A. M., Storm, J., Soceneantu, L., Linton, K. J., Gabriel, M., Martin, C., Woodhouse, J., Blott, E., Higgins, C. F., and Callaghan, R. (2001). Detailed characterization of cysteine-less P-glycoprotein reveals subtle pharmacological differences in function from wild-type protein. *Br. J. Pharmacol.* **134,** 1609–1618.

Wang, E. J., Lew, K., Casciano, C. N., Clement, R. P., and Johnson, W. W. (2002). Interaction of common azole antifungals with P glycoprotein. *Antimicrob. Agents Chemother.* **46,** 160–165.

Zhao, J., Das, A., Schatz, G. C., Sligar, S. G., and Van Duyne, R. P. (2008). Resonance localized surface plasmon spectroscopy: Sensing substrate and inhibitor binding to cytochrome P450. *J. Phys. Chem. C* **112,** 13084–13088.

Zhou, S. F. (2008). Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. *Xenobiotica* **38,** 802–832.

Zolnerciks, J. K., Wooding, C., and Linton, K. J. (2007). Evidence for a Sav1866-like architecture for the human multidrug transporter P-glycoprotein. *FASEB J.* **21,** 3937–3948.

Author Query Form

Book Series: Methods in Enzymology, 464
Chapter 11

Dear Author,

During the preparation of your manuscript for typesetting some questions have arisen. These are listed below. Please check your typeset proof carefully and mark any corrections in the margin of the proof or compile them as a separate list. This form should then be returned with your marked proof/list of corrections to Elsevier Science.

Disk use

In some instances we may be unable to process the electronic file of your article and/or artwork. In that case we have, for efficiency reasons, proceeded by using the hard copy of your manuscript. If this is the case the reasons are indicated below:

☐ Disk damaged   ☐ Incompatible file format   ☐ LaTeX file for non-LaTeX journal

☐ Virus infected   ☐ Discrepancies between electronic file and (peer-reviewed, therefore definitive) hard copy.

☐ Other: ....

We have proceeded as follows:

☐ Manuscript scanned   ☐ Manuscript keyed in   ☐ Artwork scanned

☐ Files only partly used (parts processed differently:...)

Bibliography

If discrepancies were noted between the literature list and the text references, the following may apply:

☐ The references listed below were noted in the text but appear to be missing from your literature list. Please complete the list or remove the references from the text.

☐ Uncited references: This section comprises references which occur in the reference list but not in the body of the text. Please position each reference in the text or, alternatively, delete it. Any reference not dealt with will be retained in this section.

Query Refs.	Details Required	Author's response
AU1	Please check if the sentence is correct.	
AU2	Please suggest for correct running head.	