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Reconstitution of membrane proteins into platforms suitable for biophysical and structural analyses

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Abstract

Integral membrane proteins historically have been challenging targets for biophysical research due to their low solubility in aqueous solution. Their importance for chemical and electrical signaling between cells, however, makes them fascinating targets for investigators interested in the regulation of cellular and physiological processes. Since membrane proteins shunt the barrier imposed by the cell membrane, they also serve as entry points for drugs, adding pharmaceutical research and development to the interests. In recent years, detailed understanding of membrane protein function has significantly increased due to high-resolution structural information obtained from single-particle cryoEM, X-ray crystallography, and NMR. In order to further advance our mechanistic understanding on membrane proteins as well as foster drug development, it is crucial to generate more biophysical and functional data on these proteins under defined conditions. To that end, different techniques have been developed to stabilize integral membrane proteins in native-like environments that allow both structural and biophysical investigations – amphipols, lipid bicelles, and lipid nanodiscs. In this chapter, we provide detailed protocols for the

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1.To freshly prepare BioBeads, weigh in BioBeads, wash with at least two volumes of methanol for 10 min to remove air followed by at least 4 volumes of ddH_2O. Decant the water and wash with 2 volumes of reconstitution buffer.

2.The concentration of short-chain lipids is given by the q value that needs to be determined for each protein and adjusted to the experimental needs. The q value is defined as the molar ratio of long-chain:short-chain lipids, and it determines the size and order parameter of the bicelles. For solid state NMR, q values of 3 – 6 are used, whereas q values of 0.15 – 0.5 will yield fast-tumbling bicelles suitable for solution NMR. For more details see [40] and [73].

1.The size of MSP determines to some extent the diameter of nanodiscs and thus the number of enclosed lipid molecules [54, 59]. The selection of MSP is mainly dependent on the size of the membrane protein. Ideally, the nanodiscs should be large enough to accommodate the protein and at least two layers of phospholipids to mimic the physiological environment[55, 72]. If the assembled nanodiscs are too large it may result in floating of the protein within the discs, which may obscure structural data processing and interpretation[74]. Additionally, there is an increased chance of reconstituting more than one protein per nanodisc[65].
A variety of MSP constructs with various numbers of amphipathic helices are available to generate nanodiscs with diameters between 9.8 and 17 nm[44, 54, 70]. In addition, truncated versions of MSP have been designed, allowing the preparation of nanodiscs with diameters ranging from 6 to 8 nm[72].
Circular MSP (cMSP) has been developed to improve the homogeneity of nanodiscs, which are especially crucial for structural studies. cMSP is a variant of MSP with its N- and C-termini covalently linked by sortase A[75, 76]. The nanodisc sizes produced by this technique range from 8.5 up to 80 nm in diameter. Alternatively, cMSP generated from DnaE split intein can be used for smaller nanodiscs (7-26 nm in diameter)[77].

2.Based on our experience, well-defined, published reconstitution ratios can serve as a starting point for optimization, especially those from membrane proteins of a similar size with the protein of interest, as well as similar MSP and lipid types.

reconstitution of membrane proteins according to these three techniques. We also outline some of the possible applications of each technique and discuss their advantages and possible caveats.

Keywords

Membrane proteins; reconstitution; amphipol; bicelles; nanodisc; membrane scaffold; membrane protein biophysics; lipids

1 Introduction

Membrane proteins constitute about 30 % of the proteome[1], are the connection between the inside and the outside of cells, and are entry points for pathogens and pharmaceuticals. It is thus of extreme importance to understand in molecular detail how specific proteins in the cell membrane work. In recent years, the number of high-resolution structures of membrane proteins has significantly increased mostly due to the developments in single-particle cryoEM[2, 3], novel techniques in x-ray crystallography (lipidic cubic phase)[4] and continuously improving NMR techniques[5, 6]. Drawing conclusions from structural data and understanding molecular mechanisms that govern the function and regulation of these proteins is only possible if functional and biophysical data of the same proteins under comparable conditions are available. Previously, these data have been obtained for purified proteins in detergent. However, with more complex systems under investigation, the environment of the protein becomes increasingly important, and several techniques have been developed to provide a more native-like environment for integral membrane proteins, helping their stability, structural integrity, as well as their function and regulation under purified and defined conditions (Figure 1)[7]. Although many detergents were deemed acceptable for many biophysical studies, they nevertheless display a non-native environment for membrane proteins. Their amphipathic character (Figure 2 A and B) leads to the formation of large micelles around protein molecules in order to preserve their structure even after extraction from cellular membranes. However, the presence of detergents can alter protein function and biophysical characteristics[8–12], significantly alters the surface characteristics of aqueous buffers, and, in some cases, even influences the function of extra-membraneous protein domains[13].

To that end, multiple reconstitution platforms for membrane proteins have been developed. Here, we describe three of them - amphipols, lipid bicelles, and lipid nanodiscs - together with their advantages and caveats, indicating that the best solution for individual experimental needs has to be determined on a case-by-case basis (Figure 1)[14].

Amphipols are amphipathic polymers that, in their chemical characteristics, resemble detergents, which combine a hydrophilic head group with a hydrophobic tail (Figure 2A–D)[15–17]. However, amphipols can stabilize membrane proteins more efficiently by interacting more strongly with the protein compared to detergents that form a lose micelle where single detergent molecules are in constant and rapid exchange with other detergent molecules in solution[18]. This is mostly due to the polymeric base structure of amphipols[19], which enables multiple hydrophobic chains of the same molecule to interact with the same protein (Figure 2C and D). Furthermore, due to the very slow dissociation

of the protein-amphipol complex[20], free amphipols can and should be removed from solution after reconstitution. The downsides of the approach are: 1) the protein reconstituted into amphipol is no longer accessible for ulterior functional studies, such as lipid bilayer recordings, as it is virtually impossible to remove these polymers from the reconstituted protein, 2) amphipol-reconstituted proteins may adopt different structural and functional characteristics compared to the protein in native membranes[21]. Nevertheless, amphipols have been proven to be excellent environments for protein-ligand interactions[22–25] as well as structural studies by crystallography[26] and cryoEM[27–30].

Lipid bicelles closely resemble mixed detergent-lipid-micelles and are more native-like than amphipols as they provide a lipid environment for membrane proteins (Figure 2E)[32]. The principle of lipid bicelles relies on the fact that lipid molecules in aqueous solution spontaneously assemble in bilayers (mostly in the form of liposomes) to separate their hydrophobic tails from water. By optimizing the molar ratio of long-chain lipids to short-chain lipids (a parameter usually designated as q), it is possible to induce the assembly of small patches of lipid bilayers with the protein incorporated. The long-chain lipids form the bilayer which is stabilized by a belt of short-chain lipids, which allow more curvature and protect the hydrophobic core of the bicelle (Figure 2E)[33]. Lipid bicelles can be used for 3D-crystallography[34–37] and are an outstanding tool for analyzing membrane proteins by NMR[38–43].

A more complex, but at the same time arguably a more native-like environment for membrane proteins, are lipid nanodiscs[44–48]. The detergent-free lipid bilayer is surrounded by a membrane-scaffolding protein (MSP) and the protein of interest is inserted inside the disc (Figure 3).

The MSP is a truncated form of apolipoprotein A-I. A multitude of MSPs is used to form nanodiscs[44, 45, 53], and they all share common characteristics – two monomers of MSP assemble to form a ring structure (Figure 3C)[49, 54–56]. The two subunits are held together by up to 28 inter-subunit salt bridges, and the final ring structure creates an amphipathic environment (Figure 3D–F)[57]. The outer surface of the nanodisc is fully accessible to aqueous solution, and thus is highly charged to ensure solubility. The inner surface of the ring, however, has to accommodate the lipid bilayer and accordingly carries charges along the top and bottom rims to mediate interaction with the solvent and the charged headgroups of the lipids, while in the core predominantly hydrophobic residues are exposed to provide an amenable environment for the acyl chains of the lipids[57, 58]. The high content of charged residues and the necessity of salt-bridges to stabilize the double-layered ring structure make MSP and in turn nanodiscs strongly pH-dependent assemblies. The diameter of the nanodisc can be varied by either varying the protein:MSP:lipid ratio or by selecting MSP of different chain lengths in order to accommodate the protein of interest (Figure 3E–H)[44, 51, 54, 59–62]. Nanodiscs can be assembled from various lipid types. Therefore, they are able to meet very different requirements of the protein incorporated as well as the intended applications. However, it is important to optimize the protein:MSP:lipid ratio in order to maximize the amount of nanodiscs with protein incorporated or obtain the desired nanodisc sizes (Figure 3A and B). Recently, it was shown that, once assembled, nanodiscs can tolerate significant distortion of the enclosed membrane[61, 63].

In the following sections we will provide detailed protocols for the reconstitution of integral membrane proteins into the three systems.

2 Materials

All three protocols presented here start from pure, homogenous protein in detergent.

2.1 Amphipol reconstitution

1. Amphipol stock solution (100 mg/ml in ddH_2O)
2. Detergent removal column (Pierce, ThermoScientific)
3. Superdex200 10/300 gel filtration column (GE Lifesciences)

2.2 Bicelle reconstitution

1. Lipid powder (Avanti Polar Lipids)
2. n-Octyl-β-D-Glucopyranoside (β-OG)
3. BioBeads (SM-2, BioRad)
4. Water bath sonicator

2.3 Nanodisc reconstitution

1. Lipid stock solution
2. Membrane-scaffolding protein (MSP)
3. BioBeads (SM-2, BioRad)
4. Spin-X column
5. Superose6 10/300 gel filtration column (GE Lifesciences)

3 Methods

3.1 Amphipol reconstitution

The reconstitution of membrane proteins into amphipols is the easiest of the three methods presented here and can be incorporated into any regular protein purification protocol.

1. Prepare amphipol stock solution ahead of time by dissolving 100 mg of amphipol in 750 μl ddH_2O, yielding 1 ml of 100 mg/ml. Rotate the solution at 4 °C overnight to fully hydrate the amphipol. Prepare 100 μl aliquots and store at −20 °C until needed.
2. Purify the protein of interest to homogeneity. Determine the protein concentration by absorbance and concentrate to 10 mg/ml or higher if the protein is stable at high concentrations (see Note 1).

[1]Protein should be purified to homogeneity (usually after gel filtration chromatography) to avoid reconstitution of bad particles. However, if the protein of interest displays low stability in detergent, amphipol exchange can also be performed right after affinity purification as long as the protein appears clean on SDS-PAGE at this stage.

3. Mix protein in detergent with amphipol in ddH_2O and incubate under gentle agitation at 4 °C for 2 h (see Note 2). Commonly used protein:amphipol ratios are between 1:1 and 1:3 (w/w).
4. Prepare detergent removal columns by washing with 3 column volumes of ddH_2O followed by 3 column volumes of protein purification buffer without detergent.
5. Apply the protein-amphipol mix to the pre-equilibrated detergent removal column and collect fractions of 500 μl.
6. Check fractions for their protein content by absorbance. Pool protein-containing fractions and concentrate to 10 mg/ml.
7. Further purify the protein in amphipol by gel filtration in the protein purification buffer without detergent. The reconstituted protein should elute from the gel filtration as a single, symmetric peak at a volume similar to that of the protein in detergent.

3.2 Lipid-bicelle reconstitution via liposomes

Lipid bicelles provide a more native-like environment for membrane proteins than amphipols. The reconstitution protocol for lipid bicelles is more complex and needs some optimization for each protein. The protein should be purified to homogeneity with gel filtration as last purification step since the bicelles are not further purified after reconstitution.

1. Hydrate 20 mg/ml of long-chain lipid (usually at least tetradecyl tails) in reconstitution buffer (20 mM potassium phosphate, 20 mM NaCl, pH 7) for at least 2 h at room temperature.
2. Sonicate the hydrated lipids in a water bath sonicator for 1 – 2 min.
3. Add β-OG from a 10 % stock to reach a final concentration of 0.5 % β-OG and incubate the lipid-detergent mix for 30 min at room temperature under constant agitation. Although β-OG is most widely used for bicelle reconstitutions, other detergents can be used provided that they are compatible with the structural integrity of the protein of interest.
4. Add your protein of interest to the lipid-detergent mix in a molar ratio of 1:100 protein:lipid, mix and incubate slightly above the phase transition temperature of the lipids for 1 h to avoid the lipids from entering the gel phase and to provide enough time for mixed micelles to be formed.
5. Remove detergent by adding freshly prepared BioBeads (30 mg BioBeads per mg detergent, see Note 1) and incubate for 2 h under gentle agitation.
6. Transfer the solution to a new reaction tube, add fresh BioBeads and incubate overnight under gentle agitation.

[2]Due to the free carboxylic groups, amphipols are highly water soluble at neutral pH values. The solubility is decreased at acidic pH as well as at high salt concentrations, which might lead to aggregation of amphipols.

7. Change BioBeads one more time and incubate for 2 h before transferring the liposome solution to a fresh reaction tube. Make sure to avoid transferring any BioBeads.
8. Spin down the liposomes at 40000 rpm for 1 h, 4 °C.
9. Resuspend the pellet into the final buffer of your experiment containing short-chain lipids (usually hexyl tail-lipids, see Note 2) to reach the desired protein concentration. Most prominently, lipid bicelles are used for NMR applications with a protein concentration of 0.5-1 mM.
10. Perform five freeze/thaw cycles to homogenize the bicelles. Freezing is best executed in liquid N_2. To thaw the bicelles, incubate the reaction tube at room temperature.

3.3 Lipid-nanodisc reconstitution

The most complex protocol to reconstitute membrane proteins for functional and biophysical assays is the incorporation of proteins into lipid-nanodiscs. These particles, however, represent an environment that is very close to the conditions in a cellular membrane and can be tailored to specific experimental setups.

For an optimal reconstitution, different protein:MSP:lipid ratios need to be screened to obtain nanodiscs with a single protein incorporated. Sub-optimal reconstitution conditions can lead to aggregation, empty nanodiscs, or nanodiscs containing several proteins per disc (Figure 3A and B)[64, 65]. In general, the protein:MSP molar ratio should be 1:2 for purified membrane protein[55], however, molar ratios between 1 and 25 have been applied in practice[51, 66–70]. The optimal protein:MSP:lipid ratio can be determined by systematically testing different conditions in small-scale reconstitutions and monitoring the sample quality by size-exclusion chromatography followed by SDS-PAGE and negative stain EM (Figure 3A–B).

3.3.1 Lipid preparation

1. Transfer the appropriate amount of lipids from stock solutions (typically 10 or 25 mg/ml in chloroform) to a glass tube.
2. Dry lipids to a thin film under a constant nitrogen gas stream. Remove residual organic solvent by rinsing in one volume of pentane, and dry again under the gas stream. Alternatively, the dried lipids can be placed in a vacuum desiccator over night for complete removal of the solvent.
3. Add buffer to the dried lipid film to obtain a 20 mM lipid stock. Gradually add the detergent suitable for the membrane protein while sonicating in a water bath sonicator until the lipid solution is clear. The typical final concentration of detergent is at least twice the CMC. The lipids can also be dissolved by several freeze/thaw cycles.
4. After solubilization lipid stock solutions can be stored at −80 °C for future use.

3.3.2 Bio-Beads preparation

1. Wash the Bio-Beads with two volumes of methanol, followed by extensively washing with ddH_2O to remove the organic solvent.
2. Wash the Bio-Beads with three volumes of purification buffer without detergent.
3. Bio-Beads can be used freshly or stored at 4 °C in ddH_20 and 0.01 % NaN_3 for up to three months[54].

3.3.3 Nanodisc assembly

1. MSP protein can be prepared in lab according to published protocols [71].
2. Add lipid, MSP, and the membrane protein in an Eppendorf tube at the optimized ratio. The sample volume is dependent on the application and the desired yield of nanodiscs.
3. Incubate the reconstitution mixture by gently inverting for 1-2 h at a temperature slightly above the phase-transition temperature of the lipid. In case of using a mix of lipids, the experimental temperature should be optimized according to the phase transition temperature of the main lipid components[72].
4. Start detergent removal to initiate the reconstitution by adding BioBeads (20 mg per 100 μl sample), and gently invert for ~2 h.
5. Transfer the reconstitution mixture to a new tube. Add fresh, pre-equilibrated BioBeads to the reconstitution mixture (20 mg per 100 μl). Gently invert overnight.
6. Transfer the reconstitution mixture to a new tube. Dilute the sample with sample buffer to the volume appropriate for gel filtration (typically 500 μl final volume).
7. Filter the sample through a 0.22 μm Spin-X centrifugation tube filter.
8. Apply the sample to a Superose 6 16/600 gel filtration column pre-equilibrated in sample buffer. Collect the peak fraction corresponding to the assembled nanodiscs and check for protein and MSP content by SDS-PAGE (Figure 3A).
9. The different fractions can be finally checked by negative-stain EM for evidence of nanodisc formation, presence of protein in nanodiscs, and absence of liposomes (Figure 3B).

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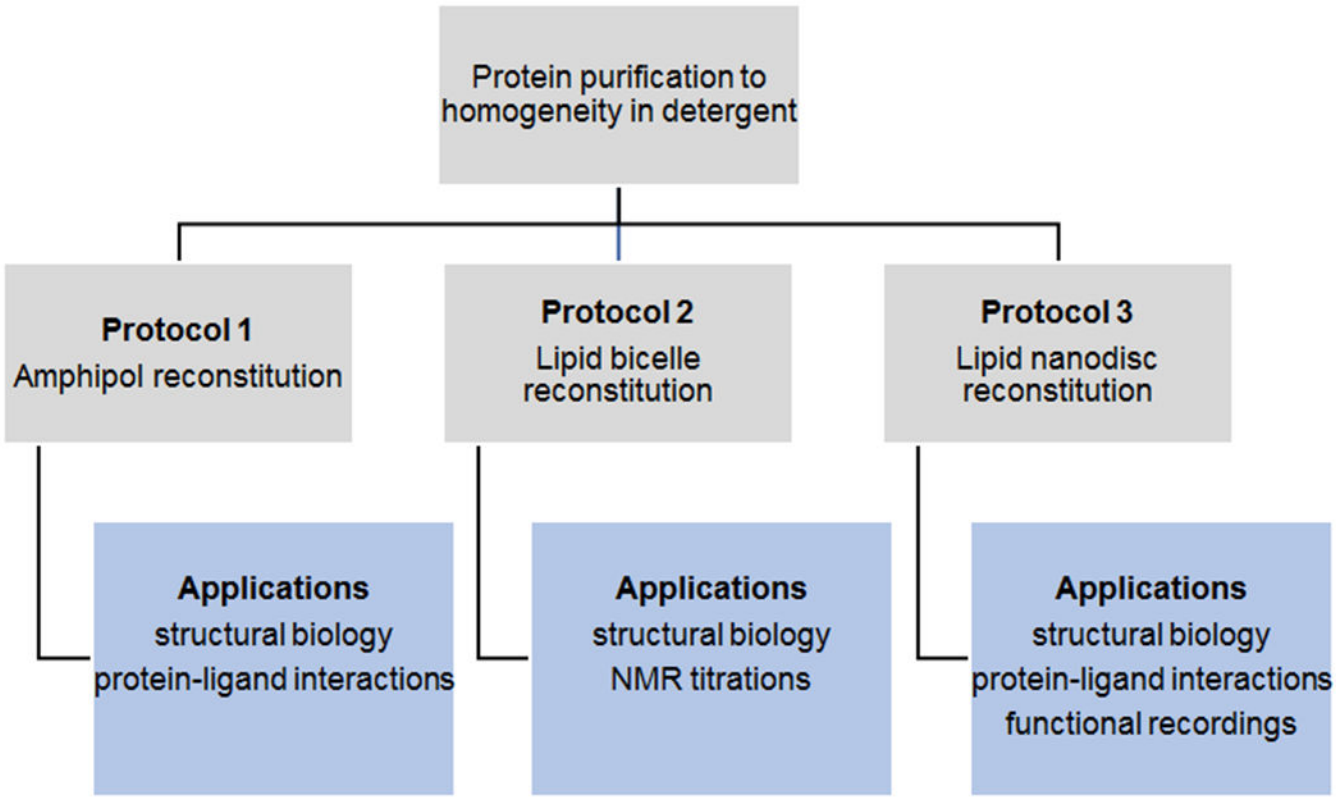

Figure 1: Reconstitution of purified membrane proteins into native-like environments.

Outline of the possibilities to transfer purified, integral membrane proteins solubilized in detergent into more native-like environments. For each platform exemplary applications are listed.

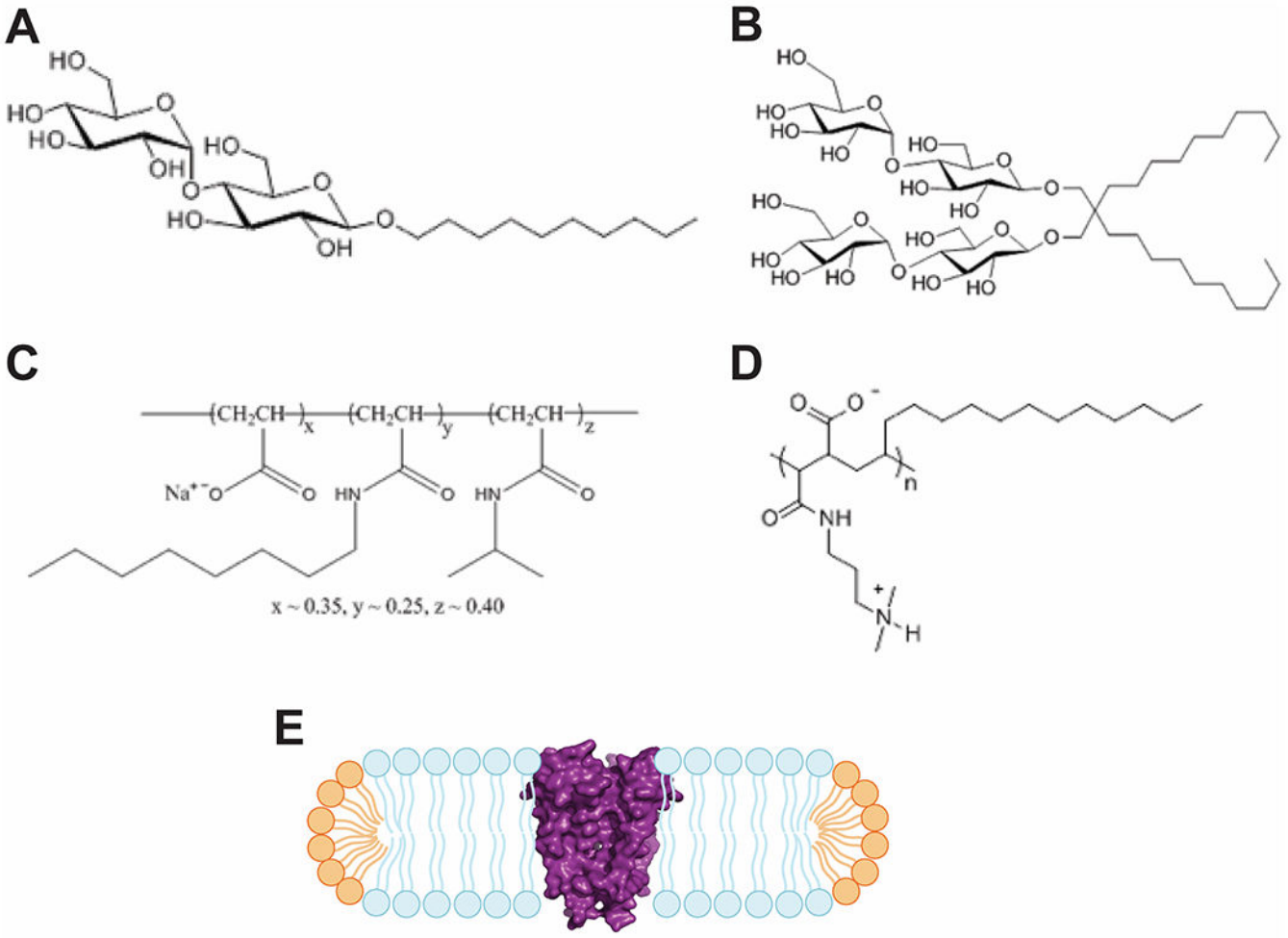

Figure 2: Characteristics of detergents, amphipols and bicelles.

Chemical characteristics of hydrophilic headgroups and hydrophobic tails are present in detergent as shown for (A) n-Decyl-α-D-Maltopyranoside - DM, (B) Lauryl Maltose Neopentyl Glycol - LMNG, and amphipols as shown for (C) amphipol A8-35, and (D) PMAL-C12. Chemical structures were adapted from www.anatrace.com. (E) Cartoon of a lipid bicelle with a protein (KcsA ion channel, PDB: 1BL8) incorporated into a lipid bicelle. Long-chain lipids in blue, short-chain lipids in orange, KcsA in magenta surface representation

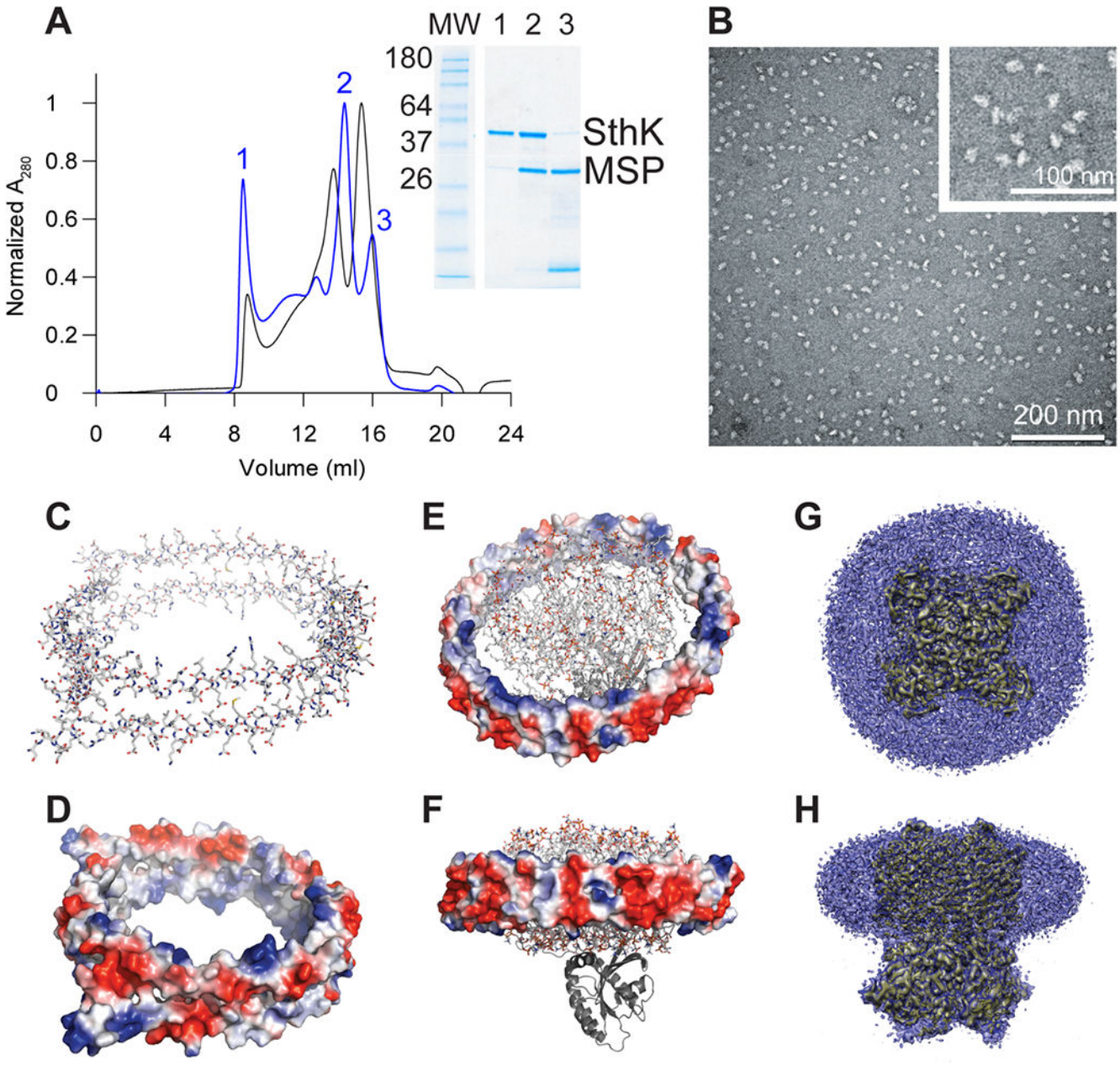

Figure 3: Structural features of nanodiscs.

(A) Gel filtration profiles (Superose 6 16/600) used to assess the reconstitution quality of the cyclic nucleotide-gated K^+ channel SthK into nanodiscs formed with different MSPs. Molar ratios of SthK:MSP2N2:POPG 1:1:125 (black) and SthK:MSP1E3:POPG 1:1:75 (blue) are shown. The bigger MSP (MSP2N2) shows an increased peak for empty nanodiscs. For the smaller MSP1E3, the SDS-PAGE clearly resolves the assembly of nanodiscs with aggregated SthK in the void peak (1), a peak for SthK inserted in MSP1E3 in nanodiscs (2) and empty nanodiscs (3). (B) Uranyl-acetate negative stain EM image of SthK in MSP1E3 (peak 2 in panel (A)) recorded on a JEM-1400 with 100 kV and a magnification of 150000. (C) Apolipoprotein A-I (PDB: 2N5E[49]) is shown in stick representation (colored by atom)

and (D) colored by the surface electrostatics. (E) top view and (F) side view of the GTPase K-RAS4B tethered to an apolipoprotein A-I nanodisc (PDB: 2MSD[50]) with the nanodisc colored by surface electrostatics, lipids shown in stick representation and the GTPase as cartoon. (G) Top view and (H) side view of the density from single particle cryoEM of a ligand-gated ion channel (yellow, EMDB: 7484) incorporated into MSP1E3 nanodiscs (blue)[51]. Panels C-F were prepared using Pymol (www.pymol.org), panels (G) and (H) were prepared using UCSF Chimera[52].