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Biomolecular membrane protein crystallization

Jani Reddy Bolla¹, Chih-Chia Su¹, and Edward W. Yu^{1,2,*}

¹Department of Chemistry, Iowa State University, Ames, IA 50011, USA

²Department of Physics and Astronomy, Iowa State University, Ames, IA 50011, USA

Abstract

Integral membrane proteins comprise approximately 30% of the sequenced genomes, and there is an immediate need for their high-resolution structural information. Currently, the most reliable approach to obtain these structures is x-ray crystallography. However, obtaining crystals of membrane proteins that diffract to high resolution appears to be quite challenging, and remains a major obstacle in structural determination. This brief review summarizes a variety of methodologies for use in crystallizing these membrane proteins. Hopefully, by introducing the available methods, techniques, and providing a general understanding of membrane proteins, a rational decision can be made about now to crystallize these complex materials.

1. Introduction

High-resolution crystal structures of membrane proteins are in great demand, as these structures often provide an immediate insight into the mechanisms on how these protein machines work. Membrane proteins play a vital role in numerous cellular functions. They act as receptors, transporters, channels, and are involved in cell adhesion, signaling, as well as a variety of metabolic pathways. About 30% of the mammalian genome encodes these integral membrane proteins [1], and more than 50% of currently administrative pharmaceutical agents are designed for targeting them [2]. Unfortunately, the understanding of the structure and function of these membrane proteins is hampered by the difficulties associated with expressing and handling them. Membrane proteins are not soluble in aqueous buffer because of their intrinsic characteristics. These proteins possess large hydrophobic surfaces, thus detergents are required to extract them from cell membranes and to maintain them in a soluble form in aqueous solution (Fig. 1). Mild detergents are widely used in handling membrane proteins, however, many of these proteins tend to aggregate or denature when solubilized by these amphiphilic chemicals. Because of the complications related to the stability and solubility of membrane proteins, it is often difficult to study these proteins using common biophysical techniques such as x-ray scattering, magnetic and optical spectroscopies.

Like many chemical compounds, proteins can be crystallized as they become supersaturated in aqueous solution. The crystallization process is often initiated by the addition of various precipitating agents, including salts like NaCl, (NH₄)₂SO₄ etc., polymers like polyethylene glycols (PEGs), and/or a variety of organic molecules like ethanol, isopropanol, 2-methyl-2,4-pentanediol (MPD), etc. These precipitating agents reduce the solubility of protein molecules and eventually supersaturate them in solution. As the pH and temperature also affect the solubility of protein samples, the process of protein crystallization is often found to be pH and temperature dependent.

*To whom correspondence should be addressed. ewyu@iastate.edu.

Different from other chemical crystals, protein crystals possess high solvent content. The solvent content of protein crystals was first studied by Matthews [3] in 1968. The study was based on the known soluble protein crystal structures at that time. It was found that the crystal volume occupied by solvent ranged from 27 to 65%, with the most common solvent content at 43%. Although the high solvent content often results in a poorer crystal quality, it provides an environment that is close to the native cellular environment for these protein molecules. Thus this high crystal solvent content indeed offers an opportunity for the protein molecule to better mimic its native structure in the cellular environment. In the case of membrane proteins, this solvent content is even higher (between 65 and 80%) because of the presence of detergent and/or amphiphilic molecules. As a result, membrane protein crystals are often found to diffract x-ray poorly, and many of these crystals can only provide low to medium resolution structural information.

Vapor diffusion is the most popular method that allows the protein samples to reach supersaturation [4]. This method is achieved by increasing the protein concentration gradually through a relatively slow dehydration process. Typically, a drop composed of a mixture of protein sample and precipitating agents is placed in vapor equilibrium with a hygroscopic reservoir solution containing two times higher in concentration of these precipitating agents (Fig. 2). As water leaves the drop, the protein concentration undergoes an increase in relative supersaturation, which eventually initiates nucleation of protein bearing crystals. Although the idea is simple, many proteins simply refuse to form crystals. Thus, protein crystallization is often found to be labor intensive and time consuming.

The principle of membrane protein crystallization is very similar to that of water-soluble proteins except that the presence of detergent, which adds another layer of difficulty, is required to solubilize the membrane protein molecules in aqueous solution. This review provides a brief overview of different approaches in the crystallization of these membrane proteins. To date, a great deal of useful information in the field can be found in internet-based resources. For example, the web sites of “membrane proteins of known structure” (<http://blanco.biomol.uci.edu/mpstruc/>) and “membrane protein data bank” (<http://www.mpd.b.ucl.ac.uk/>) [5] frequently update the lists of all known membrane protein structures, as well as their crystal growth methods and conditions.

2. Detergent-based methods

Membrane proteins can be crystallized for use in macromolecular structural determination by a variety of methodologies. The detergent-based methods (Fig. 3) employ detergent-solubilized, purified membrane protein samples directly in crystallization trials [6]. To obtain a stable protein sample suitable for crystallization, one needs to get through many obstacles, including finding an effective system for protein expression and choosing the right detergent for protein solubilization and purification. In many successful cases, the membrane proteins were produced in *Escherichia coli* cells. However, *Pichia pastoris* yeasts [7, 8], *Saccharomyces cerevisiae* yeasts [9] as well as Sf9 insect cells [10] have also been commonly used to express the proteins of interest for crystallization. After obtaining the expressed membrane proteins, the proteins have to be extracted from lipid membranes with mild detergents and then purified to stable, homogeneous samples that are essential for crystallization. Table 1 and Figure 4 summarize some of the most frequently used detergents for membrane protein purification and crystallization. The detergent-based crystallization methods include vapor diffusion [11], micro-batch [12, 13], dialysis [11, 14] and counter-diffusion [15, 16] and micro-fluidics [17]. A schematic for the detergent-based crystallization approach is shown in Figure 3. At present, the step of crystallizing purified, detergent-solubilized membrane proteins remains the major bottleneck. Nevertheless, the rate of progress is accelerating, especially in the past decade.

Among the detergent-based methods, it appears that vapor diffusion (both of the hanging-drop and sitting-drop types [11]) is the most common approach (Fig. 2). The primary advantage of this approach is that the crystallization setup is rather straightforward and relatively easy to handle. Membrane protein molecules surrounded by belt-like detergent micelles can be treated as ordinary soluble protein molecules (Fig. 1b), and thus standard vapor diffusion crystallization procedures can be used to obtain the crystals. The major drawback, however, is that the protein molecules have to withstand the harsh environment in detergent micelles. Nonetheless, vapor diffusion, which is the first to have been applied to membrane proteins, has been proven to be the most successful approach in crystallizing membrane proteins. The first crystal structure of membrane protein obtained in 1985 is photosynthetic reaction center [18]. This structure was determined using the method of vapor diffusion. To date, over 80% of membrane protein structures in the Protein Data Bank (<http://www.rcsb.org/>) were solved based on crystals grown with this method. A general protocol for the crystallization of membrane proteins using this approach can be found in an article published by Newby et al. [19].

If crystallization of particular membrane proteins does not work, one may want to try to co-crystallize the proteins with antibodies. This approach is especially useful when the membrane protein molecules contain relatively small hydrophilic domains [20]. Antibody fragments, including Fab and Fv, that recognize native protein conformations have been shown to facilitate crystallization of membrane proteins by increasing the polar surface area for protein-protein contacts. In addition, these antibody fragments can be used to stabilize specific conformations of the proteins by restricting the flexibility of mobile domains, in turn aiding the success of crystallization attempts [20]. The approach of co-crystallizing monoclonal antibody fragments with integral membrane proteins using vapor diffusion has been successfully led to several important crystal structures, including cytochrome *c* oxidase [21], KcsA potassium channel [22], ClC chloride channel [23], and most recently the human β_2 adrenergic receptor [24]. To date, more than 50 membrane protein structures have been reported in the Protein Data Bank using this approach.

Membrane protein molecules tend to be more stable in the presence of lipid. Indeed, it has been revealed that there is a direct link between the presence of lipid environment and the structure and function of integral membrane proteins [25]. Thus, one should be aware of the importance of bound lipid molecules in membrane protein crystallization. It has been shown that the addition of lipid molecules can significantly improve the quality of the membrane protein crystals and allow them to diffract x-rays at high-resolution [26, 27]. If the crystallization trials do not result in high quality membrane protein crystals, one may want to conduct crystallization by adding lipid and changing the protein:lipid molar ratio in the vapor diffusion trials. This approach has been proven to be successful in obtaining high quality crystals of the cytochrome *b₆f* complex [26, 27].

3. Crystallization of the CusBA adaptor-transporter efflux system

The approach of vapor diffusion has allowed researchers in our laboratory to determine the crystal structures of different components, either alone or in a complex form, of the CusBA heavy-metal efflux complex (Figs. 5 and 6) [28-30]. The raw proteins of the CusA transporter and CusB adaptor were individually produced in *E. coli* cells. Then, the CusA protein was extracted and purified with a detergent 6-cyclohexyl-1-hexyl- β -D-maltoside (CYMAL-6) [28]. As CusB is a soluble protein, the presence of detergent was not required for protein extraction and purification. However, it was found that the addition of a detergent *n*-dodecyl- β -D-maltoside (DDM) was helpful to produce a more homogeneous sample and avoid protein aggregation during purification procedures [29].

Crystals of the CusA transporter were grown at 298K using vapor diffusion in the presence of CYMAL-6 with PEG 3350 as precipitant and ammonium sulfate as salt [28]. Crystals of the CusB adaptor were grown using the same approach in the presence of the detergent DDM with PEG 1000 and sodium citrate as precipitant and salt [29]. For the crystallization of the CusBA adaptor-transporter complex, the crystals were produced using vapor diffusion at room temperature in the presence of CYMAL-6. The precipitant and salt for crystallizing the CusBA complex were PEG 6000 and ammonium acetate [30]. Crystals of CusA, CusB and CusBA grew to a full size in the drops within a month (Fig. 5).

For x-ray data collections, a single crystal of CusA or CusBA was flash-cooled in cryoprotectant solutions containing 30% glycerol at 100K. As for CusB, a single crystal was cryoprotected in solution containing 30% PEG 1000 before freezing at 100K. All diffraction data were collected at 100K at beamline 24ID-C located at the Advanced Photon Source, using an ADSC Quantum 315 CCD-based detector.

Crystals of the CusA transporter were found to be trigonal with space group $R\bar{3}2$ ($a = b = 178.4$ Å, $c = 285.8$ Å). In the asymmetric unit, only one CusA protomer was found. The crystal structure of the CusA transporter was then determined at a resolution of 3.5 Å [28]. For the CusB adaptor, the crystals belonged to orthorhombic space group $I222$, with $a = 85.2$ Å, $b = 113.3$ Å, $c = 258.5$ Å. The asymmetric unit consists of two CusB protomers. The structure of CusB was then resolved to a resolution of 3.4 Å [29].

The co-crystals of the CusBA adaptor-transporter complex took a trigonal space group $R\bar{3}2$ with unit cell parameters $a = b = 160.2$ Å, $c = 682.7$ Å. The asymmetric unit contains one CusA and two CusB molecules. The final structure of the CusBA adaptor-transporter complex was determined to 2.9 Å resolution [30] (Fig. 6).

4. Lipid-based methods

As the presence of phospholipids tends to stabilize membrane protein molecules, and in some cases the presence of these lipid molecules is a prerequisite for the protein activity, there is a strong rationale to crystallize membrane proteins in the presence of phospholipids. The lipid-based methods utilize biomolecular lipid bilayers to facilitate membrane protein crystallization [31]. These include the bicelle [32, 33], vesicle [34, 35], and lipidic cubic phase [36, 37] methods. In contrast to the detergent-based methods, these approaches promote the use of lipid bilayers, which mimic the native environment of membrane proteins, as an embedded system to stabilize the protein molecules. A schematic of the lipid-based crystallization is illustrated in Figure 7. The bicelle and vesicle methods are still new and have not been extensively evaluated. However, the lipidic cubic phase approach has been found to be very successful. All these methods offer an advantage that the protein molecules are removed from the harsh detergent environment and placed into a more hospitable surrounding during the process of crystallization.

Crystallization of membrane proteins in lipidic cubic phases has only been developed in the past 15 years. Nonetheless, this method has already been proven to be effective and essential for crystallizing a certain membrane proteins. The first crystal structure determined using this crystallization method is bacteriorhodopsin [38, 39]. To date, about one in ten integral membrane protein structures in the Protein Data Bank have been solved using crystals grown by this technique. Recent successes with this technique include the crystal structures of β_2 -adrenergic [40] and adenosine A_{2A} [41] G protein-coupled receptors.

In the cubic phase method, the crystallization process takes place in and from a semi-solid bicontinuous lipid matrix (Fig. 8). Thus, this technique involves making a highly ordered artificial lipid bilayer, namely cubic phase, before crystallization trials. The protein of

interest is then incorporated into the highly ordered cubic phase. This bicontinuous lipidic cubic phase contains extended water channels, which promote the detergent-solubilized membrane protein molecules to return to the lipid bilayer environment. After, precipitating agents are added to initiate protein crystallization.

1-mono-olein-*rac*-glycerol (MO) is the most commonly used lipid for forming these highly ordered cubic phases. The difficulty of this method is that it is a time consuming process in obtaining the initial cubic phase. As mentioned earlier, cubic phase is a semi-solid lipid matrix. It is also hard to work with because it is extremely viscous and sticky, making it difficult to handle and to dispense accurately in small volumes for setting up protein crystallization trials. A detailed protocol for crystallizing membrane proteins using the technique of lipidic mesophases is described in a review article by Caffrey and Cherezov [37].

Another closely related lipid-based approach is the lipidic spongy phase method [42]. A liquid lipid bilayer in the form of spongy phase can be obtained by adding an additional solvent such as PEGs, MPD, or jeffamine M600 to the MO:water system. The aqueous pores in the spongy phase can be up to three times as large as those in the cubic phase. Thus, the spongy phase method may be more useful in crystallizing membrane proteins with large extracellular domains.

One major advantage of employing lipidic spongy phase for membrane protein crystallization is that it is liquid in form at room temperature, thus it is easy to handle during the course of setting up crystallization trials. This liquid property also makes it suitable for conventional hanging-drop and sitting-drop vapor diffusion experiments. A nice summary of the lipidic spongy phase approach can be found in a literature by Wöhri *et al.* [42].

Both cubic and spongy phases form three-dimensional continuous lipid bilayers and interconnected water channels. This architecture promotes the lateral diffusion of protein molecules in the matrices. Presumably, the membrane protein molecules are first reconstituted in the lipid phase and then diffuse laterally to concentrate and assemble at the nucleation point to form small protein crystals at some point.

Recently, the bicelle method [32, 33] has been gaining popularity in the field after a few successes in crystallizing membrane proteins, including the mouse VADC₁ channel [43] and human β_2 adrenergic receptor [44], by using this approach. Bicelles, formed in a lipid-detergent mixture, have small disc-like features constituting of a planar lipid-bilayer (Fig. 9). The procedures for the bicelle approach are rather simple. First, the protein is mixed with bicelle components containing a mixture of lipid and detergent. Crystallization is then achieved using the method of vapor diffusion. Thus, the bicelle method can be considered as a compromise between the user-friendly vapor diffusion approach and the more protein-friendly lipidic mesophase technique.

The vesicle method, which makes use of successive fusion of vesicular assemblies, is a lipid bilayer approach initially developed by Takeda *et al.* [34] to crystallize bacteriorhodopsin. First, the vesicles are formed from the purple membrane fragments in the presence of detergent and precipitating agents. These spherical vesicles can be fused successively to form stacks of planar membranes by altering temperature as well as increasing the concentration of precipitating agents. Similar method has been used to crystallize the light-harvesting complex of photosystem II by reconstituting the purified membrane protein into liposomes [35]. In this case, the purified membrane protein molecules were first reconstituted in liposomes to form two-dimensional arrays. These proteoliposomes containing the two-dimensional arrays of protein molecules were then crystallized to form lattice. This approach may be useful for crystallizing membrane proteins that are unstable in

a detergent environment. Yet, it remains to be seen if this remarkable approach can be applied generally in crystallizing other membrane proteins.

5. Detergents

Choosing the right detergent has been the key success for membrane protein crystallization. It is one of the most important factors for obtaining high quality membrane protein crystals suitable for x-ray diffraction. Thus, the rule of thumb is that more efforts should be made in screening a variety of mild detergents than in optimizing other parameters when crystallizing a new membrane protein. Nonionic and zwitterionic detergents are usually used for crystallization of membrane proteins (Table 1 and Fig. 2). The most successfully used detergents are nonionic alkyl sugars with C8-C12 alkyl chains. Thus far, the most common detergent for use in crystallizing α -helical type membrane proteins is *n*-dodecyl- β -D-maltopyranoside, which contains 12 carbons in the main alkyl chain. The second most popular detergent in membrane protein crystallization is *n*-octyl- β -D-glucopyranoside, which is a C8-alkyl chained detergent.

During the past several years, the development of new detergents has been emerged as a new tool for handling these membrane proteins. Low molecular weight agents, such as hemifluorinated surfactants [45, 46] and cholate acid-based amphiphiles [47], have been proven to be promising in aiding the study of membrane proteins in aqueous solution. In addition, a new family of detergents, designated as maltose-neopentyl glycol amphiphiles [48], is of particular attractive in the field. The new generation of amphiphiles may have a wide application in solubilization, stabilization and crystallization of membrane proteins, and eventually yielding high-quality crystals for diffraction analysis.

6. Concluding remarks

This review is an attempt to outline different methods for use in three-dimensional membrane-protein crystallization. Crystallization of membrane proteins can be a rather difficult and discouraging process. It is hope that this brief summary will provide with a useful guide for researchers to tackle this problem. Particularly, the authors hope that this review will encourage more physicists to undertake research projects in this exciting field.

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Figure 1. Cartoon of an α -helical membrane protein molecule. (a) The molecule is embedded in cell membrane. (b) The molecule is surrounded by detergent micelle. (c) The molecule is surrounded by detergent-lipid mixture.

Figure 2. Vapor diffusion methods. (a) Hanging-drop vapor diffusion. (b) Sitting-drop vapor diffusion. In both (a) and (b), the drop composed of a mixture of protein sample and precipitating agents is in red. The hydroscopic reservoir solution containing two times higher in concentration of these precipitating agents is colored green. A cover slip is placed on the top of the vessel, which is sealed with a small amount of vaseline (yellow).

Figure 3. Schematic of detergent-based crystallization of membrane proteins. The membrane protein is removed from native cell membrane using detergent micelle. The purified protein is then crystallized in detergent micelles. The hydrophobic and hydrophilic domains of the membrane protein molecule are in green and pink colors. The cell membrane is colored red.

Figure 4.

Chemical structures of represented detergent molecules. (a) Nonionic detergents (OG, *n*-octyl- β -D-glucopyranoside; DDM, *n*-dodecyl- β -D-maltopyranoside; CYMAL-6, 6-cyclohexyl-1-pentyl- β -D-maltoside; MEGA-10, Decanoyl-*N*-methylglucamide). (b) Zwitterionic detergents (CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPSO, 3-[(3 Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; LDAO, *n*-dodecyl-*N,N*-dimethylamine-*N*-oxide).

Figure 5. Crystals of the *E. coli* Cus efflux proteins. (a) A crystal of the CusA heavy-metal transporter. (b) A crystal of the CusB adaptor protein. (c) A crystal of the CusBA adaptor-transporter efflux complex.

Figure 6.

Crystal structure of the CusBA adaptor-transporter complex. (a) X-ray diffraction pattern of the CusBA crystal. The crystal took a trigonal space group $R\bar{3}2$ with unit cell parameters $a = b = 160.2 \text{ \AA}$, $c = 682.7 \text{ \AA}$. (b) Electron density map at a resolution of $2.9 \text{ \AA}$. The CusA and two CusB molecules are colored yellow, green and blue, respectively. (c) Packing diagram of the CusBA crystal complex viewed along the c axis (green, CusA; blue, CusB; orange, CusB). (d) Packing diagram of the CusBA crystal complex viewed orthogonal to (c). (e) Structure of the CusBA efflux complex viewed in the membrane plane (green, CusA; blue, CusB; orange, CusB).

Figure 7. Schematic of lipid-based crystallization of membrane proteins. The purified protein is crystallized in the presence of lipid. The hydrophobic and hydrophilic domains of the membrane protein molecule are in green and pink colors. The cell membrane and lipid molecules are colored red.

Figure 8.

A cartoon representation of lipidic cubic phase. The membrane protein molecules embedded in the cubic phase are colored green.

Figure 9.

A cartoon representation of a disc-like bicelle. The membrane protein molecule embedded in the bicelle is colored blue.

Table 1

A list of commonly used detergents in membrane protein crystallization.

Detergent	Common Name	CMC ^I (mM)
NONIONIC		
<i>n</i> -octyl-β-D-glucopyranoside	OG	18.00
<i>n</i> -nonyl-β-D-glucopyranoside	NG	6.50
<i>n</i> -dodecyl-β-D-glucopyranoside	DDG	0.19
<i>n</i> -octyl-β-D-maltopyranoside	OM	19.50
<i>n</i> -nonyl-β-D-maltopyranoside	NM	6.00
<i>n</i> -decyl-β-D-maltopyranoside	DM	1.80
<i>n</i> -undecyl-β-D-maltopyranoside	UDM	0.59
<i>n</i> -dodecyl-β-D-maltopyranoside	DDM	0.17
5-cyclohexyl-1-pentyl-β-D-maltoside	CYMAL-5	2.40
6-cyclohexyl-1-pentyl-β-D-maltoside	CYMAL-6	0.56
Tetraethylene glycol monoctyl ether	C ₈ E ₄	8.50
Pentaethylene glycol monoctyl ether	C ₈ E ₅	7.10
Polyoxyethylene(5)dodecyl ether	C ₁₀ E ₅	0.81
Polyoxyethylene(8)dodecyl ether	C ₁₂ E ₈	0.09
Polyoxyethylene(9)dodecyl ether	C ₁₂ E ₉	0.05
Decanoyl- <i>N</i> -methylglucamide	MEGA-10	6.00
ZWITTERIONIC		
<i>n</i> -decyl- <i>N,N</i> -dimethylamine- <i>N</i> -oxide	DDAO	10.48
<i>n</i> -undecyl- <i>N,N</i> -dimethylamine- <i>N</i> -oxide	UDAO	3.21
<i>n</i> -dodecyl- <i>N,N</i> -dimethylamine- <i>N</i> -oxide	LDAO	1.00
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate	CHAPS	8.00
3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate	CHAPSO	8.00

^I Critical Micelle Concentration (CMC)