Crystallizing membrane proteins using lipidic bicelles

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Abstract

Crystallization of membrane proteins remains a significant challenge. For proteins resistant to the
traditional approach of directly crystallizing from detergents, lipidic phase crystallization can be a
powerful tool. Bicelles are an excellent medium for crystallizing membrane proteins in a lipidic
environment. They can be described as bilayer discs formed by the mixture of a long-chain
phospholipid and an amphiphile in an aqueous medium. Membrane proteins can be readily
reconstituted into bicelles, where they are maintained in a native-like bilayer environment.
Importantly, membrane proteins have been shown to be fully functional in bicelles under
physiological conditions. Protein-bicelle mixtures can be manipulated with almost the same ease
as detergent-solubilized membrane proteins, making bicelles compatible with standard equipment
including high-throughput crystallization robots. A number of membrane proteins have now been
successfully crystallized using the bicelle method, including bacteriorhodopsin, β2 adrenergic
receptor, voltage-dependent anion channel, xanthorhodopsin and rhomboid protease. Because of
the success with a variety of membrane proteins and the ease of implementation, bicelles should
be part of every membrane protein crystallographer's arsenal.

Keywords

membrane protein crystallization; bicelle; lipidic crystallization

1. Introduction

The pace of membrane protein structure determination is rising at a rapid rate thanks to
recent technological advances covered in this issue. The most popular and successful
approach for membrane protein crystallization so far has been detergent-based
crystallization [1,2]. Membrane proteins are generally isolated from the cell using detergents
and it is convenient to place the purified detergent-solubilized protein directly into the same
crystallization trials used for soluble proteins. Because detergents are not a perfect mimic of
a natural bilayer, however, many membrane proteins are not stable enough in detergent to
produce high quality crystals [3]. Moreover, detergent solubilization of membrane proteins
often leads to loss of structural lipids, resulting in impaired protein integrity [4]. Detergent
micelles also mask much of the surface area available for crystal contacts. Consequently,
most detergent-based crystals have Type II packing formed by contacts only between the

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hydrophilic surfaces of membrane proteins [3]. Accordingly, proteins that lack well-ordered soluble domains can be difficult to crystallize.

One strategy to improve the chances of obtaining high-quality crystals is to augment crystal contacts by expanding the soluble domains. This can be carried out using epitope-specific antibodies that provide a large stable polar domain for crystal formation. Originally developed by Hunte and Michel to facilitate crystallization of cytochrome c oxidase [5,6] and cytochrome bc1 complex [7], the antibody method involves raising Fab or Fv fragments from mice. It has also been extensively utilized by the MacKinnon lab to crystallize KcsA [8], KvAP [9] and ClC channels [10]. More recently, the structures of proton pump AdiC [11], SecYE translocon [12], nitric oxide reductase [13] and anion selective cys-loop receptor [14] were obtained using Fab fragments. Antibodies including the recently identified camelid single chain antibody fragments (nanobodies), have also played a significant role in stabilizing G protein-coupled receptors for crystallogenesis [15-18]. Although successful, the antibody approach is typically outside the scope of most laboratories because of the lengthy and challenging process of raising, selecting and producing the antibodies. A promising alternative to naturally produced antibodies is to use phage display to select binding partners [19,20]. A variation of the antibody technique involves protein engineering to introduce soluble protein domains that help extend the polar surface area for crystallization [21,22]. However, in the absence of extensive knowledge of the protein of interest, this method can potentially introduce several complications including protein instability and crystal disorder.

Another approach is to crystallize membrane proteins directly in a bilayer environment. Originally conceived by Landau and Rosenbusch, successful crystallization of bacteriorhodopsin from the lipidic cubic phase (LCP) demonstrated for the first time that membrane proteins could be crystallized from bilayers [23]. Structurally, the cubic phase consists of a single lipid bilayer bent into a highly organized three dimensional lattice pervaded by an interconnected aqueous channel system. Monoolein (9.9 MAG) is the lipid of choice for LCP, although success has also been obtained with related lipids such as monovaccenin (11.7 MAG) for sensory rhodopsins II-transducer complex [21] and 7.7 MAG for the recent crystal structure of β2 adrenergic receptor-Gs protein complex [17]. When embedded in an LCP, a protein can diffuse in three dimensions and feed any crystal nuclei that form. Crystals tend to be of the Type I variety, with extensive crystal contacts in both the soluble and membrane regions, thereby providing more potential for lattice formation than the Type II crystals usually obtained with detergents. Since its introduction in 1996, the LCP method has undergone numerous advancements [24] and has seen numerous successes including structures of the highly sought after G-protein-coupled receptors [25-28]. However, the method is still not routinely used in most laboratories due to several practical disadvantages associated with handling the highly viscous, semi-solid LCP, which requires specialized tools for all steps of crystallization from set-up to harvest.

Bicelles also represent an attractive lipidic medium for crystallizing membrane proteins in a native-like bilayer environment. Bicelles were introduced in the mid 1990's as magnetically alignable model membranes and have since been extensively used for both solid and solution state NMR [29-31]. In 2002, owing to their unique properties as an excellent membrane mimetic system, bicelles were utilized to crystallize bacteriorhodopsin, showing their potential as a convenient lipidic medium for membrane protein crystallization [32]. Structurally, bicelles can be thought of as solubilized lipid bilayer disks that are formed by the addition of an amphiphile (detergent or a short-chain lipid) to a long-chain lipid **(Figure 1)** in an aqueous medium. In most cases, the long-chain lipid is a phosphatidylcholine (PC) lipid such as dimyristoyl phosphatidylcholine (DMPC) or ditridecanoyl phosphatidylcholine (DTPC). Examples of the amphiphile are short chain lipids like dihexanoyl-

phosphatidylcholine (DHPC) or detergents like CHAPSO. These mixtures spontaneously form disc-shaped aggregates, with the long-chain phospholipid forming a central planar bilayer that is surrounded by a rim of amphiphile protecting the hydrophobic edge of the bilayer. The ratio of long-chain to short-chain lipids, also known as q, controls the physical diameter of the bicelles [33]. Importantly, it has been shown that membrane proteins can maintain their functionality upon reconstitution into bicelles [34].

Bicellar mixtures show a variety of different morphologies depending on the ratio of its components "q" and the temperature (**Figure 1**). At low temperatures and q, the system exists as a fluid and forms characteristic bicelles with the long-chain lipid residing in the planar bilayer regions and the short chain lipid or detergent in the highly curved regions at the rim of the disc [35]. Higher temperatures, in the range of the fluid-to-gel transition temperature, can transform the system into flexible worm-like quasi-cylindrical elongated micelles known as the chiral nematic phase or the cholesteric phase [35-37]. These ribbon-like structures have high concentrations of the short-chain lipid along the edges of the micelles [38,39]. As the temperature is further increased, the system transitions into the perforated lamellar phase, which consists of multilamellar vesicles with pore-like defects in the lamellar sheets. The edges of the pores are lined with the short-chain lipid [38]. The lamellar phase is a gel and is also referred to as the swiss cheese form [40].

The ease of use of bicelles as a lipidic crystallization medium can be attributed to their unique phase behavior [41]. When maintained below the transition temperature, bicelles are in a convenient, pipettable liquid state. In practical terms, bicelle crystallization is similar to detergent-based protocols. There is simply an extra step of mixing the purified detergent-solubilized membrane protein with the bicelle stock solution prior to setting up crystallization trials. Standard crystallization techniques including robotics and all commercially available screens are compatible with the bicelle method. Thus, the bicelle method can be described as an intermediate between the traditional detergent only crystallization method and the rigid LCP method, while neatly combining the practical convenience of the detergent method with the advantages of a native-like lipidic environment.

Like the LCP technique, bacteriorhodopsin provided the proof-of-concept for the bicelle crystallization method [32]. After the initial success, bacteriorhodopsin was also crystallized in other crystal forms using different bicelle formulations and conditions [42]. However, the real strength of the bicelle method was demonstrated by the crystal structure of the human β2-adrenergic G-protein-coupled receptor in complex with a Fab fragment at 3.4 Å resolution [16]. Other major breakthroughs came with the report of the structure determination of voltage-dependent anion channel 1 (VDAC1), a eukaryotic β-barrel channel solved to 2.3 Å resolution [43], and xanthorhodopsin, a member of the bacterial rhodopsin family solved to 1.9 Å resolution using bicelle crystallization [44]. Recently, the structure of rhomboid protease was also determined to high resolution from crystals grown with bicelles [45]. Table 1 summarizes all the membrane proteins solved using bicelles to date and the crystallization conditions. The growing collection of proteins demonstrates the versatility of the bicelle method for crystallizing a variety of membrane proteins that already includes colored, colorless, alpha-helical and beta-sheet proteins from both prokaryotic and eukaryotic sources including humans.

The next section focuses on the experimental details of the bicelle methodology. A step-by-step protocol is provided for setting up high-throughput crystallization trials of purified membrane proteins using standard liquid handling robots.

2. Experimental Details

The bicelle methodology comprises four main steps: i) preparation of the lipidic bicellar mixture; ii) reconstitution of purified detergent-solubilized membrane protein into bicelles; iii) manual or robotic setup of crystallization trials and crystal optimization; and iv) visualization and extraction. Each step is discussed in detail below.

2.1 Bicelle Preparation

Bicelles can be prepared from a number of lipid:amphiphile combinations and at different concentrations [42,31]. Since the majority of the proteins crystallized using bicelles have been obtained from the DMPC:CHAPSO formulation (**Table 1**), this is a good mixture to begin with. DMPC:CHAPSO bicelles can either be purchased commercially as a premixed ready-to-use formulation [46] or prepared in the lab as described below:

2.1.1 Weigh out DMPC and CHAPSO—Add deionized water to the final volume and vortex. The molar ratio of DMPC:CHAPSO can be chosen to be between 2.6 and 3.0 (**Table 1**). The final bicelle concentration can be chosen to range between 10%-40%. Keep in mind that the higher the bicelle concentration, the greater the difficulty in dissolving the lipid and the higher the viscosity of the solution. A concentrated bicelle formulation can be advantageous when the protein concentration is low, however.

2.1.2 Dissolving the lipid to obtain a homogenous mixture requires some effort—Cycles of brief heating (~40-50°C), cooling on ice, vortexing and freezing should be performed to help the lipid go into solution. This can take several hours, but it only needs to be done once and then the stock can be used for many protein samples.

2.1.3—Upon completion, the mixture should be a viscous liquid on ice, but gel upon raising the temperature to ~30 °C, which reflects the change in phase from bicelles to more organized phases such as the perforated lamellar phase (**Figure 1**). It should be stored frozen at -20°C to prevent hydrolysis of the phospholipid head groups.

2.2 Protein incorporation into bicelles

Since most bicelle structures have been obtained from ~8% DMPC:CHAPSO bicelle concentration with 8-12 mg/ml final protein concentration **(Table 1)**, a good starting point would be to set up initial screens under these conditions and then screen other concentrations at the optimization stage or if initial screens do not yield any hits. As detailed below, mixing the protein sample is much easier with bicelles compared with other lipidic crystallization methods (**Figure 2**). The protein-bicelle mixture should be made fresh for same day use in crystal trials.

2.2.1—Thaw the bicelles at room temperature until the phase changes to a clear gel. Multiple freeze-thaws will not affect bicelle behavior.

2.2.2 Place the bicelle mixture on ice to liquefy—Vortex briefly to reestablish a homogenous phase and place it back on ice. While chilled on ice, it will remain in the liquid phase making it amenable to pipetting.

2.2.3—Add the bicelle solution to the purified detergent-solubilized protein (ideally >10 mg/ml) in a 1:4 (V/V) ratio. Depending on the concentration of the bicelle stock solution (10-40%), this will give a final bicelle concentration of 2-8%.

2.2.4—Gently pipette the contents up and down until the solution becomes clear and homogenous. A quick spin with a tabletop centrifuge can help remove bubbles that may appear during mixing.

2.2.5—Incubate the mixture on ice for about 30 min to allow for complete reconstitution of protein into bicelles. The proteo-bicelle mixture is now ready for crystallization trials. Keep the protein-bicelle mixture on ice until ready to set up crystal trials.

2.3 Crystallization Trials

Trials can be carried out in either hanging drop or sitting drop format using any commercially available screen. Manual crystallization trials can be set up in the same manner as detergent based techniques. In most cases, high-throughput crystallization trials can also be performed routinely. Robots like the popular Mosquito (TTP Labtech) can easily pipette protein-bicelle mixtures without requiring any special handling for setting up crystal trials. Other robots that lack positive displacement system can also be used for bicelle crystallization with the following suggestions that will help keep the protein-bicelle mixture cool, thus lowering the viscosity and ensuring accurate pipetting.

2.3.1—Keep the plate that holds the protein-bicelle sample on ice during set-up and in between multiple runs. This plate should be the last to go on the robot before starting the run.

2.3.2—Do not program the robot to mix the reservoir and the protein-bicelle solution. This will result in heating and hence increased viscosity, which may affect dispensing of the drops.

Since the phase behavior of bicelles is temperature dependent, it may be useful to incubate the crystallization trials at different temperatures. A good starting point for the initial crystal trials is 20°C. Higher temperatures, such as 30°C and 37°C can also be tested, as they induce the lamellar phase (**Figure 1**) [40], which has the advantage of pre-organizing the protein in layers. Temperatures below 20°C can be tested but should not be less than 4°C since this will cause the lipids to precipitate over extended period of time.

As with traditional crystallization trials, the bicelle trials should be monitored on a regular basis for crystal appearance and growth. A general schedule can be checking the tray on the 1st and 3rd day followed by weekly inspection after setup.

Optimization of initial crystals can be carried out using methods routinely used for detergent based crystals such as grid screening, additive screening and different temperatures. In addition, the final bicelle percentage and the ratio of protein:bicelle can be varied. Importantly, bicelles can also be doped with specific lipids that may be necessary for protein stability and function [47-52].

2.4 Visualization and Crystal Extraction

Unlike other lipidic media that may hinder crystal visibility, even colorless protein crystals can be easily viewed using standard microscopes with the bicelle method. However, as with other lipidic media, bicelle based trials form crystalline shapes that may be confused with protein crystals. Consequently, a UV-microscope can very useful for identifying these non-proteinaceous false positives [53].

Generally, it takes 2-3 days for crystals to form and about a week or more to grow to their maximum size, as was the case for bacteriorhodopsin [32] and mouse voltage-dependent

anion channel 1 [unpublished observation]. However, for other membrane proteins it can take a longer time for crystal growth. For example, under some conditions, bacteriorhopsin crystals were only seen after ~1 month, so it is important to continue monitoring the crystal trials after the first few weeks [42].

Since the protein-bicelle mixture has a viscosity similar to detergent-based drops, crystal extraction can be carried out in a similar fashion to traditional setups. Thus, in contrast with the LCP method, no lipase or other such treatments are required for fishing crystals out of the lipidic media. In fact, the bicelle phase surrounding the protein crystal may be beneficial in providing some level of cryo-protection.

3. Conclusions

Bicelles are a unique lipidic media that provide membrane proteins with a native-like bilayer environment while offering the ease of use of detergents. Thus, in terms of ease of use, bicelles have a distinct edge over other lipidic crystallization protocols. Once bicelles are in hand, they can be mixed directly with purified detergent-solubilized membrane protein and from this step onwards crystallization setup proceeds almost exactly the same as traditional detergent-based methods.

Bicelles offer several other practical advantages compared with other lipid based crystallization methods like LCP. These include extended storage periods for bicelles, simple incorporation of proteins into bicelles, ability to use high-throughput robots, routine crystal analysis and harvesting. Furthermore, bicelles can be easily doped with lipids specific for the membrane protein of interest. In sum, bicelles are a versatile and user-friendly lipid media that should be part of all membrane protein crystallization projects.

Acknowledgments

Abbreviations

LCP	Lipidic Cubic Phase
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
CHAPSO	3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate

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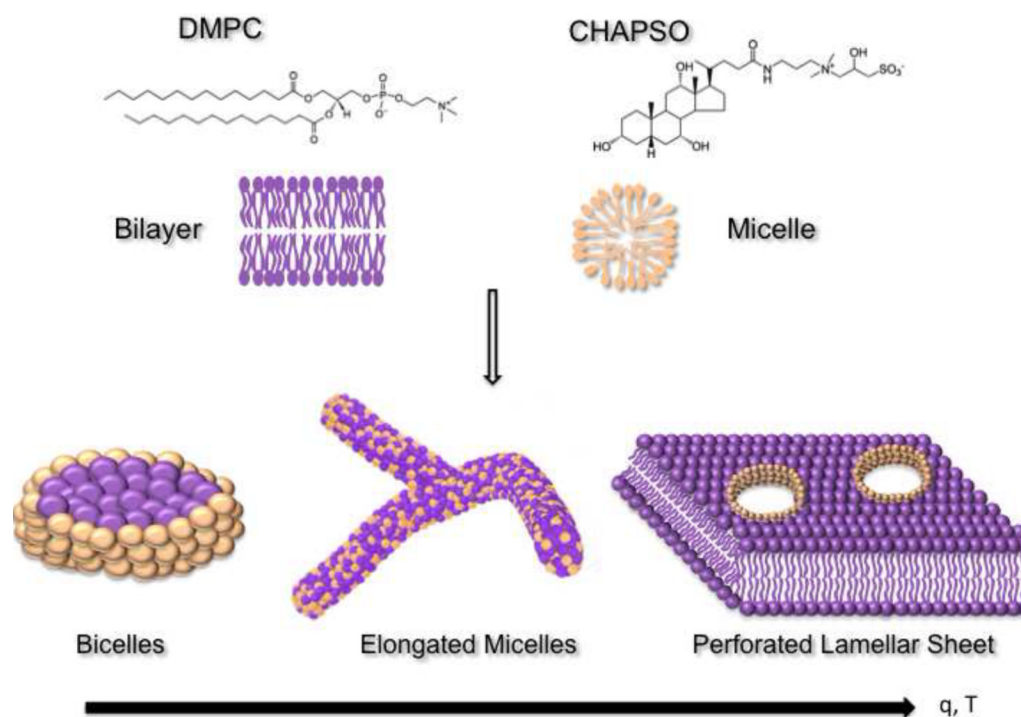
Figure 1. Phase behavior of bicellar mixtures
Bicelles are formed by mixing a phosphatidylcholine lipid such as DMPC with a detergent such as CHAPSO. The detergent shields the hydrophobic edges of the bilayer. With increasing temperatures and q, disc-shaped bicelles undergo phase transformation into worm-like cylindrical micelles and perforated lamellar sheet, which may also be beneficial for crystallization.
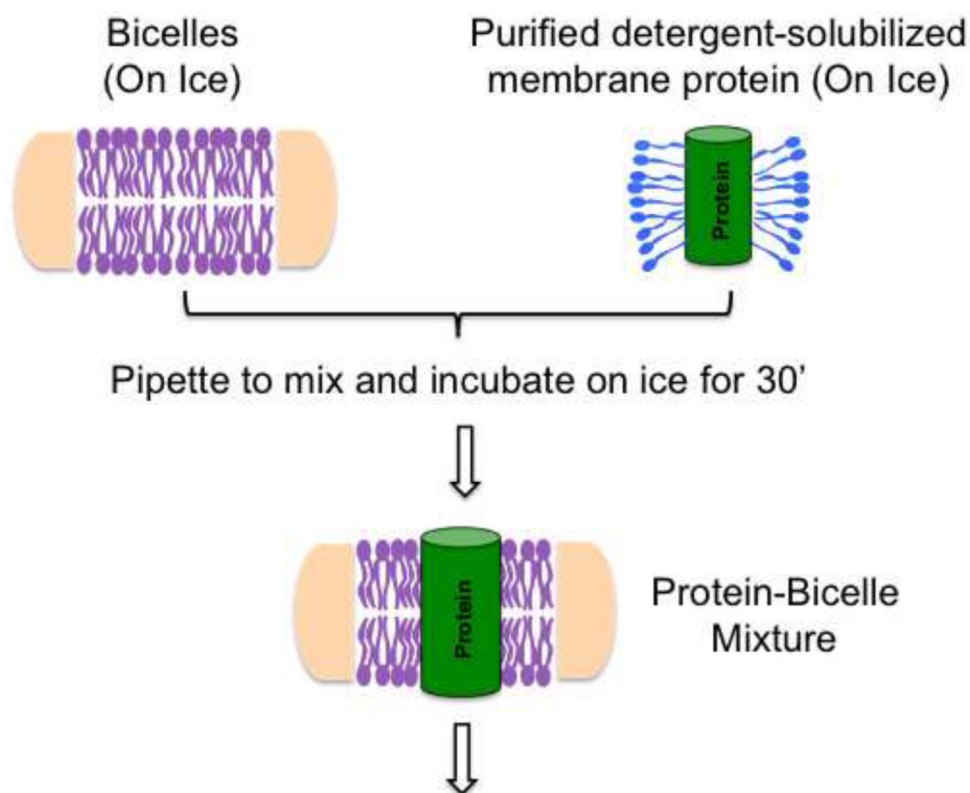
Figure 2. Bicelle crystallization
Detergent-solubilized purified membrane proteins can be mixed directly with bicelles on ice. Homogenous mixing can be achieved by simply pipetting the contents up and down. After incubating the protein/bicelle mixture on ice for ~30 minutes, crystallization trials can be carried out using any standard format including robotics.

Table 1

Membrane protein structures solved using the bicelle method.

No.	Protein	Source	Resolution (Å)	Protein Concentration (mg/ml)	Bicelle Formulation	Crystallization Condition	Reference
1	Bacteriorhodopsin	*Halobacterium salinarum*	2.0	8	8% DMPC:CHAPSO (2.8:1)	2.8M Sodium Phosphate pH 3.7, 180mM Hexanediol	Faham and Bowie, 2002 [32]
			1.8	8	8% DTPC:CHAPSO (3:1)	100mM Sodium Formate pH 4.3, 28.5% PEG2000, 280mM Ammonium Sulfate, 180mM Hexanediol	Faham *et al.*, 2005 [42]
2	β2-Adrenergic receptor	*Homo sapiens*	3.4/3.7	10	8.3% DMPC:CHAPSO (3:1)	1.85-2M Ammonium Sulfate, 180mM Sodium Acetate, 5mM EDTA, 100mM MES or HEPES pH 6.5-7.5	Rasmussen *et al.*, 2007 [16]
3	Voltage-dependent anion channel 1	*Mus musculus*	2.3	12	7% DMPC:CHAPSO (2.8:1)	18% MPD, 0.1 M Tris pH 8.5, 10% PEG400	Ujwal *et al.*, 2008 [43]
4	Xanthorhodopsin	*Salinibacter ruber*	1.9	4	4.2% DMPC, 5% NM	2.5-3.5M Sodium Phosphate pH 5.6, 2.5mM Sodium Azide	Luecke *et al.*, 2009 [44]
5	Rhomboid protease	*Escherichia coli*	1.7	9	2% DMPC:CHAPSO (2.6:1)	1.5 M NaCl, 0.1 M Bis-Tris (pH 7)	Vinothkumar, 2011 [45]