# Prospects for membrane protein crystals in NMX

Samuel John Hjorth-Jensen[a], Esko Oksanen[b,c], Poul Nissen[a], Thomas Lykke-Møller Sørensen[a,*]
[a]Department of Molecular Biology and Genetics—DANDRITE, Aarhus University, Aarhus C, Denmark
[b]European Spallation Source ERIC, Lund, Sweden
[c]Department of Biochemistry and Structural Biology, Lund University, Lund, Sweden
[*]Corresponding author: e-mail address: tlms@mbg.au.dk

## Abstract

Adding hydrogen atoms and protonation states to structures of membrane proteins requires successful implementation of neutron macromolecular crystallography (NMX). This information would significantly increase our fundamental understanding of the transport processes membrane proteins undertake. To grow the large crystals needed for NMX studies requires significant amounts of stable protein, but once that challenge is overcome there is no intrinsic property of membrane proteins preventing the growth of large crystals per se. The calcium-transporting P-type ATPase (SERCA) has been thoroughly characterized biochemically and structurally over decades. We have extended our crystallization efforts to assess the feasibility of growing SERCA crystals for NMX—exploring microdialysis and capillary counterdiffusion crystallization techniques as alternatives to the traditional vapor diffusion crystallization experiment. Both methods possess crystallization dynamics favorable for maximizing crystal size and we used them to facilitate the growth of large crystals, validating these approaches for membrane protein crystallization for NMX.

## 1. Introduction

Membrane proteins are cellular gatekeepers conveying environmental signals, translocating molecules, and generating electrochemical gradients across the hydrophobic cell membrane. Understanding how membrane proteins work will enable us to explore the evolutionary technology they represent as well as target them to combat disease (Misawa, Osaki, & Takeuchi, 2018; Ng, Poulsen, & Deber, 2012; Schmit & Michiels, 2018). Membrane proteins are very diverse with individual proteins having multiple domains adapted to reside in hydrophobic/hydrophilic environments, intra- and extracellularly. This multidomain structure make membrane proteins not only scarce but also notoriously unstable and very challenging targets to study both biochemically and structurally. But many impressive studies over the last decade have demonstrated that it can be done (see https://blanco. biomol.uci.edu/mpstruc/ for an updated list of membrane protein structures).

Numerous membrane protein structures have been determined using X-ray crystallography—and more recently cryo-electron microscopy (https://www.wwpdb.org). In combination with detailed biochemical characterization, these structures have increased our understanding of how transport and signaling across membranes work. But for many of these processes we are still short of a fundamental mechanistic understanding as we do not normally resolve hydrogen atoms and protonation states in these structures. We can infer hydrogen localization when circumstantial evidence provides a plausible model, although the importance of protonation for a given reaction mechanism may not be clear. Detailed structural views with "hydrogen resolution" would significantly change this and allow us to address unanswered questions. The method of choice for obtaining structures with "hydrogen resolution" is neutron macromolecular crystallography (NMX). Because neutrons scatter from nuclei, the coherent scattering lengths of hydrogen (and deuterium) are similar in magnitude to carbon, nitrogen and oxygen (Sears, 1992). Hydrogen and deuterium atoms can therefore be easily identified in the density maps derived from neutron diffraction data because of their increased contribution to diffraction. NMX is not a new tool in structural biology, but it is a tool that in the coming decade is going to experience a steep increase in performance once the European Spallation Source (ESS) in Lund, Sweden becomes operational (https://europeanspallationsource.se/instruments/nmx).

In structural biology, ongoing technological advancements continue to push the limit for what is possible. New synchrotrons (https://lightsources. org) and free-electron lasers (Chapman, 2019) deliver beam sizes small enough and flux densities high enough to enable useful diffraction data to be extracted from micron-sized crystals. Furthermore, recent advances in detector technology has made it possible to capture the diffraction generated from the very intense and short exposures of these tiny crystals (Forster, Brandstetter, & Schulze-Briese, 2019). Also, software capable of processing the vast amount of data generated during these experiments has now been developed (Winter et al., 2018). Advanced detector technology has also spawned the resolution revolution in cryogenic electron microscopy (cryoEM) (Cheng, 2018), allowing atomic resolution models of larger bio-molecules and their complexes to be generated without the requirement for growing crystals at all (Cheng, 2015).

With the tiny beam sizes and high flux densities now available, X-ray crystallographers have abandoned the pursuit of large crystals. Crystallization itself still presents a significant challenge, but if small crystals analyzed by micro-focused beams provide a high-resolution structure substantiating the relevant scientific question(s), the crystallographic afterthought is minimal. Only once crystal quality is poor or data analysis challenging do biochemists have to venture into the world of crystal optimization—a realm populated with empirical knowledge of dubious worth. This is also where one needs to venture on the quest to grow the large crystals required for NMX. To collect useful NMX data

requires crystals that are hundreds of microns in all dimensions, a result that requires extensive optimization of the crystallization process. The reason that crystals need to be this large for NMX is that the diffracted intensity measured is proportional to the incoming beam intensity (O'Dell, Bodenheimer, & Meilleur, 2016). Even with the predicted impressive performance of the ESS, the neutron flux to be provided is high only relative to what is currently available and will still be orders of magnitude less than the X-ray flux experienced at synchrotron facilities.

Will it then be feasible to use neutron crystallography for membrane proteins? We recently reviewed potential membrane protein targets based on reported crystal sizes and cell parameters (Sorensen et al., 2018) and found that membrane proteins should indeed be considered for NMX studies. Over the years, we have produced many well-diffracting crystals for X-ray crystallography from a number of different membrane-protein projects. Obviously, each project has presented a range of intricate challenges and required resourceful insight and hard work for a successful outcome, but in our experience, there is nothing per se limiting the size of membrane protein crystals once an ample supply of stable protein has been organized. We have had significant success with the P-type ATPases family of membrane proteins and a key facet to this success has been a thorough understanding of the biochemical properties of this class of proteins (Moller, Olesen, Winther, & Nissen, 2010).

In this chapter, we describe two of the methods that we have applied to SERCA to increase crystal size—dialysis and counter-diffusion crystallization. These methods have been reviewed elsewhere (McPherson & Gavira, 2014; Otalora, Gavira, Ng, & Garcia-Ruiz, 2009) and are known to researchers in the neutron crystallography field. Ultimately, the goal is to produce a large perfectly well-ordered crystal, but the reality may be quite different. One intuitively considers the ability for a given crystal to grow to a large size indicative of a "healthy" crystal form with a sound lattice that supports continuous growth without imperfections. But local order does not necessarily propagate across hundreds of micrometers. As a crystal grows in size so does the likelihood of crystal imperfections originating from lattice variations between crystal domains as well as lattice variations within domains (Nave, 2014).

## 2. Microdialysis crystallisation

The principle for microdialysis crystallization is illustrated in Fig. 1. The protein solution—either by itself or mixed with precipitant solution (but remaining below the nucleation zone of the phase diagram)—is separated from a larger volume of precipitant solution by a size-discriminating membrane allowing free diffusion of all components except the protein. Over time, precipitating agents diffuse across the membrane into the protein solution, and at the correct combination of concentrations, the phase enters the nucleation zone. From here, the formation and growth of nuclei results in the progressive reduction of protein concentration until the solubility limit is reached. At this point, growth is halted and crystal size remains constant over time. However, further crystal growth may be initiated by increasing the precipitant concentration within the reservoir. As this extra precipitant diffuses across the separating membrane, the protein solubility is further decreased, driving additional protein from the solution into the crystal. Expanding upon this principle are double-contained dialysis setups, in which an initial reservoir is itself dialyzed against a larger, secondary reservoir containing higher concentrations of precipitants (Thomas, Rob, & Rice, 1989).

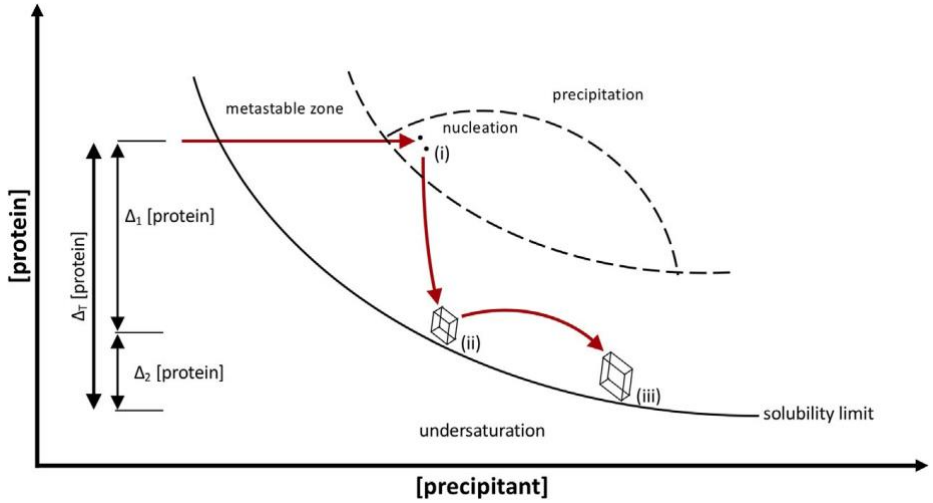


**Fig. 1** Crystal phase-time pathway within microdialysis. The mother liquor starts in the undersaturation/metastable zone and slowly moves toward the nucleation zone as precipitants diffuse across the dialysis membrane (i). As a few nuclei form, the mother liquid begins its trajectory through the metastable (i.e., crystal growth) zone, stopping at the solubility line (ii). Further grow can be induced by increasing the precipitant concentration inside the reservoir, slowly lowering the solubility limit of the mother liquor due to the diffusion of additional precipitants across the membrane (iii). A maximal amount of protein is added ($\Delta_T$ [protein]) due to the contributions of two growth phases ($\Delta_1$ [protein] and $\Delta_2$ [protein]), resulting in very large crystals.

The key advantage of the microdialysis technique is the ability to control the phase-time pathway, which is essential for producing crystals of the size needed for NMX studies. By contrast, the batch method offers very limited ability for its

saturation state to be slowly altered over time—the protein and crystallization solution are mixed together and an equilibrium is quickly reached. This most often results in uniform nucleation with many crystals of equal size that do not undergo an extended growth period. Vapor diffusion on the other hand, does offer the ability to increase or decrease the saturation state of the mother liquor over time. However, this change cannot be precisely controlled, i.e., the concentrations of each component within the mother liquor cannot be estimated at equilibrium. Also, vapor diffusion affects the concentrations of all components, with each non-volatile chemical species becoming more or less concentrated as drop volumes decrease or increase. These excessive changes in chemical composition may result in unwanted additional nucleation or osmotic shock, damaging or destroying the crystal.

Microdialysis allows each individual component within the mother liquor to be reasonably estimated and controlled over time in terms of conditions at equilibrium. For example, to increase the size of a crystal once equilibrium is reached, the polyethylene glycol (PEG) concentration of the mother liquor can be increased such that: (a) only PEG concentration significantly changes while the concentration of other components remain constant hence avoiding osmotic shock; (b) the change in PEG concentration can be accurately controlled making it feasible to reproducibly increase PEG concentration by steps of 1–2%, an increase that may extend crystals growth without inducing additional nucleation; and (c) a gradual change in PEG concentration (i.e., slow kinetics) that is conducive to the growth of an ordered lattice and less likely to result in heavy precipitation and/or nucleation.

An additional crystallization element offered by microdialysis is the ability to not just add, but to remove individual components from the mother liquor. In the case of membrane proteins, lowering the concentration of the solubilizing detergent could potentially promote crystal growth. In the case of SERCA, removing solubilizing octaethyleneglycol dodecylether ($C_{12}E_8$) may drive protein-containing micelles out of solution and into any growing crystal lattice present. We have explored this to a limited extent, and indeed crystals do appear to grow in response to the gradual removal of this detergent. However, further experiments to test the usefulness of this approach are needed to assess effects on diffraction quality, as removing detergent may affect crystal packing significantly.

A schematic presentation of the microdialysis setup we use to crystallize SERCA is shown in Fig. 2. Protein or batch solution is dispensed into the chamber of a microdialysis button, the volume of which can vary from five to several hundred microlitres. A dialysis membrane with a suitable molecular weight cut-off is placed on top, locked and sealed with an O-ring. We use PEG 6000 when crystallizing SERCA and hence use membranes with a cut-off of 12–14 kDa to allow its free diffusion across the dialysis membrane. The microdialysis button is then transferred into a reservoir chamber and fully submerged with precipitant solution. Finally, the reservoir chamber is sealed and the entire setup placed in a temperature-controlled environment.

A typical microdialysis-grown SERCA crystal is shown in Fig. 3, displaying a diamond-shaped morphology similar to the morphology observed for crystals obtained from hanging drop vapor diffusion experiments. The size of the crystal in Fig. 3 is approximately 500 X 500 X 100 μm, although we have grown SERCA crystals up to 1 mm across the largest dimension using this method. In this experiment, 12 mg/mL solubilized SERCA was mixed in a 5:1 ratio with an 8% (w/v) PEG 6000 precipitant solution creating an undersaturated batch solution as the starting point for the crystallization experiment. The 5 μL batch solution was dialyzed against 1300 μL of a precipitant solution containing 4% (w/v) PEG 6000. To further increase the size of the crystal, the concentration of PEG 6000 within the reservoir solution was increased from 4% to 6% eight days after the initial experiment was setup.

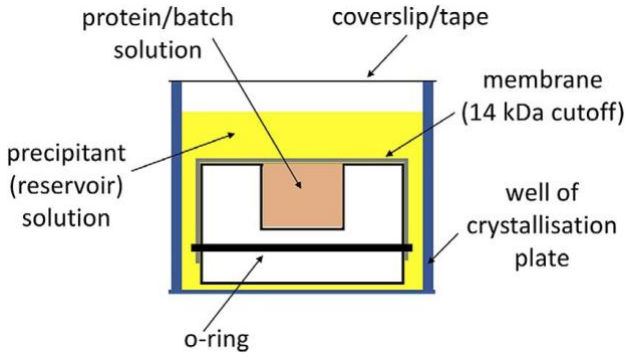


**Fig. 2** Schematic of the microdialysis format. Buttons are placed inside 24-well crystallization plates, allowing for the easy setup of replicates and parallel experiments. Reservoir solutions are able to be changed over time by first removing sealing coverslips/tape, then either adding stock straight to the existing reservoir solution (to increase component concentrations) or exchanging the entire reservoir with that of another composition (to decrease component concentrations), then re-sealing.

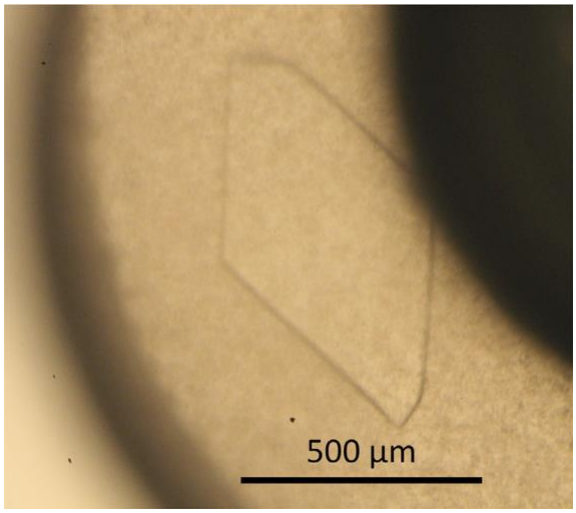


**Fig. 3** SERCA crystal grown using microdialysis. Protein chamber contained 5 µL of batch solution (11.6 mg/mL solubilized SERCA, 1.33% (w/v) PEG 6000) and was initially dialyzed against a reservoir solution containing 4% (w/v) PEG 6000. Crystal is shown 15 days after setup (the PEG 6000 concentration within the reservoir was increased to 6% at day 8).

When considering the use of microdialysis, there are a couple of challenges to keep in mind. First, microdialysis is much more challenging to set up than the more straightforward batch and hanging/sitting drop vapor diffusion crystallization formats. Particular care is needed when placing the membrane, as even the slightest movement can result in air being introduced at the protein-membrane interface. If the amount of air introduced is significant, diffusion across the membrane becomes greatly impeded as does the ability to visualize the contents of the dialysis button itself. Bubble formation is of particular concern when crystallizing membrane proteins, as the use of detergents make the occurrence of bubbles far more likely. Also, while this situation may normally be potentially reversed by removing the membrane, restoring the protein meniscus, and repeating the process, this again is more difficult when dealing with detergent-containing membrane proteins. The general advice is to practice using the actual solutions (omitting protein) to be able to handle detergent- and precipitant-containing solutions without introducing air, thereby conserving valuable protein.

Second, as microdialysis crystallization involves changing the saturation state of the mother liquor over time by dialyzing it against a reservoir of much larger volume, it is required that any ions, ligands, or other components present in the protein solution also be added to the reservoir solution. This includes additives used to stabilize the protein, to induce a specific functional state, or to generate a protein-ligand complex. These inclusions are necessary to prevent the additives from slowly diffusing out of the protein chamber over time and maintain protein stability and/or the desired functional state or complex. Again, this issue is more complicated for membrane proteins, as they often require more complex chemical mixtures to remain stable and soluble. Detergent is a key additional component that has to be included in the reservoir mixture when setting up a membrane protein microdialysis crystallization experiment. Often detergents are present in concentrations above the critical micelle concentration (CMC) to prevent the protein-containing micelles within the button from disintegrating and the protein precipitating. Consequently, expensive and highly purified detergents are often added to reservoirs in large quantities, meaning microdialysis consumes far more resources than other methods.

Finally, crystals grown within microdialysis buttons can be more difficult to harvest than crystals obtained using other methods, as the button wall surrounding the protein chamber restricts access to any crystals located inside. This is exacerbated by the fact that membrane protein crystals typically contain high levels of solvent and are therefore very fragile, making them particularly susceptible to damage during the crystal harvesting process. Again, it is worth emphasizing that practice is important, as this will maximize the chance that any prize crystal grown can be recovered safely.

Despite these challenges, microdialysis is a crystallization technique with a unique advantage in terms of the control that it offers over the crystal phase-time pathway. Results with SERCA so far indicate that it is feasible to grow NMX-compatible crystals using microdialysis. Future experiments will continue to explore this method, using protein/batch volumes of 100 µL and greater.

## 3. Capillary counter-diffusion crystallization

The principle for crystallization within the capillary counterdiffusion format is illustrated in Fig. 4. One end of the protein solution, confined within a capillary, is brought in contact with a precipitant solution, either confined within the same capillary or within a separate chamber. Immediately following setup, the two solutions begin to mix by diffusion, with the precipitating agents generally diffusing faster into the protein than vice versa. The high precipitant concentration at the liquid-liquid interface immediately results in large amounts of crystalline precipitate, and the local protein concentration begins to decline. The precipitant, which is not consumed in this process, continues to move further into the protein solution, where protein solubility is again reduced and another round of precipitation and/or nucleation occurs. As this

progressively weakening process repeats, a temporally and spatially varying supersaturation gradient forms along the length of the capillary. Farthest away from the interface, low levels of supersaturation (featuring low concentrations of precipitant and high concentrations of protein) result in fewer crystals of larger volumes (Otalora et al., 2009).

As a crystallization technique, capillary counterdiffusion possesses several inherent advantages. A major advantage with respect to diffraction data collection is that crystals, once grown, can be analyzed *in situ* with no additional handling required (Ng et al., 2003). This is very useful, as membrane protein crystals, at least in our hands, only become more fragile and difficult to handle with increasing crystal size. Another unique advantage of capillary counterdiffusion is that its dynamics are inherently favorable for generating large crystals and hence it is useful for growing crystals for NMX. Though it does not offer the precise control that microdialysis affords, the wide range of conditions and kinetics explored in each individual experiment often results in a section where only a few nuclei form, with these nuclei undergoing extensive growth to become large crystals (Otalora et al., 2009). The fact that a wide range of conditions are covered per experiment means this method in particular is able to compensate for variations between protein batches. Given the highly complex mixtures involved in membrane protein preparations, this class of proteins is particularly susceptible to batch-to-batch variations and results are often challenging to reproduce.

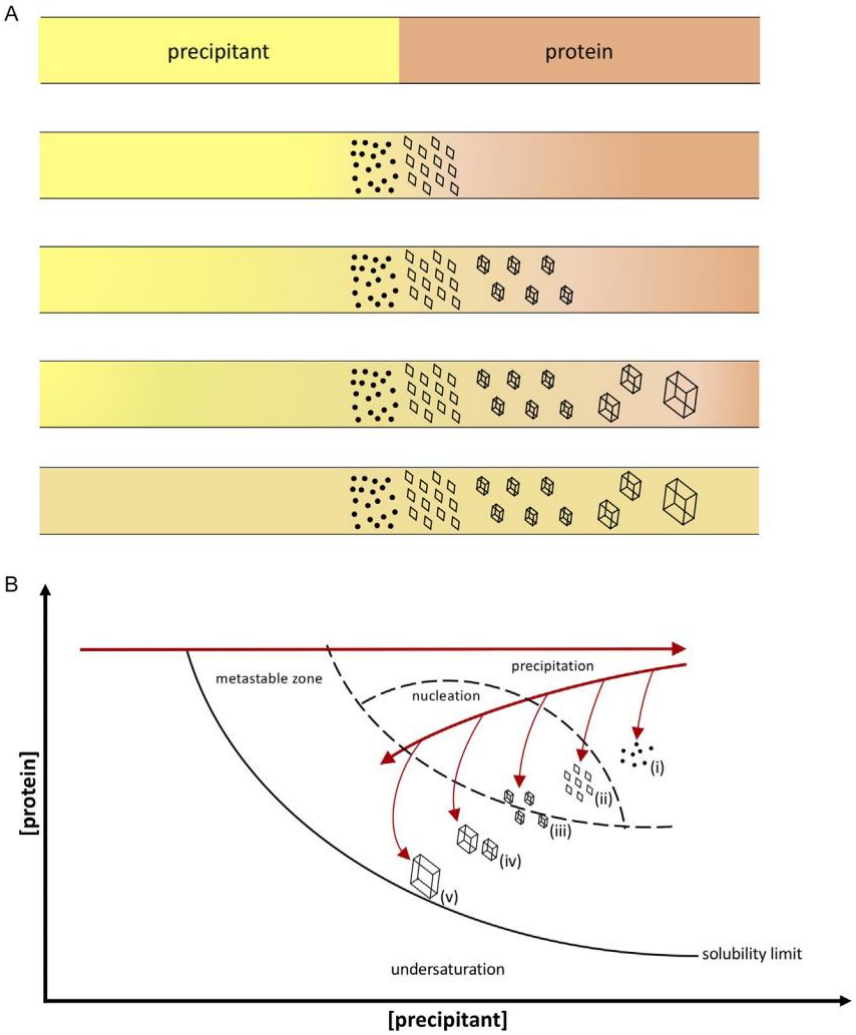


**Fig. 4** Crystal phase-time pathways within capillary counterdiffusion. (A) The protein and precipitate solutions are initially fully separated within a capillary with either the two liquids in direct contact or separated by a gel barrier. The two solutions then proceed to diffuse into one another, resulting in a wide range of conditions (and outcomes) that vary along the length of capillary (Ng, Gavira, & Garcia-Ruiz, 2003). (B) In terms of phase space, conditions at/near the interface immediately following setup consist of high precipitant and high protein concentrations, resulting in precipitate formation and the associated local reduction of [protein] (i). Farther away from the interface, the precipitant continues to diffuse into the protein (both solutions are at slightly lower concentrations as the precipitant dilutions as it is extending and the protein is both extending and precipitating/ nucleating) resulting in high density nucleation (ii). As the weakening precipitant front continues to advance, nuclei continue to form at a progressively reduced rate (iii and iv). Far into the protein solution, only few nuclei form from the now diminished precipitant wave. These nuclei spend an extended time within the metastable zone as precipitant continues to arrive over time, becoming large crystals (v) (Otalora et al., 2009).

There are several methods for setting up capillary crystallization experiments (for a detailed review, refer to the comprehensive article by Otalora et al. (2009), with the most common setups often involving the precipitant and protein solutions being separated by a physical buffer, usually a gel such as agarose, in order to delay the onset of diffusion or avoid osmotic shock. An example is the Gel Acupuncture Method (Garcia-Ruiz, Moreno, Viedma, & Coll, 1993), where a capillary filled with protein is embedded in agarose gel. Precipitant solution is added on top of or in the gel, which then

proceeds to diffuse into the protein solution and up the capillary. For SERCA, we have noted that the PEG 6000 precipitant we use to crystallize is unable to diffuse through this physical buffer at a sufficient rate to induce nucleation, and as such we have opted for a setup where both the precipitant and protein solutions are contained within the capillary and a free liquid-liquid interface exists between the two.

The setup typically used to crystallize SERCA is shown in Fig. 5. It is important to note that this particular procedure works best when using capillaries with relatively narrow diameters (≤ 0.5 mm), where geometries are restrictive enough to limit convective mixing of the protein and precipitant solution and preserve the supersaturation gradient. We are also performing experiments in large diameter capillaries (1-2 mm) in which solubilized SERCA or undersaturated batch solution is mixed with molten low-melting point agarose such that the mixture contains between 0.1 and 0.3% (*w/v*) agarose. This solution is then injected into the capillary and allowed to set before the precipitant solution is added adjacently.

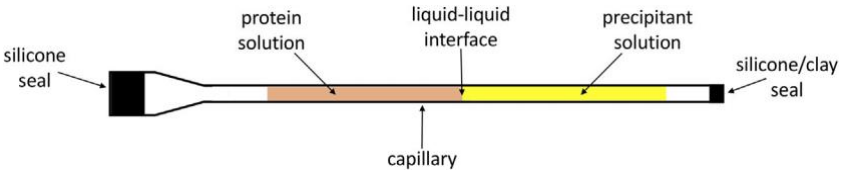


**Fig. 5** Schematic of the capillary counterdiffusion format. First protein, then an equal volume of precipitant solution is carefully aspirated into a capillary (such that the two liquids are touching). Both ends of the capillary are then sealed with silicone sealant/clay and the entire setup moved into a temperature-controlled environment to incubate.

Results from a capillary counterdiffusion experiment with SERCA are shown in Fig. 6. In this experiment, equal volumes of 12 mg/mL solubilized SERCA and a 12% (*w/v*) PEG 6000 precipitant solution were placed in liquid-liquid interface setup within a 0.5 mm diameter glass capillary. After 6 days, the supersaturation gradient characteristic of capillary counterdiffusion can be seen, with heavy precipitant becoming a few well-formed crystals as one moves along the length of the capillary.

There are a few issues to be aware of when considering capillary counterdiffusion crystallization experiments using membrane proteins. First, setting up capillary experiments require more careful handling than other crystallization methods due to the fragile nature of glass and quartz capillaries (a typical wall thickness is 0.01 mm). It is possible for capillaries to break during and after setup, resulting in the loss of significant amounts of precious protein. Second, to prevent chaotic mixing and convection from occurring within in capillaries, the protein solution sometimes needs to be gelled (Biertumpfel, Basquin, Birkenbihl, Suck, & Sauter, 2005; Gavira, de Jesus, Camara-Artigas, Lopez-Garriga, & Garcia-Ruiz, 2006; Otalora et al., 2009). However, gelling the protein solution requires mixing and dispensing at temperatures high enough to maintain the agarose in a molten state. While SERCA is able to withstand these elevated temperatures of up to 30 °C for short periods, a significant number of membrane proteins may not, and the thermal stability of your membrane protein should be demonstrated before proceeding with this technique.

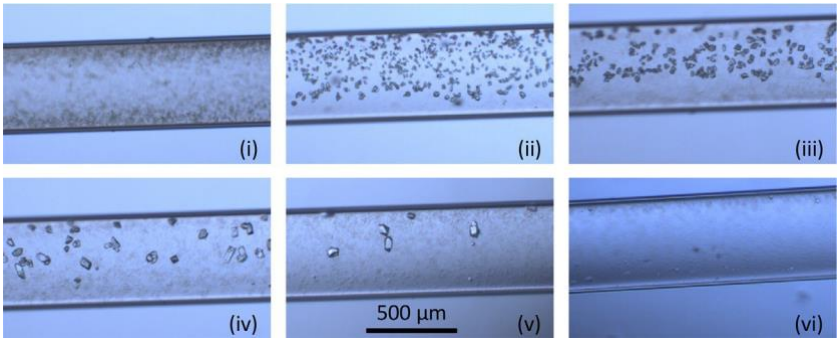


**Fig. 6** SERCA crystals grown using capillary counterdiffusion. A 0.5 mm glass capillary was filled with 4.5 µL of 12.6 mg/mL solubilized SERCA and 4.5 µL of precipitant solution containing 12% (w/v) PEG 6000, imaged 6 days after setup. Though the crystals shown are relatively small, the advantageous behavior of capillary counterdiffusion is clearly demonstrated. Heavy precipitate is seen at the liquid-liquid interface, where concentrations of both protein and precipitate were at their peak (i). Moving along the capillary one can see the results of a supersaturation gradient, with large numbers of small crystals (ii), becoming fewer in number while simultaneously increasing in size (iii–v). The final section of the capillary contains protein-rich solution free of nuclei, which over time, can serve as a protein source to grow the few single crystals adjacent.

Finally, it is important to keep the diffusion properties of the precipitants being used in mind. In the case of SERCA, we use high molecular weight PEGs which require extremely long equilibration times due to their relatively low diffusion coefficients (Carter et al., 1999). As previously mentioned, we found diffusion kinetics to be insufficient for SERCA nucleation when using setups employing a physical buffer between the precipitant and protein solutions and consequently focused on setups with a free liquid-liquid interface. This is an important issue one should consider when deciding upon the experimental setup best suited for one's particular system.

# 4. Crystallization of SERCA by microdialysis and capillary counterdiffusion

## 4.1 Equipment

**1.** Analytical balance (VWR)
**2.** TLA 100.3 rotor (Beckman Coulter)
**3.** Optima MAX-XP Ultracentrifuge (Beckman Coulter)
**4.** UV/visible spectrophotometer (ThermoFischer Scientific)
**5.** 12–14 kDa dialysis membrane discs (Hampton Research)
**6.** 30 µL dialysis kit including buttons, O-rings, and applicator (Hampton Research)
**7.** 24 well, VDX crystallization plate without sealant (Hampton Research)
**8.** High vacuum grease (Dow Corning)
**9.** 22 mm siliconized glass coverslips (Hampton Research)
**10.** 0.5 mm and 1 mm glass or quartz capillaries (Hampton Research)
**11.** 1 mL luer syringe (Chirana)
**12.** Parafilm
**13.** 1.5 mL microtubes (Sarstedt)
**14.** 250 mL Schott bottle
**15.** 250 mL beaker
**16.** Mounting clay (Hampton Research)

## 4.2 Chemicals

**1.** BME (β-Mercaptoethanol; Sigma Aldrich)
**2.** $C_{12}E_8$ (octaethyleneglycol dodecylether; Nikko Chemicals)
**3.** $CaCl_2$ (Sigma Aldrich)
**4.** KCl (Sigma Aldrich)
**5.** AMPPCP (β,γ-Methyleneadenosine 50-triphosphate disodium salt; Sigma Aldrich)
**6.** SR vesicles (sarcoplasmic reticulum vesicles from rabbit fast twitch skeletal muscle; prepared as per Andersen, Lassen and Moller (1985)
**7.** Glycerol (Sigma Aldrich)
**8.** $MgCl_2$ (Sigma Aldrich)
**9.** MOPS (3-(N-Morpholino)propanesulfonic acid; Sigma Aldrich)
**10.** PEG 6000 (polyethylene glycol 6000; Sigma Aldrich)
**11.** Sodium acetate (Fluka BioChemika)
**12.** tert-butanol (Sigma Aldrich)
**13.** Low-melting point (LMP) agarose powder (Invitrogen)

## 4.3 Protocol

### *4.3.1 Preparation of solubilized SERCA Protein ($Ca_2E1$-AMPPCP form)*

**1.1.** Prepare the detergent and buffer solutions. Calculate the volumes of stock needed to prepare a 370 mM $C_{12}E_8$ detergent solution and a 100 mM MOPS pH 6.8, 80 mM KCl buffer.
**1.2.** Working on ice, add 1 mL of the 100 mM MOPS pH 6.8, 80 mM KCl buffer to the SR vesicles vial (containing 4.7 mg of protein) and suspend the vesicles by gently pipetting up and down. Transfer the mixture into a 2 mL polyallomer ultracentrifuge tube.
**1.3.** Wash the vial with another 1 mL of the 100 mM MOPS pH 6.8, 80 mM KCl buffer and add the wash to the ultracentrifuge tube (for a total volume of 2 mL).
**1.4.** Balance the ultracentrifuge tube before placing them inside a TLA100.3 rotor. Place the rotor inside a Optima Max Ultracentrifuge and spin at 50000 RPM, 4 °C for 35 min.
**1.5.** While the suspended SR vesicles are spinning, prepare the following 2X incubation mixture in a 1.5 mL microtube. Add the required amount of each stock to produce 350 µL of solution containing the following: 2 mM AMPPCP, 10 mM BME, 20 mM $CaCl_2$, 40% (*v/v*) glycerol, 160 mM KCl, 6 mM $MgCl_2$, and 200 mM MOPS pH 6.8. *Tip: An alternative SERCA state, the $Ca_2E1$-ADP:$AlF_4^-$ form, can be prepared by replacing the 2 mM AMPPCP with 0.67 mM $AlCl_3$, 10 mM NaF, and 2 mM ADP.*
**1.6.** Recover the ultracentrifuge tube now containing a pellet of SERCA microsomes. Discard the supernatant, then add 110 µL of the 100 mM MOPS pH 6.8, 80 mM KCl buffer to the pellet and resuspend by gently pipetting up and down. *Tip: To reduce the chance of introducing bubbles, reduce the pipetting volume to ~70 µL. This will prevent air from being aspirated into the tip while drawing solution up and down over the SERCA pellet.*
**1.7.** Once the pellet has been fully resuspended, transfer 125 µL into the 2X incubation mixture along with 159 µL of $H_2O$.
**1.8.** To complete the incubation mix, add 66.2 µL of the 370 mM $C_{12}E_8$ solution (final concentration of 35 mM) and mix by lightly vortexing. The solution (final volume of 700 µL) should turn from translucent to clear.

**1.9.** Allow the mixture to incubate on ice for 1 h, then ultracentrifuge again at 50000 RPM, 4 °C for 35 min. Collect the supernatant and determine the protein concentration by measuring 280 nm absorbance with a spectrophotometer. This solution can be used directly for crystallization experiments.

#### *4.3.2 Crystallization of the SERCA $Ca_2$E1-AMPPCP form by Microdialysis*

**2.1.** Prepare the dialysis solution. Calculate the volumes of stock needed to prepare 1.5 mL of the following: 4% (*w/v*) PEG 6000, 18% (*v/v*) glycerol, 80 mM sodium acetate, 4% (*v/v*) *tert*-butanol, 100 mM MOPS pH 6.8, 80 mM KCl, 3 mM $MgCl_2$, 10 mM $CaCl_2$, 0.5 mM AMPPCP, 5 mM BME, and 15 mM $C_{12}E_8$.

**2.2.** Prepare a precipitant solution (to be mixed with the protein to form an unsaturated batch solution). Calculate the volumes of stock needed to prepare 1 mL of the following: 8% (*w/v*) PEG 6000, 8% (*v/v*) glycerol, 200 mM sodium acetate, 4% (*v/v*) *tert*-butanol, 100 mM MOPS pH 6.8, and 5 mM BME.

**2.3.** Mix the protein with the 8% PEG 6000 crystallization solution in a 5:1 ratio to produce an unsaturated batch. Make sure the volume prepared is in excess of the nominal button volume: e.g., aim to set aside ~35 µL of solution for a 30 µL microdialysis button to ensure a reasonable sized meniscus is able to be formed.

**2.4.** Half fill a 250 mL beaker with water and place a 12–14 kDa cut-off dialysis membrane inside to soak.

**2.5.** Take a microdialysis button and carefully dispense the unsaturated batch solution inside the protein chamber until a convex meniscus rises above the button surface. *Tip: As the protein solution contains a high concentration of detergent, take extra care when pipetting to avoid bubble formation. An easy way to do this is to aspirate a larger volume of solution then required and stop dispensing before the last of the liquid leaves the pipette tip. Although this method will not deliver an accurate volume of solution, this does not matter as a specific volume is not necessary so long as there is a clear meniscus rising above the button surface—minimizing bubble formation is more important.*

**2.6.** Remove the dialysis membrane from the beaker with tweezers. Using gloves, hold the membrane in both hands and carefully unfold and straighten. Remove any excess water by dabbing the membrane edge with a paper towel, then gently lower the membrane until it touches the top of the protein meniscus. The protein solution should "grab" the membrane. At this point slowly lower your fingers and allow the weight of the membrane to drape itself over the button top. Carefully release the membrane.

**2.7.** Take the applicator and place it over the top of the button. Holding the applicator in position with one hand, take an O-ring and push it down over the applicator and onto the button (making sure it locks into the groove running along the side of the button and securely holds the membrane in place).

**2.8.** Cut away the excess membrane around the sides of the button using scissors, then transfer the button into the well of a 24-well crystallization plate. Dispense the dialysis solution into the well, making sure the button is fully immersed in the liquid.

**2.9.** Seal the well using vacuum grease and a glass coverslip, then place the tray in a 20 °C environment to equilibrate.

**2.10.** To increase the size of any crystals that form, increase the PEG concentration of the reservoir (i.e., dialysis solution). Increasing PEG concentration by ~2% is a reasonable place to start. This can easily be achieved by simply removing the well seal, adding PEG stock directly, then gently mixing and replacing the seal. *Tip: The volume of PEG stock needed to be added to the reservoir to achieve the desired concentration increase can easily be calculated using the following formula:*

$$V_{PEG} = (V_R / (C_{i(PEG)} - C_{f(PEG)}))/(C_{f(PEG)} - C_{PEG})$$

*where $V_{PEG}$ = volume of PEG stock to be added, $V_R$ = current volume of reservoir, $C_{i(PEG)}$ = current concentration of PEG in reservoir, $C_{f(PEG)}$ = desired concentration of PEG in reservoir, and $C_{PEG}$ = concentration of PEG stock. This formula can of course be applied to any precipitant used to crystallize your particular protein.*

**2.11.** To harvest crystals from microdialysis buttons, first cut and remove the membrane covering the protein chamber using a scalpel. Crystals can now be fished and flash-cooled in liquid nitrogen using a standard loop, however be aware that the high walls of the protein chamber restrict access somewhat. Alternatively, crystals can be mounted within a capillary for room temperature data collection. A simple and effective method, described in detail by Mac Sweeney and D'Arcy (2003), is to place clay around the base of the magnetic cap holding the loop, then inserting the loop containing the crystal into a capillary containing some reservoir solution (a seal is formed as the capillary is pressed into the clay).

#### *4.3.3 Crystallization of the SERCA $Ca_2$E1-ADP:$AlF_4^-$ form by Capillary Counterdiffusion*

**3.1.** Prepare the precipitant solution. Calculate the volumes of stock needed to prepare 1 mL of the following: 12% (*v/v*) PEG 6000, 16% (*v/v*) glycerol, 200 mM Sodium Acetate, 6% (*v/v*) *tert*-butanol, 100 mM MOPS pH 6.8, and 10 mM BME.

**3.2.** Take a 0.5 mm glass or quartz capillary and open it just above the narrow sealed end (opposite of the bulb end) by scoring with a glass cutting stone and then snapping. Solutions are now able to be aspirated into the capillary.

**3.3.** Dispense 5 µL of protein onto a siliconized glass coverslip. Holding the capillary at the bulb end, aspirate the protein into the capillary by touching the drop with the opened narrow end (through capillary action).

**3.4.** Dispense 5 µL of precipitant solution onto a clean section of the coverslip. Carefully aspirate the drop as per *3.3*.

The precipitant solution should directly follow the protein solution, i.e., the two solutions touching with no air between them. Both solutions should now be contained within the capillary with a liquid-liquid interface existing between the two.

**3.5.** Seal the opened narrow end of the capillary by pressing into clay (this seal can be reinforced by applying silicone sealant on top of the clay to make an air-tight seal that is stable over time).

**3.6.** Seal the bulb end of the capillary with silicone sealant and place the capillary in a 20 °C environment to equilibrate (horizontal position, with the bulb end slightly raised to allow any air pockets that form to rise to the end of the protein solution away from the interface).

**3.7.** To prepare any grown crystals for data collection, first open the capillary at both ends (the silicone sealing the bulb end may be cut away with a scalpel and other end may be cut through). Immobilize the opened capillary and use another capillary of narrower diameter (e.g., 0.1 mm) to remove the material surrounding the crystal to be isolated (keeping some solution on either side to maintain a stable environment for the crystal). The capillary can then be resealed at both ends with clay and mounted directly onto a goniometer for X-ray/neutron analysis.

### *4.3.4 Alternative method for crystallizing SERCA $Ca_2E1$-ADP:$AlF_4^-$ form by Capillary Counterdiffusion (with gelled batch solution)*

**4.1.** Prepare the precipitant solution. Calculate the volumes of stock needed to prepare 1.5 mL of the following: 18% (*w/v*) PEG 6000, 15% (*v/v*) glycerol, 100 mM sodium acetate, 4% (*v/v*) *tert*-butanol, 100 mM MOPS pH 6.8, 40 mM KCl, 1.5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.5 mM ADP, 0.167 mM $AlCl_3$, 2.5 mM NaF, and 7.5 mM BME.

**4.2.** Prepare another precipitant solution (to be mixed with the protein and molten agarose to form an unsaturated gelled batch). Calculate the volumes of stock needed to prepare 1 mL of the following: 5.7% (*w/v*) PEG 6000, 14.3% (*v/v*) glycerol, 286 mM sodium acetate, 11.4% (*v/v*) *tert*-butanol, 100 mM MOPS pH 6.8, and 10 mM BME.

**4.3.** Prepare a custom syringe to dispense the solutions inside the crystallization capillary. First cut a small rectangular section of parafilm (measuring approx. 10 x 50 mm) and pre-stretch it to about twice its initial length. Attach one end of the parafilm to the end of a 1 mL syringe and wrap it 1–2 times around the syringe end (leaving the remaining length of the parafilm hanging). Take a 0.5 mm capillary and cut off the narrow sealed end (removing as little length as possible). Hold the bulb of the capillary against the syringe end with one hand. Use your free hand to carefully wrap the loose end of the parafilm around the capillary bulb while applying constant tension to attach it to the syringe. To make sure the seal is airtight, pre-stretch and wrap another piece of parafilm around the joint between the capillary bulb and the syringe end.

**4.4.** Prepare a 1% (*w/v*) solution of low-melting point agarose by adding the agarose powder to a buffer of 100 mM MOPS pH 6.8, 80 mM KCl in a Schott bottle. Dissolve the agarose by repeatedly heating the solution in a microwave, stirring every 30 s until the liquid is completely clear. Allow the solution to start cooling down.

**4.5.** Take another capillary (of 1 mm diameter) and lay it flat on the bench next to a ruler. Using a permanent marker, make a mark 15 mm from the narrow sealed end of the capillary. From this point make another two marks along the capillary shaft, at distances of 20 and 40 mm respectively. These mark the sections where the protein and precipitant solutions will be dispensed. Immobilize the capillary in a horizontal position, with the open bulb end slightly raised, using a portion of modelling clay.

**4.6.** Mix 100 µL of the protein with 70 µL of the 5.7% PEG 6000 precipitant solution in a 1.5 mL microtube to create an undersaturated batch, then place inside a 30 °C incubator. Also transfer the custom syringe, a 200 µL pipette tip, and 30 µL molten agarose (within its own pre-warmed microtube) into the incubator.

**4.7.** Once thermally equilibrated, remove the batch solution, the molten agarose, and the pipette tip from the incubator. Using the warmed pipette tip, add the batch solution to the molten agarose and mix thoroughly by gently pipetting up and down. Remove the custom syringe from the incubator and aspirate the batch-agarose mixture. Quickly but carefully, insert the custom syringe inside the 1 mm crystallization capillary and dispense the mixture between the two marks nearest the sealed end.

**4.8.** Allow the batch-agarose mixture to set, then use the cleaned custom pipette to carefully dispense the 18% PEG 6000 precipitant solution inside the crystallization capillary, from the gelled batch to the mark farthest away from the sealed ended. Make sure that there is no air present at the interface between the gelled batch and the dispensed precipitant solution.

**4.9.** Seal the open end of the capillary with silicone and place the capillary in a 20 °C environment to incubate (in a horizontal position).

**4.10.** To prepare any grown crystals for data collection, first open the capillary at both ends (the silicone sealing the bulb end may be cut away with a scalpel and other end may be cut through). Immobilize the opened capillary and use a custom pipette of the variety previously mentioned to remove the material surrounding the crystal to be isolated (keeping some solution on either side to maintain a stable environment for the crystal). The capillary can then be resealed at both ends with clay and mounted directly onto a goniometer for X-ray/neutron analysis.

## 5. Summary

Growing large membrane protein crystals is challenging, but here we demonstrate that it is possible in the case of SERCA. Working toward producing crystals of membrane proteins for NMX, it is essential that we learn from past experience and how the first membrane protein structures were solved using MX. The early targets were available in sufficient amounts and biochemically well-described. This highlights the importance of starting with well-characterized targets to be able to take a systematic reproducible crystallographic approach. Crystallographic success is most often built on a foundation of biochemical insight, and for SERCA we have benefitted from decades of biochemical characterization, which, combined with a good deal of crystallographic understanding, has enabled a successful outcome. We suggest that a similar approach is considered for other membrane protein projects pursuing large crystals for NMX.

There are many approaches to solubilizing and purifying membrane protein for crystallization. The crystals of SERCA described here have been solubilized directly from the native membrane and hence crystallized in the presence of high concentrations of lipid and detergent (HILIDE) (Gourdon et al., 2011). The technique has been described as mimicking solubilized membranes and appears to be able to "re-form" repetitive bilayers in the crystal when stimulated by crystallization conditions. This approach has worked in numerous cases and here we have demonstrated that it also is compatible with microdialysis and capillary crystallization techniques.

Other approaches are being explored and we are working to test the applicability of other techniques for membrane protein crystallization. There are aspects of crystallization space we do not explore in any great detail during standard crystallization. For example, the effect of temperature on crystallization is explored rather coarsely by placing duplicate experiments at 4 °C and 20 °C. Recent studies (Junius et al., 2016) have shown that continuous monitoring and adjustment of temperature enables careful control of nucleation and crystal growth. Going forward, it will be attractive to further explore continuous monitoring of parameters in crystallization space to extend stable growth from MX- to NMX-sized crystals.

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