

## Vapor Diffusion-Controlled *Meso* Crystallization of Membrane Proteins

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### Abstract

The presented method to crystallize membrane proteins combines the advantages of the meso-phase crystallization method and the classical vapor diffusion crystallization. It allows fast screening of crystallization conditions employing automated liquid handlers suited for the 96-well crystallization format.

**Key words:** Membrane protein crystallization, In meso crystallization, Lipidic cubic phase, Meso phase, Vapor diffusion, Monoolein

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### 1. Introduction

The fact that there is only a small number of membrane protein structures known can be directly traced back to the common problems in obtaining membrane protein crystals for structural investigations. Methods developed for soluble protein crystallization are inefficient for membrane proteins. Landau and Rosenbusch (1) used lipidic meso-phases to accommodate the specific needs of membrane proteins in a way compatible with crystallization: the lipidic component monoolein is an amphiphile and therefore self-organizes in water into complex structures, the meso-phases (2) (Fig. 1). The cubic phase Pn3m consists of a bi-continuous bilayer, that separates two channel systems of aqueous phase (depicted as circles in picture of Pn3m phase in Fig. 1). The bilayer locally appears two dimensional similar to a cell membrane and therefore allows the incorporation of membrane proteins. However, it extends continuously through space and therefore supports diffusion of the protein in three dimensions and crystallization upon dehydration.

Dehydration of the meso-phase can be achieved by lowering the level of humidity. In vapor diffusion experiments this is

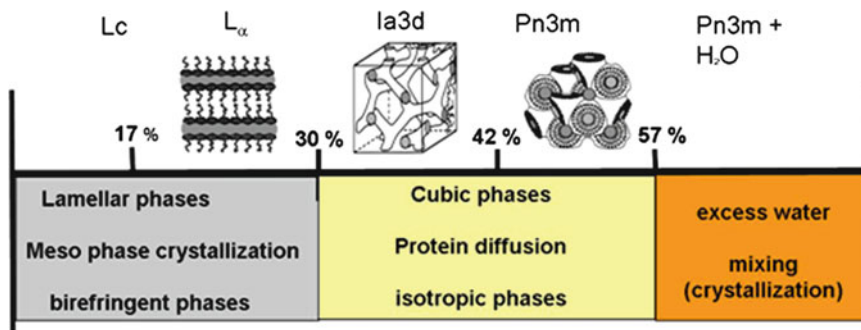


Fig. 1. The monoolein–water isotherm at 22°C (2). With increasing water content the layered birefringent lamellar phases (Lc, L<sub>α</sub>), the optically isotropic cubic phases (Ia3d, Pn3m), and the swollen cubic phase in the presence of excess water (sponge) phase are formed reversibly. The added amount of water determines the maximal hydration level. Upon dehydration by vapor diffusion the maximally hydrated system reverts back to the final meso phase which is determined by the dilution of the added screening solution (Fig. 2).

experimentally realized by enclosing the wetted monoolein together with a reservoir solution that takes up water from the gas phase that separates the two condensed phases (3).

In contrast to (1) in meso crystallization in batch (1), no weighing of mg amounts of monoolein or dehydrating salt is required. Furthermore, in contrast to (2) sponge phase crystallization (4), we are not limited to a small number of screening solutions. Also, in contrast to (3) the active mixing approach (5), we find the protein–monoolein ratio in the crystallization experiment not to be limited by the solubility of the protein in detergent solution when targeting a certain meso-phase because excess water can be removed via vapor diffusion. Finally, the vapor diffusion-controlled *in meso* crystallization experiment can be set up with the same equipment used for the crystallization of soluble proteins.

Similar to the crystallization of soluble proteins the likelihood of crystallization will depend on the presence of a crystallizable species, which may be controlled by the addition of specific lipids to the protein solution or other stabilizing compounds to the diluted screening solution (6).

In the typical setup, 132 µg solid monoolein is hydrated with 900 nl aqueous solution in total: (1) the membrane protein in 450 nl protein solution will dissolve in the meso-phase matrix that acts as a solvent. This matrix will also separate excess detergent from the protein. (2) The content of undiluted screening solution in the added 450 nl of diluted screening solution will determine the final dehydration level that can be reached upon equilibration against the undiluted reservoir and thereby the final meso-phase, as well as the protein concentration in the aqueous phase above the monoolein (Fig. 2).

Crystals may be obtained with excess water conditions, in the cubic phase or in the presence of a lamellar phase (Fig. 3).

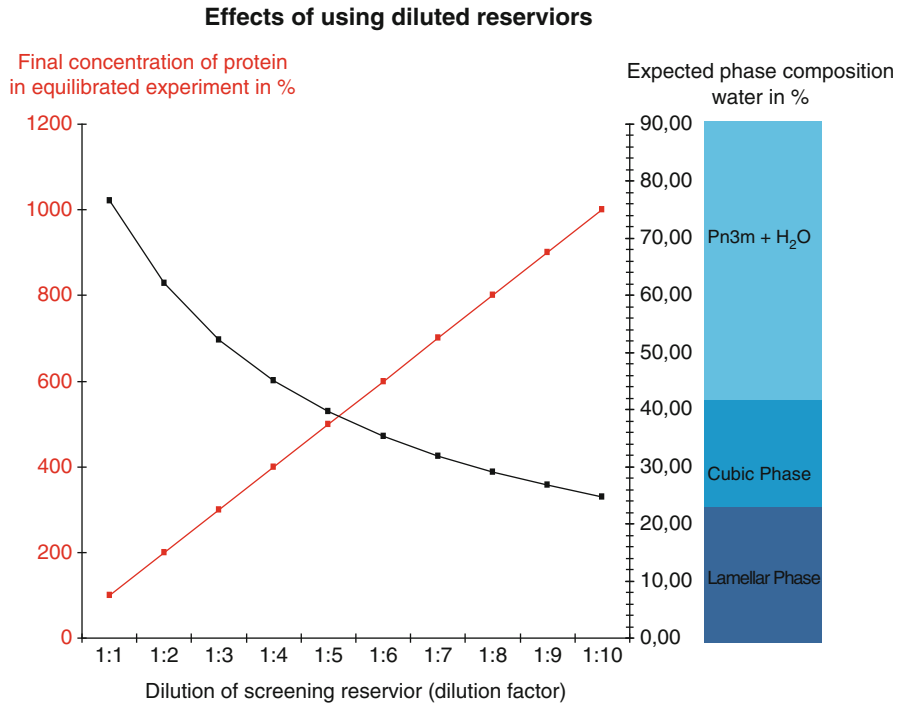


Fig. 2. Targeting final hydration levels and meso-phases using dilutions. *Red curve*: Expected final protein concentration (stock solution 100%) depending on dilution of screening solution for a vapor diffusion experiment without transfer of protein or water from aqueous solution into monoolein, if a mixture of equal volumes of protein solution and (diluted) screening solution is equilibrated against undiluted screening solution. *Black curve*: Expected final hydration level of meso-phase depending on dilution of screening solution, if the final water volume of the ideal vapor diffusion experiment (*red curve*) is available for meso-phase formation. *Right*: Targeted type of meso-phase as a function of expected hydration level (*black curve*) based on the phase isotherm of the monoolein–water system at 22°C.

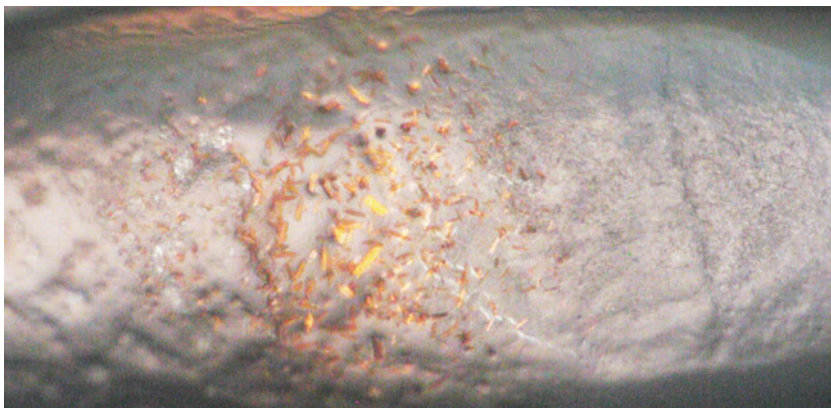


Fig. 3. Crystallization of a naturally colored rhodopsin. The largest yellow protein crystals appear in the *center* where the protein droplet was set. At the right side of the experiment birefringent speckles of lamellar phase can be detected in the overall isotropic cubic phase.

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## 2. Materials

1. Monoolein pre-coated crystallization plates (see Note 3b)  
Either 132  $\mu\text{g}$  monoolein prefilled evolution microplate (see Note 14) or 29  $\mu\text{g}$  prefilled crystallization plate in MRC or Intelli format (Qiagen GmbH, Germany).
2. Screening solutions (see Note 2b).  
Cubic Phase I and Cubic Phase II (Qiagen GmbH, Germany) or other commercially available screening solutions in Deep Well Blocks.
3. Plate sealing.  
AMP Liseal, Transparent Microplate Sealer (Greiner bio-one, Germany).
4. Anti-evaporation layer.  
Perfluoropolyether (PFO-X175/08, Hampton Research, USA).
5. Other  
Deep Well Blocks.
6. Instruments and equipment
  - (a) Polarization microscope.
  - (b) Incubator (22–24°C).
  - (c) Fluorescence microscope (optional).
  - (d) Automatic liquid handler (optional).

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## 3. Methods

- (a) Sample preparation
  1. Prepare a membrane protein solution with a concentration of 2–35 mg/ml of known detergent content (see Note 1).
  2. Prepare the 96 screening solutions that are to be used for the crystallization plate in a 96-well Deep Well Block or use a block of premade solutions. Prepare the required (50–100  $\mu\text{l}$  each) dilution of the screening solutions in a second block (see Note 2).
- (b) Setup of the crystallization experiment
  1. The ready-to-use microplate, pre-coated with 132  $\mu\text{g}$  (29  $\mu\text{g}$ ) monoolein, is unfrozen for 10 min at 22°C (see Note 3).
  2. To the experimental wells with monoolein 450 nl (100 nl) of protein solution is added (see Note 4).

3. The plate is sealed and incubated for 3 h at 22°C (see Note 5).
  4. Fill the reservoir wells with 75  $\mu$ l of undiluted screening solution (see Note 6).
  5. Add 450 nl (100 nl) of diluted screening solution to the experimental well (see Note 7).
  6. Reseal the plate and incubate at 22°C (see Note 8).
- (c) Monitoring progress
1. Monitor the formation of cubic meso-phase initially every day until the meso-phase becomes optically isotropic when viewed with a polarization microscope (see Note 9).
  2. Monitor for dehydration as indicated by loss of optical isotropy when viewed with a polarization microscope and for protein crystal formation twice a month for 3–4 months (see Note 10).
- (d) Harvesting crystals
1. Add 0.5–2  $\mu$ l of screening solution or a water-immiscible liquid (e.g., perfluoropolyether) to the experiment to minimize evaporation (see Note 11).
  2. Remove monoolein from the crystal with a mounting loop without actually touching the crystal (see Note 12).
  3. Transfer the crystal with a mounting loop into suitable cryoprotectant and freeze the crystal without monoolein (see Note 13).

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## 4. Notes

1. (a) Though protein purity appears to be a less important factor in the case of meso-phase crystallization when compared to crystallization of soluble proteins (7), detergent concentration is an additional search parameter: if the protein is concentrated by ultrafiltration it should *not be assumed* that detergent micelles pass through the membrane into the filtrate. If the detergent content of the protein sample after concentration is high, the compatibility of a protein-free detergent solution with meso-phase formation should be tested to avoid waste of protein. Generally the concentration of detergent should be controlled, e.g., by thin-layer chromatography to insure the reproducibility of crystallization experiments (8).
- (b) The presence of glycerol will reduce dehydration and lengthen the equilibration time.

- (c) Organism-specific lipids may be added after concentrating the protein.
- 2. (a) For initial experiments dilutions of 1:1 (undiluted), 1:4, and 1:7 are reasonable starting points to target different meso-phases.
- (b) Commercially available screening solutions for membrane proteins are a good starting point.
- (c) Often a screening for an additive may be required to succeed in crystallization. Therefore a salt and a PEG screen are recommended to us as dehydrating screens and mix these (90%, v/v) with other screens (10%, v/v) (e.g., anion screen, cation screen, etc.) when searching for additives. The addition of a strongly binding ligand that stabilizes a certain protein conformation is recommended in any case.
- 3. (a) Pre-coated plates should be stored at  $-20^{\circ}\text{C}$  to avoid oxidation of monoolein.
- (b) Only the evolution plate prefilled with 132  $\mu\text{g}$  monoolein (see Subheading 2) is suitable for manual dispensing of solutions (see Note 14).
- 4. (a) If the protein concentration is very low ( $<2\text{ mg/ml}$ ) or a high ratio of protein/monoolein is targeted, the dispensed volume of protein solution can be increased.
- (b) If the protein is dispensed by an automated liquid handler the actual release of the protein droplets should be monitored. Problems with droplet release can be solved by (1) changed ejection speed and (2) change of detergent or salt content of protein solution.
- 5. This incubation step can be omitted for experiments with 29  $\mu\text{g}$  monoolein (see Note 14).
- 6. If it is desired to slow the kinetics of dehydration, some  $\mu\text{l}$  of a low density, low vapor pressure, with water-immiscible liquid can be added on top of the screening solution in the reservoir well (9).
- 7. If a different final hydration level is required, another dilution of the screening solution can be used.
- 8. (a) If evaporation of reservoir solution or salt crystal formation is observed the plate was not properly sealed.
- (b) Incubation temperature should be between 22 and  $24^{\circ}\text{C}$ . During incubation any variation of temperature should be avoided.
- 9. (a) The time for the complete transformation of solid monoolein into cubic phase varies between 20 min and 3 days, depending on the composition of the aqueous phase.



Fig. 4. Effect of screening solution on meso-phase formation upon hydration of solid monoolein. *Left*: Arrest of phase transformation at strongly polarizing lamellar phase due to incompatible screening solution. *Right*: Complete transformation into isotropic cubic phase with cubic phase salt screen I.

- (b) If no formation of optically isotropic cubic phase is observed (Fig. 4), the protein or the screening solution contains components or concentration of components incompatible with meso-phase crystallization: check compatibility of detergent concentration and/or concentration of screening solution without protein to establish a proper concentration range for your new screen or detergent.
- 10. (a) If an increased volume of protein solution has been used (see Note 4a) crystallization of optically isotropic crystals of monoolein may be observed.
- (b) Formation of lamellar phase from cubic phase upon dehydration occurs typically in form of birefringent speckles or whiskers (Fig. 3).
- (c) Discrimination between very small birefringent protein crystals and local transformation of isotropic cubic phase into birefringent lamellar phase may be difficult using a polarization microscope. Monoolein phases and protein phases can be distinguished by tryptophan-fluorescence (10).
- 11. If complete removal of monoolein from the protein crystal is desired, better results will be obtained with the anti-evaporation layer, e.g., of PFO (11).



12. (a) Meso-phase turns opaque on freezing. Typically protein crystals frozen with large excess of monoolein become undetectable.
- (b) In the case crystals stick to the loop when removing the meso-phase, it will be necessary to have a second loop at hand.
13. Meso-phases frozen at 100 K show pulver diffraction rings upon data collection which may be treated like ice rings.
14. The addition of 450 nl protein solution to 132  $\mu$ g monoolein and subsequent incubation generate a protein gradient in the meso-phase. Under this condition all protein concentrations down to almost zero are screened in the same experiment at the same time (Fig. 3).

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