PROTEIN CRYSTALLIZATION

1. Protein Crystallization Methods

1.1 Typical Vapor Diffusion

Vapor diffusion is the method most frequently used to crystallize proteins. As the name indicates, it makes use of the diffusion of vapor – water and other volatile components – present in a closed system. In this method, the crystallization solution containing the precipitating agent is placed in a reservoir and left to equilibrate with a drop of protein + crystallization solution. The difference in precipitant concentration between the drop and the reservoir drives the system towards drop evaporation and, consequently, concentration. In optimum conditions, the protein enters a supersaturated state close to the point of equilibrium and crystals start to form. The most common vapor diffusion techniques are sitting and hanging drop (Figure 1):

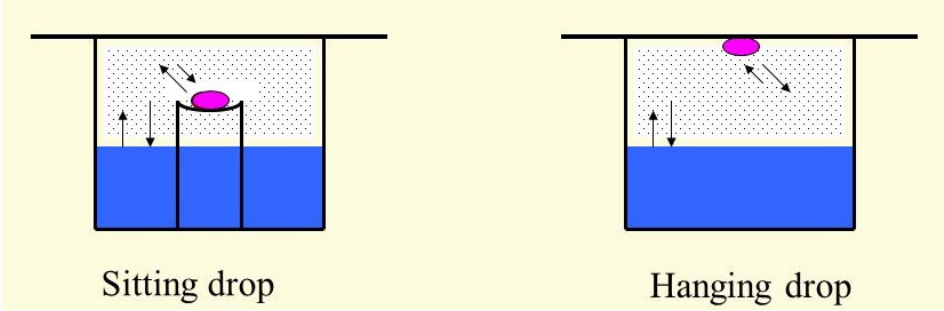


Figure 1 - The Sitting and hanging drop techniques of the vapor diffusion method for protein crystallization.

The vapor diffusion principle applies both in sitting drop and in hanging drop, and the difference is in the experimental setup which can affect the equilibrium. Sitting drops can be used for larger volumes and will equilibrate at a slower rate than hanging drops, in an equivalent experiment.

1.2 Other Crystallization Methods

Besides vapor diffusion, several methods can also be used:

Microbatch - the protein solution and the precipitant are mixed and supersaturation is achieved due to lower solubility of the protein in the presence of the precipitant rather than by vapor diffusion. The drop is dispensed under oil, to prevent evaporation (Figure 2).

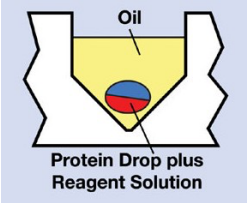


Figure 2 – Microbatch protein crystallization method.

Microdialysis - the protein is placed inside a dialisis button which is then covered with a dialysis membrane and placed in a reservoir containing the crystallization condition. The membrane allows controlling the ionic strength of the protein solution (not possible in vapor diffusion) since small molecular weight substances can diffuse in while preventing the protein from diffusing out (Figure 3). The exchanged carried out through the membrane will promote to the supersaturation state and the formation of crystals

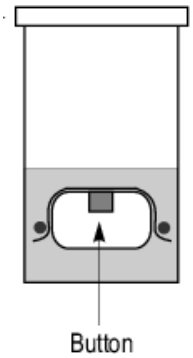


Figure 3 – Microdyalisis method for protein crystallization

1.3 Phase diagram

Crystallization phase diagrams are frequently used to understand the processes taking place during the crystallization of macromolecules. Normally a phase diagram is commonly obtained after finding a hit during the initial screens; several (10-30) drops are prepared varying the protein and precipitant concentrations in small steps. These trials allow drawing the solubility curve which separates the nucleation region where small nuclei form, from the metastable region which is ideal for crystal growth. Crystallization methods follow different paths on the phase diagram (Figure 4).

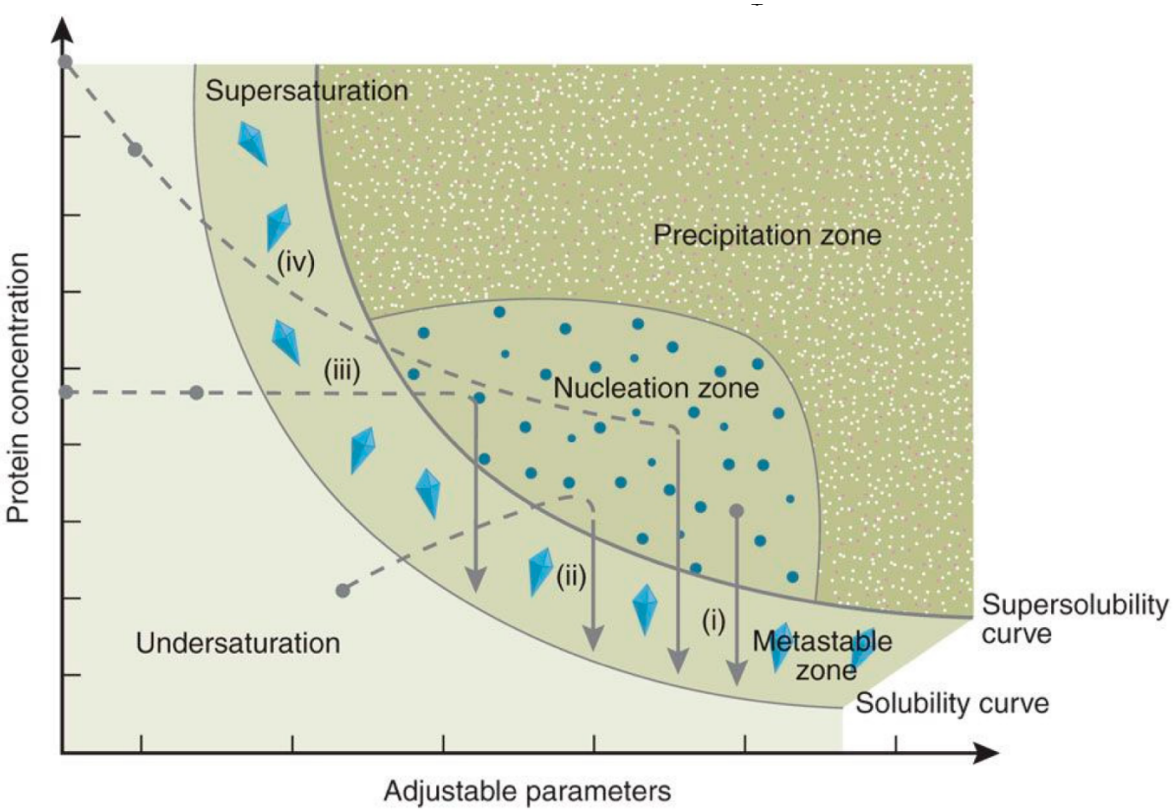


Figure 4. Phase Diagram illustrating the different routes of attaining supersaturation. (i) Micro-batch trials where instant supersaturation is reached. (ii) Vapour diffusion (iii) Dialysis (iv) free interface diffusion trials. The super-solubility curve separates the conditions under which spontaneous nucleation occurs and the metastable zone, ideal for crystals growth [1].

2. Experiment

Materials & reagents

- Protein solution (50 mg/ml of Hen Egg-White Lysozyme in 0,1M Sodium Acetate Buffer pH 4.5)
- Crystallization stock reagents: 10%NaCl, 1M Sodium Acetate buffer pH 4.5, ultrapure water
- Automatic micropipettes
- 24-well crystallization plates
- Cover slips (regular and silanized)
- Crystallization plastic bridges
- Graduated tubes
- Crystallization score sheets
- Dialysis button and membrane
- Syringe with silicone paste
- Mineral oil

2.1. Exercise 1 – Crystallization of Lysozyme through vapor diffusion

Hanging drops:

1. Identify the 24-well crystallization plate with the group number and date;

2. Gently spread silicone paste on the edge of 4 reservoirs of the crystallization plate;

3. Prepare the following precipitant solutions using the stock reagents (final volume = 2000 µl) and pipette 700 µl of each into a reservoir of the crystallization plate:

	H_2O (Ultrapure)	10% NaCl (Final [NaCl])	1M Acetate Buffer (Final [acetate buffer])
1	1400 µl	400 µl (2%)	200 µl (0,1M)
2	1000 µl	800 µl (4%)	200 µl (0,1M)
3	600 µl	1200 µl (6%)	200 µl (0,1M)
4	200 µl	1600 µl (8%)	200 µl (0,1M)

4. Pipette 2 µl of Lysozyme solution (50mg/ml) and 2 µl of precipitant solution from the reservoir into a silanized cover slip. Pipette-mix the two solutions, avoiding air bubbles. Turn the cover slip and seal the reservoir;

5. Repeat the process for all the prepared precipitant solutions.

Sitting drops:

1. Place the small plastic bridges inside 4 different reservoirs;

2. Spread silicone paste on the edge of those reservoirs;

3. Pipette 700 µl of each of the previously prepared precipitant solutions into a reservoir of the crystallization plate;

4. Repeat the procedure done for the hanging drops (steps 4-5) but this time pipette the protein and the precipitant solutions on top of the bridge inside the reservoir. Seal the reservoir with a regular cover slip.

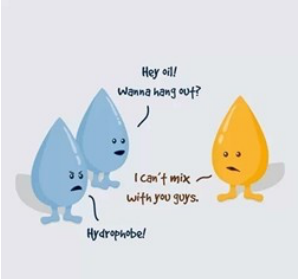
2.2. Exercise 2 – Crystallization of Lysozyme using other methods

Microbatch:

1. Pipette 500 µl of mineral oil into a reservoir of the crystallization plate;

2. Prepare a drop of 2 µl protein solution + 2 µl precipitant solution (choose one from the 4 different conditions prepared above);

3. Carefully pipette this drop into the mineral oil. Spread silicone paste on the edge of the reservoir and seal with a regular cover slip



Microdialysis:

1. Pipette 5 µl of protein solution into the dialysis button;
2. Close the button with a dialysis membrane using an O-ring to keep it in place;
3. Put the button in a reservoir of the crystallization plate and fill with the precipitant solution. Spread silicone on the edge of the reservoir and seal with a regular cover slip.

2.3. Exercise 3 – Drop Visualization

After setting up the crystallization drops (hanging, sitting, microbatch and microdialysis) the results need to be visualized under the microscope regularly:

1) Immediately after setup;

2) Each day in the first week;

3) Once a week for several weeks.

Here are some examples of what you can observe in your crystallization drops:

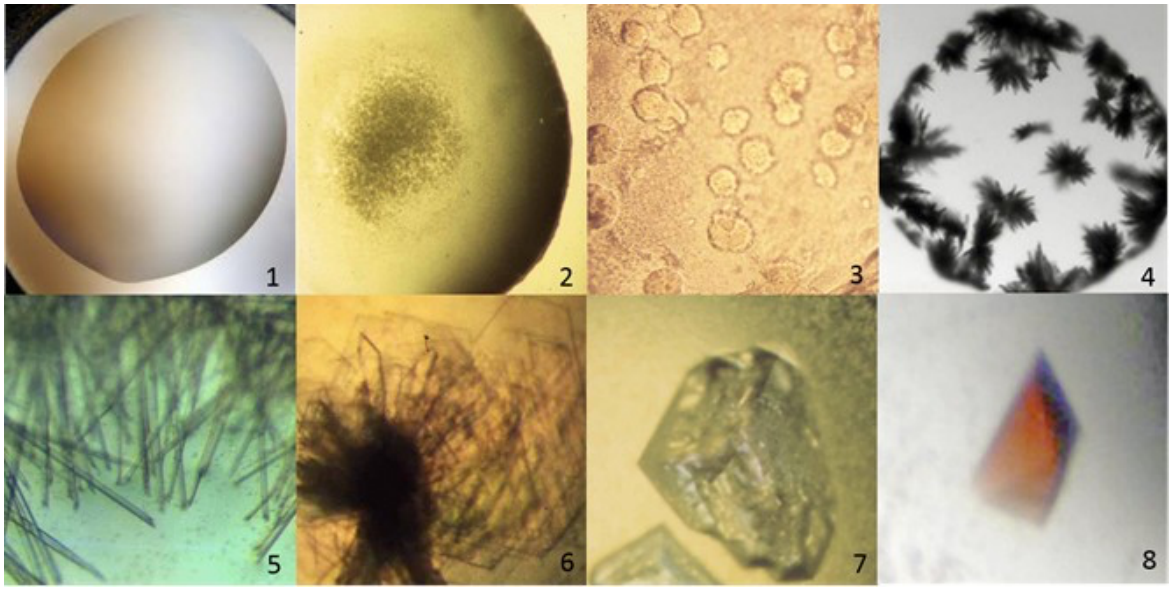


FIG1 – Crystallization drop phenomena and interpretation: (1) drop is clear; (2) amorphous light precipitate; (3) spherulites; (4) sea urchins; (5) needles; (6) plates; (7, 8) crystals.[3,4]

2.4. Exercise 4 – Cryo-preservation of protein crystals

1. Observe under the microscope lysozyme crystals previously prepared and made available by the professor.

2. Choose a single crystal suitable for the diffraction experiments and incubate it in a cryoprotectant solution.

3. Mount the crystal in a nylon loop, under cryogenic conditions:

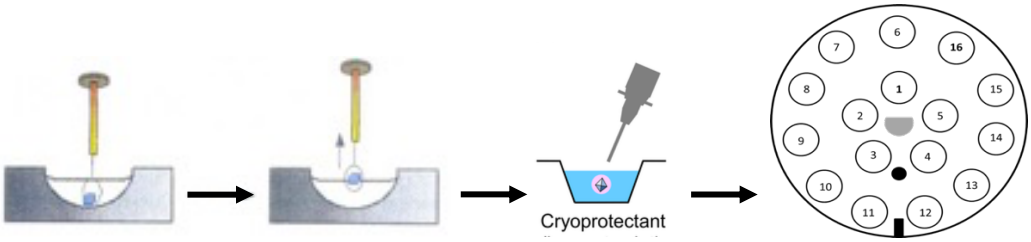


Figure 5 - Steps in mounting and cooling protein crystals

Figure 6 – Tools for cryocooling protein crystals

References

[1] Govada, Lata and Chayen E., Naomi (2019), ***Choosing the Method of Crystallization to Obtain Optimal Results***, Crystal, *9*(2), 106
[2] Terese M. Bergfors (1999). ***Protein Crystallization***. USA: IUL Biotechnology Series, 10
[3] Bernhard Rupp (2010). Biomolecular Crystallography: ***Principles, Practice, and Applications to Structural Biology***. USA: Garland Science, Taylor & Francis Group.