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):

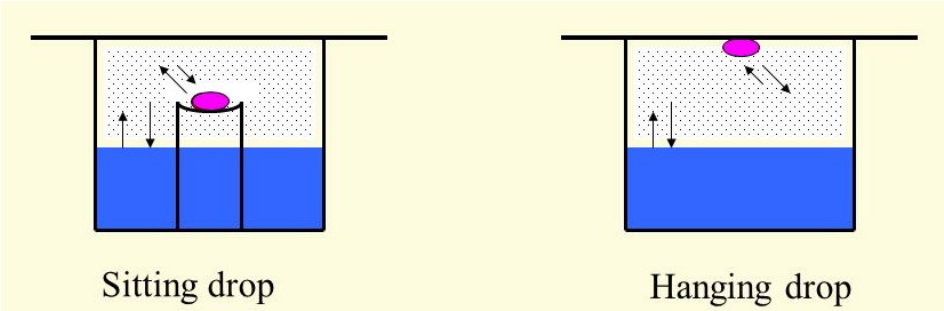

Figure 1 - The Sitting and hanging drop techniques of the vapor diffusion method for protein crystallization.

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).

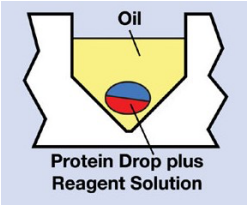

Figure 2 – Microbatch protein crystallization method.

Microdialysis - the protein is placed inside a dialysis 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

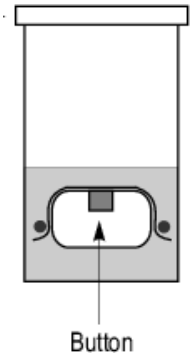

Figure 3 – Microdialysis method for protein crystallization

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).

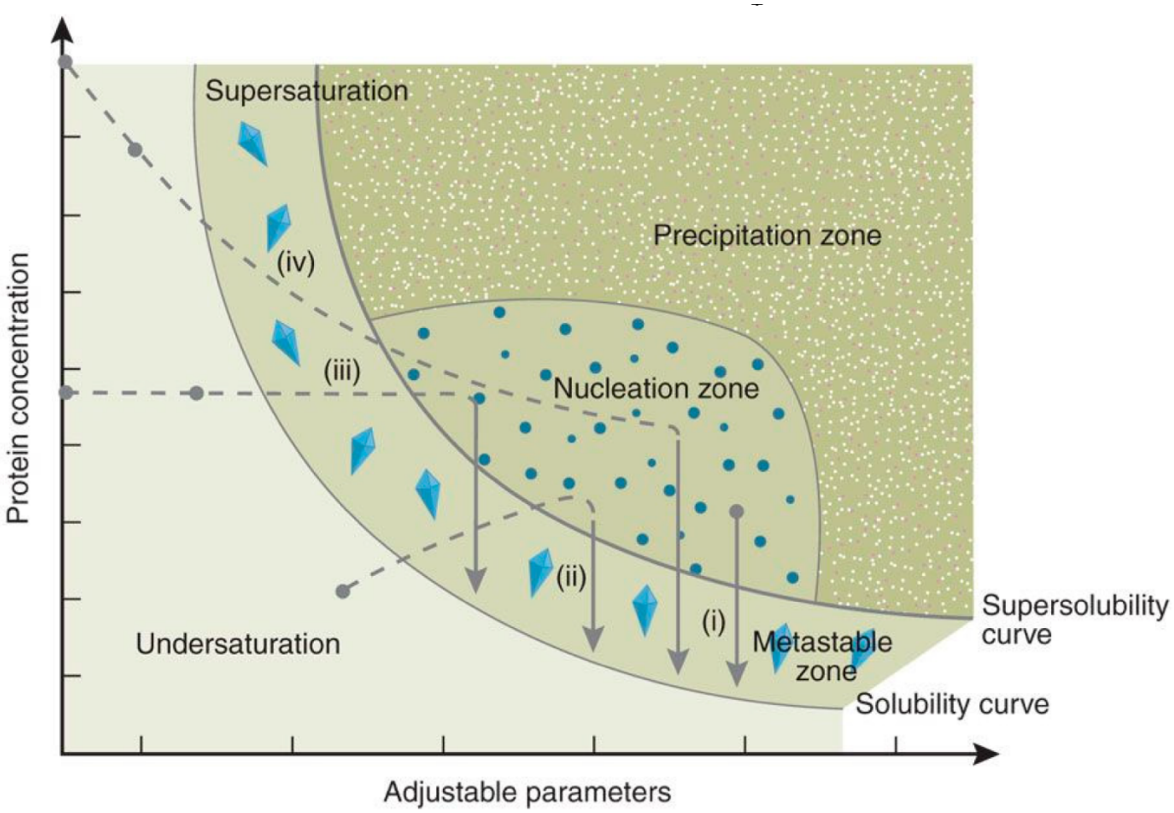

Figure 4. Phase Diagram illustrating the different routes of attaining supersaturation. (i) Micro-batch trials where instant supersaturation is reached. (ii) Vapour diffusion (iii) Dialysis (iv) free interface diffusion trials. The super-solubility curve separates the conditions under which spontaneous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

2. Crystallization of protein-ligand complexes

Structures of protein-ligand complexes are important for understanding the protein function and can be essential in guiding drug discovery projects. Ligands are usually well tolerated in crystal structures and can even be a requirement for the protein crystallization, purification and expression processes. There are several ways of generating crystals of protein complexes that can vary depending on the protein and ligand properties. The more used are co-expressing, co-purifying, co-crystallizing and co-soaking the ligands [2].

2.1 Co-Crystallization of protein-carbohydrate complexes

Protein-carbohydrate complexes are typically obtained by co-crystallization. In this procedure, the protein is pre-incubated with the ligand for a few hours or overnight at 4ºC before crystallization experiments. The ligand:protein molar ratios will depend on the complex affinity/dissociation constants (1:1, 2:1, 4:1 and 10:1) This technique is preferred to co-soaking because usually, the protein binding sites in crystals are not accessible to the large carbohydrate ligands (oligosaccharides).

The carbohydrate ligands can have low solubility or be challenging to obtain. Due to these ligand restrictions, one usually tries to obtain the crystallization conditions for the protein alone before making crystallization trials for the protein-carbohydrate complex. The usual crystallization conditions set for the complex are related to the protein crystallization condition where the concentration of the precipitant or the drop ratios (2:1, 1:1 and 1:2 protein:well solution) can be varied. However, in some instances, the crystallization of the protein is only possible when complexed with the ligand or the crystallization condition of the complex is different from the crystallization condition of the protein. In these cases, a crystallization screening needs to be performed for the complex.

3. Experiment

Materials & reagents

- Protein solution (18mg/mL of the carbohydrate recognition domain (CRD, L114-I250) of human galectin 3 (hGal-3) in in 50 mM sodium phosphate buffer pH 6.8, 1 mM DTT, and 0.1 % NaN_3)
- Crystallization Precipitant Solution: 32 % (w/v) polyethylene glycol (PEG) 4000, 100 mM Tris-HCl (pH 7.5), 100 mM MgCl2 and 8 mM β-mercaptoethanol
- Automatic micropipettes
- 24-well crystallization plates
- Cover slips (regular and silanized)
- Crystallization plastic bridges
- Graduated tubes
- Crystallization score sheets
- Syringe with silicone paste

2.1. Crystallization of human Galectin-3 using the vapor diffusion method

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. Pipette 700 µl of the precipitant solution into a reservoir of the crystallization plate.

4. Pipette 2 µl of the hGal-3 solution (18mg/ml) and 2 µl of precipitant solution from the reservoir into a silanized cover slip, forming a single 4 µl drop. Turn the cover slip and seal the reservoir;

Sitting drops:

1. Place the small plastic bridges inside 1 reservoir.

2. Spread silicone paste on the edge of this reservoir.

3. Pipette 700 µl of the precipitant solution into the reservoir of the crystallization plate.

4. Pipette 2 µl of the protein and the precipitant solutions on top of the bridge inside the reservoir, forming again a single 4 µl drop. Seal the reservoir with a regular cover slip.

2.2 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:

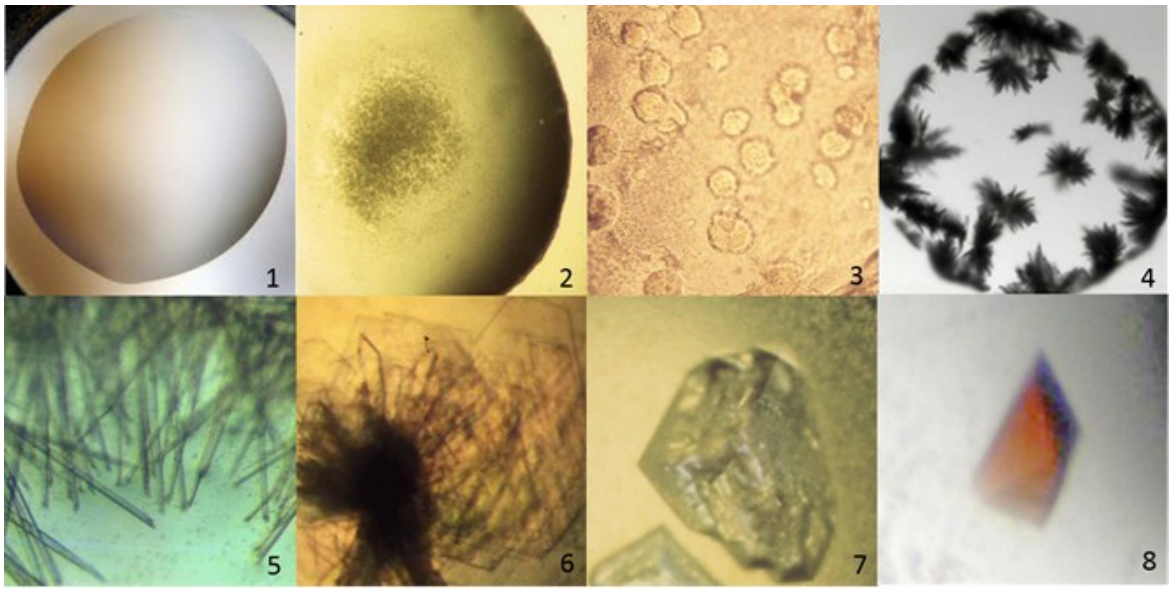

Figure 5 – Crystallization drop phenomena and interpretation: (1) drop is clear; (2) amorphous light precipitate; (3) spherulites; (4) sea urchins; (5) needles; (6) plates; (7, 8) crystals.[3,4]

2.3 Cryo-preservation of protein crystals

1. Observe under the microscope galectin-3 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:

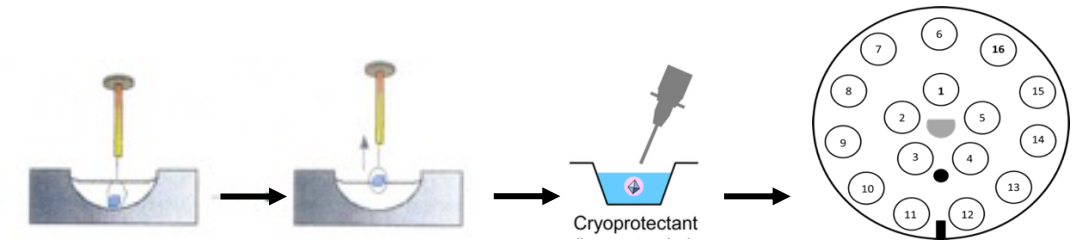

Figure 5 - Steps in mounting and cooling protein crystals

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] Ilka Muller (2016), Guidelines for the successful generation of protein–ligand complex crystals, Acta Cryst. D73, 79-92

[3] Bernhard Rupp (2010). Biomolecular Crystallography*:* ***Principles, Practice, and Applications to Structural Biology***. USA: Garland Science, Taylor & Francis Group.

[4] Terese M. Bergfors (1999). ***Protein Crystallization***. USA: IUL Biotechnology Series, 10

[5] C. D. L. Lima, H. Coelho, A. Gimeno, F. Trovão, A. Diniz, J. S. Dias, J. Jiménez-Barbero, F. Corzana, A. L. Carvalho, E. J. Cabrita, F. Marcelo, Chem. Eur. J. 2021, 27, 7951.

Supplementary Material

Strategies for successful crystallization of protein-glycan complexes