Introduction

HAMPTON RESEARCH
Solutions for Crystal Growth

Introduction to the Crystallization of Biological Macromolecules

The Crystal Growth 101 series prepared by Hampton Research presents an overview of the preparation of the sample, methods, screening, optimization, reagent formulation, and other aspects of protein crystallization. We hope Crystal Growth 101 will prove a useful resource and inspiration in your crystal quest. Best of success with your crystals!

The First Protein Crystal & Beyond

The first record of crystals of biological macromolecules were those of hemoglobin, reported by Hunefeld around 1840.[1,2] During the 1880s, crystallization moved from being a mere curiosity to a method for purification.[3,4] During the 1920s and 1930s crystallization grew in popularity, with the crystallization of insulin by John Jacob Abel and colleagues, as well as work by James B. Sumner, demonstrating enzymes could be obtained as crystalline proteins, alongside the work with a number of important crystalline enzymes by John Northrop and colleagues.[5-7] It was not until the late 1930s that crystalline proteins were introduced to X-rays, beginning a torrid affair that shines bright to this day.[8]

Much has changed with how biological macromolecules are sourced. Early on, and to a small extent today, samples were obtained by protein chemists through extraction and purification from natural sources, including plants, as well as various organs and tissues of pigs, cows, and other animals. In the 1980s, with the near cataclysmic death of heavy metal and the fortuitous end of disco, geneticists and molecular biologist rose above the fog and hair spray, allowing DNA technology to integrate with structural biology, totally accelerating and transforming the field of structural biology.

It's Simple, But Complicated

Although much change, and the numerous advancements in molecular biology and crystallography has reduced many of the arduous, laborious, math and physics infused tasks to, in some cases, the mere push of a button, crystallization remains at the crossroads where science meets art. The growth and optimization of crystals of biological macromolecules remains largely empirical in nature. There is no comprehensive theory to guide efforts and experiments related to the crystallization of proteins, nucleic acids, and other biological macromolecules. Although much knowledge and experience has been accumulated, the crystallization of a protein involves collected wisdom, intuition, creativity, patience, and perseverance.

Crystallization of biological macromolecules composed of many thousands of different atoms, bound together with many degrees of freedom, is a complex task. Confounding this many variables and factors influencing the crystallization experiment (Tables 1 & 2).[9-11]

This extensive number of variables confounded with typically limited sample material negates a precise and reasoned strategy typically applied to a scientific problem. Instead, crystallization is often a matter of searching, as systematically as possible, through crystallization experiments, to identify those variables key to success, as well as their ranges. Initially, one employs crystallization screening, typically to identify a hit, an association of variables that produces a crystal. In some instances this will produce crystals with the desired characteristics. More often than not, a series of successive experiments, termed optimization, will need to be carried out, in order to produce crystals with the desired properties, be it for structural biology, purification, formulation, or the delivery of a biological therapeutic.

Table 1.
Biochemical & Chemical variables that could or do affect protein crystal growth

Purity of the sample	Genetic modifications
Conformational flexibility of the sample	Symmetry of the molecule
Homogeneity of the sample	Stability and level of denaturation of the sample
pH and buffer	Isoelectric point
Type and concentration of the precipitant (reagent)	His tags and other purification tags – presence or absence
Concentration of the sample	Thermal stability
Purity of the sample	pH stability
Additives, co-factors, ligands, inhibitors, effectors, and excipients	History of the sample
Chaotropes	Proteolysis
Detergents	Microbial contamination
Metals	Storage of the sample
Ionic strength	Handling of the sample and associate cleanliness
Reducing or oxidizing agents	Anion and cation type and concentration
Source of the sample	Degree of relative supersaturation
Presence of amorphous or particulate material	Initial and final concentration of the reagent
Post-translational modifications	Path and rate of equilibration
Chemical modifications	

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Table 2. Physical variables that could or do affect protein crystal growth	
Temperature	Electric and magnetic fields
Rate of equilibration	Surface of the crystallization device
Method of crystallization	Viscosity of the reagent
Gravity, convection, and sedimentation	Heterogeneous and epitaxial nucleants
Vibration and sound	Geometry of crystallization device
Volume of the sample and reagent	Time
Pressure	Dielectric property of the reagent

Despite the largely empirical nature of crystallization, today's crystal grower may choose from a readily available collection of screens, plates, and tools for identifying initial crystallization conditions. These tools are accompanied by a portfolio of methods (Table 3), screens, and reagents (Table 4) for crystal optimization. These tool chests, together with a rich pool of crystallization literature to explore, along with a caring and sharing group of mentors and instructors offering wisdom and advice through meetings, workshops, and the internet, provide a tremendous resource for today's crystal grower.

Table 3. Crystallization Methods – Achieving Supersaturation	
Vapor Diffusion (Sitting, Hanging, Sandwich)	Sequential Extraction
Batch (Microbatch with or without oil)	pH Induced
Dialysis (Microdialysis)	Temperature Induced
Free interface diffusion (Counter diffusion, liquid bridge)	Effector Addition (Silver Bullet)
Controlled Evaporation	

Table 4. Examples of reagents used in protein crystallization	
Salts (Ammonium sulfate, Sodium formate, Ammonium phosphate…)	Non-Volatile Organics (+/-)-2-Methyl-2,4-pentanediol, Glycerol, 1,6-Hexanediol…)
Polymers (Polyethylene glycols (M_r 200 – 20,000), Ethylene imine, Jeffamine®…)	Buffers (HEPES, Tris, Sodium acetate, MES…)
Volatile Organics (2-propanol, 1,4-Dioxane, Ethanol…)	Additives (Calcium chloride, Sodium chloride, TCEP, n-Octyl-ß-D-glucoside…)

Alas, there is much more to crystallization than making, formulation, or buying and using tools. So before heading off to grab a pipette or pressing go on the robot, consider some important principles as a foundation of your crystallization strategy, as presented originally by the giant of crystallization, Alex McPherson.[12-13]

The Sample
The sample is the most important variable in the crystallization experiment. Prepare, purify, handle, and store the sample with only the greatest care and respect. Manipulate the sample and refine its environment (buffer, reagent) as needed to produce the desired crystals.

Homogeneity
Purify, purify, then purify some more. Start with a pure, uniform population of the sample.

Solubility
Solubilize the sample in a sample buffer that is optimized with regard to pH, buffer, and excipients that dissolve the sample to high concentration free of aggregates, precipitate, or other phases. Pursue monodispersity not polydispersity.

Stability
Prepare and maintain the sample in a chemical and physical solution that promotes optimal stability of the sample. Do not allow the sample to go to the dark side, form oligomers, undergo significant conformational change, denature or change in any way before and during crystallization. Pursue a stable and unchanging sample.

Supersaturation
Find and pursue ways to move the sample into a supersaturated state. Using reagents, pH, temperature, and other variables to move sample equilibrium from a solution to a solid.

Association
Promote the orderly association of the sample molecules while avoiding non-specific aggregation, precipitate, or phase separation. Manipulate the chemical and physical environment to facilitate positive molecular interactions.

Nucleation
Promote and induce a few nuclei in a controlled manner. The number, size, and quality of the crystal depend upon the first nuclei and the mechanism of their growth. Manipulate the chemical and physical environment to produce limited nucleation and controlled growth.

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Variety
Pursue everything. Explore as many chemical, biochemical, and physical options and opportunities as possible for the growth and optimization of the crystal. Be thorough and relentless.

Control
Maintain control of the experimental system, at an optimal state, free of unknowns, perturbations, and fluctuations, from start to finish.

Impurities
Keep it clean. Avoid and discourage the presence, inclusion, and formation of impurities in the sample, reagent, and containers. This can minimize the incorporation of impurities into the crystal lattice, as well as minimize problems with reproducing experimental results.

Preservation
Take care of the crystal, protect them from shock, as well as chemical, biochemical, and physical change or disruption.

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