GROWTH OF PROTEIN SEED CRYSTALS WITH HIGH-STRENGTH HYDROGELS


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X-ray protein crystallography is a valuable tool for gaining the three-dimensional (3D) structure of proteins at the atomic level. The 3D structures of proteins offer important information for the structure-function relationship and the structure-based drug design. However, X-ray crystallographers rarely use hydrogen atoms and hydration water molecules to obtain an in-depth understanding of the protein 3D structure. Neutron crystallography provides insights into the protein structure protonation details. One important purpose of neutron macromolecule crystallography is to obtain extremely large protein crystals to obtain an analyzable diffraction pattern from the available neutron beam. So far, investigators have developed several growth techniques for large protein crystals, including macroseeding, the slow-cooling method, the floating-and-stirring technique, the large-scale hanging drop method, and top-seeded solution growth. However, several obstacles remain with these methods. For example, with the macroseeding technique that is often employed to grow protein single crystals, we must pay scrupulous attention when handling the seed crystal. A seed crystal is introduced into a pre-equilibrated protein solution, and this cycle is repeated several times. However, it is often difficult to use a seed crystal because protein crystals are usually very small and fragile. To overcome this difficulty, we used hydrogels [1]. We previously developed a new method for growing protein crystals in high-strength hydrogels [2, 3]. Our study demonstrated that the high-strength hydrogels increase the mechanical stability of the protein crystals while considerably reducing osmotic shock, in part because incorporating hydrogel fibers into the crystal during growth significantly strengthens the crystal. Here we report the novel combinatorial technique of seeding and reinforcing hydrogel-grown protein crystals for neutron crystallography [4].


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Neutron crystallography is an important complementary technique to X-ray crystallography since it provides details of the H-atom and proton positions in biological molecules and from crystals that are free from radiation damage. Historically, the use of neutron crystallography was limited because very large crystals of several cubic millimeters were required, due to the low fluxes of even the most intense neutron sources. In more recent years however, crystal volume requirements have been reduced via the use of new and improved neutron instrumentation, and the creation of dedicated deuteration facilities for the production of perdeuterated samples. These developments now allow crystals with volumes from ~0.1 – 1 mm³ to be used for high-resolution neutron diffraction studies of biological macromolecules with unit-cell edges from ~ 50 - 150 Å, respectively [1]. Nevertheless, further reductions in crystal volumes are certainly required before the application of neutron crystallography can become commonplace. In this talk I will speak about progress in the development of neutron cryo-crystallography, which until recently had been rarely attempted [2, 3] and yet can allow smaller crystal volumes to be used than at room temperature, and permit a wider array of studies to be performed, such as cryo-trapping studies of enzyme reaction intermediates [4].


Fungal polysaccharide monoxygenases (PMOs) are a recently discovered family of extracellular enzymes that break glycosidic bonds at the surfaces of crystalline carbohydrates, and enhance the susceptibility of the substrate to further enzymatic degradation by “classical” glycoside hydrolases. PMOs are copper containing metallo-enzymes which require input of two electrons and one oxygen molecule to achieve hydroxylation of one carbon in the glycosidic bond.

X-ray crystallographic studies to date have failed to unambiguously determine the chemical nature of the oxygen species bound to the catalytic center. This is due in part to metal photo-reduction by exposure to the X-ray beam leading to a mix Cu(I)/Cu(II) catalytic center.

In marked contrast to X-rays, neutrons do not cause radiation damage and are therefore well suited to study the enzymatic mechanism of redox enzymes. Neutron crystallography is however inherently limited by the flux of neutron beams available. Large crystals are required to compensate for the weaker neutron beam intensities. We will discuss methods used to grow PMO crystals suitable for neutron data collection on the IMAGINE instrument at the High Flux Isotope Reactor and our recent neutron crystallography structure of a fungal PMO.
CRystallization with an automated apparatus for temperature-controlled flow-cell dialysis with real-time visualization

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Much instrumentation developments in crystallization have concentrated on massive parallelization assays and reduction of sample volume per experiment to find initial crystallization conditions. Yet improving the size and diffraction quality of the crystals for diffraction studies often requires decoupling crystal nucleation and growth. This in turn requires controlling variables such as precipitant and protein concentration, equilibration rate, temperature, that are all difficult parameters to control in the existing setups.

We have developed a temperature controlled dialysis button for our previous device [1] and also constructed a prototype of an automated integrated apparatus for the rational optimization of crystal growth by mapping and manipulating temperature-precipitant concentration phase diagrams [2]. Moreover as the crystallization process is based on dialysis, it allows to explore crystallization conditions without consuming the protein sample, so the conditions can be changed in a reversible fashion.

This new crystallization bench (Figure 1) comprises a flow-cell dialysis set-up in thermal contact with Peltier modules. A microscope-mounted video camera allows for real-time observation in the dialysis chamber. There is also a dry air system preventing condensation. Each parameter is controlled using the user-friendly supervision software, resulting in reducing any handling on the bench during the experiment. The control software of the apparatus, written under LabVIEW (Figure 2), enables real-time visualization of the dialysis chamber, direct measurements of crystal size, temperature control thanks to a PID electronic temperature controller in the range of 233-353±0.1K and control of the chemical composition thanks to a pressure-driven flow controller.

This instrument, makes possible the rational optimization of large crystal growth based on knowledge of a multi-dimensional phase diagram.
