Crystallization of biological macromolecules in microgravity

by Edward H. Snell, Naomi E. Chayen and John R. Helliwell

Introduction

Why microgravity for crystallization?

Imagine a hot-air balloon in a field on the ground. As the air is heated the balloon rises because the hot air expands and has less density than the surrounding air. The balloon would not fly without gravity in much the same way as the apple that hit Newton on the head would not fall. Similarly, in crystallization on the ground, as the molecules move from the solution to the crystal, the solution around the crystals becomes less dense, depleted of these molecules. This fluid starts to rise, forming a convection plume that can degrade the quality of the crystal (Figure 1). Microgravity alters, and can indeed remove, this buoyancy-driven convective flow of fluid during crystallization: depleted regions of the fluid form 'halos' around the crystals, termed the depletion zones. Removal of this flow and the resulting depletion zones are the prime reason for using microgravity, as it is thought to benefit the quality of the crystal. If we go back to the floating apple (and Newton!) there is another obvious effect: the apple does not fall, it only moves when some force acts on it. With a crystal this is akin to a lack of sedimentation as it grows. This keeps the whole surface area of the crystal in the nutrient solution and a larger crystal volume results.

There is evidence that microgravity is beneficial to both the short-range and long-range order of the crystal, leading to more strongly diffracting crystals increasing the detail seen (the resolution) and an increase in the physical perfection. However, experimental evidence, supported by previous fluid physics calculations, shows that in many cases the potential benefits of the microgravity environment have not been exploited fully. More strongly diffracting crystals and increases in crystal volume do result from

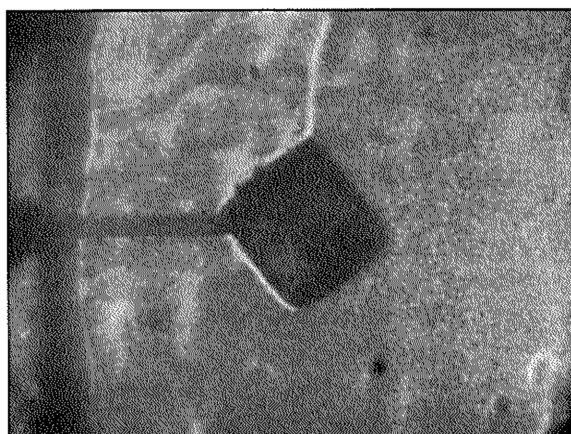

Figure 1. Schlieren photograph of an on-ground growing crystal of lysozyme showing the 'convection' plume. Courtesy of Marc Pusey, NASA, Huntsville, USA.

microgravity studies; however, so do smaller crystals and poorly diffracting samples. Observed improvements show us that microgravity can have a positive effect but also tell us that we might not know yet how to best to use it. In this article we describe the history of diagnostic experiments that we have carried out and thereby address the question: how successful can microgravity be?

Why do we want to crystallize a protein and where does microgravity come into the equation?

Macromolecular crystals can be just as beautiful as diamonds, and to the researcher are just as priceless. They allow us to solve the three-dimensional structure of a biological molecule through X-ray crystallography. From the structure we gain an understanding of how the molecule works, how it interacts with other molecules and, increasingly, how best to modify its action through rational drug or agricultural chemical design. On the biochemistry side tremen-

dous advances in the fields of expression and purification are allowing us to get our raw material in ever-increasing quantities. In crystallography, X-ray sources, methods and computation have evolved over the years, enabling structure to be inferred from diffraction data more rapidly than ever. Despite this, crystallization still remains the major bottleneck. That is, before X-ray or neutron crystallography techniques can be used you have to have grown the crystal. The use of microgravity is seen as a possible way to try and widen this bottleneck, by improving crystals that grow but diffract poorly and by perhaps providing conditions in which crystals that won't grow on the ground (under normal gravity conditions) will form. Does this sound far-fetched or hyped? Let's look at the evidence.

An early analysis of 16 NASA Space Shuttle missions flown between 1985 and 1993 showed that 20% of the proteins grown by vapour diffusion exhibited improvements, i.e. better morphologies or better quality diffraction data than their Earth-grown counterparts[1]. This percentage may seem low but in these initial missions equipment was being developed and the primary goal was not usually to establish a microgravity environment. A different apparatus, using temperature-induced crystallization, flew on four of the 16 missions and showed better results[1]. More recent missions devoted to microgravity, with more advanced hardware, are producing encouraging results, including structures deposited in the Protein Data Bank from insulin, bacteriophage λ lysozyme, satellite tobacco mosaic virus, antithrombin III, serine protease and pike parvalbumin. On average, to date, the equivalent of two laboratory crystallization plates (or 50 experimental wells) have been flown for each individual sample. This is in contrast to typical laboratory work where tens or hundreds of these plates are used to get a good crystal. In some senses the use of microgravity for crystal growth is similar to the initial parasitic use of synchrotron radiation for structural X-ray studies, i.e. non-ideal experimental conditions, maturing hardware and few opportunities. Despite the oft-quoted 20% success rate, it is not surprising, given the parasitic mission profile and the low number of samples that have been flown, that many scientists are still very interested in the subject, and the opportunities of the International Space Station.

Factors affecting microgravity crystallization

The apparatus that we have used in our research is called the Advanced Protein Crystallization Facility (APCF), designed by the European Space Agency (ESA) and built by Dornier. This is a sophisticated piece of apparatus that has been designed with simple operation in mind. Once in microgravity it can be operated simply by pressing a single button, which activates the crystallization experiments. Pressing the button a second time deactivates the system before the Orbiter comes back to Earth. A total of 48 experiments are carried out in this apparatus during a flight, with CCD video and interferometry to allow monitoring of the growth process. This has enabled simple but revealing diagnostic studies. Previous trials relied heavily on observation of the final result, not the process. Consequently, with the data from the APCF, researchers are now beginning to find the crucial factors on which to focus when conducting experiments in microgravity.

The optimal conditions for crystallization in microgravity are not necessarily identical with those on Earth and need to be determined in order to ensure that we gain the full benefit of the microgravity environment. So far, no general experiments have been flown to see how the optimum conditions shift and, with so few samples per flight, this is not altogether surprising. The limited number of experimental opportunities have then been given over to experiments that are already successful on the ground. Access to the new International Space Station will greatly increase the number of experiments possible and the available microgravity time, allowing optimization of conditions.

Since vapour diffusion is the most widely used technique for crystallization on the ground, it naturally became the dominant method for crystallization in microgravity[1]. Recently though, different methods of crystallization have been compared while monitoring the crystal process by CCD and interferometry. We have demonstrated that the method of crystallization in microgravity may be as crucial to the results as the determination of the optimal conditions.

Finally, the term microgravity implies a condition whereby the acceleration in which the experiments are carried out is one-millionth that experienced on Earth. This is not always the case. Many things happen on an orbiting spacecraft, especially when astronauts are on board!

The optimum conditions of crystallization are experiment-dependent, although general trends do exist, i.e. higher concentrations of the sample are usually needed. The lack of flight opportunities has prevented systematic studies of crystallization conditions and one hopes that this will change with

the International Space Station. In the following sections we will now focus on the method of crystallization and the microgravity environment.

The method of crystallization

There are several ways to grow crystals[2], all of which involve decreasing the solubility of the macromolecule until it no longer stays in solution. The trick is to get it to come out of solution and form a crystal, not a precipitate. In the APCF experiments described here three techniques are used, vapour diffusion, dialysis and liquid–liquid diffusion. Vapour diffusion involves the crystallizing solution being separated from the precipitate by a vapour pathway. The crystallizing solution loses water and the solubility of the protein molecules is decreased and, hopefully, crystals are formed. Dialysis and liquid–liquid diffusion are based on similar principles but there is no vapour path. In the dialysis method the two solutions are separated by a dialysis membrane, impermeable to the protein molecules. The liquid–liquid diffusion method just allows the two liquids to come into contact and again, hopefully, crystallization proceeds. If this sounds somewhat like alchemy then we do not apologize. Crystallization is predominately an empirical science of rational trial and error guided by past results.

The method of crystallization has quite an impact on the results of the experiment. We grew crystals of crustacyanin, lysozyme and apocrustacyanin C_1 in microgravity by the three different crystallization methods described above[3–6]. Vapour diffusion and dialysis methods made use of the APCF and the Protein Crystallization Facility (PCF), a predecessor of the APCF, was used for liquid–liquid diffusion. The APCF was flown on two dedicated microgravity missions: the second International Microgravity Laboratory (IML-2) and the United States Microgravity Laboratory (USML). The PCF was flown in a European free-flyer satellite called EURECA (European Retrievable Carrier). A fault with the cooling system on EURECA 'cooked' the crystals, preventing useable samples from being retrieved for X-ray analysis. Crystals grown in the APCF were all successfully returned, analysed and compared with respective ground controls. Overall, the best crystals of these proteins were microgravity-grown[3]. However, even when improvement in diffraction quality was found in the case of apocrustacyanin C_1, which was grown by the vapour-diffusion method, it was not as pronounced as for the microgravity-grown lysozyme crystals, which were grown using the dialysis method[3]. In the case of lysozyme, all the microgravity-grown crystals tested were superior to their Earth-grown counterparts[2] (Figure 2). In the case of apocrustacyanin C_1, several Earth-grown crystals were as good as microgravity-grown ones.

The partial improvement in quality of the microgravity-grown apocrustacyanin C_1 crystals, compared with the Earth-grown controls, may be explained by correlation of the X-ray analysis results with CCD observations of apocrustacyanin C_1 crystals during the IML-2 flight. The crystals displayed a cyclic motion within the hanging drop[4], which was caused by Marangoni convection[6]. This convection results from surface-tension gradients along the interface between the crystallizing solution and the precipitant. The gradients are then relieved by cyclic flow. In contrast, for lysozyme grown by dialysis (without a liquid–vapour-phase boundary), such crystal motion was not visible[8] and there was a very clear increase in crystal quality. The EURECA experiment involved CCD video monitoring of crystallization of crustacyanin by free interface diffusion. The microgravity crystals were stationary during the long mission[6] and they were far larger than their Earth-grown counterparts[6], for which contin-

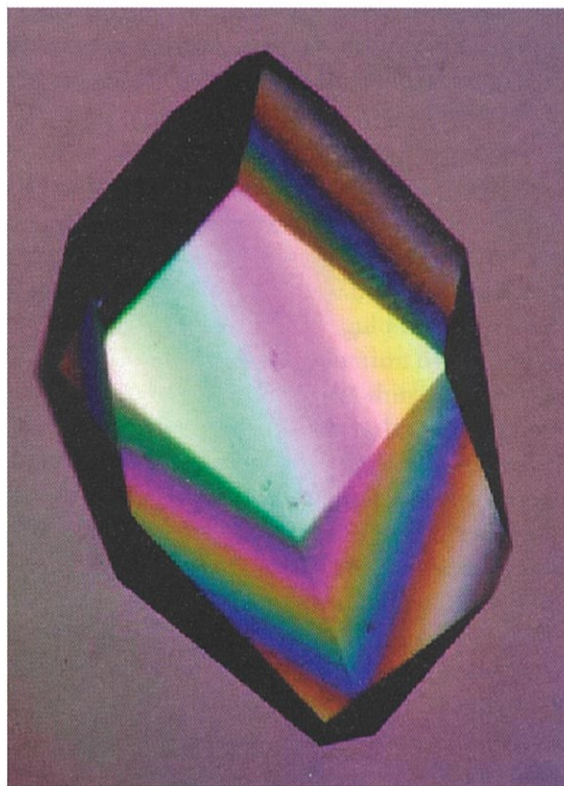

Figure 2. A large lysozyme protein crystal (2.5 mm long) grown on a short Space Shuttle mission[5]. Large microgravity-grown crystals are especially suited for use in neutron protein crystallography data collection.

uous turbulence throughout growth was observed. Cyclic movement of the crystals may well be a limiting factor then the ultimate perfection of the protein crystals that can be obtained.

Experiments conducted recently on the Life and Microgravity Science (LMS) mission compared the quality of crystals of alcohol dehydrogenase grown in microgravity in APCF dialysis chambers with those grown in the APCF using vapour diffusion[7]. It was shown that crystals grown by the dialysis method were better-ordered than their Earth-grown controls, whereas the vapour-diffusion ones were no different from the Earth-grown controls. The lesson to be learnt is that vapour diffusion, although commonly used on the ground, is not the best method to use in microgravity. It would seem that dialysis or liquid–liquid methods are preferable to vapour diffusion in order to maximize microgravity benefits.

The microgravity environment

Other causes of crystal motion during growth, besides Marangoni convection in vapour diffusion, have been witnessed on CCD video. For example, a very large and sudden movement occurred on SpaceHab-01, late in the mission, and after crystal growth had essentially stopped. This motion was attributed to preparation for the retrieval of the EURECA satellite. As has been pointed out previously, this is an example where the crystal-growth experiment was parasitic to the overall mission, in this case retrieving the satellite. Approaching the satellite requires sudden changes in acceleration, enough that the astronauts have to brace themselves, as the Orbiter manoeuvres. The lysozyme crystals (grown by the dialysis method), examined by ultra-long-distance synchrotron Laue diffraction, were the most perfect protein crystals ever grown[3]. Hence, sudden movements, and also re-entry into the Earth's atmosphere, did not affect these already-grown crystals. Those crystals were surrounded by fluid in the growth chamber, but it may well be that sudden motion may affect vapour-diffusion geometries or be detrimental where grown crystals are especially fragile.

It is a different matter during the growth of crystals. In another experiment on IML-2 involving lysozyme, correlation of CCD-video with accelerometer (G-jitter) data, (Figure 3) showed spurts and lulls of crystal growth coincident with G-jitter bursts caused by astronaut exercise periods[5]. Such spurts and lulls in crystal growth may then explain the residual mosaic character of protein crystals seen in X-ray topographs[2] (where the crystal is made up of many separate regions). These accelerations increase the level of perceived gravity experienced by the crystals and hence detract from the overall quality that could be obtained.

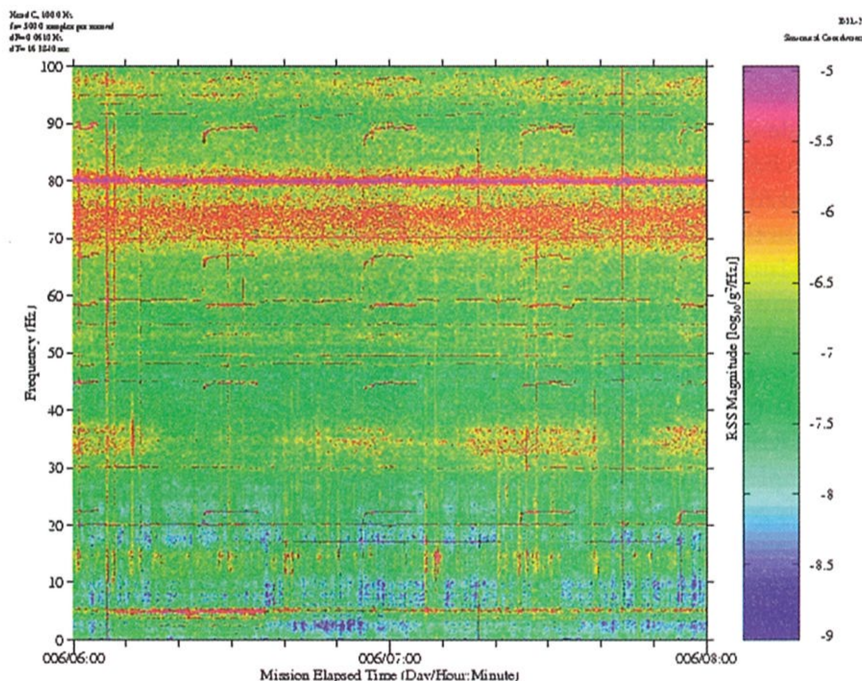

Figure 3. In microgravity Space Shuttle Orbiter flights, astronaut exercise periods produce G-jitter and can thereby induce re-stimulated growth[5]. Seen here in the spectrogram from accelerometers on the Orbiter is astronaut exercise at about 5 Hz in the lower left of the graph. Low frequency is most detrimental to crystal growth.

Mimicking microgravity

A way of partially mimicking the microgravity environment with respect to creating a contact-free container for a crystal is by suspending a crystallization drop between two oils of different densities. The oils are not miscible and the crystallization solution floats at the interface[8]. Improved crystals have been produced by this method but it still does not overcome the issue of convection. Crystals grown in gels on Earth can also help counter this problem. However, experiments with gels on specific molecules have shown that, although an Earth-based gel-grown crystal is better than an normal Earth-grown one, a microgravity-grown crystal still beats them both in terms of quality. Recently, gel-based experiments have been flown in microgravity and produced enhanced results over the ground gel controls[9].

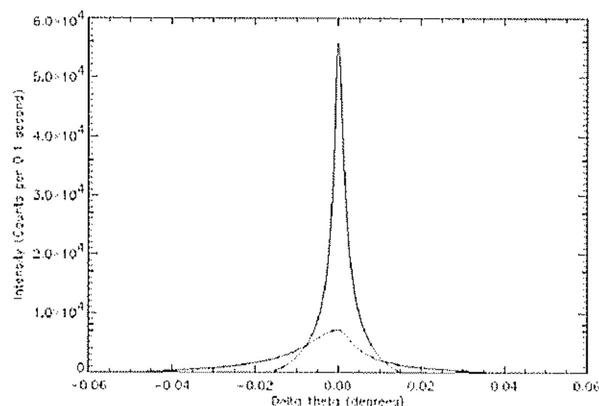

Figure 4. Ultra-fine mosaicity scan obtained from a room-temperature lysozyme crystal grown in microgravity in comparison with one on the ground, demonstrating the superior X-ray signal-to-noise ratio achievable when combined with fine X-ray-beam perfection from the European Synchrotron Radiation Facility X-ray source[2].

How best to make use of microgravity

In our experiments we have seen the benefits of simple diagnostics and have found the importance of paying particular attention to the environment. As referred to above in detail, we have made us of accelerometer data to measure G-jitter. Our experiments in the APCF were conducted under temperature-controlled conditions but many microgravity experiments are carried out at 'ambient' temperatures. This ambient temperature has been known to fluctuate as much as 10°C during a mission. One needs to always control these parameters in any study.

Two major lessons come from our results: try to use a crystallization method that avoids fluid flow effects and remove the influence of acceleration on the sample. With regard to crystallization method this would ideally be a batch (pre-mixed) or liquid–liquid diffusion experiment. As to removing the influence of acceleration we can foresee a micro experiment, with a free-flying satellite orbiting with the Space Station (perhaps tethered on a line to it). To grow crystals the experiment is charged with fresh solutions by retrieving it to the Space Station. It is then released, and then weeks or months later brought back to harvest the resulting samples for analysis.

A note of caution needs to be sounded here. While recommending that vapour diffusion not be used we have ignored the fact that even with vapour diffusion the sedimentation-free environment still remains — the apple floats. Motion of the crystals through the solution, via flow effects, causes new nutrient to come into contact with the crystal. This is ideal for neutron crystallography where large samples are the prime need. Neutron-crystallography studies allow hydrogen–deuterium-exchange sites to be mapped on to a protein and/or bound-water deuterium positions defined, e.g. for molecular-recognition studies. Because of the need for large-volume crystals there are relatively few neutron studies and microgravity can make a big impact.

What the future holds

In the Introduction to this article we said that microgravity can help produce better crystals or that it may produce crystals that could not be grown on Earth. Evidence for the production of new crystals in microgravity comes from the shift of biochemical conditions needed to grow crystals in microgravity. So far, a requirement of all the agencies concerned to fly experiments is that you must have grown crystals on the ground before a flight. This makes sense for the limited amount of experimental space available and the demand for that space. It has not allowed us to address the question of producing new crystals in microgravity. With the International Space Station coming on line, far more experimental space and time becomes available in microgravity. Crystallization experiments are small, can be quite easily automated, followed remotely by video and have low mass. The International Space Station can literally accommodate thousands of screening trials (the crystallographer's equivalent of multiple choice) and searching for the best conditions and optimization become a reality.

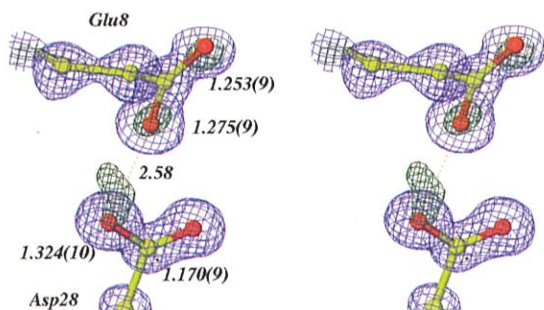

Figure 5. Ultra-high-resolution (0.92 Å) structure study of concanavalin A, using a ground-grown, frozen, crystal showing (in stereo) resolved single, double and mixed bond carboxyl side-chain distances (standard uncertainties, on the last digit, in parentheses). Microgravity improves protein crystal perfection and diffraction data for room-temperature structural studies (see Figure 4), i.e. near to physiological temperatures, for rational drug design. Reproduced from reference 10, with permission of the Royal Society of Chemistry.

Microgravity for crystallization has given positive results despite scientifically compromised conditions. We think that microgravity crystallization will be successful in improving crystals and perhaps producing crystals where none is found on the ground. With the potential pharmaceutical, agricultural, and not to mention scientific payoffs of even one of these experiments, the future looks bright indeed and it behoves us as scientists to make use of this opportunity.

A goal that we have emphasised is to make more perfect protein crystals and harness that property by better measurement physics to improve X-ray reflection signal to noise (Figure 4). Thus the weaker, higher-resolution diffraction data can be better measured. Obtaining ultra-high resolution protein diffraction data and structures has, so far, been largely the province of cryo-frozen (Earth-grown) crystals (Figure 5). There are differences in protein stucture between cryo (100 K) and room (293 K) temperatures[10], and to try and improve room-temperature diffraction data, based on better microgravity-grown crystals, is a way forward.

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