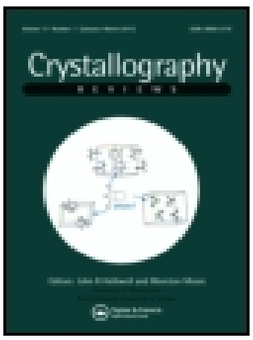

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REVIEW ARTICLE

Microgravity as an environment for macromolecular crystallization – an outlook in the era of space stations and commercial space flight

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ABSTRACT

In 2005 we reviewed microgravity for macromolecular crystallization, four years after the final flight of the Space Shuttle Orbiter, and five years before the first commercial flight to the International Space Station. Since then, there have been developments in access to space and advances in technology. More regular space flight is becoming a reality, new diffraction data detectors have become available that have both a faster readout and lower noise, a new generation of extremely bright X-ray sources and X-ray free-electron lasers (XFELs) have become available with beam collimation properties well suited geometrically to more perfect protein crystals. Neutron sources, instrumentation, and methods have also advanced greatly for yielding complete structures at room temperature and radiation damage-free. The larger volumes of protein crystals from microgravity can synergise well with these recent neutron developments. Unfortunately, progress in harnessing these new technologies to maximize the benefits seen in microgravity-grown crystals has been patchy and even disappointing. Despite detailed theoretical analysis and key empirical studies, crystallization in microgravity has not yet produced the results that demonstrate its potential. In this updated review we present some of the key lessons learned and show how processes could yet be optimized given these new developments.

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Contents

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1. Introduction

The environment of an orbiting spacecraft provides a method to achieve a reduction in forces on the ground associated with gravity as the spacecraft orbits the earth in free fall. This environment, commonly called microgravity, has significant impacts on processes that involve fluid physics. For many years, macromolecular crystal growth has been one of the areas where this environment has been thought to offer a positive impact. As an application for spaceflight, the mass requirements are small, the experiments can be conducted with a high degree of automation, the potential value is high, and the samples can be brought back to the ground where sophisticated instrumentation is available and analysis can take place.

Our interest in the area has been in understanding the differences that the microgravity environment makes to the crystallization process and the structural outcome. We have

pioneered the field in this aspect with several notable findings and in this article update our comprehensive review published in 2005 [1].

The fluid physics environment of microgravity has the potential to yield convection-free, diffusion-limited, and sedimentation-free conditions for crystallization. While we will explain these in detail later, the primary outcomes from these conditions in terms of macromolecular crystallization are:

- diffusion-controlled growth
- increased spatial coherence length for the ordering of a crystal
- increased crystal volume

From these outcomes there can accrue derived benefits:

- potential filtering of larger oligomers of the macromolecule in the mother liquor and time for disordered molecules to dissociate
- increased diffraction signal-to-noise due to reduced mosaicity
- increased signal-to-noise from the increased volume and/or ability to spread dose across a larger area of the crystal

There is experimental evidence demonstrating that these benefits occur. However, it is important to note that detailed efforts to understand and use the microgravity environment require both a knowledge of how to maximize any benefits through the subsequent analysis on the ground as well as an awareness of investigator expectation bias. Experimentally, there are many examples of crystals grown in microgravity where their improved quality is subsequently destroyed by cryocooling on the ground or where improvements are masked by the use of an inappropriate diffraction instrumental setup. There are also many cases where a small positive effect of the environment is noted, but not at a level of significance that demonstrates efficacy.

Our efforts in this area have stemmed from a curiosity-driven hypothesis [2]: could a microgravity environment enable a macromolecular crystal to reach the perfection of a non-biological crystal such as silicon for example? This hypothesis presented a simple target: a calculable crystal rocking curve based on diffraction grating theory to be compared with a measured value. It also introduced a detailed X-ray analysis of the outcome, an area missing in the field to that point.

We comprehensively reviewed macromolecular crystallization in microgravity four years after the final flight of the Space Shuttle Orbiter and five years before the first commercial flight to the International Space Station [1]. Since that review there have been several significant developments: (i) Regular commercial space flight is becoming a reality; (ii) New X-ray diffraction data detectors have become available that have both a faster readout and lower noise – critical to achieve an X-ray diffraction data enhancement when mosaicity is reduced; (iii) There has been a renewal of interest in X-ray crystal structure analysis at physiologically relevant temperatures and; (iv) A new generation of X-ray sources, X-ray free-electron lasers (XFELs) have become available with extremely good beam collimation properties well suited to geometrically more perfect protein crystals. Interestingly XFEL studies show that information can be obtained from crystals consisting of just a few unit cells [3]. This has helped prompt developments at synchrotron source beamlines that

greatly enhance collimation and brightness thereby enabling the study of crystals measured in a few microns or less.

Unfortunately, progress in harnessing these new technologies with microgravity-grown crystals for structural biology studies has been patchy and even disappointing. Despite detailed theoretical analysis and key empirical studies, crystallization in microgravity has not produced the improved X-ray crystal structure results that might be expected. In this updated review we present some of the key lessons learned and show how processes could yet be optimized given these new capabilities. We also discuss potential synergies with neutron crystallography.

1.1. What is microgravity?

We have already introduced microgravity as a description commonly used for free fall in an orbiting spacecraft. If we take, for example, the International Space Station (ISS) orbiting approximately 400 km above mean sea level, the gravity experienced at a point that height above the ground is about 90% of sea level – not even milligravity. The weightlessness commonly used to describe the environment on the ISS comes from the fact that the space station is in continuous free-fall as it orbits the earth. This reduces accelerative forces such that the acceleration perceived at the centre of gravity of the space station is less than 10^{-6} that of acceleration on the ground, $\sim 9.8\ \mathrm{ms}^{-2}$, hence the term microgravity. Because the space station is in continuous orbit, processes that take a significant time, e.g. crystallization of macromolecules, can be conducted completely under reduced acceleration for as long a period as is needed. The acceleration sensed increases as distance increases from the centre of mass. For every 1 m away from the centre of mass of the ISS (approximately 100 m in the largest dimension) an object experiences a 10^{-7}g increase in force to constrain it to a fixed position relative to that centre of mass. The acceleration is also not a constant vector, several additional forces such as those arising from orbital mechanics, atmospheric drag, thruster firings, and equipment and astronaut disturbances impact the acceleration environment. While the constant acceleration seen on the ground is eliminated, forces that are ideally masked can become apparent and a dynamic understanding of the ISS's acceleration environment may be required to interpret crystallization outcomes.

1.2. Can a reduced acceleration environment impact crystallization?

Macromolecular crystallization is the process of producing an ordered three-dimensional array of macromolecules. Successful crystallization does this with enough precision that the crystals diffract X-rays or neutrons such that the resultant structure is produced in sufficient detail to answer questions about biological mechanism and molecular interactions. The more ordered the crystal, the higher the resolution information that results. There are a number of fundamental processes involving fluid physics that may impact crystallization when accelerative forces are changed. These factors include transport of the macromolecules in the solution, formation of the initial crystal (nucleation), crystal growth, and sedimentation. There are fundamental factors that are not impacted by a reduction in acceleration, e.g. Brownian motion and interatomic forces, as distinct from others that are impacted by the on earth acceleration due to gravity when the samples are returned to the ground and no longer in free fall. We briefly consider each of these factors here.

1.2.1. Transport in the fluid

Crystallization setups have liquid–liquid and in the vapour diffusion case, liquid–vapour interfaces. The liquid–vapour interface becomes important in a process called Marangoni convection described later, for our purposes here, we will consider transport in the liquid case. For crystallization to occur, macromolecules in solution have to come into contact. This involves transport processes in solution to the crystal nucleation site and subsequent transportation of more material to the growing crystal faces. It has been proposed that diffusion-controlled flow of macromolecules in solution may reduce the incorporation of large impurities, namely natural aggregates formed as the sample solution ages. Evidence for this was provided by studies on lysozyme where a 1% dimer impurity was incorporated with the monomer. In this case, the ground-grown crystals contained 4.5 times the amount of this dimer than their microgravity-grown counterparts [4]. In a reduced convection environment, the growing lysozyme crystal depletes the protein solution around it and the transport of new protein to the face is dominated by diffusion processes. Larger aggregates diffuse slower than the monomer and are hypothesized to be incorporated at a lower rate due to this transport limitation. In the original study, there were some inconsistencies in the analysis [4]. Probing these results further showed a concentration dependency [5]. At 0.5% dimer impurity concentration, the microgravity-grown crystals had almost 7 times the dimer concentration as ground control samples. As the impurity concentration increased to 3.6%, the incorporation of dimer converged with the microgravity-grown crystal samples containing 1.4 times the dimer concentration of the ground-grown ones. Whilst there is an effect involving the incorporation of impurities, it is not a simple transport effect. In this case, ground crystal growth did not seem to be as sensitive to this impurity effect. As the gains obtained in microgravity are easily lost if this dimer is incorporated into the crystal lattice, optimizing the improvement of the crystal grown in microgravity, requires the purification of the macromolecule solution to the highest level possible. This is obviously an important conclusion.

A later study, also using lysozyme but with different protein impurities [6] showed that in the presence of impurities that are not easily segregated, the diffraction quality of crystals grown under diffusion-limited conditions is better than those grown in the presence of natural convection. There was also a crystal space group impact suggesting a possible influence on crystallization contacts. An elegant study of the same effect used fluorescently tagged aggregates in the growth of lysozyme and *Plasmodium falciparum* glutathione S-transferase which saw the aggregates incorporated [7]. Unfortunately, the study did not quantitatively compare the space and earth-grown crystal groups. It is not clear how common this situation of large aggregate 'impurities' is and all efforts should be made to produce as pure a sample as possible before resorting to microgravity let alone to help that purification process. Certainly, samples can be characterized on the ground to understand if oligomerization occurs over time and if microgravity may be needed to grow crystals of single components. Suffice to say the partitioning process in solution is complex and seems to be heavily condition- and technique-dependent. Another benefit of slower diffusion-limited growth is the potential for molecules that are not as well ordered to dissociate from the growing crystal, the crystal itself being in a dynamic state throughout the experiment. This is shown in elegant experiments using Atomic Force Microscopy [8]

Preparation of pure samples on the ground for the flight experiments is a key step. This has been identified as extremely significant in improving the success rates in experiments on the ISS [9].

1.2.2. Nucleation

The initial process in crystallization is nucleation which involves solute-solvent/precipitant interactions. If no nucleus is formed, crystal growth does not take place. For microgravity to have a direct effect on nucleation, it would imply that gravitational forces at the molecular scale are comparable in magnitude to the intermolecular forces. If this were so, other physical properties such as boiling and freezing points, enzyme kinetics, etc., would also be affected. This is unlikely and has not been observed to date [10]. This is a positive, as optimized crystallization conditions on the ground, all other factors being equal, are probably well suited as a starting point for crystallization in microgravity.

While there is no direct effect on the initial nucleation there can be effects associated with nucleation once the nucleation process and growth has occurred. Crystal numbers and sizes can be used to examine the macro-effect of microgravity upon crystal nucleation. A suppressed nucleation rate results in fewer and larger crystals, an increased rate results in a larger number of smaller crystals or even crystalline precipitate. Secondary nucleation, the formation of nuclei in solutions that already contain growing crystals, can occur. In a 1 g field and a crystal of size $\sim$10–100 μm, buoyancy-driven fluid flows develop which not only maintain a high crystal growth rate, but may also produce increased secondary nucleation [11–13]. Secondary nucleation is thought to be caused by the removal of partially solvated clusters from near the surface of the crystal (the absorbed layer) by this flow [14]. Reduced buoyancy fluid-driven flows in microgravity reduce this effect. While a reduced number of nuclei are commonly thought to be produced, based on reduced crystal number and increased crystal volume, this is due to fluid flow rather than any impact on intermolecular forces or nucleation directly.

1.2.3. Crystal growth

The standard model for understanding the effects of microgravity on macromolecular crystal growth is based on the concept of a depletion zone [15]. In the absence of acceleration, a crystal is subject solely to Brownian motion and remains in solution with no sedimentation. As macromolecules leave the solution and add to a crystal, a region of solution depleted in protein is formed around the crystal. Usually, this solution has a lower density than the bulk solution and will rise upward in a 1 g field [11]. In zero gravity, the buoyancy force is eliminated and no buoyancy-driven convection occurs. Because the position of the crystal and its depletion zone are stable, the crystal can grow under conditions where its growing surface is in contact with a solution that is only slightly supersaturated. In contrast, the sedimentation and convection that occur under 1 g place the growing crystal surface in contact with a bulk solution that is typically several times supersaturated. Lower supersaturation at the growing crystal surface allows more high-energy mis-incorporated growth units to disassociate from the crystal before becoming trapped in the crystal by the addition of other growth units. Since microgravity is not zero gravity, the buoyant convection and sedimentation are severely attenuated rather than eliminated. We call this *promoting the ideal*. In short, the promotion of a stable depletion zone in microgravity is postulated to provide a better-ordered crystal lattice and benefit the crystal growth process. This depletion

zone has been seen in interferometer-based observation [16] and the convective plumes observed on the ground [11]. More detailed discussions of the depletion zone and other fluid flow effects are available elsewhere [17–20].

1.2.4. Reduced sedimentation

All of these processes in a fluid can be described as large-scale, i.e. they have little impact on the amino acid residue to amino acid residue ordering that leads to improved diffraction resolution indicated by a reduced Wilson B factor and therefore detail in the resulting structural model. There is evidence to suggest that crystallization contacts can be impacted [21] but structural detail is most likely impacted by Brownian motion and interatomic forces.

Similarly, the average density of a protein is about 1.35 gcm^{-3} and water is 1.00 gcm^{-3} respectively. Given that crystals contain between 30% and 70% solvent and that the aqueous solution typically contains buffer and precipitation components, the density of the crystal and solution are very close in value once crystals are grown. This is why the acceleration forces experienced with return to the ground, for the most part, have a limited negative impact on the crystalline order. There are bigger factors that can have a negative impact and have to be avoided which we will discuss later.

1.2.5. Impact on crystal perfection outcome and structural information

Figure 1 graphically illustrates the impact of microgravity crystal growth on the physical perfection of crystals and how that impacts the resultant X-ray data. In Figure 1(a,b), a topograph of an X-ray reflection from a microgravity-grown lysozyme crystal is shown side by side with a detailed rocking curve analysis of identical reflections from microgravity and ground-grown lysozyme crystals [22]. A similar topograph from a ground-grown crystal shows a grainy grey image and lacks the clear domains present in the microgravity-grown example. In Figure 1(c,d) data from microgravity and ground-grown insulin are shown. A statistical analysis of reflections showed a significantly reduced mosaicity correlated with increased signal-to-noise and an overall higher resolution diffraction limit [23]. Putting aside observations of larger crystal volume and tighter crystal volume distributions in the case of microgravity grown crystals, this figure demonstrates the experimental impact that microgravity can have on crystallization and the resultant diffraction quality, i.e. the larger, more perfect, domains yield narrower rocking widths which can enhance the diffraction signal-to-noise if appropriate instruments and experimental techniques are used. This reduction in mosaicity and enhancement in signal-to-noise is for all reflections and in turn, promotes an increase in diffraction resolution for the data.

1.3. History

In evaluating the success of crystallization in microgravity it is useful to understand some of the metrics used. The principal metric is the diffraction resolution of the resulting model. A lower number for the resolution indicates more detail and precision of the resulting model. The relationship is not linear, with the number of recorded reflections proportional to the inverse cube of the diffraction resolution. Small improvements at higher resolution can have larger impacts on the data to parameter ratio than the same resolution improvements at lower original resolution. There is no ideal resolution allowing an understanding of the biological question being asked. For subtle details, it is possible to estimate the resolution

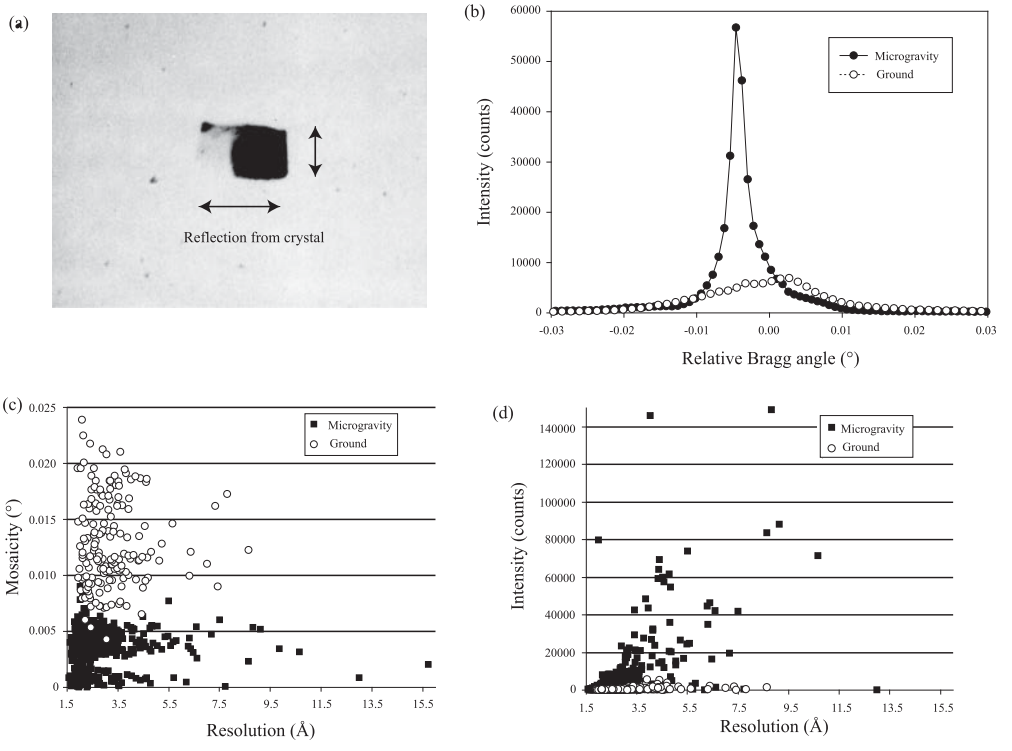

Figure 1. Detailed and well-controlled experimental studies of crystals grown in microgravity show (a) larger uniform domains producing a dramatically reduced mosaicity (well-ordered internal alignment of the crystalline lattice) with (b) detail from rocking width studies and (c) statistical reflection analysis. The result is (d) an improved intensity recorded for the reflections and therefore signal-to-noise in the overall data. The sample in (a) and (b) is lysozyme [22] and in (c) and (d) insulin where an improved methodology allowed a statistical analysis of outcome [23] confirming the lysozyme result.

required based on that question with the use of the diffraction precision index that allows the errors in non-covalent bond distances to be estimated [24,25]. For others, the difference may be between observing a key structural feature or not.

Another metric is mosaicity, a measure of crystal perfection derived from the angular spread of reflections. Microgravity-grown crystals were surprisingly perfect when studied at the temperature at which they were grown [1]. Until recently it was not possible to routinely harness this property to improve the signal-to-noise for diffraction data collection, and while it is a measure of physical crystal perfection improvement, in most cases this advantage was mostly discarded. This has changed as we will discuss later.

Visual observation of the resulting crystals has also noted crystal volume improvements and more uniform crystal volume distributions. However visual characteristics often do not correlate with diffraction properties, the main use for the crystals that are grown.

The history of macromolecular crystallization in microgravity through to 2005 is extensively reviewed in our previous work and references therein [1]. We summarize this briefly in the next section and then bring the historical picture up to date with some of the more recent work conducted on the ISS.

1.3.1. The Space Shuttle Orbiter era

Littke conducted the first microgravity protein crystallization in 1981 using Germany's TEXUS sounding rocket. The protein β-galactosidase was crystallized by liquid–liquid diffusion. In microgravity, strictly laminar diffusion was observed, in contrast to turbulent convection on the ground. Several single crystals approximately 100 μm in length grew in the six minutes of microgravity. These crystals were described as of inferior but comparable visual quality to those grown on the ground [26]. Two years later the NASA Space Shuttle program flew its first macromolecular crystal growth experiment where days of microgravity instead of minutes were available. This was a joint NASA – European Space Agency (ESA) science mission carrying Spacelab. The apparatus was based on the TEXUS liquid–liquid diffusion hardware design but incorporated eight growth cells, four in a freezer and four in an incubator. Later, the vapour diffusion method was used for the first time in microgravity in 1985. This is the most common method of macromolecular crystallization on the ground. Two Vapour Diffusion Apparatus (VDA) were flown and a later mission flew that same year with 48 individual growth cells and crystals of C-reactive protein, bacterial purine nucleoside phosphorylase, and lysozyme were produced [27]. Diffraction resolution analysis of the samples indicated they were as good as the best ground-grown crystals.

The first unmanned macromolecular crystallization experiments were carried out on the Photon satellite mission, launched in 1988 which carried five proteins in a total of 21 liquid–liquid growth cells. A 30S ribosomal subunit from *Thermous thermophilus* crystallized in microgravity but not on the ground, and catalase produced larger crystals in microgravity [28]. However, experiments under optimal laboratory conditions, rather than in ground control hardware, produced larger crystals of catalase. In 1988 COSIMA-1 (Crystallization of Organic Substances in Microgravity for Applied research) flew on a Chinese rocket. On re-entry, the payload experienced a 13 g force culminating in a 60 g jolt when the parachute opened. A total of 101 samples were flown of 7 different proteins. The microgravity crystals generally diffracted to equal or higher (5 out of 7 samples) resolution than the ground controls grown in the same apparatus and had a greater volume (6 out of 7 samples) [29]. Crystals grown under optimal conditions on the ground in standard laboratory apparatus were better than the microgravity or ground-controls. COSIMA-2 launched with a Soviet Earth observation satellite in 1989. Thermolysin and a lysozyme from *Streptomyces coelicolor* were crystallized in orbit but displayed much weaker diffraction than crystals grown on the ground [30]. Asano et al. [31], however, grew ribonuclease S crystals that diffracted to 2.2 Å, with the best ground-grown crystals (by any method) diffracting to only 3.0 Å.

Sweden's MASER (Material Science Experiment Rocket) was used in 1989 to study the growth of bovine ribonuclease (RNase-A) and bovine pancreatic trypsin inhibitor (BPTI). Despite the short duration, flight RNase-A crystals were almost 3.0 mm^3 in volume compared to 0.5 mm^3 for those grown on the ground. The flight samples yielded diffraction data beyond 1 Å, unlike the Earth-grown samples that diffracted to 1.26 Å [32].

The first Space Shuttle Orbiter flight to have the maintenance of a microgravity environment as its primary mission was the International Microgravity Laboratory (IML-1) in 1992. This mission carried both the German Cryostat hardware and the VDA. The Cryostat has two thermal enclosures, each with 7 growth cells for liquid–liquid diffusion experiments. Satellite Tobacco Mosaic Virus grown in this resulted in a 1.8 Å structure [33]. Later that year, the first crystallization experiments conducted by a person mixing solutions in

orbit occurred with payload specialist Dr Lawrence (Larry) DeLucas, enabling iterative techniques in protein crystal growth [34]. Stoddard et al. [35] developed a new vapour diffusion device (VD) reproducing sitting drop vapour diffusion crystallization techniques rather than the hanging drop geometry mimicked by VDA. This vapour diffusion flew on Mir from December 1989 to February 1990. The design was further developed into the Protein Crystallization Apparatus for Microgravity (PCAM) [36]. PCAM first flew as a hand-held device on STS-62, (March 4th-18th, 1994), and evolved into the current design that has flown on multiple Space Shuttle missions to date.

Our experience in the field started with the Spacehab-1 mission in 1993 which retrieved the European Retrievable Carrier (EURECA) long-duration satellite launched almost a year earlier. This mission also flew ESA's Advanced Protein Crystallization Facility (APCF) with 48 individual growth cells that could operate, **most importantly**, so as to investigate the fluid physics of each, namely in a dialysis, liquid–liquid, or vapour diffusion geometry. We had experiments on both the EURECA and Spacehab-1 missions. The EURECA mission had crystallization apparatus that recorded CCD video of free interface crystallization experiments on apocrustacyanin and showed that crystal positions remained fixed over the series of observations [17,18]. Unfortunately, the temperature control failed after crystal growth and before it could be retrieved thereby destroying the resulting crystals. Vapour diffusion crystallization experiments on the same protein carried in the Orbiter provided the first observation of Marangoni convection in macromolecular crystallization [17,37]. Lysozyme crystals grown in the APCF by free-interface diffusion showed a slow drift in position over 15 days of observation unlike the video for experiments on the free-flying satellite. The APCF facility on the orbiter was temperature-controlled to ± 0.1°C and allowed CCD video observation of 12 of the experiments [17,37,38]. The APCF has flown on a large number of missions and an excellent review of those results is available [39]. Lysozyme was crystallized in the APCF and the crystals produced were visually comparable (average dimension 0.7 mm). X-ray diffraction analysis of the crystals with those grown in identical apparatus on the ground showed that the mosaicity of the microgravity crystals was on average 0.0015°, approaching the theoretical diffraction grating value, and the earth-grown crystals mosaicity values were 0.0047° on average with a corresponding increase in diffraction intensity for the space-grown [22].

A similar experiment, also with lysozyme, was conducted by us the following year on the IML-2 mission, a mission dedicated to maintaining the microgravity environment for a longer period. The microgravity-grown crystals produced were on average 1.8 mm in length (with the longest being 2.5 mm) in comparison to the ground-grown crystals which averaged 0.8 mm in length. Detailed examination of identical X-ray diffraction reflections from an earth-grown and a microgravity-grown crystal took place. The results were dramatic. After deconvolution of the instrument divergence and spectral contributions to the rocking width, the earth-grown crystal exhibited mosaicity ranged from 0.0067° to 0.0169° (average 0.0120°) measured at full width at half maximum (FWHM) while the microgravity crystal exhibited mosaicity from 0.0017° to 0.0100° (average 0.0047°). The integrated intensities of the microgravity-grown crystal reflections were approximately double that of the earth-grown crystals which corresponded to the almost doubling in volume. However, the dramatic effect was in the eight-fold increase in peak height. The reduction in mosaicity caused a corresponding increase in signal-to-noise. This provided the first evidence of how crystallization in microgravity could enhance the physical perfection of crystals

and how that increase in perfection could enhance the diffraction data that might result. The lysozyme experiments on crystals grown in the APCF were detailed studies of single reflections using techniques more appropriate to solid-state crystal analysis such as silicon or germanium crystal rocking widths. Indeed, the surprise was that protein crystals were of a physical quality close to those of solid-state crystals.

The APCF was a sophisticated and productive facility. Our experiments in it were the first to see: Marangoni convection (see later) in vapour diffusion protein crystal growth, a process inducing fluid flow in a reduced convection environment [37]; to discover the increase in long-range order and improvement in mosaicity and domain coherence [22,40,41]; and to correlate disturbances in the microgravity environment with changes in crystal growth rates [38]. We were also fortunate to help in expanding the APCF's capabilities incorporating interferometer methods to monitor protein and precipitant concentration in solution [42]. The APCF was used by many groups on both the Space Shuttle Orbiter and later the ISS. A review of the biological results from the facility is presented by Vergara et al. [43,44] which also describes results that confirmed our early studies in the facility.

Another method of crystallization based on temperature flew in 1991. The Protein Crystallization Facility (PCF) [45,46] consisted of four cylinders each containing 20–500 ml of solution, over which a temperature gradient could be established. Insulin was crystallized in this facility and the resulting crystals were on average 34 times larger than the ground-grown crystals and had fewer visual flaws. X-ray diffraction data were collected on multiple Bragg reflections for each of six microgravity-grown insulin crystals and six earth crystals. The best microgravity crystals had an average mosaicity of 0.002° with a standard deviation (s.d.) of only 0.001°. Two of the earth crystals had fairly low mosaicity with average mosaicity values of 0.013° (s.d. 0.004°) and 0.017° (s.d. 0.005°), respectively, yet these mosaicity values were 6.5 and 8.5 times higher than the best microgravity crystals and both crystals were relatively poor diffractors. For any given earth crystal, the mosaicity values for individual reflections varied over a surprisingly large range, with standard deviations ranging from 0.004° to 0.024°. The spread in mosaicity for microgravity crystals was 4–5-fold narrower with standard deviations ranging from 0.001° to 0.005°. This was the first study that statistically confirmed the reduction in mosaicity and the corresponding increase in signal-to-noise of the X-ray diffraction data from both the volume increase and mosaicity reduction [47].

The insulin crystals that showed such a dramatic improvement from microgravity grown on the Space Shuttle Orbiter were also studied after cryocooling [48]. Cryocooling caused a 43-fold increase in mosaicity for the microgravity-grown crystals and an 8-fold increase for the ground-grown crystals (average 0.217° and 0.246° respectively) – **cooling effectively destroyed any advantage in crystal perfection produced by crystal growth in orbit**. Interestingly the cryocooling did not cause the observable formation of any additional scattering domains ('mosaic blocks') in the crystals. Once the domains in the crystals have formed they appear to remain, even during cryocooling.

Macromolecular crystal diffraction data is typically collected at cryogenic temperatures (100 K) to mitigate radiation damage and help sample handling. Despite the damage that cryocooling causes to physical perfection resulting from microgravity crystallization, most of the crystallographic analyses of samples grown on orbit have been performed under cryogenic conditions. This is the case for the majority of the studies described here unless

otherwise stated. While cryocooling destroys much of the advantage due to the improvement in crystal quality, the volume enhancement can still play a beneficial role, in that the specific radiation damage (i.e. the absorbed dose per unit volume of the crystal) can be reduced by e.g. helical data collection methods. We will discuss how the physical quality may also be exploited later.

The first macromolecule crystallization experiments on a Space Station, Mir, came in 1992 when a Progress supply rocket carried up a vapour diffusion device (VD) [35]. Chicken egg white lysozyme and D-amino transferase were grown. The size and diffraction characteristics of the crystals were superior to those grown in identical hardware on the earth. Using standard laboratory techniques to grow the same crystals on earth, the improvement was small but still measurable [35]. An experiment named the Gaseous Nitrogen-dewar (GN2) [49] first flew on the first Shuttle Orbiter docking with Mir in 1995. Experimentally, the precipitant solution was loaded into Tygon tubing sealed at one end, frozen, then the protein solution added, frozen again and the tube sealed. The frozen sample was transferred to a liquid nitrogen dewar which was taken to Mir. Over time the liquid nitrogen evaporated, the dewar warmed, and the samples thawed allowing crystallization by free interface diffusion to commence. On this mission, GN2 contained 183 samples of 19 proteins (spanning a range of molecular weights, biological functions, and physical properties) of which 17 were crystallized. The third Shuttle Orbiter mission to Mir in 1996, STS-76, introduced the Diffusion-controlled Crystallization Apparatus for Microgravity (DCAM) [50]. DCAM consists of two cells containing protein and precipitant solutions, separated by a gel plug that controls the equilibration rate. It requires no activation or deactivation by the crew. Mir ceased continuous operation in 1999 and was deorbited in 2001.

The International Space Station (ISS) was assembled in orbit starting in 1998 with it being largely complete in terms of internal space and facilities in 2010. Subsequent additions included external elements and docking adapters, as well as testing alternative module designs. There have been a number of crystallization experiments conducted on the International Space Station that have been reported [39,51–57]. E. coli manganese superoxide dismutase (MnSOD) crystals grown on the International Space Station from December 2001 to April 2002 were 80 times greater in crystal volume than earth-grown crystals. Diffraction spots to 1.26 Å resolution were observed providing significantly improved data than that obtained from crystals grown on the ground [56]. Crystals of thaumatin were grown on the ISS in September-October of 2000 (STS 106 mission). Synchrotron diffraction data collected from the best space-grown crystal extended to 1.28 Å, compared to the best ground-control crystal at 1.47 Å [51].

In 2003, the Space Shuttle Orbiter Columbia disintegrated on re-entry. Flights did not resume until 2005 with the majority being devoted to the assembly of the International Space Station and one a Hubble Space Telescope servicing mission. The last Space Shuttle Orbiter mission was in 2011. During the Space Shuttle era, Kundrot et al. [58] reported that 20% of the macromolecules ever flown until then had obtained their highest diffraction resolution to date from the microgravity crystals. If the analysis is limited to those proteins that flew four or more times the success rate based on the criteria of improved diffraction resolution however increases to 60%. It is difficult, however, to unequivocally demonstrate clear cases where microgravity crystallization fundamentally enhanced knowledge of biological mechanisms. Many of the studies were focused on developing the appropriate

techniques to use the environment and understanding the advantages that the microgravity environment might provide.

1.3.2. The post Space Shuttle era

Betzel et al. [59] reviewed protein crystallization experiments performed on the ISS during the Space Shuttle program and beyond. When the Space Shuttle program ended experiments continued on the ISS using Russian vehicles to send and retrieve samples. A Japanese facility, JAXA-PGC (Japanese Aerospace Exploration Agency Protein Crystal Growth) [9], was initially used in the Russian section of the ISS and then in the Japanese Kibo module. This was based on the counter diffusion technique. It yielded many results in the post Shuttle Era and arguably established a Russo-Japanese lead in the area of macromolecular crystallization in microgravity. The apparatus was developed from the Granada Crystallization Box design [60] that has been used on the ISS. An analysis of 215 proteins flown in the JAXA-PCG as of 2013 showed an overall success rate of 28% of samples improved compared to growth of the crystals on the ground measured by diffraction criteria comparing both resolution and mosaicity [9]. Examining the data in detail showed that those proteins that had well-optimized crystallization conditions on the ground were improved in 50% of the cases. Purification of the sample prior to flight seemed to be a key element required to improve the success rate.

Analysis of experiments flown on the Space Shuttle Orbiter showed that cryoprotection destroyed much of the physical quality that microgravity crystal growth had produced [48]. Similar to the previous section, the majority of experiments conducted in the post Space Shuttle Era have used cryocooling and benefit from volume increases in the resulting crystals but do not show the full potential that can be achieved by harnessing the increased perfection. There are a number of examples in the literature resulting from the JAXA-PCG facility flights. Native bleomycin N-acetyltransferase was solved to 2.5 Å from microgravity-grown crystals [61]. The transcription termination factor NusA was crystallized on the ground and in orbit [62]. The microgravity-grown crystals diffracted to 2.29 Å compared to 3.0 Å on the ground. Interestingly the ground grown crystals had twinning present (18.3%) which was significantly reduced in the microgravity-grown samples (1.0%). While crystal length remained similar, the microgravity-grown crystals doubled in their depth and breadth. The beam collimation is not described, so as to calculate the difference in X-ray beam exposed volume of the crystal, but overall crystal volume increased by $\sim$5 fold [62]. Mouse wild type and mutant lipocalin-type prostaglandin D synthase crystals were grown on the ISS after optimization of crystallization conditions and purification of the samples on the ground [63]. Wild type and mutant microgravity grown crystals diffracted to 1.06 and 1.16 Å respectively compared to the ground grown samples at 1.20 and 2.0 Å. Diffraction data collection took place on the same beamline. Microgravity-grown crystals in both cases were of larger volume.

Human haematopoieic prostaglandin D synthase complexed with an inhibitor was crystallized in two separate missions on the ISS in 2007 over 12 weeks for the first, and 11 weeks for the second, with controls grown on the ground. Microseeding was used in the JAXA counter diffusion apparatus. The microgravity-grown crystals were visually of larger volume and morphology and diffracted to 1.1 Å compared to 1.5 Å for the best earth-grown ones. Mosaicity was quoted but used the estimate produced by data processing software

without extraction of the instrument or spectral contributions. However, due to the cryocooling approach used, this mosaicity value was likely true but almost certainly masking any benefit from microgravity growth [64]. Crystals of Pz peptidase B were grown on the ground and on orbit [63]. The ground grown native crystals diffracted to 2.2 Å and a SeMet substituted crystal was used to collect MAD data to 2.4-2.2 Å and solve the structure. The microgravity grown crystals diffracted to 1.6 Å. However, it should be noted that different synchrotron sources were used for ground and microgravity crystal analyses [65].

Uridine phosphorylase was crystallized on the ground in both the native form and complexed with uridine, and crystallized in the native form on-orbit using the JAXA apparatus [64]. The native form yielded ground-grown crystals diffracting to 1.9 Å and microgravity-grown ones diffracting to 0.95 Å [66]. Crystals of the dipeptidyl peptidase 11 from *Porphyromonas gingivalis* complexed with citrate and potassium ions were grown by the counter diffusion method in the JAXA-PCG [9]. The structure was determined at 1.50 Å resolution using diffraction data measured at a synchrotron and with a pixel area X-ray detector [67]. A previous study determined the structure to 1.66 Å also using microgravity-grown crystals but with a CCD detector [68]. The initial structural solution came from 2.5 Å data from a ground-grown crystal. The study was not designed to compare the efficacy of the different crystallization environments. A slightly different method made use of seeding within the JAXA-PCG apparatus [69]. Human MutT homologue-1 was crystallized over six weeks and the structure was determined to 1.04 Å on return to the ground. An earlier structure from ground grown crystals was determined to 1.2 Å but a mutant complex showed similar diffraction resolution to the microgravity grown crystals [69].

Thermostable T1 lipase was crystallized in orbit and on the ground and crystals were analysed at the same synchrotron beamline sequentially [70]. The microgravity-grown crystals diffracted to 1.1 Å, with the ground-grown ones diffracting to 1.3 Å. The microgravity crystals were significantly larger than the ground-grown ones and displayed a reduced Wilson B-factor. A dramatic result was reported with the crystallization of mitogen-activated protein kinase kinase-7, producing a structure to 1.3 Å resolution, compared to the previous 2.1 Å structure [71]. Another notable result from the JAXA apparatus was the crystallization of native human erythrocyte band 3 and the diffraction to 6.7 Å compared to the best ground grown samples at 8.7 Å, a significant enhancement but unfortunately not sufficient enough to produce detailed structural information [72]. Other studies have directly used the JAXA apparatus to obtain structural information of ligand binding in mistletoe lectin 1 [73], the structure of phosphopantetheine adenyltransferase [74], the ultra-high resolution structure of native *Shewanella oneidensis* MR-1 uridine phosphorylase [75], and the structure of feline serum albumin [76] among others. The JAXA-PCG facility has then been as important to microgravity crystallization as the APCF was during the Orbiter Era.

Free diffusion was used to crystallize an insulin polysialic acid complex on the ISS in the Modul′-1 protein-crystallization apparatus [77]. Crystals were also grown on the ground using the vapour diffusion method. The microgravity-grown crystals reached a maximum dimension of greater than 0.4 mm with the ground-grown crystals reaching 0.2 mm. A control experiment using the same apparatus as in microgravity but on the ground, it yielded crystals of a maximum dimension of $\sim$0.1 mm. The microgravity-grown crystals diffracted to 1.55 Å compared to the vapour diffusion ground-grown ones at 1.84 Å, using synchrotron and a rotating anode source respectively. The authors do not provide details on

the temperature for data collection in the publication or the PDB deposition. It is difficult to compare diffraction properties based on this analysis but the results are notable in illustrating the difference in crystal volume for microgravity growth and how it is important to compare the same apparatus and the best crystals grown on the ground, irrespective of the method used, to study the efficacy of the use of the microgravity environment to improve crystal properties.

In early 2012 the SpaceX Company launched a Dragon 1 capsule on a Falcon 9 rocket which provided supplies to the ISS. The capsule remained docked for about two and a half weeks then returned to the earth landing in the Pacific Ocean. Commercial space flight to the ISS is now a reality. A high-throughput approach was trialed using microfluidic techniques with a commercial product called a CrystalCard^{TM} system. A total of 25 CrystalCards^{TM} containing $\sim$10,000 experiments were launched to the ISS on a SpaceX Dragon capsule launched on a Falcon 9 rocket in 2013 [78]. The apo ligand-binding domain (amino acids 315-505) of the regulator of glucose metabolism and adipogenesis: peroxisome proliferator-activated receptor gamma (apo-hPPAR-γ LBD) and model proteins chicken egg white lysozyme, glucose isomerase, lipase B, and xylanase were used. The samples were frozen and then thawed for filling then refrozen. Unfortunately, the commercially available glucose isomerase at the time was a thermally stable mutant and cold denatures so that sample was unable to be used. The samples were launched, thawed on-orbit, and returned approximately 70 days later. They were returned on a Soyuz capsule that landed in Kazakhstan. The samples experienced high acceleration several times on the return (possibly up to 9 g) and measured and unmeasured temperature excursions, the measured ones being up to 32°C. Control samples were grown on the ground. A visual analysis took place during the mission and after the return of samples to Houston, Texas. Lysozyme showed similar results under both conditions, thermoysin produced larger crystals in orbit, and a single xylanase crystal resulted in orbit with none in the ground controls. The apo-PPAR-γ LBD displayed the best visually determined crystals in the microgravity case. X-ray analysis was not conducted on any of these and would be difficult to interpret if it had due to the variability of temperature and acceleration conditions over the experiment. The experience clearly demonstrated the need for environmental control of the crystallization experiments.

Some macromolecules have a propensity to form disordered aggregates and present a good candidate 'use case' for assessing any impact of the reduced convection characteristics of microgravity crystallization. Examples of this were tested by growing crystals of an anti-polyQ antigen-binding fragment, MW1 Fab, and attempting to grow crystals of human huntingtin exon 1-thioredoxin (TRX) fusion proteins (HD-16Q, HD-25Q, HD-39Q and HD-46Q) GFP-huntingtin and the Fab from an MW1-related antibody called 3B5H10 [79]. The experiment was conducted in the High-Density Protein Crystal Growth (HDCPG) hardware that used a vapour diffusion method of crystallization. The samples were due to be launched to the ISS on a Dragon spacecraft. The launch in 2014 was rescheduled multiple times and most notably on 12 March was moved to 30 March or 2 April. A further delay occurred due to range issues with the new launch dates being either 14 April or a backup date on 18 April. The mission ultimately launched on 18 April but required repeated reloading of fresh samples for each delay. The crystals were grown at 4°C and returned on the Dragon spacecraft parachuting into the Pacific after approximately 6 months of crystallization in microgravity. Control experiments were conducted in the

same apparatus on the ground, even including the delays between preparation and activation. We give the dates in this case specifically to illustrate the challenges of undertaking microgravity experiments. Microgravity crystals were on average larger and fewer in number in each crystallisation well. Interestingly, additional crystals grew on the ground in the microgravity apparatus after it was returned to earth. These additional crystals had the best diffraction quality (1.59 Å) and were characterized in a different space group, $P2_1$, compared to the $P2_12_12_1$ of the other samples. Between the well-controlled ground and flight samples, the ground-grown crystals diffracted to a maximum resolution of 1.98 Å and the microgravity ones to 2.47 Å. The average resolution in each case was 2.58 and 2.73 Å with standard deviations of 0.07 and 0.51 Å for the microgravity and ground-grown crystals respectively. While the highest quality data was from a single ground crystal, overall, the microgravity-grown crystals were remarkably consistent in the quality achieved with the population showing generally improved quality over the ground-grown crystals. Data were collected at cryogenic temperatures and the mosaicity was analysed but presumably, the best values were masked by the instrument resolution function given the experimental setup used. Two different space groups were seen in microgravity and ground grown crystals. The best diffraction to low resolution, 3.2 and 4.05 Å for microgravity and ground respectively. The experiment used multiple conditions for the crystallization of each protein studied and successfully produced crystals of the one protein that had already been crystallized on the ground. It did not produce crystals of the other targets that had proved recalcitrant to crystallization on the ground but represents one of the few times crystallization screening rather than optimization has been carried out in microgravity. No structural studies are reported on the $P2_1$ samples formed on the ground in the experiments that were flown. It is not clear if these crystals are from the full-length native sample, if the protein had been proteolytically attacked, or if they formed from aggregates thought to be excluded due to diffusive-only fluid flow on orbit. Another facility was launched on this mission, the Granada Crystallization Box [60]. This consists of a sample containing column separated from a precipitate solution by a gel fuse through which the precipitant diffuses over time. Three different studies were due to be performed in this apparatus but because of launch delays and the time-delayed activation which could not be paused once samples were prepared, the experiments performed were sub-optimal with some reduced in scope due to sample consumption over several reloads.

Not all post Space Shuttle experimentation has been done on space stations. One example involved experiments conducted on a miniature satellite system called CubeSat on the model protein lysozyme. The study illustrated many of the undesirable features associated with crystallization on a spacecraft, in this case, a rocket that returned the sample to the ground without orbiting. Careful monitoring of the environment and protein concentration in the crystallization cell during crystal growth and X-ray diffraction analysis of the resulting crystals was carried out [80]. The authors do not provide specific details on the launch but showed that the experiment experienced a moderate increase in acceleration, peaking at 6.8 g on launch during the protein mixing period. They note that on launch temperatures can reach more than 35 °C and that they may descend to −45 °C when the rocket has reached altitude. To mitigate this effect, a simple but elegant method was developed with polymer ice packs combined with a heater. The temperature control appears to have spanned a region where lysozyme is particularly temperature-sensitive [81]. It is difficult to comment on the resulting X-ray diffraction analysis due to uncertainties in the

crystal growth regime and the subsequent crystal cryocooling step damaging long-range order. The study is notable for the demonstration of the practical difficulties of translating a ground-based experiment into a microgravity environment.

The majority of microgravity crystallization experiments conducted are to produce crystals that have improved diffraction properties for structural studies that then take place on the ground. However, biological macromolecules are also directly used for pharmaceutical purposes, most famously insulin micro-crystal preparations for diabetics. Another example are monoclonal antibody (mAb) therapeutics being used to treat oncological, cardiovascular, metabolic, and neurological diseases and disorders. Pembrolizumab (Keytruda®) is a humanized mAb approved for several cancer therapies. A crystalline suspension would improve the quality of care and allow treatment by subcutaneous injection over time-consuming intravenous infusions. Understanding crystallization is critical for such processes and microgravity has been used to identify key variables that drive the crystallization process [82] building on studies on the Space Shuttle Orbiter with interferon [83] and insulin [46]. Pembrolizumab crystals were grown with the batch method launched to the ISS and returned on a SpaceX Dragon capsule in 2017. Particle size analysis on ground and microgravity grown samples showed that the ground-grown crystals had a bimodal size distribution while the ones grown on orbit had a monomodal distribution and lower overall viscosity, important for dosing purposes. The crystals grown in orbit showed uniform sedimentation over time. They were more suitable from a pharmaceutical perspective but the experiment identified sedimentation and temperature gradients as parameters that could also be manipulated on the ground via rotational growth reactors or stirring to improve the overall outcome.

Of note in the post Space Shuttle Era, was the shift from studies designed to understand how to best use the environment to those that made use of it to understand biological mechanism in systems of wider biological interest. Even so, while resolution enhancements have been seen in many cases, it is hard to find many examples where the environment has developed new biological information and previous lessons may have been lost.

2. Design of experiments in a spacecraft

2.1. Physically different from the ground

A spacecraft is a self-contained system that has limitations not applicable to an experiment in a laboratory on earth. These limitations can be divided by the key elements of the mission, i.e. launch, on-orbit operations, and return to earth. Experiments have to be loaded onto the transport going to orbit well in advance of the planned launch window. The initial launch window is the period of time where the capabilities of the launch system match with the orbital mechanics necessary to reach the space station. For missions to the ISS and also those that may not have to rendezvous in orbit, the window is also constrained by other aerospace operations that may be underway at the launch site. This launch window dictates the earliest time the mission will launch, but not necessarily the actual time. Unanticipated delays due to mechanical, electrical, or natural elements such as the weather, etc. may close the time-frame or dictate considerable delays until the next launch window or flight opportunity occurs. During the launch window, the sample sits in the vehicle.

The transport vehicle itself has limited power which may constrain temperature control. Any experiment has to be designed with potential delays in mind and potential issues with temperature fluctuations.

Once launched there is a delay until a space platform is reached and significant disturbances in the acceleration environment can occur due to vehicle manoeuvring and docking. Once docked, more disturbances can occur due to the transport of any material to the platform. These build in delays to the ideal time that an experiment can be initiated. The environment of a space platform is a closed one. Chemicals and materials that may be acceptable on the ground, could build up in orbit and pose health risks to the crew if the platform is manned. Similarly, if chemicals leaked or the apparatus shattered, the hazardous material does not fall to the floor. These hazards could easily get in an astronaut's eye or be pulled into delicate instrumentation by airflow from the platform's cooling fans. Temperature control for the experiment is also challenging – forced air, fluid, or temperature-controlled plates are sometimes required.

The acceleration environment of orbiting spacecraft was not experimentally characterized during the initial studies of protein crystallization on orbit. The work of fans, compressors, and other equipment contributes micro-accelerations on the level of 10^{-6}–10^{-3} g [84]. These manifest as forces that can be transmitted through the entire space platform. Our work first demonstrated the impact of perturbations in the environment illustrating the impact of astronaut exercise on crystal growth spurts [38]. By comparing missions dedicated to maintaining a microgravity environment and those that required significant in orbit manoeuvring, it is clear that maintaining a quiet environment is critical [85]. The ISS is also a vehicle, which means it has an orientation system and requires occasional orbital corrections. Shifts in orientation impart accelerations of 10^{-3}–10^{-2} g and orbital correction can be more severe with accelerations of 10^{-2}–10^{-1} g [84]. The size of the ISS, compared with e.g. a satellite or the Space Shuttle Orbiter, also limits the reduction in acceleration achievable with the forces increasing dependent on the experiment distance from the ISS centre of mass.

An unexpected surprise was the observation of Marangoni convection during vapour diffusion crystallization [37] although obvious in retrospect. The most common crystallization method on the ground is that of vapour diffusion where the macromolecular sample is kept in a drop of buffer and precipitant solution with a vapour pathway to a reservoir of more concentrated precipitant solution. Over time, the drop containing the protein sample loses some of its water slowly increasing the concentration of protein and precipitant and driving the system to a region where crystallization is favoured. In microgravity, acceleration-based forces are reduced and other forces, otherwise masked on the ground, become apparent. In the vapour diffusion case, the motion of growing crystals was seen in microgravity due to surface tension differences between the part of the drop closest to the precipitant reservoir and the edge of the drop furthest from it (see Figure 3). Interestingly, while this drove crystal motion, it also resulted in increased crystal volume presumably due to bringing more sample to the growing crystal surface and keeping the crystals suspended in solution. Some experimental hardware has tried to reduce this Marangoni effect by using reservoirs with porous materials that conform to the initial shape of the drop. But, as liquid is removed, the geometry changes over time, and forces are invariably introduced. Such Marangoni convection is masked on the ground and not observed in shorter time periods or acceleration levels achieved by parabolic flights [86].

Once an experiment has been completed the samples are returned to the ground for evaluation. Crystallization experiments have an optimum duration, beyond which there is the possibility that the crystals may degrade through various processes. For experiments conducted on an orbital platform, the return of samples also has a time element. Return by the same capsule is usually not delayed too much, however, if another mission has to be flown to retrieve the sample, it is also subject to all the launch delay possibilities that the original launch could be affected by. Unfortunately, these practices preclude experiments that are sensitive to timing. Once an experiment is completed, there are few if any options to mount and preserve crystals or even to store them at a precisely controlled temperature storage comparable to those in the laboratory on the ground.

Now that the Space Shuttle Orbiter is no longer available, the return of samples to the ground relies on capsules entering the earth's atmosphere. These capsules are slowed by atmospheric drag, and then by deployment parachutes to land in the ocean or on the ground. In some cases, rockets may be used in the final stage to slow landing on the ground. This imparts significant acceleration forces, up to ~ 9 g, and typically returns the capsule to a location distant from support laboratories. Recovery of the capsule from the ocean or land delays the time that samples are under active temperature control.

2.2. Choice of type of experiment

The use of microgravity for crystallization is not recommended as a routine part of the structural determination process. There are significant practical limitations and delays, and many additional potential failure points. With that stated, we lay out some of the practical considerations that would enable the successful use of a microgravity environment, and in the section that follows, ideal cases are presented where microgravity provides an environment that yields proven benefit.

2.3. Considerations for preparation, storage, and activation

Before an experiment can be launched to the ISS there are unique biohazard and materials' safety requirements to satisfy due to the closed environment in orbit. Each aspect has to be assessed before launch. Biohazard considerations classify samples into five categories (0–4) based on Bio-Safety Levels (BSL) common across many countries [87]. Only BSL-1 and -2 samples are allowed on orbit. BSL-1 are well characterized and not known to cause disease in healthy adults. A single layer of containment is required. BSL-2 samples, which are agents known to be associated with human disease but are not life-threatening, are divided into two classes depending on the infectious dose. For infectious doses that are high, with moderate health risk, two levels of containment are required. For infectious doses that are low, with high health risk, three levels are required. Materials' safety requirements involve both the hardware and the solutions used during the experiment. Guidance from the flight agencies around this area is opaque in the authors' opinion. A safety review is held for each launch to the ISS, and each mission is considered independently – meeting safety requirements for one mission does not guarantee meeting those for the next. Materials are classified according to toxicity level and material required for the safety review is submitted

a month in advance. The assessment of toxic hazards for a spacecraft is different from a laboratory on the ground as in addition to the toxicology hazard, it has to consider potential modes of exposure (liquids do not fall to the ground), and the rate of removal in a closed system. To illustrate the differences between a laboratory on the ground and in space, a salt solution, NaCl, with greater than 1M concentration is rated at a toxicology level 1 as it can produce transient, mild eye irritation in some individuals. Toxicity levels range from 0 to 4 and toxicity levels higher than 1 are not flown in the current crystallization apparatus on the ISS.

Another aspect of experiments on the ISS is materials testing. It is required to test the solutions used for crystallization with the materials that make up the apparatus, especially any containment or seals. This has to be conducted for a period equal to or longer than the maximum duration of the planned mission. This adds a significant time delay into any experimental protocol that is inconsistent with many of the iterative processes in the laboratory precluding the routine and efficient use of microgravity as a tool for crystallization.

We do not advocate the removal of these safety requirements but merely note them to illustrate some of the more unique factors that must be considered when conducting experiments in a self-contained microgravity environment.

Key elements of microgravity crystallization experiments are storage before launch, activation, and if necessary, deactivation before the return to the ground. Astronaut time is very limited, so ideally this process should be automatic. Two approaches have been used, passive activation through a time delay, and active methods allowing sample and precipitant solution to come into contact, either directly or by a vapour pathway. Our previous review contains details on many of the methods used to do this [1].

Passive activation has used gel fuses so that the experiment is activated when the precipitant diffuses into the sample solution and crystallization proceeds. This provides the easiest operational mode and has been quite successful in a number of apparatus, e.g. the DCAM [50], the GCB [60], and the JAXA-PCG apparatus [9] (based on the GCB). The diffusion time can be designed based on the length of the gel pathway and the makeup of the gel. A disadvantage is that once the instrument is loaded with sample and precipitant, there is no way to prevent activation, e.g. if there is a significant launch delay, crystallization will be initiated on the ground. It is recommended to prepare at least twice the material needed for the mission, if not more, to mitigate against such a delay, and provide the material for a simultaneous control experiment on the ground if a delay does not occur.

An activation mechanism allows the experimental samples to be stored until they reach a microgravity environment and while requiring an activation step, is more suitable for samples that are only available in small volumes and/or expensive to produce. There are many physical mechanisms for activating experiments which involve the motion of a valve or removal of seals to enable a vapour pathway. Examples of these include those used in the APCF [88], PCAM [36], VDA [27], and the HDPCG [54] apparatuses. Another method of activation is thermal where the experiment is frozen then thawed on-orbit to activate. This requires active temperature control on the pad and during launch to orbit. An early example of this is the EGN [49] which was developed for joint US/Russia efforts on the Mir space station. Samples are loaded into tygon tubes, sealed, and rapidly frozen then placed in the liquid nitrogen in the Dewar. The nitrogen is allowed to boil off on

orbit warming the system by ~ 100 K per day until it reaches ambient temperature. This was able to fly many samples but care needs to be taken in the loading profile. Different solutions, e.g. of sample and precipitant, can melt at different rates and air bubbles can result [51]. More recently, in some of our own studies, samples have been loaded in rectangular profile quartz glass capillaries, frozen on the ground, then thawed and observed microscopically. The experiment studied the growth rate of crystals but unfortunately, the freeze thaw cycle also generated air gaps and, in some cases, cracks in the capillary were seen.

2.4. On-orbit issues

There are unique considerations to experiments as they are conducted in microgravity. These include the acceleration environment, but there are considerations beyond the effective reduction of acceleration forces that unmask other acceleration effects collectively known as g-jitter. Another effect masked on the ground is flow induced by differences in surface tension, referred to as Marangoni convection. Finally, temperature control can be very different from the ground due to the reduction of convective effects.

2.4.1. g-jitter

In the Space Shuttle era, missions dedicated to microgravity were shown to produce improvements in significantly more samples than those that were not dedicated to maintaining a microgravity environment [85]. The International Space Station is dedicated to microgravity experiments with a measured quasi-steady acceleration on the order of 10^{-5} g [89]. However, as noted above, the acceleration sensed increases as distance increases by 10^{-7}g for every 1 m from the centre of mass. The acceleration is also not a constant vector with forces arising from orbit correction, maintaining orientation, fans, compressors and other equipment, the activities of the crew, and other smaller and more transient influences [84].

The influence of g-jitter can be seen in Figure 2 where accelerometers were on board and the growth of lysozyme crystals was monitored with CCD video observation over time [38]. Crystals moved in the growth chamber when the Orbiter's attitude was adjusted by several firings of the Vernier Reaction Control System. There were clear differences in the acceleration environment when astronauts were awake, Figure 2(a). Initially, crystals grew rapidly and then the growth rate slowed. However, several peaks in the growth rate were seen over the experiment duration [38]. A careful analysis of the flight log and accelerometer data showed that crew exercise occurred with prolonged periods of up to 500 μg directly preceding these growth spurts. Acceleration vectors can then significantly complicate the analysis of the outcome.

The spectrum of acceleration forces on the ISS is complex and while some of the same forces are common in laboratories on the earth, they are masked by the magnitude of the gravitational force. The acceleration environment on the ISS is well characterized and its influence on convection is modelled [90]. Low-frequency g-jitter has a higher impact on convection than higher frequencies [89]. Fortunately, the acceleration environment is routinely characterized but it adds an additional element to the analysis of outcome.

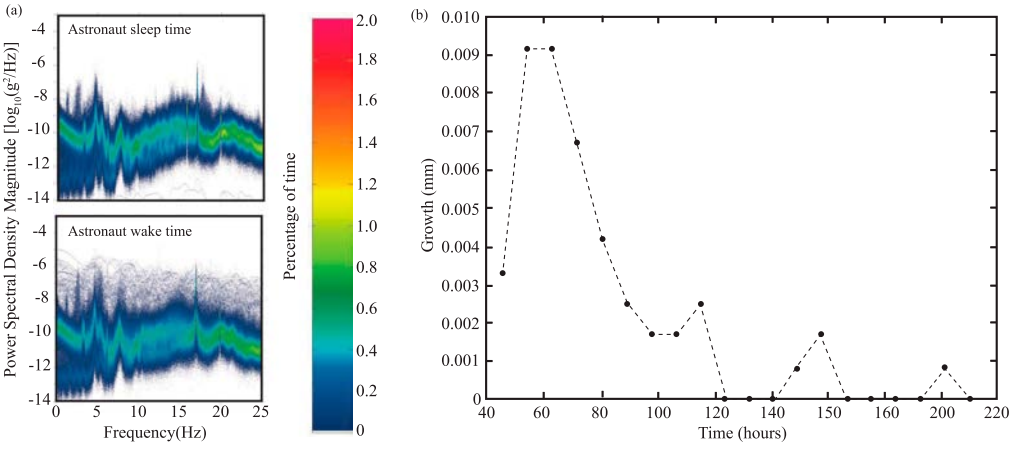

Figure 2. The acceleration environment on the Space Shuttle Orbiter showing (a) the magnitude of the Power Spectral Density during the sleep and wake cycles for the crew and (b) spurts in crystal growth correlate with the times when astronauts exercised and produced their significant g-jitter accelerations [38].

2.4.2. Marangoni convection

Many of the techniques used to crystallize samples on the ground were used to develop apparatus for microgravity crystallization. The major crystallization technique used on the ground is vapour diffusion. As described above, in this method the solution containing the sample to be crystallized is mixed with the precipitant solution and placed in a drop. Another drop containing only precipitant solution is linked to the first drop by a vapour pathway and the concentration difference causes water vapour to be lost from the drop with the sample. The concentration of both the sample and the precipitant then increases, impacting the solubility and shifting the conditions toward nucleation and growth. There have been several ways to keep the vapour path closed until the experiment reaches orbit and then activate the experiment. These include valves, compressible seals, and the alignment of two chambers so they are open to each other. For all of them, the drop has a semispherical shape. On the ground the drop can sit on a surface and as crystals grow, they can sediment to the bottom surface, or the drop can hang from a coverslip as an example, and as crystals grow the crystals sediment to the bottom of the drop.

In a microgravity environment, sedimentation is reduced and other forces become apparent. Marangoni convection describes the convective flow that is induced due to surface tension differences in the drop because of the variation in distance between the precipitant solution reservoir and the drop and the slight difference in concentrations that result. While well known in the fluid physics community, this effect was a surprise for the crystallization community. We were the first to observe this in a study on the protein apocrustacyanin, a pigment that is involved in the blue to red colour change when a lobster is cooked. Figure 3(a) shows images of three crystals growing in the APCF. They were imaged over time and the centroids of position plotted in Figure 3(b). Individual images were recorded several hours apart so that while the positions are known, the sequence of those positions is not and we cannot calculate the rotational velocity of the motion.

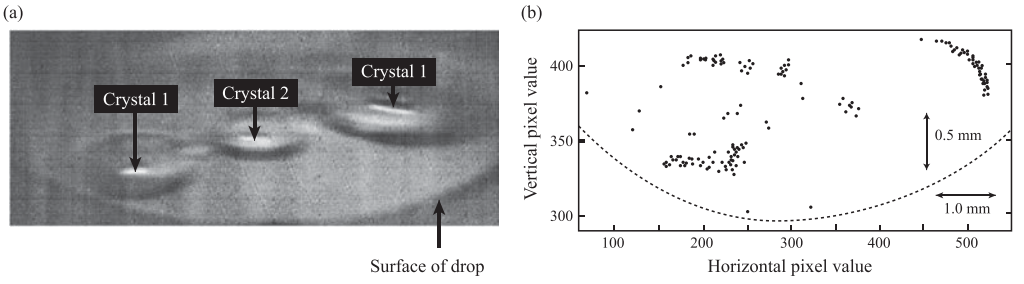

Figure 3. Observation of Marangoni convection in the APCF flown on the Space Shuttle IML-2 mission. A video still is shown in (a) with a plot of centroid positions for three crystals seen in (b). Full details are available elsewhere [37].

However, the positional shape is striking, reproducing the expected Marangoni flow in this situation.

In the original work on this Marangoni convection observation [37] we noted the halos that appeared around the crystals and how the crystals' motion was undesirable given the hypothesis that a reduction in convective flow could lead to improved physical perfection. Observation of several experiments with this type of illumination suggests that the halo initially thought to be the presence of a depletion zone could just be an optical effect of the illumination on the small rod-like crystals. The presumably detrimental convective flow may actually have a benefit in keeping the crystals suspended and flowing through the medium bringing fresh material to the crystal face. While it is detrimental to physical perfection, it may well be favourable for enhancing volume. At the time of the experiment where crystal volumes of several hundred μm were typically needed for a synchrotron study, this may explain some of the positive results from microgravity-grown samples where Marangoni convection was present. We are unaware of any well-constructed physical studies of crystal quality from samples grown by the vapour diffusion technique and can only hypothesize that this is not the ideal method of growth to maximize all the benefits that a microgravity environment can provide. It should be noted that synchrotron experiments on crystals as small as a few μm are possible today and with XFEL sources, some studies have used crystals where the size is measured in terms of unit cells [3].

2.4.3. Temperature control

Accurate temperature control is possible on the ISS and has been achieved for different instrumentation using Peltier devices, forced airflow, or fluid-based cooling. In Figure 4(a) the temperature was measured for one of our crystallization experiments in the Light Microscopy Module (LMM). In this experiment, we imaged the growth rate of crystals over time. The experiment was initiated simply by thawing pre-mixed protein and precipitant solutions and then transferred to the LMM where the temperature was controlled through a water-cooled plate. Temperature control over the initial observation time was maintained at 21.5°C with only small fluctuations. The actual temperature was determined in discussion with the payload planners at NASA to remain within the power budget for the ISS. As crystals grew, observation times became periodic. The LMM was powered down between experiments to reduce power demands and reactivated for observations. The temperature excursions are significant with fluctuations between 20°C and 26°C. The

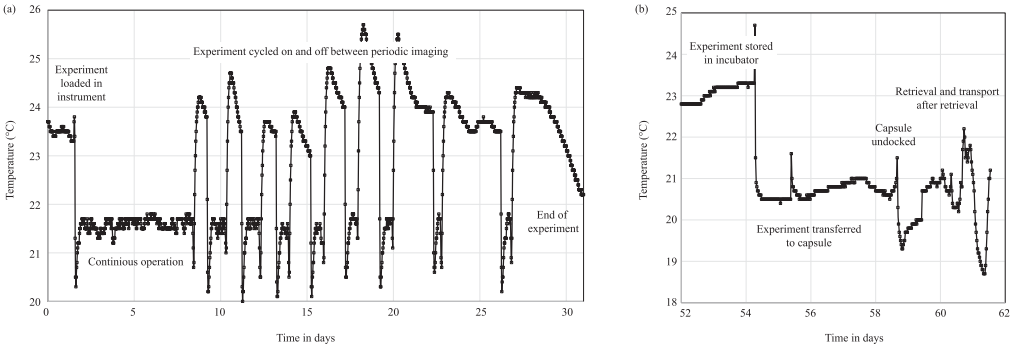

Figure 4. Temperature profile for (a) crystallization experiments conducted in the LMM on the ISS and (b) the storage and return to the ground of the same experiments from our studies.

experiment ran for 30 days and the crystal samples were transferred to an onboard incubator at 23°C for storage until they could be returned to the ground. Figure 4(b) shows the temperature profile for the samples as they are removed from the incubator and transferred to a Dragon capsule for return to earth. The capsule temperature is about 20.5°C and drops as it is undocked and returned to the ground. For capsule retrieval and transport, there is considerable fluctuation in temperature as no active temperature control is used. Temperature control can be critical for successful crystallization experiments and each instrument is different. For example, the APCF that flew on the Space Shuttle Orbiter and ISS had active temperature control from the point where samples were loaded through to their return to the investigator. Other systems, e.g. the Enhanced Gaseous Nitrogen Dewar rely on thawing to ambient conditions and are then subject to variation in those conditions. Another example is the PCAM instrument. In our previous review, we provided data from this system flown on the Space Shuttle Orbiter. The instrument had one wall that was maintained at the desired temperature. For our case, it was 22°C. Three sets of cylinders are carried in the instrument, one next to the wall and the others at successive distances from it. Those next to the temperature-controlled wall remained within 0.5°C of the setpoint, those in the middle within 1.0°C, and those furthest away, within 1.5°C.

2.5. Return of samples

To maximize the benefit of crystals grown in microgravity the most brilliant X-ray or neutron sources need to be used on the ground. There were considerations made to build an X-ray facility onboard the ISS that could harvest crystals, place them in front of an X-ray beam, and record diffraction data [91] due to perceived disadvantages with returning samples to the ground. The physical improvement in quality seen from crystals grown on orbit and then examined on the ground suggests that any damage due to the return of samples can be mitigated. During the Space Shuttle Orbiter Era samples were returned with the Orbiter landing on a runway. As the Orbiter left orbit, the forces gradually increased to a maximum of less than 2 g for approximately 10 min as the Orbiter entered the atmosphere and reduced speed. Peak acceleration occurs during the approach manoeuvres with

transient spikes above and below 1 g over a couple of minutes. Capsules reentered the atmosphere in a similar manner but did not have the ability to land on a runway and used a parachute during the last stages of descent either onto land or into the ocean. The deceleration due to a parachute was significant and forces up to 9 g have been noted. The disadvantages of returning samples to the ground seem more than compensated by X-ray sources on the ground providing orders of magnitude more brilliance, as well as the ability to tune the wavelength, and the capacity to use large detectors.

3. Analysis of outcome

Beyond the visual observations confirming that crystals have resulted, as well as their sizes including the range of sizes, diffraction analysis is necessary when the crystals are grown for structural analysis as visual appearances are not necessarily linked with the diffraction properties. The structural analysis of crystals grown in microgravity falls into two main categories. Firstly, there is the careful analysis of the impact of the environment or developments in apparatus used in that environment, and secondly, there is the use of microgravity as a medium to extend the resolution of any structural model that has been achieved on the earth. We make some observations on the analysis for these outcomes.

3.1. To cryocool the crystal or not?

Cryocooling is a critical technique that has had a dramatic impact on the success of structural biology. While originally used to help mitigate the effect of radiation damage from X-ray beams, it has eased the workflow in the structural biology process enabling routine preservation, shipping of crystals, and remote data collection. However, it can also limit biologically meaningful conformations [92,93] and produce structural artefacts [94,95]. Critically, for crystals of high-physical perfection, cryocooling destroys much of the long-range order resulting from growth in microgravity [48]. An enhanced resolution has been reported by many investigators for crystals grown in microgravity and then cryopreserved for data collection. However, our studies on insulin crystals grown both in microgravity and on the ground show that cryocooling is extremely detrimental to macromolecular crystal quality [48]; microgravity grown crystals comprised of a single well-ordered domain and exhibited an average mosaicity of 0.005°. On cryocooling there was a 43-fold increase in this mosaicity, broadening the reflections but not impacting the single domain. The cryocooling conditions were already extensively optimized and resulted in diffraction data to 1.0 Å for the microgravity-grown crystals. The ground-grown crystals in comparison displayed an average mosaicity of 0.031° which increased eight-fold on cooling. The ground-grown crystals contained multiple domains within the crystal which separated from each other after cryocooling. The ground crystals diffracted at best to 2.0 Å. For comparison the average mosaicities of the microgravity and ground grown crystals after cryocooling were 0.217° and 0.246° respectively. Cryocooling almost completely destroyed one aspect of the advantage of growing a crystal in a reduced convection environment provided from microgravity. The enhancement in resolution was presumably a result of the diffracting volume with microgravity grown crystals averaging more than 40 times the volume of the ground-grown controls.

Cryocooling can take advantage of the larger crystal volume that results from microgravity growth but is a poor choice of analysis to harness the maximum benefit that microgravity-based crystallization has been shown to produce.

3.2. Choice of instrumentation

To make the optimal use of microgravity-grown crystals consideration has to be made to matching the beamline and detector to the quality of the sample [96] and consideration has to be given to the experimental protocol of diffraction measurements [97]. Our studies on microgravity-grown lysozyme crystals built on the hypothesis of highly perfect crystals resulting [2] and knowledge of the beamline parameters used [98]. To make full use of the enhancements in X-ray diffraction signal-to-noise that result from a greatly reduced crystal mosaicity, the X-ray beam geometric properties have to be matched to the crystal sample, and also the crystal's rotation range has to be matched to the crystal mosaicity. For the perfection achieved this results in using crystal oscillations of fractions of a degree. This is challenging for the goniometer instrumentation and also time-consuming due to the finite readout time of the detector. Experiments to probe a statistical number of reflections took many hours. Since our original experiments, there have been dramatic developments in source beamline instrumentation eclipsing developments in spaceflight. Firstly, the development of pixel area detectors now allows continuous crystal rotation and thereby almost continuous readout of the diffraction data. The readout dead time of the detector is no longer a significant issue and the mechanics of sample motion is greatly simplified. Diffraction data can be collected maximizing the impact of reduced mosacity. Secondly, the brilliance of synchrotron sources has improved so that the same or increased flux can be incident on a smaller area of the crystal sample.

We have mentioned XFELs in the text and microgravity grown samples are well matched to these sources with their high-quality and narrow distribution of crystal volumes. XFELs are highly brilliant sources with short exposure times but doses orders of magnitude above synchrotron doses within that time [99]. Data collection is usually via a stream of crystals flowing through the beam or crystals sequentially rastered through the beam. Images are typically 'stills' due to the short exposure time and effective motion of the crystal during that time. For this reason, crystals with a small mosaicity have a higher probability of providing full reflections rather than partial ones. To build up a complete data set many images are recorded and the data treated to produce a complete set of reflections. The narrower distribution of crystal volumes and reduced mosaicity benefits this analysis and reduces the sample size needed.

The rapid exposure time for XFELs is used to study dynamic biological mechanism. Light or flowing a substrate into the crystals is used to initiate this mechanism and again, a series of observations used to build up the complete structural data set of various steps in the molecular function i.e. its chemical mechanism. The reduced distribution of crystal volume also helps this case with subsequent crystals reacting at similar rates due to the illuminated volume or transport distance within the crystal.

To the best of our knowledge, no experiments have made use of microgravity-grown crystals with XFELs whereas many have used synchrotrons [100]. XFELs are a new X-ray source, there are far fewer facilities than synchrotrons (less than ten), and each XFEL

accommodates far fewer users. As the number of sources grows and capabilities for simultaneous use of stations become available, this avenue can be explored. Synchrotron and XFEL beamlines suitable for crystallography are summarized elsewhere [101,102].

3.3. Comparing crystals, like with like

In describing the history of microgravity experiments a factor that stands out is the difference in the analysis carried out for the microgravity and the ground grown samples. If one is trying to achieve the highest diffraction resolution possible, then the combination of the use of microgravity with the best analysis techniques on the ground is justified. This approach can then answer a molecular structural question but it is not an analysis strictly comparing the two crystal growth environments. On the other hand, if the study is designed to compare and contrast the crystal growth environments, the analysis must be conducted in an identical manner, or if that is not possible on the first occasion it must be repeated until identical conditions can be obtained. The point being that there is always a risk of expectation bias due to the amount of effort expended to conduct an experiment in microgravity and the search for a result that may justify that effort.

4. A prescription for effective use

We have found that there can be clear and common benefits that arise from macromolecular crystallization in microgravity. Crystals grow with a larger volume, reduced mosaicity, and more isotropic in size. These benefits drive the effective use appropriately taking advantage of the improved perfection, or using the crystal improved volume for techniques that benefit from it, or harnessing the tighter distribution of crystal volumes. Additionally, each advantage can build on the other for both providing new structural knowledge and a better understanding of how to make advantageous use of the microgravity environment.

We have also identified major considerations that would need to be addressed to optimize the use of microgravity. Ideally, there would be rapid, frequent, and routine access to space and the return of samples to the ground. Access needs to be temperature-controlled and acceleration forces minimized on the return. Facilities used for crystallization should allow activation and deactivation and provide growth geometries that minimize or eliminate Marangoni effects. The facilities should offer levels of containment that can accommodate a wider range of chemicals involved with crystallization screening on the ground. For diagnostic and production processes there are differing requirements – detailed observation capabilities competing against large numbers of experiments respectively. A paradigm shift is that the experiment does not end when the crystals are returned to the ground. Good temperature control from launch to analysis is needed and the analysis should be enabled as rapidly as possible once the samples are returned. These considerations would optimize the outcomes but even without all of them for many studies, enhancements have been seen.

In conducting an experiment in microgravity there can be an investigator bias that some improvement is expected, especially given the effort that has to be expended. Any improvement that results should be clear and convincing to make a valid case for its use.

By understanding the nature of the environment and how it can influence the process a hypothesis can be constructed in advance of the study and that hypothesis tested. Psychologists have a term for the reverse – hypothesizing after the results are known [103]. There is always the danger of trying to prove a hypothesis rather than test one.

Understanding the physical improvements that have occurred and the fluid physics mechanism behind those also allows us to better understand the analysis techniques used. In many cases, the returned-to-earth-samples' analysis techniques themselves can significantly reduce the advantages that microgravity crystallization provided. While we have described many historical studies, we argue that no single study has yet harnessed the full benefits of microgravity-grown crystals properly, as we have defined it in this review article. Ignoring the major considerations above, which require efforts in developing instrumentation and procedures we can define prescriptions to exploit the outcomes that have been seen. We do this for the categories of physical perfection of crystals; using the improved crystal volume; studying the crystallization process itself; obtaining new structural knowledge; and comparing the environments. We summarize each of these in turn below.

4.1. Increased physical perfection of a crystal

The typical minimum mosaicity recorded for a crystal sample by current standard diffraction data processing software is on the order of $\sim$0.1-0.2° which simply represents the limits due to the geometrical and spectral parameters of the typical beamline. When a crystal is cryocooled, this estimate by the software, not disentangling the various factors of crystal and beam, is reasonable as the cryocooling destroys the initial perfection such that the crystal is now the dominant factor. When crystals are X-rayed at ambient temperatures, there is no cryocooling-induced increase but then the instrument contribution is significantly greater than the quality of the crystal. So, the mosaicity estimate is misleading. To maximize the signal-to-noise the beam geometrical and spectral parameters need to be minimized to match the intrinsic sample quality. In many cases the available monochromator cannot be improved upon, however, it may be possible to defocus the convergent-beam to be a parallel beam. The instrument contribution to the rocking curve can be calculated [98].

To maximize the diffraction signal-to-noise, reflections should be collected over their rocking width and ideally no more. This requires matching the oscillation to the mosaicity and collecting in a fine-slicing mode [97]. Since the initial experiments establishing the physical quality of macromolecular crystals, pixel area detectors have become available for macromolecular crystallography [104]. These have high efficiency, no noise, rapid readout, high dynamic range, and low point spread. They provide the perfect detector to take advantage of intrinsically high-quality crystals.

To probe the physical perfection of samples cryocooling must be avoided, the instrument smearing contribution should be minimized and an optimal detector used. Some studies, including our own, have explored a crystal using X-ray diffraction topography imaging, each reflection shows the diffracting area satisfying the Bragg equation within the crystal.

Figure 1 illustrates two studies in this area where specialized instrumentation was used to directly compare the physical quality of crystals in the most controlled way possible and

in the case of insulin, in a statistically valid manner i.e. involving a large number of crystals for standard 'student t-test' type of evaluations including 'the null hypothesis'.

4.2. Improved crystal volume

Neutron crystallography is well suited to fully understand ordered waters i.e. as neutral or hydroxyls or hydronium ions, and the protonation states of ionizable amino acid residues, often the key to enzymatic mechanisms. It also allows the use of room temperature ('physiologically relevant') and causes no ionization damage. In practice though, neutron sources are many orders of magnitude weaker than synchrotron X-ray sources and the scattering power of the sample for neutrons is less. Consequently, despite a program on increasing the flux of neutron sources and the availability of sensitive detectors large volume crystals, compared with those used at X-ray synchrotron or XFEL facilities, are required for successful neutron studies. This makes for a clear application for microgravity crystals where volume enhancement occurs.

To enhance the neutron flux available a wider wavelength band is often used than typical for X-ray sources. This impacts systems where unit cell parameters, and instrumental limitations, could cause the overlap of diffraction reflections. In the context of the increased crystal sample volume, the improved physical perfection also contributes to success in reducing any spot-to-spot overlap.

A disadvantage of microgravity crystallization is the delay introduced to the process, both known and unintentional. In the case of neutron studies, this delay is not as critical. There are few, sometimes no, alternative experimental probes that could be used to determine an answer beyond neutron diffraction. Similarly, the need for cryocooling to minimize radiation damage is eliminated. Samples can be studied at temperatures closest to or at physiological temperatures which are increasingly emphasized by the biological structure research community.

Neutron studies with microgravity-grown crystals have been reported. Ho et al. [105] describe the neutron structure of monoclinic lysozyme crystals to 2.1 Å as a test of the environment to enable neutron studies. It was noted that the non-exchangeable hydrogen atoms could be resolved. Unfortunately, the data were not deposited in the protein data bank [106]. Similarly, inorganic pyrophosphatase from *Thermococcus theoreducens* was crystallized on the ISS using the counter diffusion method and neutron data collected to 2.3 Å. The structure was deposited in the protein data bank as PDB ID 5TY5 but no publications have so far resulted. A ground-grown crystal diffracted neutrons to 2.5 Å [107] but the difference in neutron sources used was significant. For the $\sim$176 neutron-derived structural models deposited to date, microgravity has had little application to neutron crystallography, despite its possible advantages; as far as we can tell from the literature and the PDB there are just those two above cases, one unpublished.

4.3. Studying the crystallization process

The major use for crystals is to conduct diffraction experiments that reveal the three-dimensional structure of the macromolecule making up the crystal. However, there has always been an interest in the use of crystallization for pharmaceutical dosing purposes. An example of this is interferon α-2b [83] and insulin [46]. In the interferon case,

microgravity-grown crystals showed a 2.4 times increase in length and width and remained stable in a free-flowing suspension for over two years [108].

Microgravity provides a diffusion-controlled growth environment with reduced sedimentation. For systems where crystal growth is controlled by transport rather than kinetic processes, microgravity may have a role in the production of crystals for pharmaceutical dosing. As mentioned previously, pembrolizumab crystals were grown in microgravity producing a monomodal distribution and lower overall viscosity, important for dosing purposes [82]. The crystals grown in orbit showed uniform sedimentation over time and were more suitable from a pharmaceutical perspective than those grown on the ground. The microgravity experiments identified sedimentation and temperature gradients as parameters that could also be manipulated on the ground via rotational growth reactors or stirring to improve the overall outcome.

4.4. Obtaining new structural knowledge

Developing new biological structural knowledge from microgravity crystal growth requires maximizing all the advantages that microgravity is known to provide. This includes the increased physical perfection, the increased volume, and if necessary, the more uniform population of sample sizes. To develop new structural knowledge the aim is to record the most complete diffraction data set as possible at the highest resolution while minimizing radiation damage.

For the X-ray approach, the samples should be returned as rapidly as possible once the growth is complete under temperature-controlled conditions. Data should be collected on a synchrotron source configured so that the geometrical and spectral divergence either match or are less than the crystal quality [96–98]. It is not recommended to cryocool the crystals so as to preserve the crystal perfection. A continuous rotation should be used where the readout time is equal to or less than the rotation equivalent to the angular rocking width. Low noise or noiseless pixel area detectors are recommended rather than earlier detector technologies. The crystal volume enhancements should be used so that the X-ray exposure dose is distributed across the entire crystal, not simply through one slice. If multiple crystals are available, a serial crystallography approach can be utilized. For crystals that diffract to high resolution, multiple data passes can be used with the detector at different distances and the low-resolution data collected first. This is also useful because, despite the advances that cryo-cooling has enabled, the technique can also mask biologically meaningful conformations [92,93], yield non-native structural artefacts [94,109], and may not be amenable to every system.

There are surprisingly few studies where new biological information can be attributed solely to the growth of crystals in microgravity compared to those on the ground. Certainly, resolution enhancements are stated in many cases, but for the most part, those enhancements have not yielded new knowledge. One example where microgravity crystallization has provided new information includes mistletoe lectin I in complex with the photohormone zeaton [110]. There was a notable difference between the microgravity and ground-grown control crystals although specific details of the X-ray analysis of the control samples are not provided. The structure has revealed a distinct single binding site within the two subunits, almost opposite to the active site region. The X-ray structure of the complex provided new information on parasite–host interactions.

The potential is clearly there as is the case of the dramatic mosaicity enhancements reported with insulin crystals [47] with microgravity samples having a mosaicity of 0.002°. A theoretically ideal X-ray diffraction data set for 180° of coverage, with the images matching the smallest oscillation angle, would have almost a million raw diffraction images. This is approximately three orders of magnitude larger than the largest typical synchrotron-based raw diffraction data sets but not outside of the range used for XFEL-based studies with single exposures on multiple crystals. With modern detectors and X-ray sources a dataset can be collected in less than 30 min but it does present a computationally challenging storage and analysis problem, but not insurmountable ones.

While we strongly advise against cryoprotection for microgravity-grown crystals, there are still small advantages to be had [48]. If cryoprotection is necessary, we recommend the inclusion of a small percentage of the cryoprotectant in the protein solution before crystallization. Cryoprotectants can sometimes bind to regions in the protein and when introduced after growth and before vitrification, can disrupt the crystal lattice [111]. We also recommend cooling methods that rapidly reduce the temperature [112] rather than standard plunge cooling.

An alternative approach is serial crystallography. In this approach, a slurry of crystals can be fired through an X-ray beam or deposited on a fixed target that is rapidly rastered through an X-ray beam [113]. Microgravity samples with improved volume and a tighter distribution of the overall volume make the analysis of these cases easier. In the slurry approach, the crystals are studied at ambient temperatures but could be prone to potential damage via the flow. In the fixed target approach crystals are typically cryoprotected.

In the neutron case, radiation damage is not a significant factor but matching the beam geometric and spectral divergence to the sample is. We recommend that the main advantage that is harnessed is the crystal volume and to maximize signal-to-noise and minimize impacts to the crystals post-growth, we strongly suggest growth in deuterated conditions which increases the overall scattering strength and considerably reduces incoherent scattering from hydrogens. We note that heavy water is allowed on manned space flights because in small quantities it is safe to drink.

4.5. Comparing crystallization environments

The comparison of crystallization environments can target any, all, or a combination of the categories above. However, for a valid comparison, the sample treatment, the X-ray source, detector, and diffraction data collection methods have to be carefully controlled. Many crystallization experiments record small improvements in the resulting diffraction resolution but used beamlines that have different brilliance, different types of detectors or are collected in a different manner. Also, true comparison can be hard if experiments are conducted at different times as experimental setups and capabilities change as well as sample shelf life might then have to be considered. In all cases, the instrument and experimental parameters have to be fully described.

4.6. Improving effective use

The use of microgravity for crystallization purposes poses significant challenges for the investigator and there can be a temptation to report results that while positive, do not

reflect an appropriate return on the effort extended. A study that demonstrates the efficacy of crystallization in microgravity needs to show results that are significantly better than the best experiment that could be conceivably done on the ground. As the access to space platforms becomes easier or at least with fewer burdens and fewer faults than currently, the benefits may become more time and cost-effective. This might be expected with the commercialization of access as long as there is a laboratory facility in orbit. There is a historical analogy for this. In the early days of synchrotron radiation, there was a transition from the parasitic use of high-energy physics research first-generation machines to the first dedicated synchrotron X-ray source 'SRS' at Daresbury UK – a 'second generation' source dedicated to providing synchrotron radiation for multiple research users. The initial design had a source size of 14.0 by 0.4 mm, suited to spectroscopists but not crystallographers. It took 5 years of operation before this was corrected with a 'high brightness lattice' and the source size became more suitable for protein crystal samples. The third generation of synchrotrons, defined by the introduction of insertion devices, with the first being the European Synchrotron Radiation Facility, greatly reduced source sizes. Even at ESRF, it took time to optimize the use of the insertion devices and develop optimized detectors. The development of space stations can also be considered generational. Excluding Skylab, which was produced from modified components of the Apollo program, there have arguably been two generations of manned space stations, Mir and the ISS. Synchrotron science moves faster than space science and we hope that the lessons learned to date, can be used to optimize scientific applications on future space facilities.

In our review in 2005, we reported on many fundamental studies that explored the microgravity environment and that were based on a strong theoretical background established by a research community on the ground supported by NASA. Historically NASA is an engineering-focused organization and priorities have shifted so that access to scientific facilities on the ISS are supported, but the basic research that enables the effective use and analysis is not. There has been a considerable loss of expertise in the scientific community in this area. Access to the ISS in the USA is made available for scientific experiments with resources primarily covering the flight of samples and instruments but not the preparation or analysis of those samples. While there has been a reduction in NASA research capabilities, other partners on the ISS are moving forward. There is clear evidence for the improvements microgravity can produce on macromolecular crystals but many practical barriers to the successful use of the environment.

5. Discussion and outlook for the future

From the experimental data available, crystallization in microgravity clearly demonstrates that the microgravity environment can enhance the spatial coherence length of the arrangement in three dimensions of macromolecular molecules improving the overall physical quality of crystals. That improvement in quality manifests itself in narrower X-ray diffraction rocking widths in the resulting Bragg reflections and thereby the diffraction spot sizes. Harnessing this to improve the diffraction data quality requires matching the experimental method and instrumentation used to such crystal quality. In many cases, after the return of crystals to earth, this has not been done effectively, and at the time of our original review in 2005, it was difficult to harness this approach routinely. Now, the

X-ray source and detector technology are available to do this. Modern rapid readout low-noise detectors allow the oscillation angle to be matched to the crystal quality (so-called fine phi slicing mode) and indeed, continuous rotation of a crystal with fast enough readout to enhance the overall signal-to-noise of the Bragg reflections. In terms of harnessing the ultimate crystal quality achieved, it is disappointing to see so many studies where the improved crystal quality is then reduced or even destroyed by cryocooling. Fortunately, driven by the interest in structural studies of systems nearer to physiological temperatures and developments in serial and XFEL-based crystallography, as well as with neutrons, there are an increasing number of studies being done without resorting to cryogenic treatments. The new fourth-generation synchrotron sources such as the ESRF Extremely Bright Source are also adopting serial (femtosecond) crystallography (SFX) approaches.

Another observation is that the volume of the resulting crystals invariably increases in microgravity, an observation that is not surprising given the reduction in sedimentation and access of the macromolecules in solution to all faces of the crystal for a longer time as it grows. Enhanced crystal volume can be harnessed for neutron diffraction where weak flux from neutron sources requires, compared to X-ray sources, large volume crystals to obtain measurable diffraction data in reasonable time periods and from larger molecular weight systems. Neutrons yield not only room temperature, damage-free structures but also complete protonation state details, as well as the orientation of bound waters. This is even at quite modest neutron diffraction resolutions. The same crystals are of more than sufficient size that a laboratory X-ray source, installed at the neutron facility support laboratory, can be used for the X-ray diffraction data collection. This can be a significant consideration where a neutron and a synchrotron source are not co-located. In the X-ray case, the larger volume can also be used to spread dose across the crystal thereby reducing radiation damage effects.

In terms of lessons learned and applied on the ground, an important one has been the industrial interest in the observation of more uniform crystal populations produced in space. This has provided information on how to achieve this on the ground and is useful for patient dosing purposes. This is also an indicator of the types of samples that could benefit from microgravity growth, namely decoupling kinetically controlled growth from diffusive transport control. An as yet untapped application for the uniform size distribution of crystal samples is in serial crystallography, either at XFELs or synchrotrons, where a smaller distribution of crystal volume aids the diffraction data analysis and increase overall quality and information content of the X-ray diffraction data.

Investigating protein crystal perfection required new methods, or rather the transfer of methods used to evaluate such as perfect silicon crystals. Thus protein crystals were evaluated both in real space by X-ray topography and in reciprocal space by rocking curves and reciprocal space mapping [114,115]. The evolution of adopting these methods and applying them took a decade or more and was prolonged by further factors. One was the need to assemble a statistically significant number of measured crystals. Another was the need to understand the fluid physics of microgravity such that vapour diffusion should be avoided and liquid–liquid diffusion should be used. Overall a key guide throughout was the hypothesis of the theoretical reflecting range of a perfect protein crystal. The lessons learned from crystal quality evaluation of crystals grown in microgravity have led to benchmarks of how to better undertake on-earth crystallization. Gels and ever-smaller liquid

volumes suppress convection and sedimentation which we discuss in our previous review [1]. The hypothesized perfect protein crystal is increasingly there for the harnessing for more precise protein crystal structures via the improved signal to noise of sharper rocking widths, especially benefitting high diffraction resolution. The X-ray laser offers room temperature, secondary damage-free diffraction measurements along with a very narrow beam divergence sufficient to harness that improved protein crystal perfection.

In our original review of Macromolecular Crystallization in Microgravity [1] we made several observations. The first was the lack of a predictive capability to determine which systems might benefit from crystal growth in microgravity. The second was that, despite many experiments, the fundamentals of the impact of microgravity on crystallization were still not completely understood. We also noted that many of the experiments conducted, with a few exceptions, were done in apparatus that built on methods designed for the ground and not designed around the unique characteristics of the microgravity environment. We also discussed cost. Advances in all these areas are occurring. Experiments based on the kinetics of crystallization, studying systems controlled by diffusive transport versus kinetic attachment process, are looking to develop predictive characteristics. The flow around growing crystals has been studied in microgravity, and the effect of impurity partitioning is being analysed. In terms of cost, the NASA/Air Force Cost Model is no longer publicly available but SpaceX commercially quote about $2000 per kg to fly a payload to orbit. This is between 5 and 10 times less expensive than the costs at the time of our original review where the only launch systems available were government vehicles. We have explored the link between crystallization and the resulting crystal perfection in general elsewhere [116]. The experimental evidence for the improvement of crystal quality in microgravity is well established. However, the biological impact has been marginal at best. The potential is there but the practical barriers are steep. The improvements that can be expected also come with multiple mechanisms of failure in the process. We are still far from the point where microgravity could be used as a routine element in improving the structural detail we can see of the biological world around us and there is powerful competition. Other methods offer the ability to determine structure without a crystal. Cryo-electron microscopy is now able to produce structural data for larger molecules with comparable resolution as crystallography [117], Nuclear Magnetic Resonance is accessing larger and larger macromolecules [118], and when combined the methods can be very complementary [119]. There is even the possibility of in-silico approaches that may rival the experimental [120,121]. To use microgravity most efficiently, the focus should be on the problems that cannot be tackled easily by any other methods. We assert that there is space for microgravity in structural biology.

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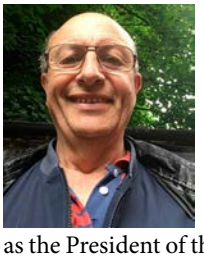

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