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Investigation of Microgravity Grown Organic Crystals by Diffusion Techniques over the Course of Thirty-one Years

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Investigation of Microgravity Grown Organic Crystals by Diffusion Techniques over the Course of Thirty-one Years

A Thesis

Presented to the Department of Chemistry and Biochemistry

College of Liberal Arts and Sciences

and

The Honors Program

of

Butler University

In Partial Fulfillment

of the Requirements for Graduation Honors

Ashley Renee Wilkinson

In Partnership With

Dr. Anne Wilson

May 5, 2023

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Acknowledgements

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Abstract

The effect of microgravity on crystallization experiments has been a topic of interest over the past fifty years. The first microgravity crystallization experiments have been dated back to the early 1970s, and since then hundreds of crystals have been grown in microgravity. Crystallization experiments have been conducted on multiple flights and a variety of spacecraft, using an assortment of techniques, and funded by several different countries. Scientists have been and continue to examine how macromolecular crystals grow in microgravity and how the structures determined from crystals grown in microgravity compare to the structures determined from Earth grown crystals. Parameters such as size, uniformity, mosaicity and resolution limit can give the scientists insight into whether or not there are marked improvements in crystals. The ability to grow high quality crystals can lead to many developments in the electronics, healthcare and pharmaceuticals, and metal industries along with a variety of areas of research. This study is an analysis of publicly available data on organic macromolecular crystals grown by diffusion techniques over a thirty-one year period of time (1988-2019). The hypothesis is that experimental techniques and experience in microgravity crystallization have provided improved crystal growth throughout the analysis time period.

Keywords: microgravity, crystallization experiments, macromolecular organic crystals, diffusion techniques

Introduction

Protein Crystallization

Protein crystallization can be a very difficult process and ultimately does not always result in high-ordered protein crystals. Sometimes the proteins cannot be crystallized or the crystals produced are not of sufficient quality for X-ray crystallography.[1] High-ordered protein crystals are extremely crucial for X-ray crystallography. X-ray crystallography is used to give information about a protein's atomic and molecular structure. High-ordered protein crystals can give clearer images of a protein and more detailed information. Obtaining a protein's three-dimensional structure allows scientists to understand how proteins function and interact.[2] Ultimately, characterizing a protein can lead to many advancements, specifically in the technological, manufacturing, pharmaceutical and healthcare industries.

Protein crystallization has been used since the 19th century for purification of proteins. Crystallization of proteins is the process of mixing protein samples and crystallization reagents. This process reduces the solubility of the solution of proteins. Specifically, a precipitant in the crystallization reagent is what reduces the solubility of the protein by interfering with the proteins ability to interact with the solvent or water. Examples of precipitants that are typically used include salts, polymers, organic solvents and alcohols. The precipitant often causes the protein to precipitate. Crystallization is a form of precipitation, but in the case where precipitation is slow and ordered, it can form crystals of high-ordered phases and well-aligned molecules. Well-aligned molecules along with identical growth units and directional forces help to produce high-quality crystals. There are three main causes that interfere with high-quality protein crystallization: uniformity, conditions, and preservation. First, uniformity takes into account purity and 'structural' uniformity of the protein of interest. If the protein is too dynamic

and exists in many conformations, then there will be no crystal formation. A non-uniform protein sample that is used for protein crystallization will cause homologous impurities. Impurities are anything that are not ideal, which affect the growth, structure, and size of crystals. One or more of these will greatly reduce the growth rate of crystals, which is why a uniform protein sample is desired. The second factor correlates to crystallization conditions. Finding ideal crystallization conditions creates optimal forces which allows for better alignment between protein molecules.[3] The crystallization conditions are going to differentiate depending upon the type of diffusion technique being used and the protein being studied. Factors that are important to consider when developing the conditions are pH, precipitant concentration, ionic strength, temperature, sample (protein) concentration, etc.[4] The final factor of producing high-quality crystals is to correctly preserve these crystals. If not preserved well, deterioration of crystals can occur.[3] Deterioration of crystals can be for instance fractures, cracking, pitting, or erosion of the edges. Overall optimizing sample preparation, crystallization reagents and methods, and correct preservation will result in high-ordered protein crystals.

While protein crystallization techniques have improved and grown rapidly to identify many crystal structures efficiently and effectively, there are still hundreds of protein structures that need to be characterized, such as proteins and genes involved with life-threatening illnesses like cancer.[2] This is specifically why scientists started investigating the use of a microgravity environment for protein crystallization. Microgravity conditions are distinct from the conditions on Earth and can have a large, positive impact on crystal formation and results. In a microgravity environment, there is an absence of buoyancy and sedimentation, convection, and hydrostatic pressure. The lack of buoyancy and sedimentation in microgravity allows substances of different relative densities to disperse evenly.[5] Buoyancy refers to the natural tendency of objects to float

in a liquid due to the upward exerted force, known as buoyant force. An example of buoyancy and sedimentation in microgravity is that water and oil would disperse evenly.[5] Sedimentation is the process of heavier particles settling at the bottom of a liquid mixture, instead of mixing together. An example of sedimentation on Earth would be sand settling at the bottom of a water and sand mixture, instead of how salt and water would mix. The absence of convection is also due to different relative densities, since convection is the heat transfer. Convection is the transfer of heat by the movement of a liquid or gas where the hotter or less dense materials rise and the cooler, denser materials sink to the bottom. Due to the absence of gravity in a microgravity environment, the relative densities of liquids and gasses are lighter which inhibits convection and can result in better manufacturing of crystals. An example of convection on Earth would be boiling a pot of water. Finally, since there is little to no hydrostatic pressure in microgravity, hydrostatic pressure does not increase as material gets deeper in liquid. Microgravity also has a containerless float characteristic, meaning that liquids can float in the air without a container unlike on Earth.[5] All of these conditions make microgravity favorable for crystallization experiments and can help produce materials and crystals of higher quality.

Microgravity crystallization experiments began in 1973 with Apollo and Skylab. Microgravity experiments have continued with additional spacecraft like Mir, the Space Shuttle, International Space Station, and Tiangong as well as other rockets and satellites. Microgravity crystallization experiments can last anywhere from seconds, on a sounding rocket, to months on the Space Station. The experiments are extremely versatile in the types of material that have been flown and produced. This includes, but is not limited to, macromolecules, conventional crystals, inorganic crystals, semiconductors, and ZBLAN (a specialized glass). This thesis project is specifically looking at macromolecules, such as proteins, nucleic acids, viruses, etc.

Macromolecular crystallization experiments present their own challenges when compared to crystallization of small molecule crystals. According to McPherson and Gavira [ref #1], there are three main reasons that make macromolecular crystallization more difficult than conventional crystallization. Due to their large and dynamic nature, macromolecules have several different solid states, the crystals nucleate at very high levels of supersaturation, and growth is much slower than for conventional crystals.[1] While macromolecules are unique in their characteristics and present challenges, it can lead to a lot of breakthroughs to understand the structures of these crystals and how they function. For example, the mutated LRRK2 gene that plays a role in developing Parkinson's disease has been flown in microgravity.[6] By crystallizing this protein under microgravity without the impacts of buoyancy and sedimentation, convection and hydrostatic pressure it can give a higher quality structure. With that, having a more in depth understanding of the structure of this gene could potentially help find a cure for Parkinson's disease. A high-resolution structure could give insight into the cause of Parkinson's disease and to help create a drug to treat this disease. Although an attempt at both Earth-grown and microgravity-grown, there still has been no high-resolution structure of this protein produced.[6] Crystallizing the LRRK2 gene has been a challenge for researchers, but each experiment done helps them to learn more about the conditions needed to crystallize this protein.

Diffusion Techniques

This thesis project is exploring diffusion techniques as a method for crystallization of macromolecules in microgravity. There are a wide variety of techniques used to crystallize proteins, different diffusion techniques were common methods that were seen during the analysis of macromolecular microgravity crystallization experiments. The six diffusion techniques that were seen in the research are capillary counter diffusion, free interface diffusion, hanging drop

diffusion, liquid diffusion, sitting drop diffusion, and vapor diffusion. While all of these diffusion experiments are very similar, there are small differences that separate them. Some of the techniques are older than others, which is why some are more frequently used in the experiments evaluated in this study. The more popular techniques have an established track record, and it is easier for researchers to use the same conditions that have already been created and successful. Understanding the uniqueness of each method can be beneficial when developing an experiment.

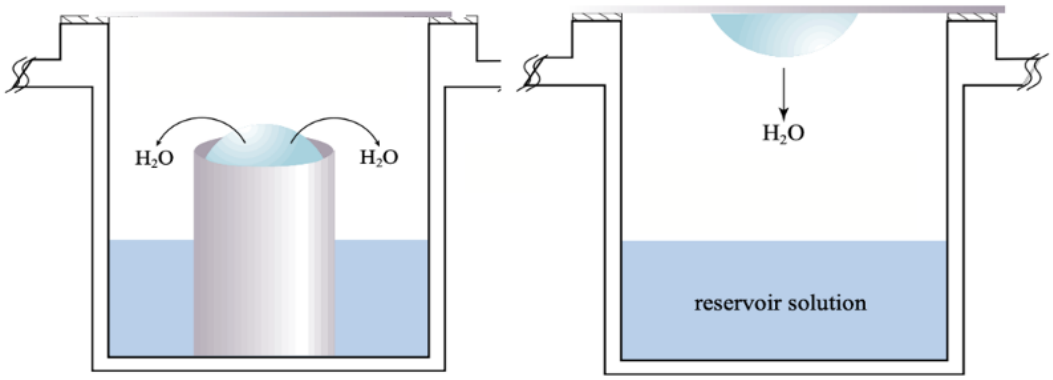

Figure 1. Diagrams of Sitting Drop and Hanging Drop Vapor Diffusion Techniques. Sitting drop is on the left and hanging drop is on right.[8]

Vapor diffusion is one of the most popular, and also one of the most studied methods. In the vapor diffusion method, a one to one solution of protein sample and a crystallization reagent (reservoir solution) is made. The protein sample and crystallization solution are mixed 1 to 1 and suspended above a larger reservoir of crystallization solution.[3] The sample solution vaporizes such that the solute concentrations in the drop equilibrate with those in the much larger volume reservoir. The two different techniques employing the vapor diffusion method are the hanging drop and the sitting drop methods. Hanging and sitting drop diffusion techniques are very popular due to how simple they are to perform. During hanging drop diffusion, a drop of sample and reagent are put on a siliconized glass cover slide inverted over the reservoir in vapor equilibration (Fig. 1).[7] The vapor equilibration contains a liquid reservoir of reagent, which has a

higher reagent concentration than the drop. As the water from the drop leaves, the sample becomes more supersaturated. Once the reagent concentration is the same as the reservoir, equilibration is reached.[5] Sitting drop is an advantageous technique because it is very simple and fast. During sitting drop diffusion, a drop of sample and crystallization reagent are mixed on a platform in vapor equilibration with the reagent. The reservoir has a higher concentration than the reagent concentration in the droplet. This causes the reservoir to pull water from the droplet and will increase the supersaturation of the sample in the drop (Fig. 1).[8] Vapor diffusion, hanging drop, and sitting drop methods are all very similar.

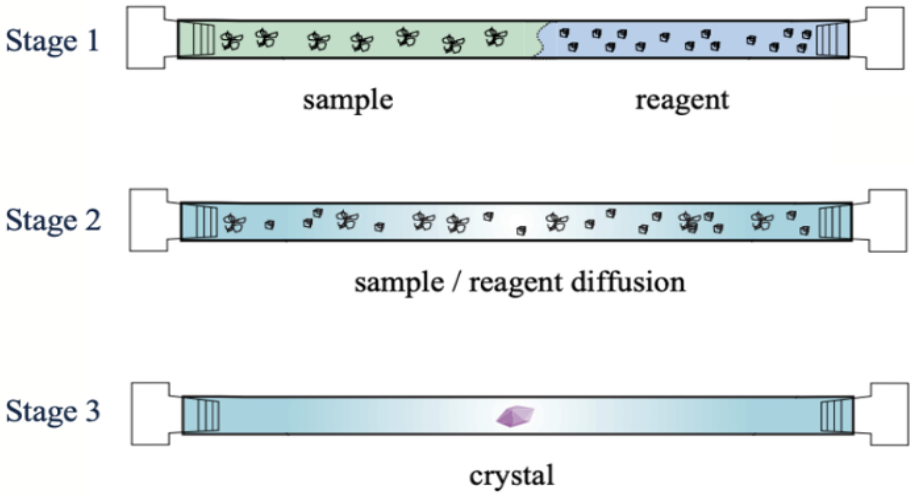

Figure 2. Representation of Free Interface Diffusion. Three stages are shown above. Stage one the sample and reagent are separated. Stage two the sample and reagent diffuse into each other. Stage three crystallization occurs to create a crystal.[8]

The other three diffusion methods are slightly different from hanging drop, sitting drop, and vapor diffusion. Free interface diffusion is not as popular as other diffusion methods. During free interface diffusion, the sample is placed in liquid contact with the precipitant in an attempt to create an interface between the sample and the precipitant. Eventually, the sample and precipitant diffuse into each other and crystallization occurs at the interface (Fig. 2).[8] Liquid diffusion is similar to free interface diffusion in the way that it also forms crystals at the interface. Liquid

diffusion, however, uses a concentrated solution of the compound to be crystallized and a precipitating solvent where the compound is insoluble. The solutions are then added to a vial and mixed until crystals are formed (Fig. 3).[9] Capillary counter diffusion, also known as counter diffusion, is a little different from free interface and liquid diffusion. During capillary counter diffusion, a protein sample and a precipitant are filled in contact with one another in a narrow capillary tube. Capillary counter diffusion looks for high values of supersaturation to provoke formation of amorphous precipitates early. Using a capillary and early supersaturation allows for diffusion and crystallization along the length of the crystallization chamber (Fig. 4). This method looks for the best crystallization conditions.[10]

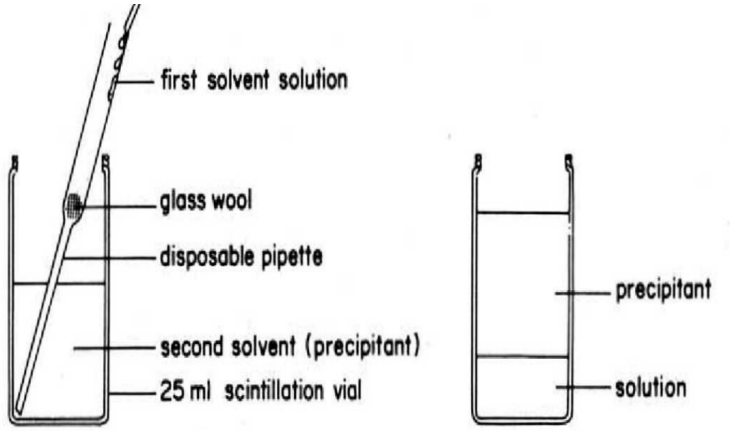

Figure 3. Diagram of the Liquid Diffusion Technique. The image on the left shows a concentrated solution of the compounded being added to the precipitating solution, in which the compound is insoluble. The image on the right shows the precipitant on top of the solution after all the first solvent solution has been added and before crystallization has occurred.[9]

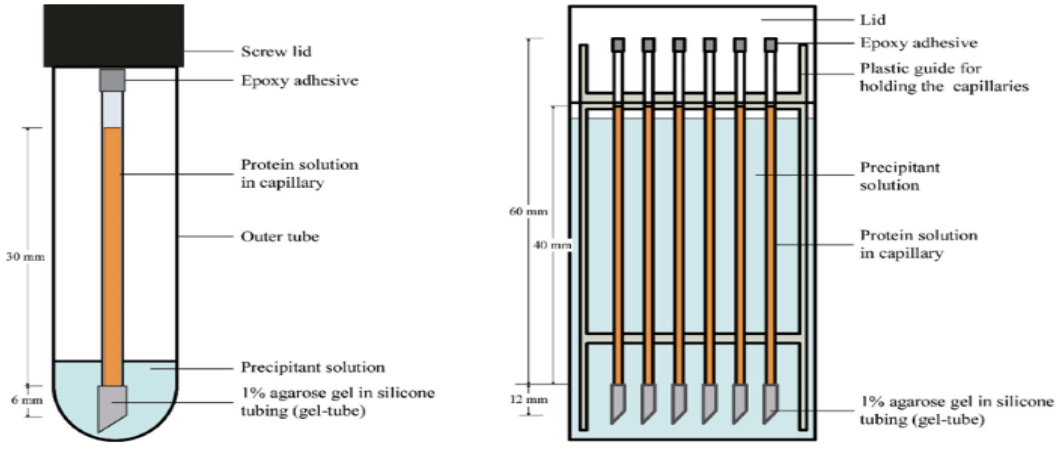

Figure 4. Diagram of Capillary Counter Diffusion. On the left is a singular capillary, while on the right are multiple capillaries. The capillaries are filled with a protein solution and then placed in a precipitant solution.[10]

All six diffusion methods have advantages and disadvantages to them, but the set up and the conditions of the experiment really determines whether crystallization is achieved or not. Advantages and disadvantages of each of the six different diffusion techniques can be found in Table 1, shown below. These are just some of the advantages and disadvantages that were found throughout the research process, which means there are still plenty others.

Methods	Advantages	Disadvantages
Vapor Diffusion	• Very popular technique that is widely used. • Straightforward process.[3]	• Difficulty controlling concentrations individually, which can affect crystal production.[3]
Hanging Drop	• Able to view the drop through glass. • Reduced chance of crystals sticking to the hardware. • Easy access to the drop.[8]	• Extra time is required for set ups.[8]
Sitting Drop	• Quick • Simple set up • Excellent method for screening and optimization.[8]	• Crystals can adhere to the surface making removal difficult.[8]
Free Interface	• Can be readily performed in small capillaries. [8]	• Not as frequently used, with less information available for planning. [8]
Liquid Diffusion	• Often one of the most successful because crystallization typically will occur.[9] • Samples can be frozen immediately after they are prepared and not un-thawed until starting the experiment.[12]	• Not as widely used. • The preparation can be difficult due to finding a perfect intersolvent meniscus. • Can take several days to achieve complete mixing.[9]
Capillary Counter	• Looks for initial high supersaturation values preventing amorphous precipitates. • Minimizes supersaturation and impurity levels. • Simple and practical. • Cost-effective.[10]	• Can be a difficult set up. [10]

Table 1. Advantages and Disadvantages of the Methodologies. The six methods, previously discussed, are shown above with advantages and disadvantages found throughout research.

Materials and Methods

Microgravity experiments are extremely expensive making it critical that these experiments are successful or at least contribute information to microgravity crystallization research. This research is specifically an in-depth analysis of microgravity crystallization experiments. No microgravity crystallization experiments were conducted by my research team and I. Before 2022, there was no searchable database of the publicly available microgravity

crystallization experiments. Without this database, researchers are not able to quickly and efficiently refer to previous experiments to help further their own studies. Previous crystallization experiments can provide a foundation for other scientists during the implementation of their own research project as well as ensuring experiments are not rerun with identical conditions. The database can give insight and information about the crystals, methods, parameters and critically, results.

During the summer and fall of 2022, a database of all the publicly available microgravity crystallization experiments was created by my research team and I. My research team consists of Dr. Anne Wilson, Amari Williams, Hannah Wright, and Frannie Brewer facilitated at Butler University under the instruction and contract of Aerospace Corporation. The database was generated by searching for publicly available articles and journals on microgravity crystallization experiments. A lot of the experiments were found on journal article databases like *ScienceDirect*, *Springer Link*, *PubMed,* etc. All of the journals were read and the data from each experiment was recorded into an excel sheet. Included in the database are compounds by name, the DOI and journal title for reference, mission flown, year flown, methodology of crystallization, crystal parameters and metrics, conditions and the application. As of August 30, 2022, the original database included 322 microgravity crystallization experiments. Excluded from the database are experiments that are not publicly available or that have occurred after August 2022. These

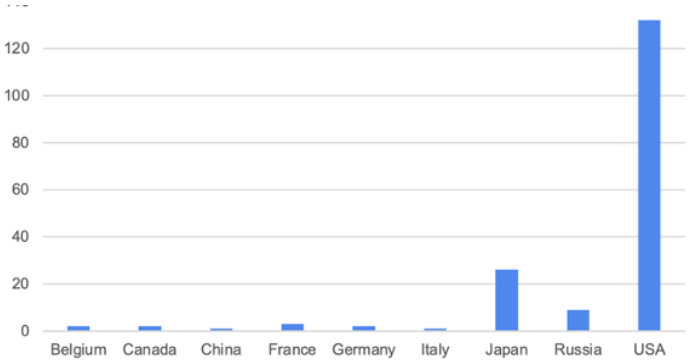

Figure 5. Distribution of sponsoring countries. The distribution above includes 178 microgravity experiments with the known sponsoring country. The USA has sponsored the most at 132 experiments and Russia is the next leading country at 26 experiments.

scientific publications were available through our library system, via interlibrary loan, or from the scientists who performed the experiments. The database included 212 macromolecules and 110 inorganic compounds. This database has been made publicly available and results have been recorded for the use of other researchers. The database is continuously being updated as more microgravity crystallization experiments are being conducted and documented.

For this thesis project, the original database of 212 macromolecular organic crystals was used as a starting point, but was reduced to a total of 178 microgravity experiments to fit the parameters of interest. The complete list of macromolecular crystals used for this thesis project can be found in Table 2.

- (Pro-Pro-Gly)10]3
- 20S Proteasome
- 30S Ribosomal Subunits (*Thermus thermophilus*)
- 3902c (TB)
- 5S rRNA (*Thermus flavus*)
- Alcohol dehydrogenase (*Solfobus solfataricus*)
- Anti-HPR Fab fragment
- Anti-polyglutamine Fab Crystals
- Antigen- antibody complex (Camelid) cAb-CA05
- Apocrustacyanin C1
- B-subunit of V-type ATPase
- Bacteriophage lambda lysozyme
- Bacteriorhodopsin-mixed Micelle
- Bovine brain prolyl-isomerase
- Bovine Insulin
- Bovine Trypsin
- Brefeldin A-ADP ribosylated substrate (BARS)
- BTB domain of the CP190 protein
- Canavalin
- Carbohydrate-Binding Fab
- Carboxypeptidase B
- Carboxypeptidase T
- Cellulase
- Chaperonin-60
- Chicken liver basic fatty acid binding protein complexed with cholic acid
- Chitinase- ChiW
- Collagenase
- Concanavalin B
- Connexin
- Cratylia mollis seed lectin
- Cystic Fibrosis NBD1
- D-amino transferase
- D-tagatose 3-epimerase C66S (*Pseudomonas cichorii)*
- Dipeptidyl aminopeptidase BII (DAP BII)
- Dipeptidyl peptidase 11 *(Porphyromonas gingivalis)*- PgDPP11
- E13- Ribosome Recycling Factor
- E14- Aspartate Carbamoyl transferase
- E16- Nucleoside-triphosphatase
- E17- Putative Citrate Synthase
- E18- Acyl-CoA dehydrogenase 1
- E19- Acyl-CoA dehydrogenase 2
- E2- O-methyltransferase family protein 1
- E21- FadE1_3
- E22- FAD9
- E25- Pyridoxal-phosphate-dependent transferase
- E27- Enoyl-CoA hydratase
- E28- Putative Acyl-CoA dehydrogenase
- E29- Cysteinyl-tRNA synthetase
- E3- O-methyltransferase family protein 2
- E7- aminotransferase
- Eco RI Endonuclease
- EF- hand protein
- Epidermal Growth Factor Receptor
- Factor D
- FeADH
- Fenna Mathews Olsen protein
- Gamma0Interferon D1
- GBS Sortase
- Glucose Isomerase
- Goat Hemoglobin
- GrpE
- Haematopoietic Prostaglandin D Synthase
- Haloacid Dehalogenase
- Hen Egg-White Lysozyme (HEWL)
- Herpes Simplex Virus 1 (ICP8)
- HIV-1 Reverse Transcriptase
- Horse Hemoglobin
- Human a-thrombin
- Human Bence Jones Protein
- Human Erythrocyte Band 3
- Human Recombinant Insulin
- Human Serum albumin
- Human T6 Insulin
- Human α-interferon
- Hydrogenase Maturation Factor (HypF)
- Inorganic Pyrophosphatase
- Insulin Hexamer
- Interferon alfa-2b
- Isocitrate Lyase
- Isolectin I
- LRRK2
- Lysin-49 Phospholipase A2 Protein
- Macrophage Migration Inhibitory Factor (MIF)
- Malic Enzyme
- Manganese Superoxide Dismutase
- Methyl Transferase Fusion Protein
- Mistletoe Lectin I
- Mouse Lipocalin- Type Prostaglandin D Synthase
- Myoglobin Triple Mutant
- NAD Synthetase
- NBD1
- NovoNordisk Lipase
- Nucleosome Core Particle
- Orthocanavalin
- Outer Surface Glycoprotein (*Methanothermus fervidus*)
- Platelet Adhesion Protein A (PadA)
- Parvalbumin
- Pesticin
- Phospholipase A2
- Phosphopantetheine Adenylyltrasnferase (PPAT)
- Plasma Antithrombin III
- *Plasmodium falciparum glutathione S-transferase* (PfGST)
- PlsC
- Porcine Carboxypeptidase B
- Porcine Elastase
- PPL3 Isoforms
- Proteinase K
- Purine Nucleoside Phosphorylase (E. coli)
- Recombinant Human Insulin
- Recombinant NAD+ -Dependent Formate Dehydrogenase *Arabidopsis thaliana* (AraF DH)
- Recombinant Phosphoribosyl-Pyrophosphate Synthetase 2
- Rhodopsin (Bacterioelectrogenic membrane)
- Ribonuclease S (Beef)
- Satellite Tobacco Mosaic Virus
- Schmallenberg Virus
- Sortase
- Thaumatin (*Thaumatococcus daniellii*)
- Thermolysin
- Thermostable T1 Lipase
- Thymidine Phosphorylase
- Topoisomerase Poison, CcdB from E. Coli
- Triose Phosphase Isomerase (TIM)
- Turnip Yellow Mosaic Virus
- Type II 3-dehydroquinate dehydrates (Actinobacillus pleuropneumoniae)
- Vibrio cholera
- Zn-Insulin

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Table 2. List of Macromolecular Organic Crystals. A list of 126 different macromolecular organic crystals that have been grown in microgravity.

It is important to note that 58 total experiments are from a singular study, the Larry DeLucas double-blind study that took place in 2014.[12] There are a total of 178 unique experiments on 126 different macromolecules that all fit within the parameters of interest. The first parameter of interest was that the experiments had to be macromolecular microgravity crystallization experiments. Some experiments were conducted both on Earth (in a normal gravity environment) and in space (in a microgravity environment). Other experiments were only done in microgravity. Regardless, the experiment needed to include a microgravity conducted crystallization experiment. The microgravity crystallization experiments have been performed by a variety of countries, such as the United States, Russia, and Japan (Fig. 5). The country, however, was not a limiting factor and all experiments were used. Specifically, this research is intended for macromolecules that were grown in microgravity. Macromolecules are large organic molecules that include proteins, nucleic acids, lipids, and carbohydrates. The four categories encompass other molecules such as enzymes and viruses. Although macromolecules are considered "large," it is important to note that these molecules cannot be seen with the naked eye. Due to investigating only macromolecular microgravity crystallization experiments, this eliminated experiments that focused on inorganic molecules and ZBLAN, along with any other materials and molecules that have been grown in microgravity.

The second parameter of interest were macromolecular microgravity experiments that were crystallized using diffusion techniques. Macromolecules that were crystallized using techniques other than diffusion, such as dialysis

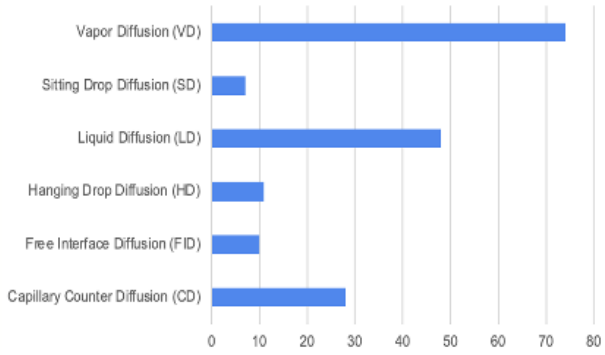

Figure 6. Array of Diffusion Techniques. The distribution above includes 176 macromolecular crystallization experiments.

and seeding were omitted from the database. Diffusion is a broad category that encompasses 6 individual techniques: capillary counter diffusion, free interface diffusion, hanging drop diffusion, liquid diffusion, sitting drop diffusion, and vapor diffusion. Of the 178 microgravity crystallization experiments, 28 are capillary counter diffusion, 10 are free interface diffusion, 11 are hanging drop diffusion, 48 are liquid diffusion, 7 are sitting drop diffusion, and 74 are vapor diffusion (Fig. 6). While the number of diffusion techniques is widely dispersed, there are a couple of explanations for this. For example, vapor diffusion is one of the most widely practiced crystallization techniques. Sitting drop and hanging drop diffusion techniques are subcategories of vapor diffusion, so while research journals documented that the experiments are vapor diffusion, they could fall into one of the subcategories. Free interface diffusion, however, is not as popular in comparison to the other diffusion methods.[7] This could be due to the fact that not as many free interface crystallization experiments have been documented, which makes the experiments harder since the crystallization conditions are unknown. Theoretically, this would mean the scientists are starting from scratch when performing their experiments. In *A Comprehensive Evaluation of Microgravity Protein Crystallization*, Dr. DeLucas and Dr. McPherson, commented on how there was little to no information on liquid diffusion crystallization conditions before performing their microgravity crystallization experiments.[12] Lack of information can make crystallization experiments harder. It also can be not as appealing to scientists to use the techniques that are documented less frequently on.

The third parameter was looking at microgravity crystallizations grown during the time period of 1988 to 2019. Experiments that were conducted outside of this time frame were not included in the research because a large majority of experiments were done during the analysis time period. Of the 178 microgravity crystallization experiments, 87 experiments were done

between the years 1988 and 2003 and 91 experiments were conducted between 2004 and 2019 (Fig. 7). The experiments were split into an earlier and a latter half, so the tests ran could conclude whether there were marked improvements in microgravity crystallization data over the thirty-one year period. Finally, the last parameter of interest was looking at experiments that documented resolution, structure, or uniformity. While not all of the experiments in this study documented on all three, all of the experiments did document on at least one. These three parameters (resolution, structure, uniformity), if documented, were used to determine comparisons between the analysis time period. Resolution, structure, and uniformity were chosen because these criteria can show whether there was a marked improvement in crystal structure between Earth-grown and microgravity-grown crystals.

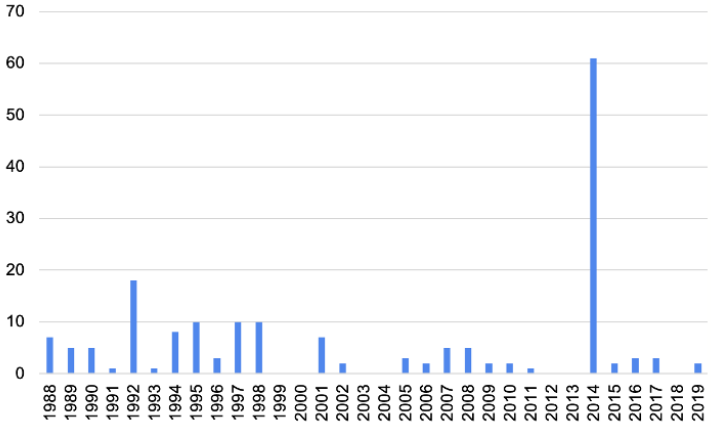

Figure 7. Distribution of Macromolecular Microgravity Crystallization Experiments based on Year Flown. The distribution above includes 178 unique crystallization experiments ranging from the year 1988 to 2019. Some years have no experiments documented on. The 2014 data is primarily from the Dr. Larry DeLucas double blind study.[12]

Results

A total of twelve tests were performed to determine if experimental techniques and experience in microgravity improved crystal growth between 1988 to 2019. Out of the twelve tests, nine of the tests determined percentages specifically to examine whether crystals were improved or not during microgravity crystallization based upon resolution, structure quality, and uniformity. Not all crystallization experiments documented on all three parameters, so the experiment that did not were omitted from that specific test. Out of the twelve tests, the last three tests were two proportion z-tests to determine whether or not there was a statistically significant

difference between the earlier half experiments (1988-2003) and the latter half experiments (2004-2019).

The first set of three tests were of the entire crystallization database without division of time period. Of the 176 crystallization experiments, 150 experiments documented resolution improvement (Chart 1A). Of 150 macromolecular microgravity crystallization experiments, 121 crystals had improved resolution (81%), 18 crystals had unchanged resolution (12%), and 11 crystals had worsened resolution in microgravity (7%). This is a very high percentage of crystals that had improved resolution when flown in microgravity. The next comparison was made to determine if the crystals grown in microgravity were structurally better. A total of 141 crystallization experiments documented on structure improvement (Chart 1B). Out of 141 microgravity crystallization experiments, 124 crystals were structurally better (88%), 2 crystals were structurally the same (1%), and 15 crystals were structurally worse (10.6%). This is also an extremely high percentage of crystals that were structurally better after being crystallized in microgravity. The last parameter looked at was uniformity and 120 crystallization experiments documented on crystal uniformity (Chart 1C). Of the 120 microgravity crystallization experiments, 99 crystals had improved uniformity (83%), 5 crystals had unchanged uniformity (4%), and 16 crystals had decreased

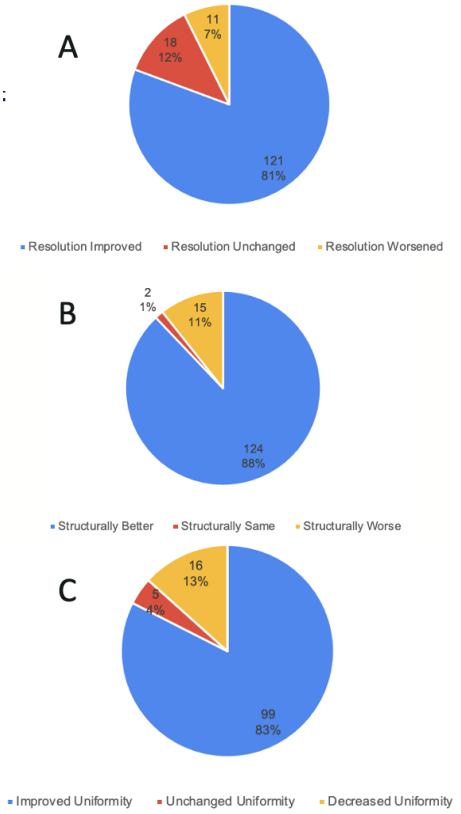

Chart 1. Percentages of Microgravity Experiments based on Resolution, Structure Quality, and Uniformity. Chart A includes 150 microgravity experiments, Chart B includes 141 microgravity experiments, and Chart C includes 120 microgravity experiments.

uniformity (13%). From the first three tests, it is conclusive that crystals grown in microgravity had improved resolution and uniformity and were also structurally better.

The next 6 tests were divided into two time periods, three tests from the earlier half and three tests from the latter half. The earlier half of the data included the years 1988 to 2003. During this time period, there were a total of 87 macromolecular crystallization microgravity experiments documented on. The latter half of the data included the years 2004 to 2019 and a total of 91 macromolecular crystallization microgravity was documented during this time period. A big proportion of the later half experiments are from the DeLucas double-blind study, which was conducted in 2014.[12] Three two proportion z-tests were performed to determine whether there was a statistically significant difference between the earlier and later data.

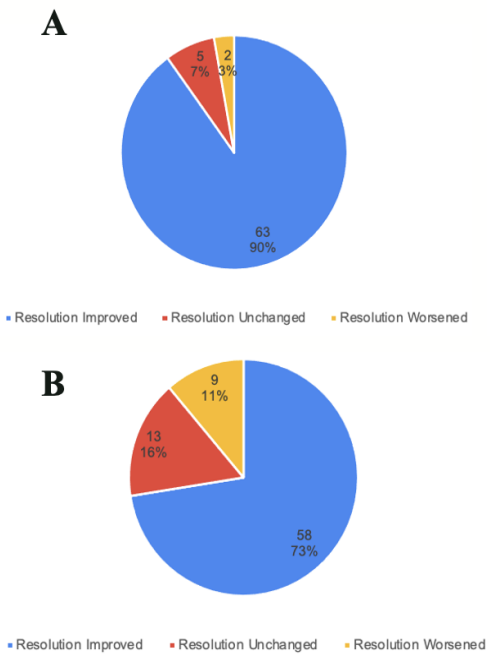

Chart 2. Percentages of Organic Microgravity Experiments based on Change in Resolution Limit (s) Compared to Terrestrial Experiments. Chart A is based off data from years 1988-2003 and includes 70 different experiments. Chart B is based off data from years 2004-2019 and includes 80 different experiments.

1988-2003	2004-2019
N_1=70	N_2=80
X_1=63	X_2=58

Table 3. Two Proportion Z-test Comparing Resolution Data. A two proportion Z-test was performed to compare resolution data. N stands for total number of microgravity crystallization experiments and X stands for the number of experiments that documented improved resolution. A p-value of 0.00678 was calculated.

Resolution of microgravity grown crystals was the first parameter that was examined (Chart 2). Articles that did not document the resolution of crystals were omitted from the tests. The resolution of crystals grown in microgravity was documented as improved, unchanged, or worsened when compared to terrestrial counterparts. During the years 1988 to 2003, 70 microgravity experiments were documented on

resolution and of the 70 experiments, 63 experiments reported improved resolution of crystals (90%), while only 2 experiments reported crystals having worsened resolution (3%) when grown in microgravity. During the years 2004 to 2019, 80 experiments were documented on resolution of the crystals. Of the 80 different experiments, 58 experiments reported the microgravity grown crystals having improved resolution (73%), while 9 experiments documented that the crystals did not have improved resolution (11%). A two proportion z-test was performed to compare the data of the resolution of crystals grown in the earlier time frame and the resolution of crystals grown in the later time frame (Table 3). N stands for the total number of microgravity crystallization experiments and X stands for the number of experiments that documented improved resolution during the designated time period, which can be found in the table. A p-value of 0.00678 was calculated from the two proportion z–test, which is less than the alpha value of 0.05. A p-value that is less than the alpha value represents that there is a statistically significant difference between the number of crystals with improved resolution during 1988 to 2003 versus improved resolution of crystals during 2004 to 2019.

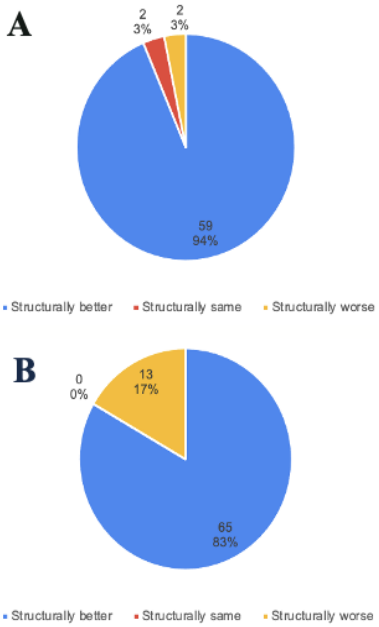

Chart 3. Percentages of Organic Microgravity based on Structural Quality of the Crystal Compared to Terrestrial Experiments. Chart A is based off data from years 1988-2003 and includes 63 different experiments. Chart B is based off data from years 2004-2019 and includes 78 different experiments.

The second parameter that was investigated was the structure quality of crystals. Crystals grown in microgravity crystallization experiments were documented as structurally better, same, or worse than terrestrial crystallization counterparts (Chart 3). During 1988 to 2003, 63 total experiments reported on structure quality and during 2004 to 2019, 78 experiments documented

1988-2003	2004-2019
N_1=63	N_2=78
X_1=59	X_2=65

Table 4. Two Proportion Z-test Comparing Structural Data. A two proportion Z-test was performed to compare structural data. N stands for total number of microgravity crystallization experiments and X stands for the number of experiments that documented improved structure quality. A p-value of 0.0614 was calculated.

on structure quality. During the earlier years, 59 out of 63 (94%) microgravity crystallization experiments reported that structure quality was improved and only 2 out of 63 (3%) reported worsened structure quality when compared to terrestrial experiments of the same crystals. During the later years, 65 out of 78 (83%) microgravity crystallization experiments documented improved structure quality and 13 out of 78 (17%) reported worsened structure quality when compared to terrestrial crystallization experiments. A two proportion z-test was performed to compare the data of structure quality of crystals grown during the earlier time frame and those grown during the latter period (Table 4). From the table, N stands for the total number of microgravity crystallization experiments for the specified time period and X stands for the experiments that have improved the structure quality of crystals grown in microgravity during the specified period. A p-value of 0.0614 was calculated, which is greater than the alpha value of 0.05. A p-value greater than the alpha value suggests that there is not a statistically significant difference between the number of crystals with improved structural quality from earlier experiments and later experiments.

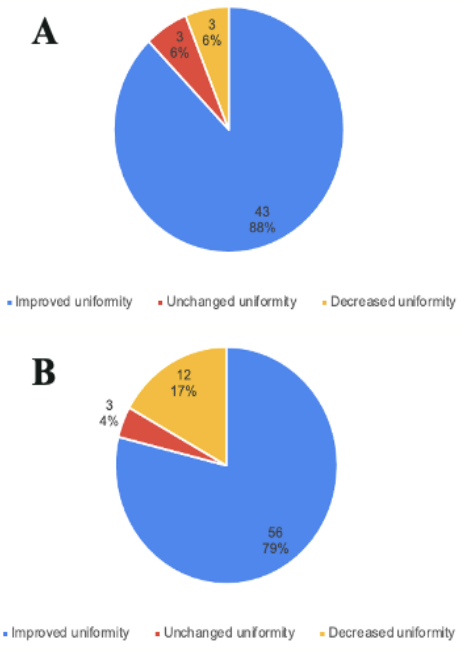

Chart 4. Percentages of Organic Microgravity Experiments based on Uniformity Compared to Terrestrial Experiments. Chart A is based off data from years 1988-2003 and includes 49 experiments. Chart B is based of data from years 2004-2019 and includes 71 different experiments

The final parameter that was investigated was the uniformity of crystals. The crystals grown in microgravity were documented as having improved, unchanged, or decreased uniformity when compared with terrestrial counterparts (Chart 4). A total of 49 experiments documented on uniformity in the years 1988 to 2003. Of the 49 microgravity crystallization experiments, 43 experiments reported having crystals with improved uniformity (88%) and only 3 experiments reported having crystals with decreased uniformity (6%). A total of 71 microgravity experiments documented on uniformity in the years 2004 to 2019. Of the 71 experiments, 56 experiments reported the crystals having improved uniformity (79%) and 12 experiments reported having crystals with decreased uniformity (17%). A two proportion z-test was conducted to determine whether there was a significant difference between the data of crystal uniformity between earlier and later experiment years (Table 5). N stands for the total number of microgravity experiments for the specified time period and X stands for the number of experiments that documented improvement of crystal uniformity from microgravity. A p-value of 0.2082 was calculated, which is greater than an alpha value of 0.05. With a p-value greater than the alpha value, this suggests that there is not a statistically significant difference between the data of crystal uniformity from the earlier and later experiments.

1988-2003	2004-2019
$N_1=49$	$N_2=71$
$X_1=43$	$X_2=56$

Table 5. Two Proportion Z-test Comparing Uniformity Data. A two proportion Z-test was performed to compare uniformity of crystals. N stands for total number of microgravity crystallization experiments and X stands for the number of experiments that documented improved uniformity. A p-value of 0.2082 was calculated.

Discussion

The hypothesis is that experimental techniques and experience in microgravity will provide improved crystal growth throughout the analysis time period. While on the surface it may appear that this hypothesis was disproved based on the results, there are some explanations as to why this occurred. One major explanation as to why there are not higher percentages of crystals that have marked improvements during later experiments in comparison to earlier experiments is because scientists started evaluating crystals that had not been crystallized before and more challenging experiments. Once scientists figured out that they could crystallize macromolecules in microgravity, they started performing novel diffusion experiments. Since researchers had demonstrated that they could crystallize macromolecules at normal (Earth) gravity, scientists began to evaluate new techniques with new crystallization conditions to figure out if they could produce more uniform crystals with better structural quality and improved resolution. Another explanation for why there are not higher percentages is due to the fact that conditions of the experiments were not always perfect. For example, if there are flight delays this can extremely affect crystallization of the conditions, especially crystals grown by vapor diffusion. Flight delays affect the loading and activation of crystals, which can impact crystal growth. Microgravity experiments are difficult, and in order to produce better crystals, there is little room for error in the crystallization environment and set up. If one factor is off, it can influence the whole process. Another possibility for the weaker more recent data is that scientists also got better at growing crystals on Earth because of more accurate methods, crystal screening set up, better controlled environments, etc.

A final explanation is the fact that not all of the crystallization experiments performed later in the study have Earth-grown counterparts, meaning some crystals have not even been

crystallized on Earth before attempting to crystallize them in microgravity. There are 11 crystals that have been attempted to be crystallized in microgravity with no documented corresponding Earth-grown counterparts. The crystals that have been attempted at crystallization in microgravity, but not on Earth, include antibody complex (camelid) cAb-CA05, bacteriophage lambda lysozyme, cratylia mollis seed lectin, dipeptidyl peptidase 11 from porphyromonas gingivalis, haloacid dehalogenase, human erythrocyte band 3, human T6 insulin, lysozyme (bacteriophage), methyl transferase, plasmodium falciparum glutathione S-transferase, and recombinant phosphoribosyl- pyrophosphate synthetase. There are 123 crystals that have been crystallized on Earth and in microgravity. It is important to note that these are reported attempts of crystallization and not all of them were successful. Finally, there are 7 crystals where it is not clear if there are Earth-grown counterparts. These crystals have been reported as crystallized in microgravity, but the articles do not specify if there were Earth-grown counterparts.

A detailed evaluation in the improvement in the metrics can shed some light on how microgravity experiments changed over time. From the historical results, 63 experiments reported improved resolution of crystals (90%) during the years 1988 to 2003, while only 58 experiments reported having improved resolution (73%) during the years 2004 to 2019. The two-proportion z-test showed that there was a statistically significant difference between these two values based upon the total number of experiments that were conducted. This demonstrates that fewer experiments reported crystals having improved resolution during the later experiments than the earlier experiments. It is important to note, however, that there are still more crystals with improved resolution (73%) than crystals having worsened resolution (11%) during the latter experiments. A total of nine crystals had worsened resolution during the later experiments, so a detailed analysis was done of these particular crystallization experiments. The crystals which had

worsened resolution include plasmodium falciparum glutathione S-transferase (pfGST), LRRK2, horse hemoglobin, goat hemoglobin, glucose isomerase, E28 putative acyl-CoA dehydrogenase, E22 FAD9, E19 acyl-CoA dehydrogenase 2, and connexin. Of the 9 crystals, 6 of them were crystallized by liquid diffusion. The crystals grown by liquid diffusion are horse hemoglobin, goat hemoglobin, E28 putative acyl-CoA dehydrogenase, E22 FAD9, E19 acyl-CoA dehydrogenase 2, and connexin, which are from the article, *A Comprehensive Evaluation of Microgravity Protein Crystallization*. In the article, Dr. DeLucas notes that little or no data for microgravity liquid diffusion crystallization conditions was previously reported, so he had to make educated guesses as to appropriate crystallization conditions.[12] Utilizing untested crystallization conditions can provide an explanation as to why more crystals had worsened resolution during later experiments. A lack of prior knowledge of crystallization conditions means the scientists were defining new protocols in these experiments. One of the nine crystals, glucose isomerase, was grown by vapor diffusion. Dr. DeLucas reports in *A Comprehensive Evaluation of Microgravity Protein Crystallization* that there were multiple flight delays, which can cause adverse effects on proteins flown by vapor diffusion and is a major factor for the excessive precipitation for some of the proteins crystallized by vapor diffusion techniques. This demonstrates that microgravity was likely not the cause of worsened resolution for glucose isomerase, but rather flight delays that affected the loading and activation of vapor diffusion experiments.[12] One of the crystals, LRRK2, was grown by free interface diffusion. The article, *Crystallizing the Parkinson's Disease Protein LRRK2 Under Microgravity Conditions,* discusses that shaking occurred when transporting the plates, which could have led to preventing crystal formation and that crystal growth had to be delayed until samples arrived at the ISS.[6] The final crystal, pfGST, was grown by capillary counter diffusion. The article, *Effect of Macromolecular*

Mass Transport in Microgravity Protein Crystallization, reported as having lower resolution due to the gradient of crystallization conditions produced along the capillary.[13] Similarly to other crystals, crystal success is strongly dependent upon the crystallization conditions.

The explanations continue to hold true when examining the metric of structure quality. The older studies reported a 94% improvement in structure quality of crystals when grown in microgravity compared to an 83% improvement rate from the more current experiments. The two proportion z-test calculated a p-value of 0.0614, which is greater than an alpha of 0.05. Since the p-value is greater than the alpha value, there is not a statistically significant difference between the number of experiments reporting improvement in crystal structure quality of older and newer studies. This shows that although there was a higher percentage of improvement in structure in older studies when compared to newer experiments, statistically the difference is insignificant and the percentages are comparable. This means that there truly is not a decrease or increase in the values, rather the percentages are staying consistent. When examining the three metrics of resolution, structure quality, and uniformity, structure quality had the largest amount of crystals reported with worsened structural quality. There were a total of 13 crystals documented as being structurally worse from the newer experiments. Of the 13 crystals, 8 of them were also reported as having worsened resolution. It is likely that many of the crystals that did not improve in one category also did not improve in another category. The crystals reported as not having improved structure quality includes bovine trypsin, canavalin, connexin, E13-ribosome recycling factor, E17-putative citrate synthase, E19 acyl-CoA dehydrogenase 2, E22 FAD9, E28-putative acyl-CoA dehydrogenase, glucose isomerase, goat hemoglobin, horse hemoglobin, orthocanavalin, and LRRK2. Out of the 13 crystals, 10 of them were crystallized using liquid diffusion. The crystals, bovine trypsin, canvalin, connexin, E13-ribosome recycling factor,

E17-putative citrate synthase, E19-acyl CoA dehydrogenase 2, E28-putative acyl-CoA dehydrogenase, goat hemoglobin, horse hemoglobin, and orthocanavalin, were all apart of Dr. DeLucas's double-blind study. Similarly to before, little or no data for microgravity liquid diffusion crystallization conditions was previously reported, so DeLucas had to develop his own microgravity conditions.[12] Glucose isomerase and E22 FAD9 were also crystallized by Dr. DeLucas, but by using vapor diffusion. As mentioned previously, there were multiple flight delays that affected the proteins. Making sure that discrepancies of the crystallization environment are controlled and limited can lead to better results of the crystals. Finally, the LRRK2 crystals, crystallized by free interface diffusion, also had worsened structure quality. Likewise, it was also due to environmental conditions. Understanding the conditions that can affect protein crystallization in microgravity is crucial for success.

The final metric that was evaluated was the uniformity of crystals. The older studies, 1988 to 2003, reported an 88% improvement in uniformity of crystals grown in microgravity. Similarly the more recent studies, 2004 to 2019, documented a 79% improvement in uniformity of crystals. It is worth noting that only 49 experiments documented on crystal uniformity in historical studies, while 71 experiments reported in more current studies. A final two proportion z-test was conducted to figure out if there was a difference in the experiments. A p-value of 0.2082 was calculated, which means there is also not a significant difference in between the crystal uniformity from the earlier and later experiments. Although there is a decrease in improvement of uniformity of crystals over the years, it does not prove to be significant enough. There were a total of 12 crystals in the recent studies that reported having decreased uniformity. The crystals include canavalin, connexin, E16 nucleoside-triphosphatase, E28 putative acyl-CoA dehydrogenase, E3 O-methyltransferase family protein 2, horse hemoglobin, methyl transferase

fusion protein, novo nordisk lipase, orthocanavalin, platelet adhesion protein A (padA), satellite tobacco mosaic virus, and LRRK2. A large number of the crystals (10) were crystallized by liquid diffusion and a part of the DeLucas double-blind study. The crystals that were a part of the double-blind study are canvalin, connexin, E16 nucleoside-triphosphatase, E28 putative acyl-CoA dehydrogenase, E3 O-methyltransferase family protein 2, horse hemoglobin, methyl transferase fusion protein, novo nordisk lipase, orthocanavalin, and satellite tobacco mosaic virus. As stated previously, the liquid diffusion crystallization conditions were newly developed for these experiments. Although a fair amount of the crystals crystallized by liquid diffusion did not have improved results, learning about the experimental setup and conditions that were used can be beneficial for other microgravity crystallization experiments performed by liquid diffusion. Documenting the crystals that do not have improved results in microgravity can contribute to the field and help scientists to learn more. PadA was also a part of the DeLucas double-blind study, however, and it was crystallized using vapor diffusion. All of the crystals from this study that were flown as vapor diffusion techniques suffered from the flight delays. While some of the crystals were not affected by the flight delays, others were and it caused excessive precipitation for them. The excessive precipitation of crystals affected the growth of the crystals.

A final evaluation was done of all the crystals that did not improve in the more recent experiments (Table 6). The crystals that were seen as being worse in all three metrics are LRRK2, horse hemoglobin, E28 Putative acyl-CoA dehydrogenase, and connexin. These crystals were crystallized in microgravity using different diffusion techniques and conditions. It is worth noting that some of these proteins have never been successfully crystallized on earth and there still are no known structures of them. Some proteins present more challenges than others when

trying to crystallize them. The crystals that were seen as being worse in two of the three metrics are goat hemoglobin, glucose isomerase, E22 FAD9, E19 acyl-CoA dehydrogenase 2, canavalin, and orthocanvalin. These crystals at least improved in one metric, which could signify the crystallization conditions need to be slightly altered, but not completely changed. A lot more crystals, however, were seen worsening in only one of the three metrics. The crystals are pfGST, bovine trypsin, E13-ribosome recycling factor, E17-putative citrate synthase, E16 nucleoside-triphosphatase, E3 O-methyltransferase family protein 2, methyl transferase fusion protein, novo nordisk lipase, PadA, and satellite tobacco mosaic virus. This is promising because the protein crystals are still improving in some metrics, and ultimately will lead to better structures.

Protein	Technique	Metric	Reason
Plasmodium falciparum glutathione S-transferase	Capillary Counter Diffusion	Worsened Resolution	Due to the gradient of crystallization conditions produced along the capillary.
Bovine Trypsin	Liquid Diffusion	Structurally Worse	Little or no data for liquid diffusion crystallization conditions.
E13-ribosome Recycling Factor	Liquid Diffusion	Structurally Worse	Little or no data for liquid diffusion crystallization conditions.
E17-Putative Citrate Synthase	Liquid Diffusion	Structurally Worse	Little or no data for liquid diffusion crystallization conditions
E16 Nucleoside-Triphosphatase	Liquid Diffusion	Worsened Uniformity	Little or no data for liquid diffusion crystallization conditions.
E3 O-methyltransferase family protein 2	Liquid Diffusion	Worsened Uniformity	Little or no data for liquid diffusion crystallization conditions.
Methyl transferase fusion protein	Liquid Diffusion	Worsened Uniformity	Little or no data for liquid diffusion crystallization conditions.
NovoNordisk Lipase	Liquid Diffusion	Worsened Uniformity	Little or no data for liquid diffusion crystallization conditions.
Platelet Adhesion Protein A (PadA)	Vapor Diffusion	Worsened Uniformity	Multiple flight delays affected the proteins.
Satellite Tobacco Mosaic Virus	Liquid Diffusion	Worsened Uniformity	Little or no data for liquid diffusion crystallization conditions.
Goat Hemoglobin	Liquid Diffusion	Structurally Worse Worsened Resolution	Little or no data for liquid diffusion crystallization conditions.
Glucose Isomerase	Vapor Diffusion	Structurally Worse Worsened Resolution	Multiple flight delays affected the proteins.
E22 FAD9	Vapor Diffusion	Structurally Worse Worsened Resolution	Multiple flight delays affected the proteins.
E19 Acyl-CoA dehydrogenase 2	Liquid Diffusion	Structurally Worse Worsened Resolution	Little or no data for liquid diffusion crystallization conditions.
Canavalin	Liquid Diffusion	Structurally Worse Worsened Uniformity	Little or no data for liquid diffusion crystallization conditions.
Orthocanavalin	Liquid Diffusion	Structurally Worse Worsened Uniformity	Little or no data for liquid diffusion crystallization conditions.
LRRK2	Free Interface Diffusion	Structurally Worse Worsened Uniformity and Resolution	Shaking occurred when transporting the platers, which led to preventing crystal growth and formation.
Horse Hemoglobin	Liquid Diffusion	Structurally Worse Worsened Uniformity and Resolution	Little or no data for liquid diffusion crystallization conditions.
E28 Putative Acyl-CoA dehydrogenase	Liquid Diffusion	Structurally Worse Worsened Uniformity and Resolution	Little or no data for liquid diffusion crystallization conditions.
Connexin	Liquid Diffusion	Structurally Worse Worsened Uniformity and Resolution	Little or no data for liquid diffusion crystallization conditions.

Table 6. Protein Crystals that had Worsened Results during 2003 to 2019. The table lists the protein, the technique used to crystallize the protein, the metric(s) in which the protein worsened, and the reasoning given behind why the protein had worsened metric(s).

Conclusion and Future Studies

Scientists have devoted their efforts to understanding how organic macromolecular crystals develop in microgravity and how they compare to crystals grown on Earth. Parameters, such as resolution, uniformity, and structure quality determine whether the improvements in crystals are notable or not. While it may seem statistically that over the analysis time period experimental techniques and experience in microgravity have not yielded improved crystal growth, it is important to remember that the metrics for these microgravity-grown crystals are still improved.. While percentages are not as high as they once were, a considerable number of crystals have been successfully grown under microgravity conditions using diffusion techniques. The ability to cultivate high-quality crystals can lead to many advancements in the fields of electronics, metals, and healthcare and pharmaceuticals. This is why my research team and I are still exploring other materials grown in microgravity. As microgravity crystallization experiments become publicly available, data for these crystallization conditions can continuously be updated. Materials grown in microgravity have proven to be improved making microgravity experiments worth exploring.

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