Protein Crystallization

Team 21
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Engineering 339/340 Senior Design Project
Calvin College

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Photo taken by Preston Ji

http://journals.iucr.org/d/issues/1998/01/00/gr0693/gr0693fig1mag.jpg

http://www.embl-hamburg.de/services/spc/Services-and-resources/protein-crystallization/crystal2.jpg
Contents

Table of Figures ........................................................................................................... 4
Executive Summary ....................................................................................................... 5
Introduction .................................................................................................................. 6
Project Management ..................................................................................................... 7
  Team Organization ...................................................................................................... 7
  Schedule ..................................................................................................................... 9
  Budget ....................................................................................................................... 12
  System Architecture ................................................................................................ 12
Approach ...................................................................................................................... 13
  First Experimental Procedure – Lysozyme Crystallization ...................................... 14
    Equipment ............................................................................................................... 14
    Materials ................................................................................................................ 15
    Procedure .............................................................................................................. 15
  Future Direction ........................................................................................................ 17
Research ....................................................................................................................... 17
Design .......................................................................................................................... 21
  Criteria ...................................................................................................................... 21
    Cultural appropriateness ....................................................................................... 21
    Transparency ......................................................................................................... 22
    Stewardship .......................................................................................................... 22
    Integrity .................................................................................................................. 23
    Justice .................................................................................................................... 23
    Caring .................................................................................................................... 24
    Trust ....................................................................................................................... 24
  Alternatives ................................................................................................................ 24
  Decisions .................................................................................................................... 25
Conclusion ..................................................................................................................... 26
Acknowledgments ........................................................................................................ 27
References ..................................................................................................................... 28


Table of Figures

Figure 1: Gant chart of project tasks and their duration. ................................................................. 9
Figure 2: Displays the system architecture of Team 21’s project. The bioscience community consists of
many facets with protein crystallization being one of them. Proteins are crystallized with many different
methods, and one of these methods has different control variables (i.e. temperature, pressure, pH, and
relative humidity). Once crystals are formed, they can be analyzed via x-ray crystallography to
determine the macromolecular structure. .......................................................................................... 12
Figure 3: Linbro plates being filled with stock salt solution.................................................................. 15
Figure 4: Cartesian variability matrix .................................................................................................... 20

Table 1: Dates and duration for tasks leading to project completion. ...................................................... 10
Executive Summary

Protein crystallization is relevant in biomedical and pharmaceutical research. Team 21, The Crystal Method, plans to research and test new or unproven protein crystallization equipment and techniques under the oversight of Professor Jeremy VanAntwerp of Calvin College and Professor Mo Jiang of Virginia Commonwealth University. The Crystal Method is made up of senior Calvin engineering students Zachary Ardavanis, Braxton Bliss, Benjamin Feikema, Jose Abraham Mena, and Ross Kieser. The team has researched existing methods and purchased equipment to repeat and learn from current methods, and we believe that it is a reasonable goal to develop and optimize new equipment by the end of the spring semester. The team plans to acquire a protein upon which there is minimal research, but one that is also safe and easy to purchase, to perform new work in both technological improvement and crystallization parameters. The team has acquired permission to use lab space during second semester, and is in the process of acquiring equipment and supplies for experimentation. Success will be determined by the team’s outcome in adding new knowledge to the research community, likely in the form of better experimental equipment set-up.
Introduction

Protein crystallization was discovered around 150 years ago\(^1\). Since then it has become an important tool in understanding macromolecules’ structures; determining a macromolecule’s structure is crucial for the safe development of drugs and treatments that target and interact with it. Protein crystals are produced when the solution the protein is dissolved in becomes supersaturated. Currently four major techniques have been developed: vapor diffusion, micro batch, micro dialysis, and free interface diffusion. Among the major methods, vapor diffusion is the most used in industry and scientific research. Vapor diffusion accomplishes supersaturation using concentration gradients as the driving force.

Protein crystallization experiments are hard to generalize because each molecule behaves differently under different conditions; therefore, optimization is based on a per-case basis. Take lysozyme for instance; the conditions under which crystallization of lysozyme occurs have been optimized to the point that commercial kits are available. The variables that affect the rate of crystallization include pH, relative humidity, protein concentration, solvent, presence and concentration of precipitating agent and/or salts, and temperature, among others.

This design project aims at investigating the effects of relative humidity in a vapor diffusion, hanging-drop, crystallization technique, with the end goal of developing an optimized technique for the crystallization of a protein of interest.

Team 21 is made up of Ben Feikema, Braxton Bliss, Ross Kieser, Zac Ardavanis and Jose Abraham Mena, all Engineering majors with a Chemical Concentration at Calvin College. Jeremy VanAntwerp\(^2\)

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\(^1\) McPherson, *Current approaches to macromolecular crystallization*, 1989.
\(^2\) Professor of Chemical Engineering at Calvin College.
performs the role of faculty supervisor. Mo Jiang serves as an outside consultant. Several other chemistry and biochemistry professors have been contacted for their expertise on the subject.

Project Management

Team Organization

The roles of each team member are all intertwined and constantly evolving; however, some general roles have been adopted by each member according to their interests and skills. Ben Feikema manages the administrative aspects of the team, such as official communication with Professors VanAntwerp and Jiang.

Abraham Mena and Zac Ardanavis focus on research and experimental procedures. Mena and Ardanavis’ role entails looking for updated and relevant scientific papers, understanding the material and assessing the relevance of its material for the rest of the team.

Ross Kieser’s main official role is to guarantee that the budget is being executed appropriately and within means. Ross updates the budget as purchases and expenses are approved by the team and Professor VanAntwerp during weekly meetings; after approval, purchases are carried out by Calvin’s lab managers.

Braxton Bliss’s role is to manage team deliverables; these are those documents required by the Engineering Faculty as part of the Engineering 339 and 340 courses. Braxton also maintains the team’s official webpage.

The team meets twice a week, every Thursday morning with the student members and every Friday with Professor VanAntwerp. Thursdays meetings’ agendas start with each team member

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3 Professor of Chemical Engineering at Virginia Commonwealth University.
4 Robert DeKraker (Engineering Department) and Dave Ross (Chemistry Department)
5 http://engr.calvinblogs.org/17-18/srdesign21/
introducing the team to the work done in the tasks completed the week prior. The discussion then shifts towards an evaluation of what the next steps that need to be considered. Each team member decides his next step within the task, however the team input must be considered. Friday meetings begin similarly to Thursday meetings; all team members update Professor VanAntwerp on their respective tasks, then one team member updates Professor VanAntwerp on the team decisions made the day prior. Technical questions addressed to Professor VanAntwerp are also shared in this meeting.

Meeting minutes are recorded by Ben Feikema and stored within a privately shared OneDrive folder consisting of research notes, deliverables, presentation, and other digital elements.
Figure 1: Gant chart of project tasks and their duration.
Table 1: Dates and duration for tasks leading to project completion.

<table>
<thead>
<tr>
<th>Task Name</th>
<th>Start Date</th>
<th>End Date</th>
<th>Duration</th>
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<td>12/3/2017</td>
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<td>12/12/2017</td>
<td>1/24/2018</td>
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<td>1/3/2018</td>
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<td>4/5/2018</td>
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<td>5/4/2018</td>
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Budget

Ross Kieser is responsible for recording all expenses and tracking the budget percentage available. All expenses are discussed as a team first; final decisions are made after consulting with Professor VanAntwerp. Requests for additional money will be submitted and recorded by Ross Kieser and evaluated by Professor Jeremy VanAntwerp. Orders and receipts will be recorded on an Excel spreadsheet available on the team’s OneDrive folder. The team has applied for roughly $300 for equipment and supplies for the first round of experimentation through the fund available to all senior design teams.

System Architecture

Figure 2: Displays the system architecture of Team 21’s project. The bioscience community consists of many facets with protein crystallization being one of them. Proteins are crystallized with many different methods, and one of these methods has different control variables (i.e. temperature, pressure, pH, and relative humidity). Once crystals are formed, they can be analyzed via x-ray crystallography to determine the macromolecular structure.
Approach

To comprehend the team’s approach is it important to consider that the scientific community is the client, and a better understanding of protein crystallization is the product. To a certain extent, Professor Mo Jiang is a client, as his own research could potentially use the team’s product.

Initially, the team has researched the field of protein crystallization starting with academic material provided by Professor Jiang and immediately after using scientific research databases available through Calvin College’s subscriptions to scientific journals. Other sources of knowledge were academic materials produced by other universities’ chemistry departments describing protein crystallization of well-known proteins such as lysozyme for use in undergraduate and graduate laboratories. Vendors also produce academic material that was used by the team to better understand the field. It is important to note that given the nature of this design project, research is an ongoing process that continuously redirects the team’s focus. Our research and reporting depend mainly in individual accountability and responsibility; it is not a task that is designated and supervised, yet it is essential to the continuous betterment of the project path.
First Experimental Procedure – Lysozyme Crystallization

Throughout the project the team expects to crystallize two different proteins, the first of which is lysozyme. Lysozyme will be crystallized first because it is a well-documented protein allowing the team to practice crystallization while comparing results to existing data.

The next protein to be crystallized and used in technological development will be determined as a group following advice from Professor Jeremy VanAntwerp and Professor Mo Jiang. The procedure for the second protein will likely be optimized using the same hanging drop technique like the one below, though it is subject to change.

**Equipment**

1 – 0.1-2.0 μL micropipette
1 – 1 - 5 μL micropipette
1 – 100-1000 μL micropipette
1 – box low-retention micropipette tips
1 – syringe (for sealant application)
1 – 24-well hanging-drop crystallization plate
24 siliconized cover slides
1 – 40x magnification microscope
1 – pH meter
1 – forceps/tweezers
1 – container syringe filters
Access to 5-50mL beakers
Professional wipes
Access to -80°C freezer for protein storage
Materials

- Immersion oil, vacuum grease, Lubriseal, or equivalent coverslip sealant
- 1 gram hen egg white Lysozyme protein (99% purity ideal)
- 10 g of sodium chloride
- 0.5 L of 1M sodium acetate solution
- Purified water for solution preparation and cleaning

Procedure

1. Mix 0.1 L of the 1M Sodium Acetate solution with 1.9 L of deionized water to produce a 2 L of a 50mM Sodium Acetate solution.

2. Dissolve hen egg white lysozyme in 50 mM Sodium Acetate (NaAc) buffer solution at pH 4.6 (measured with pH meter) to obtain a 35 mg/mL protein concentration stock solution.

3. Dissolve NaCl in 50 mM NaAc buffer solution at pH 4.6 to obtain a 2 M NaCl stock solution.

4. Pipette 0.5 mL of salt solution in each well in the linbro plate, Figure 3 shows a linbro plate set. Apply enough oil to the rim of each well in the linbro plate for it to act as a seal using a syringe. The ring of sealant around the edge of the well should have a small gap to prevent air pressure buildup as the well is sealed.

Figure 3: Linbro plates being filled with stock salt solution.
5. Take a cover slide and clean it with kemwipes to prevent contamination.

6. Pipette 5 µL of protein solution onto the center of a cover slip; pipette an equal volume of salt solution using a fresh tip. Homogenize both solutions by pipetting up and down gently.

7. Pick up each cover slip with forceps and invert it without losing the drop; place it on top of each well.

8. Let the wells sit over a shock absorbing material such as packing foam, and check for crystals periodically for 1 week in 5-hour intervals.
Future Direction

Once the crystallization of lysozyme has been achieved, a less studied protein will be chosen. The next protein to be investigated should be the following criteria: easily and economically accessible, representative of an interesting group, and relatively less studied. The chosen protein should also be one that belongs to a group of proteins that are of interest to the scientific community. The last criteria simply mean that something novel should be researched.

The next step is to determine the hardware\textsuperscript{6} variables that will be considered. The team has considered the use of relative humidity as a tool to optimize a crystallization method. The team is currently working on developing a mathematical model for the equilibrium between vapor, well fluid, and droplet and their relationship with relative humidity of the chamber.

Once the chosen protein is crystalized, the team will consider the process of controlling crystallization with relative humidity. Once this is achieved, the team with find ways to optimize a built device or create a new one with the hopes to achieve larger crystals with high purity. The team’s success will come from either by adding knowledge to the selected protein, or the development of new crystallization equipment.

Research

McPherson and Gaviria describe the crystallization process concisely and successfully in their 2013 paper.

\textit{“Macromolecular crystallization (...) is based on a rather diverse set of principles, experiences and ideas. There is no comprehensive theory, or even a very good base of fundamental data, to guide our efforts, although they are being accumulated at this time.\textsuperscript{6}”}

\textsuperscript{6} Equipment used for crystallization
As a consequence, macromolecular crystal growth is largely empirical in nature, and demands patience, perseverance and intuition.”

Protein crystallization is an art as much as it is science given the vast number of variables and the even wider range of sensitivity that each protein shows to every technique and variable. After consulting with Professor Jiang, the team considered lysozyme as the most appropriate protein to practice the hanging-drop crystallization method because lysozyme is relatively easy protein to crystallize and the process is well understood, making it quite easy to discern good results from bad ones.

Initial screening taught us that companies such as Hampton Research, Fluka Analytical and Sigma-Aldrich sell prepared protein crystallization kits that guarantee the success of easy crystallization (i.e. lysozyme); similarly, the team encountered plenty of research papers and educational sources that outline the general method for the crystallization technique. The team relied mainly on the papers by Iwai W. at al, the lab manual published by the University of Michigan, the research procedure by Carvajal and McDonald, and Hampton Research’s Siliconized Glass Cover Slides kit documentation to create the procedure and material list specified above. This was then cross-checked with Professor Rachael Baker’s lysozyme procedure to confirm that the team had written a well-designed procedure.

Vapor Diffusion Technique

In McPherson’s 1889 paper, Current approaches to macromolecular crystallization, he introduces the general theory behind vapor diffusion techniques. The general approach is to bring a solution of protein to supersaturation through the diffusion of water, or another solvent, out of the

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7 McPherson and Gavira, Introduction to protein crystallization
8 Hampton Research, Siliconized Glass Cover Slides
9 Fluka Analytical, Crystallization Cryo Kit for Proteins
10 Sigma-Aldrich, High Throughput Crystallization Kit for Nucleic Acids
11 Iwai W. et al, Crystallization and evaluation of hen egg-white lysozyme crystals for protein pH titration in the crystalline stat
12 University of Michigan, BCH 6744C: Molecular Structure Determination by X-Ray Crystallography
13 Carvajal and McDonald, Growth and Characterization of Lysozyme Crystals in Varying Precipitants.
protein. Two solutions are prepared: One with protein, precipitants, and buffers, and another with precipitants, buffer, and no protein. The two solutions are introduced in a closed system separated by air. The solutions must vary in concentration in some respect to create the driving force necessary to promote effective diffusion. For the crystallization of Lysozyme, sodium chloride (NaCl) is dissolved in both solutions, but at a higher concentration in the solution not containing the lysozyme. Because the concentration of NaCl is higher in the solution without protein, the system will tend toward equilibrium via diffusion of water out of the lysozyme solution, increasing the concentration of salt and lysozyme. The increased concentration of salt acts to decrease the solubility of the lysozyme, pushing the system into the supersaturated region until nucleation is achieved.

**Determining Saturation Curves**

In their 2007 paper “Crystallization and evaluation of hen egg-white lysozyme crystals for protein pH titration in the crystalline state”, Iwai et al describe a method for determining a protein’s saturation curve that relies on the micro-dialysis method. The team, although excited to have discovered a detailed description of the process flow, was also faced with the difficulty of another learning curve in addition to all others contained in this design project.¹⁴

Two lessons were taken from the paper by Iwai and peers. The first is that linbro plates can be set up in such a way that the equipment resembles a cartesian plane where one variable changes horizontally and another vertically allowing. Variables X and Y can be anything the team choses if it is variable that can be controlled in either one of the solutions.

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¹⁴ Rachael Baker, *Chemistry 383 Independent Project: X-Ray Crystallography of Lysozyme*
The second lesson learned from Iwai et al was the magnitude of the steps taken for each variable. Adequate steps across a variable should not surpass 1% of the total range and can be as small as 0.1% of the total range.
Design

Criteria

To engage in a discussion of ethics, the team must define what and for whom we are building. The team intends to add knowledge about a protein and a protein crystallization method to the scientific community. This is different than a typical design-and-build project in that our customer isn’t a purchasing consumer but an individual or team of researchers. Our project is less about a saleable consumer product than a functional scientific device, so conventional design criteria like aesthetics and ergonomics have little to no influence compared to whether the device functions. The team will use the design norms as presented in Ermer and VanderLeest’s 2002 paper on engineering ethics in education as a method for examining the moral aspects of our project. These norms are qualifications for proceeding with a project, normative principles in the design and research process, and qualities of the final product. Throughout this discussion, it will be helpful to remember that where Ermer uses the word design and the concept of a tool or vehicle or building comes to mind, we will take that to mean both our construction in the lab and the presentation of our results.

We now have a conception of what we are building and for whom we are researching, so we can continue with a discussion of the relevance and application of design norms to our project.

Cultural appropriateness

Ermer defines cultural appropriateness by saying, "The design ought to fit the culture into which it will be introduced." If we call "design" the actual physical construction and experimentation we do, the culture into which it is introduced is only us five students. This is an unhelpful conception of design in that there’s not much else we can say, so let’s instead replace "design" in that definition "knowledge."

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15 Ermer G., Using design norms to teach engineering ethics
16 Ibid
The culture into which our knowledge will be applied is that of the scientific community, specifically those interested in protein crystallization methods. It is the team's and advisors' conviction that this level of research at an undergraduate level is appropriate. The research we've done is not beyond our comprehension, and the field of protein crystallization is both undeveloped and broad enough to be accessible to college students.

Transparency

Ermer defines transparency by saying, "The design ought to be understandable to the user." As shown in the discussion of cultural appropriateness, it's most fruitful to think of "design" instead as "knowledge." And we'll again redefine "user" as a scientist with reasonable background in crystallization science and methods. In this respect, the team will make every effort to make our final research report understandable to anyone with some basic knowledge of chemistry, biochemistry, and/or chemical engineering. Our target reader will be someone in who has done work in protein crystallization, but we will strive to make our report accessible to entry-level scientists (junior level chemical engineers might be a good target), as we were when starting out this project. This means that the language used will be accessible to the intended audience; we'll thoroughly define terms before using acronyms and make the basic science approachable to someone with a basic college-level of science or engineering education.

Stewardship

Ermer defines stewardship by saying, "The design ought to carefully use earth's resources, frugally and thoughtfully." For this analysis, I'll define design as our experimental set-up. The team was forced to wrestle with this question when asked to predict how much waste would be produced in experimentation and check if any special disposal methods were required. The team concluded that, at least for the first round of experimentation, no special disposal methods would be required. As for the total amount of waste, this is incredibly difficult to predict, but a rough estimate would be 1 liter of liquid, rounded quite generously up. This waste consists almost exclusively of purified water, but would
contain trace amounts of lysozyme protein, NaCl, sodium acetate, and small concentrations of other reagents. One concern is number of plates we’re purchasing. The minimum order is 24, but we will likely only use 2-4 at one time. Unfortunately, there doesn’t seem to be a way around this apparent waste, and we can only hope that the plates will be put to good use after we leave.

Our research will hopefully lead to improved efficiency in the drug development process, reduced petroleum use, and medicines with more specific targeting capabilities, all of which are desirable under the lens of stewardship.

**Integrity**

Ermer defines integrity by saying, “The product must do the job that it was created to do, do it in a way that makes it pleasing to use, and should promote human values and relationships.” The relevance of this design norm is tougher to identify. Since our goal is the creation of a new or improved crystallization method, we likely won’t have nicely packaged, easy-to-use, saleable device, but rather a complex collection of lab glassware, tubes, and chambers. The primary goal isn’t to improve human experience, but to accomplish a necessary function. If we instead define “product” as “research report,” then the report will be written in such a way to be accessible, educational, and concise to the intended audience. See *Transparency* or further discussion.

**Justice**

Ermer defines justice by saying, “The engineer must consider not only the user, but also others who are directly or indirectly affected by the design.” An understanding of the “Cultural Appropriateness” section is relevant here to understand those who are directly or indirectly affected by the design. We’ve already discussed the distant relationship between our work and the what it might someday be used for, namely drug development.
Caring

Ermer defines caring by saying, “The design ought to show due care for persons.” Protein crystallization does not involve problematic supply chains or dangerous reagents to the best of our knowledge. It is hard to think of a less morally contentious use of technological progress than medical care. While there are many difficult ethical questions in some areas of healthcare (assisted suicide, abortion, embryonic stem cell use, among others), protein crystallization is far detached from any of those. The team believes that drug development is generally a good, moral thing to do, and that the pursuit of further understanding of biological systems is a noble goal.

Trust

Ermer defines trust by saying, “Designs and designers should be trustworthy.” She goes on to discuss conflicts of interest and competency in the field of development. While these concepts are more relevant in a design-and-build project that has human users and an employer, they are still relevant if we again define “designs” as “knowledge” and “designers” as “researchers.” In this way, the team will strive to present accurate, coherent reports and to support any claims we make with evidence and reason since researchers may not have reason to trust us based on our (lack of) previous research or credentials.

Alternatives

The team had several options to consider. We could have chosen to crystallize a protein that had little to no successful crystallization methods developed. We could have chosen to develop a new or modified crystallization technique and equipment alongside some well-documented protein, or we could do a combination of both: a new device and methodology, and a somewhat unresearched protein but still one that was relatively easy to acquire and posed no usage restrictions.
Decisions

The team struggled some in identifying a path for research due to several factors but mostly an unfamiliarity with protein crystallization. It was only near the end of the semester that team decided to more seriously pursue the hardware option, as it will be called, instead of the protein research option. The main driver of this decision was an unfamiliarity with biochemistry, but a protein that little to no research would likely be expensive or impossible to purchase. In that case, even producing and purifying the protein would be a project more suited to biologists or biochemists. Our education is first with hardware methods, and second with chemistry and biochemistry, so a project that pulls more heavily on the hardware side of things is more suited to our educational background.

Another factor in our decision making was the likelihood of success. Using an entirely un-researched protein would mean that anything we learn would be new and useful, whether we successfully produce a diffraction-quality crystal or not. But, this would come at the cost of a steep learning curve and a huge time and money investment to make any progress. On the other hand, if we had chosen to crystallize a well-researched protein, it would be difficult to add anything of value to the research community. Since there has been little research into novel hardware for crystallization, we are ensured success in that anything we try will be new and interesting, and it is something that is intellectually and financially accessible.
Conclusion

Team 21 aspires to adding to the bioscience community wherever they can. So far, this semester consisted of establishing a strong basis for the design project by determining design methods and criteria, navigating through obstacles of lab space and equipment, and setting schedule and design structure to ensure the success of next semester. Team 21 is scheduled to crystallize lysozyme this interim to establish a basic biochemical understanding of the processes. We believe it to be feasible to optimize or create equipment currently established in the protein crystallization field to further its methods. The teams successful will come from adding any new understanding to the field, mostly likely in the area of experimental set-up.
Acknowledgments

Professor Mo Jiang – advisor, chemical engineering
Professor Jeremy VanAntwerp – advisor, chemical engineering
Professor Eric Arnoys – consultant, biochemistry
Professor Larry Louters – consultant, biochemistry
Professor Dave Benson – consultant, crystallization
Professor Rachael Baker – consultant, crystallization
Dave Ross – chemistry lab manager
Bob DeKraker – engineering lab manager
Professor Douglas VanderGriend – chemistry department chair
References


