

Analyzing Ice Recrystallization Inhibition Using Amino Acids in High Pressure
Conditions and Implementing Spatially Resolved Chord Length Distribution

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Abstract

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Even today, ice crystallization is still a research subject that is studied in various fields of science but has been barely explored. Within the ice crystallization research, one of the most common research subjects is ice recrystallization inhibition (IRI). Within this research, the influence of peptides and peptoids on IRI has been an emerging research subject. However, there has still not been much successful results on how peptides and peptoids affect or enhance IRI. Another subject that has not been explored is the effects of ice crystallization at high pressure. The first part of this study aims to study and

compare the IRI effects of amino acids and peptoids to peptides in different ice phases, specifically ice VI. The effects of amino acids on ice VI were studied utilizing a method known as the “squeeze” assay. A solution of alpha-alanine and sodium chloride were loaded into a Merrill-Bassett diamond anvil cell which was utilized for ice crystallization at high pressure. The IRI effects of alpha-alanine were observed during ice crystallization. Another obstacle in IRI research is that there is still no analytical method that provides precise quantitative data for non-uniform crystal structures. Adding impurities to water leads to non-uniform ice crystallization. The second part of this study focuses on proving how effective a novel quantitative data analysis method known as Spatially Resolved-Chord Length Distribution (SR-CLD), which measures the spatial variation of non-uniform ice crystals, can be useful. The accuracy of SR-CLD was tested by utilizing a preprocessing method, then analyzing the spatial variation of the non-uniform ice crystals using the SR-CLD algorithm. Results showed that the amino acids were just as highly effective as ice recrystallization inhibitors as peptides on ice VI and that SR-CLD is confidently capable of quantifying spatial variation of the ice crystals.

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Secondly, I would also like to thank both Dr. Baptiste Journeaux and Chris Woodburn for their help in my research. I am greatly indebted to both as they have helped me with my research despite their busy schedules. Dr. Journeaux and Chris truly helped me to learn and perform successful research experiments. From obtaining lab materials to teaching integral techniques, my thesis would not have been possible without the aid of both Dr. Journeaux and Chris.

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Dedication

To my loving MOM and DAD

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Chapter 1: Introduction

1.1 Ice Crystallization and Ice Recrystallization Inhibition

Ice crystallization has become a growing interest in many science fields. One of the most studied subjects on ice crystallization by scientists is ice recrystallization inhibition.

Unfortunately, scientists are still struggling to understand the chemistry of ice to develop efficient ice recrystallization inhibition (IRI) methods. A few science fields that have gained much interest in studying ice crystallization and pursuing IRI research are biochemistry, materials science, and planetary science. IRI has become a significant research topic in the fields of both biochemistry and materials science due to practical applications. A very common practical application of IRI is antifreeze agents, which are used to prevent ice formation, such as plane wing icing and organ cryopreservation. Unfortunately, antifreeze agents have still shown to be ineffective in both applications. Another major problem with currently used antifreeze agents is that they produce toxic byproducts.

Another significance of IRI is that it can help scientists learn more about unexplored subjects, such as deep sea creatures, subducting oceanic plates, and icy moons. Studying IRI activity has shown that it can be used to help understand how to predict volcanic activity and understand the habitability of organisms in cold icy environments. Even on Earth, there are species that have been found living under the ocean in deep sea levels that are capable of naturally producing antifreeze proteins to survive extreme cold temperatures and prevent ice formation. Outside of Earth, one of the subjects that scientists at NASA have been studying is if there is life existence in icy moons. NASA scientists have also performed studies to understand

cryovolcanic behavior of icy moons, which is a phenomenon where water, ammonia, or methane-rich fluids rather than molten rock, cause volcanic eruptions.

1.2 Peptides

A peptide is a sequence of amino acids that are formed by the reaction between carboxyl and amino groups of each amino acid. **Figure 1** shows a simple structure of a peptide sequence. Thus, nitrogen is able to become a part of the backbone of the peptide, which is the significance of peptides in the study of IRI. Chemistry and materials science fields have found the application of peptides to be much more effective in IRI and do not produce harmful byproducts.

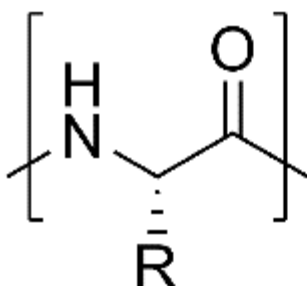


Figure 1. Structure of a simple peptide

A major subject that scientists have been studying is how to develop effective peptides that can enhance IRI. Experiments have shown that when peptides are applied to a water sample, the IRI occurs because the water particles attach to the peptides instead of other water particles, which inhibits ice crystallization. Many peptides have been created by synthesizing a sequence of amino acids that mimics antifreeze proteins that are naturally developed by animals and plants. In many science fields, the interest in synthesizing more efficient IRI peptides is because

it has shown to help with many practical applications, such as improving cryopreservation and high altitude ice crystallization inhibition on airplane wings

1.3 Peptoids

Peptoids are synthetically developed chemicals that are derived from peptides. The main difference between a peptide and a peptoid is that while a peptide has a side chain group on the central carbon backbone, a peptoid has a side chain group on the nitrogen of the backbone.

Figure 2 shows a simple example of a peptoid structure. Peptoids have been gaining much interest due to their enhanced IRI effectiveness compared to peptides.

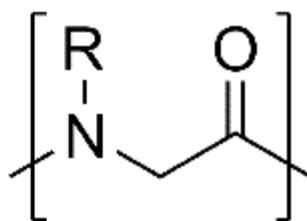


Figure 2. Structure of a simple peptoid

Peptoids are not naturally produced in nature. New peptoid sequences that are being studied are structured based on preexisting peptide structures, which are then modified so that the functional groups are attached to the nitrogen atom instead of the central carbon of the peptoid backbone. One of the most common methods that is currently being used to develop effective and environmentally friendly antifreeze agents is by studying biomimetic proteins to synthesize peptides and peptoids.

1.4 Amino Acids

Meanwhile, there has not been much study on the effects of amino acids on IRI. Amino acids are simple sequences that contain a central carbon, amino group, side chain group, carboxyl group, and a hydrogen atom. **Figure 3** shows a simple structure of an amino acid.

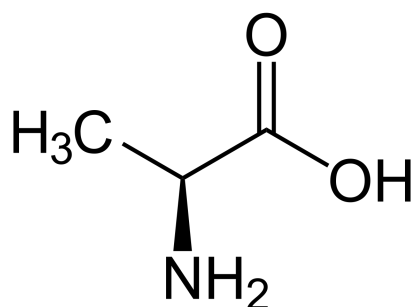


Figure 3. Structure of a simple amino acid

It has been shown that even a simple amino acid known as alpha-alanine in a 10 mM sodium chloride solution greatly increases IRI. The paper, *Ice Recrystallization Inhibition by Amino Acids: The Curious Case of Alpha- and Beta-Alanine*, discusses the effects of alpha-alanine and beta-alanine as a IRI substance and shows that amino acids, themselves have the potential to perform significant ice crystallization inhibition depending on the structure of the amino acid. The two amino acids that were tested in the study were alpha-alanine (α -alanine) and beta-alanine (β -alanine). The two amino acids correspond to which carbon the amino group is attached to in **Figure 4**.

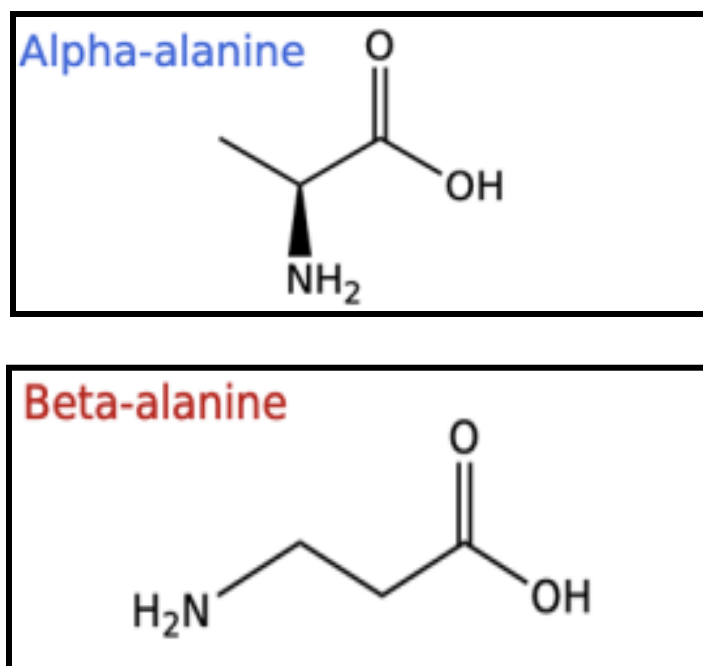


Figure 4. Structures of Alpha-alanine (α -alanine) and Beta-alanine (β -alanine). Figures from: *Ice Recrystallization Inhibition by Amino Acids: The Curious Case of Alpha- and Beta- Alanine. The Journal of Physical Chemistry* (2022) 13(9), 2237-2244.

The main method that this research utilized to analyze ice recrystallization inhibition is known as the “splat” cooling assay. The paper describes the method as a way to rapidly cool a droplet of solution on a platform that is -8°C to form a polycrystalline ice monolayer. The comparison of mean grain size was used to determine ice growth. In addition, a “sucrose-sandwich” assay was utilized to study if α -alanine has any effect on ice crystal morphology. The study showed that α -alanine did not exhibit morphologies that are characteristic of ice-binding. However, β -alanine did not show ice recrystallization inhibition.

This study takes a different approach from the “splat” cooling assay in the sense that it utilizes the “squeeze” assay that has been termed by Peter Pauzauskie. The squeeze assay relies on pressure to form a polycrystalline crystal structure rather than temperature. The motivation for using the squeeze assay in this study was to observe the IRI effects of a simple amino acid on a

different ice crystal structure. At high pressure, different ice crystal structures can be formed. Thus, one of the main parts of this study was to compare the effects of IRI of α -alanine on an ice crystal structure that is formed at high pressure. To observe the effect of IRI of amino acids on a different ice crystal structure, ice VI was used as the main test subject for this research project. Ice VI has a tetragonal ice crystal structure. The main goal was to observe if there will be any significant in IRI activity between a single α -alanine amino acid and in a different ice crystal structure using high pressure.

1.5 Ice Phases at High Pressure

Another subject that has not been explored is ice crystallization in high temperatures and pressures. Until now, most research on ice crystallization has only been applied to hexagonal structured ice crystals, which are designated as I_h . Hexagonal ice is the typical ice that is present on Earth, such as snow. However, there has not been research that has focused on the application of ice crystallization on other ice crystal structures, such as tetragonal and cubic structures, which are known as ice VI and ice VII, respectively.

The different structures are produced when hexagonal ice is exposed to high pressure. The bonds between the hydroxide groups in the water molecules become deformed, which causes the bonding patterns of the ice crystal structure to change. There has not been much research conducted on how amino acids and peptides can affect the IRI activity of different ice crystal structures. However, this subject of IRI has become a growing interest in planetary science due to the question of if there are living organisms in icy planets, such as the moon of Jupiter, Europa, and the moon of Saturn, Titan, which all contain different ice crystal structures.

In addition, a significant question in space exploration and technology is if peptides can prevent ice formation on spacecraft and instruments in icy environments.

Until now, only sodium chloride (NaCl) has been used as the basic approach to studying the effects of salt on ice crystallization at high pressure. It has already been shown in previous studies that salt in water can perform IRI. This study observed how ice crystallization at high pressure can be enhanced for NaCl solutions when α -alanine is added. This research project studied the efficiency of amino acids based on the results that amino acids can produce efficient IRI in a NaCl solution and apply it to a different ice crystal structure, which is found in an ice phase that is exposed to a higher pressure than I_h .

1.6 Spatially-Resolved Chord Length Distribution

Both quantitative and qualitative data are crucial to understanding the attributes of a crystal structure. Through the advancements of technology, improved automated methods for quantitative data analysis have been a major focus for performing more efficient data analysis. Unfortunately, currently utilized automated methods have shown to produce inaccurate data analysis for non-uniform microstructures, and human intervention has been required to correct the inaccurately measured data. It has been found that the inaccuracy of quantitative data analysis stems from estimated measurements that do not account for non-uniform microstructures. Therefore, the second focus of this research is on evaluating a novel automated quantitative data analysis method known as spatially-resolved chord length distribution (SR-CLD).

The SR-CLD method developed by Sheila Whitman from the University of Arizona was applied to this experiment due to its ability to analyze the spatial variation of non-uniform

microstructures of heterogeneous crystal structures. The main reason for applying this method to my research was to compare the efficiency to previous algorithms, and whether it can accurately measure spatial variations of the non-uniform ice crystal structures. SR-CLD was used in this research to calculate the spatial uniformity and spatial variation of the ice crystals to determine the effectiveness of α -alanine as an ice recrystallization inhibitor.

Determining spatial differences is important because microstructure description is a significant method for understanding process–microstructure–property relationships. Whitman (2024) specifically states that

a microstructure with a significant gradient is a result of a particular process and will have properties different from those of a uniform microstructure. Yet, microstructure descriptions that focus on overall size distributions or mean values without spatial sensitivity will fail to reflect such differences. The SR-CLD approach that resolves spatial microstructure variations can therefore serve as a statistically rigorous microstructure description for quantitative process–microstructure–property relationships in materials with significantly non-uniform microstructures. For example, SR-CLDs or their reduced-order representations (e.g., from principal component analysis could be used as microstructure ‘features’ for machine learning, as previously shown with traditional (non-spatiallyresolved) CLDs. (p. 8)

SR-CLD’s ability to help understand process–microstructure–property relationships is what makes it a significant tool to help explain the relationship between the effects of amino acids on the IRI of an ice crystal structure other than hexagonal ice.

Chapter 2: Experimental Methods

2.1 Ice Crystallization

The same α -alanine concentration and NaCl concentration was used from *Ice Recrystallization Inhibition by Amino Acids: The Curious Case of Alpha- and Beta-Alanine* as a reference for this research. A Merrill-Bassett diamond anvil cell (MDAC) was utilized to perform the squeeze assay. Rhenium gaskets were utilized for the metal gasket. To prepare the MDAC, a 30 mm thick rhenium disk was brushed with sandpaper to remove the oxidized surface of the alloy and any other impurities. After the disk was brushed, the MDAC was fully assembled with the disk between the DACs for the dimpling process. The schematics shown in *Figure 5* can be used to see how the MDAC was assembled.

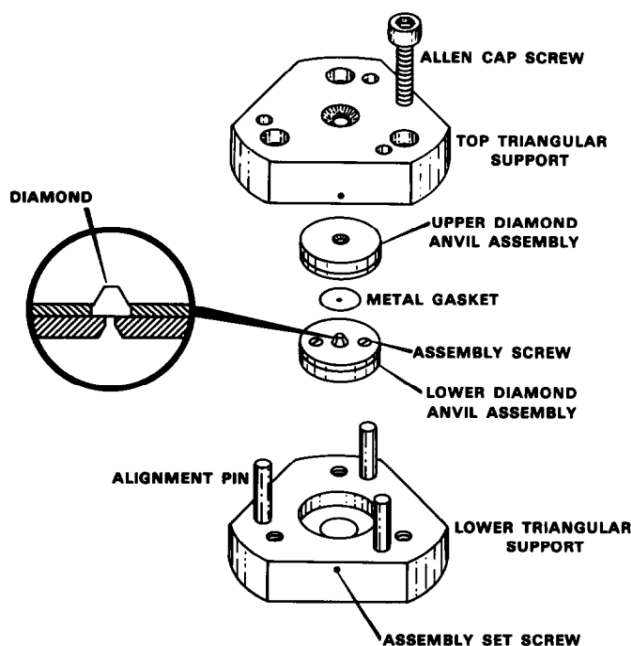


Figure 5. Schematics of Diamond Anvil Cell (DAC)

After the MDAC was fully assembled, an allen wrench was used to tighten and apply pressure to the metal disk. Once the MDAC was tightened securely, the Allen wrench was further used to tighten the MDAC until the disk was dimpled. For this experiment, the disk was dimpled to 50 microns. Once the disk was dimpled, a food coloring was applied to the center of the gasket so that the drilling process would be easier. After the dimpling process, a drill with a diameter of 400 microns was used to drill a hole through the disk to create a gasket. The gasket was then washed using methanol to prevent impurities during the experiments.

A few things that were considered as most important in the process of preparing the MDAC experiment was that the diamonds needed to be aligned parallel to each other. Furthermore, it was important to make sure that the distance between the DACs are precise in order to perform experiments without damaging the diamonds. A Nikon microscope and micrometer were used to measure the distance between the two DAC and to make sure that the lower and upper cells of the MDAC were parallel to each other.

For the experimentation, a solution containing α -alanine and sodium chloride solution was exposed to high pressure by using the MDAC. A single drop of 50 mM α -alanine and 10 mM NaCl solution was loaded onto the DAC within the hole in the gasket. After each solution was loaded, the MDAC assembled and tightened using an Allen wrench to control the amount of pressure and crystallization of the sample. The pressure of the solution was controlled by how tight the MDAC was. The MDAC was tightened until the ice phase was in the ice VI phase. After the pressure was adjusted to ice VI, ice crystallization was observed throughout a period of 60 minutes. The ice crystallization and IRI results were recorded at 5, 10, 30, and 60 min. Photoluminescence and Raman spectroscopy were used to calculate the pressure of the ice crystallization of the sample at 300 K.

2.2 Preprocessing Images for Spatially-Resolved Chord Length Distribution

After acquiring the ice crystallization images, the images were used to evaluate SR-CLD. Image preprocessing was required before running the SR-CLD code. A preprocessing algorithm was developed so that the image showed just the crystal structure in binary format. The main tools used for the preprocessing step were Adobe Photoshop, ImageJ, and Python was used to develop the algorithm. Adobe Photoshop was used to brighten excessively dark areas of the image being processed. For the first part of the preprocessing OpenCV and Numpy were used to convert the image to binary. First, the image was converted to a grayscale image so that the image is analyzed based on the intensity of patterns, instead of color. Next, a histogram equalization function was utilized to improve the contrast of the images. Then, Gaussian Blur was applied as a smoothing filter to reduce noise and smooth transitions between each ice grain. After that, a Canny edge detection algorithm and hysteresis thresholding were utilized to convert the raw image into a binary image, where the zero value indicates an empty area and the one value indicates the presence of a crystal surface.

The preprocessed image then had to be inverted using ImageJ because the preprocessing code outputted the grains as black and the edges as white. The algorithm converts the image to a binary image with opposite binary values because the algorithm views the black areas in the image as areas with color. Then, the preprocessed image was then inverted using ImageJ so that the white areas indicated the crystal surfaces, while the black areas indicated the separation between the crystals. This process was able to show ice deformation in addition to the

crystallization, which was shown to depend on surface area. **Figure 6** shows the fully preprocessed images at each captured time.

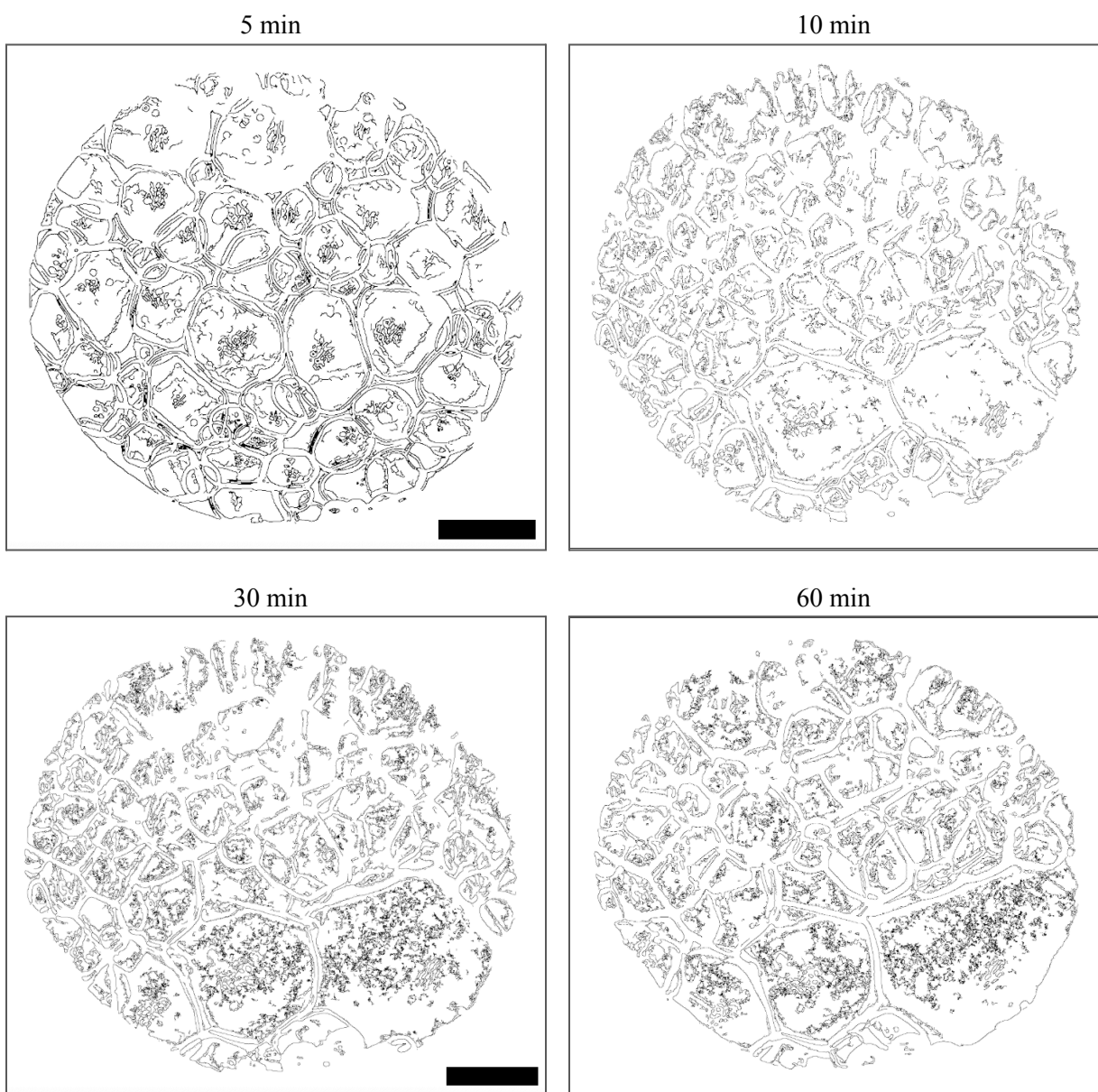


Figure 6. Preprocessed images of the sample at 5, 10, 30, and 60 minutes. The scale bar is 100 μm and consistent for all images

2.3 Testing Spatially-Resolved Chord Length Distribution

The most significant aspect of the first part of this research is the change in ice crystal structures by adding amino acids. It was important to acquire data of the spatial distance of the ice crystals in order to determine how the amino acids affect ice crystallization and IRI. In addition, determining the spatial variation of the ice crystals was crucial in order to compare ice crystallization in a different pressure environment. Thus, this research analyzed quantitative data, in addition to qualitative data. Python scripts and ImageJ were the main methods used to obtain quantitative data for this research. **Figure 7** shows an example of how SR-CLD is calculated to generate heat maps that show the spatial variation of a non-uniform microstructure.

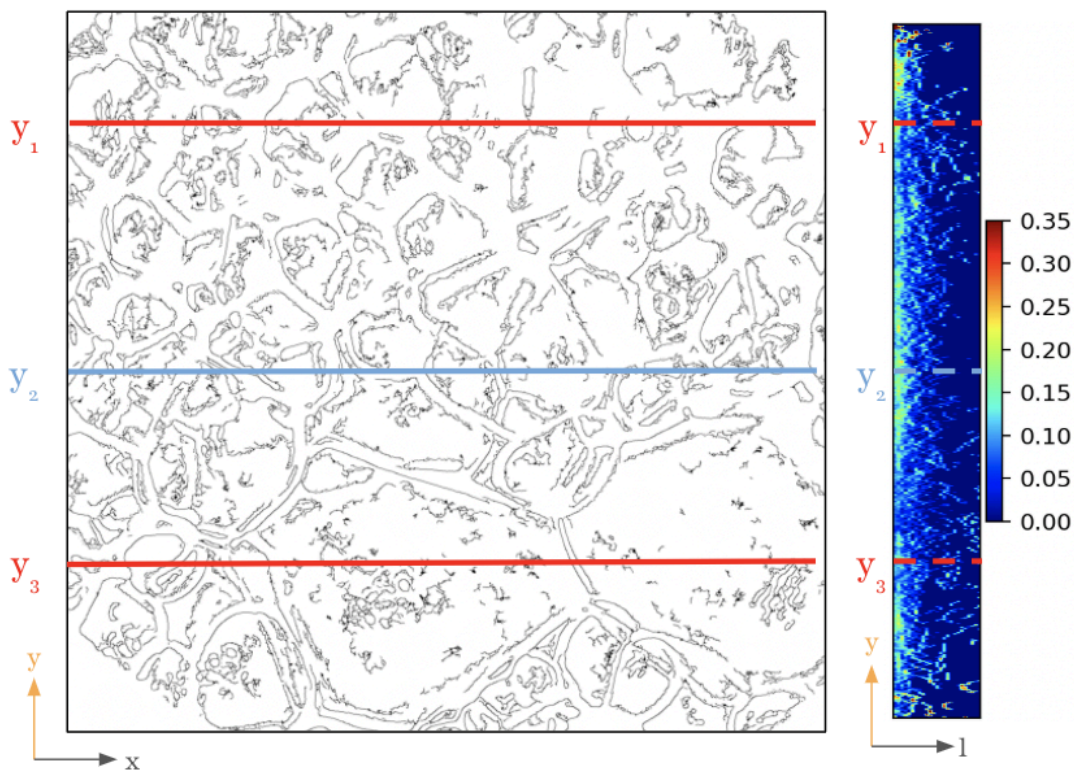


Figure 7. SR-CLD measurements using the scan-line method after 10 minutes of ice crystallization.

SR-CLDs are numerically represented by two-dimensional probability density arrays (matrices) of size $K \times B$, where K corresponds to the number of pixel rows or columns in the microstructure map, and B is the number of chord length bins. In **Figure 7**, the horizontal chord lengths of ice crystals, B , at each row, K , are being measured. Each chord length along the scan-line across the row K of the image is measured by the length of each intercept, in pixels, of each grain in the crystal, and stacked, which is depicted in the heat map by the different colors. The heat map shows a visual representation of the spatial variation of the crystal structure of ice VI at 10 minutes.

Chapter 3: Results and Discussion

3.1 Ice Recrystallization Inhibition Using Amino Acids

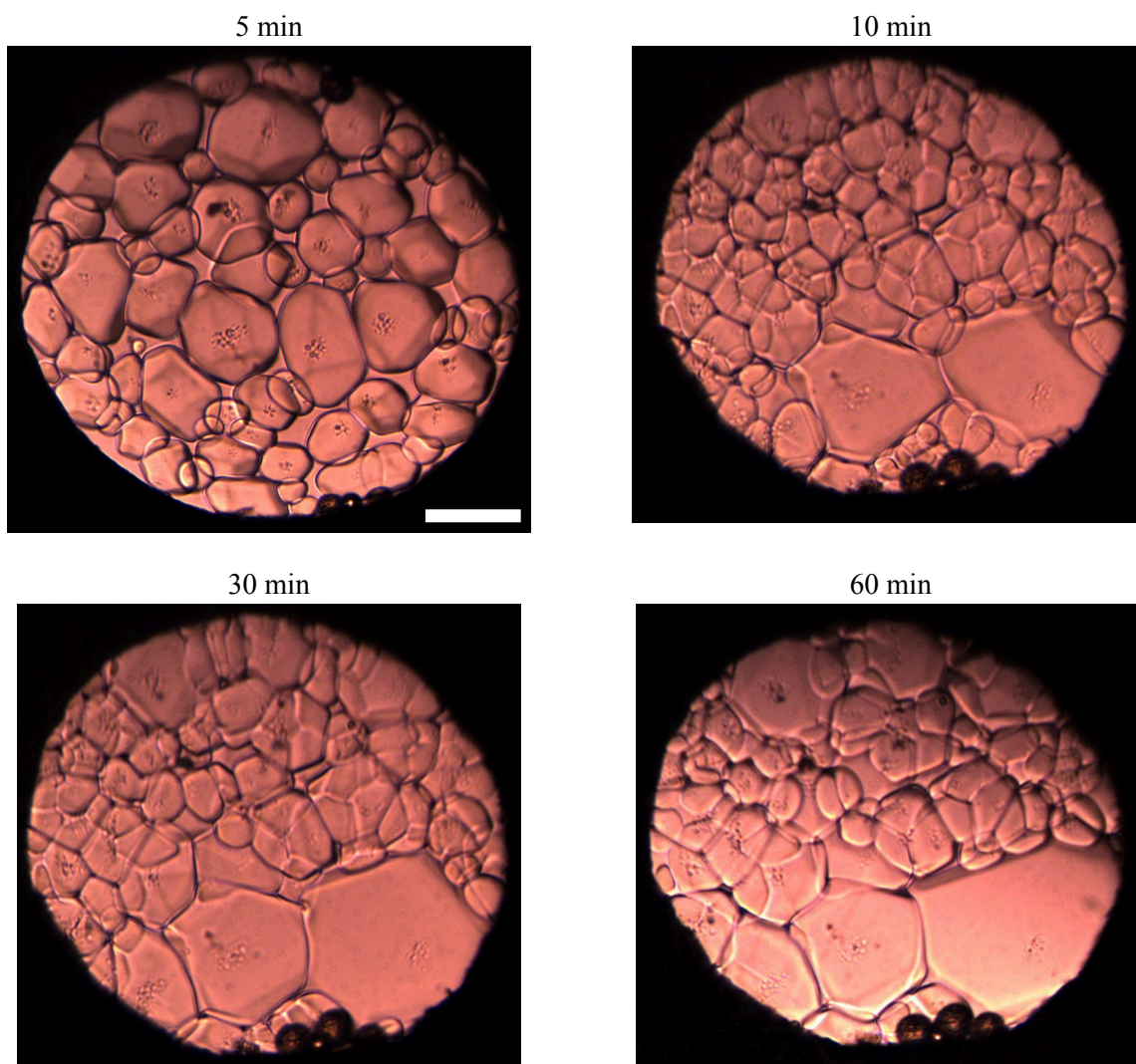


Figure 8. Micrographs of squeeze assay results at 5, 10, 30, and 60 minutes. The scale bar is 100 μm and consistent for all micrographs

The experiments visually show that α -alanine is able to show significant IRI. **Figure 8** shows the ice crystallization of ice VI at each time stamp, which was observed with simple direct

brightfield illumination. It was also observed that ice crystallization is highly dependent on the surface area of the ice grain. The image at 5 minutes shows that ice crystallization is beginning. This is shown by the fact that there is more overlap compared to the other images. In addition, the image at 5 minutes has a combination of smooth edges and very defined edges. The fact that the small ice grains crystallize into bigger ice crystals show that α -alanine shows significant IRI ability. The results also show that different ice structures don't seem to have a big difference in terms of IRI ability, in this case tetragonal versus hexagonal ice structures. **Figure 9** shows the Raman spectra of ice VI at 60 min. The Raman spectra was obtained using a ruby crystal within the MDAC gasket and then used to characterize ice VI at 60 minutes. The two peaks were used to calculate the pressure of the ice crystal, which was 1.1 GPa at 300 K. The first peak on the left indicates the ruby crystal reference, and the second peak to the right indicates the Raman spectra of ice VI with α -alanine and NaCl.

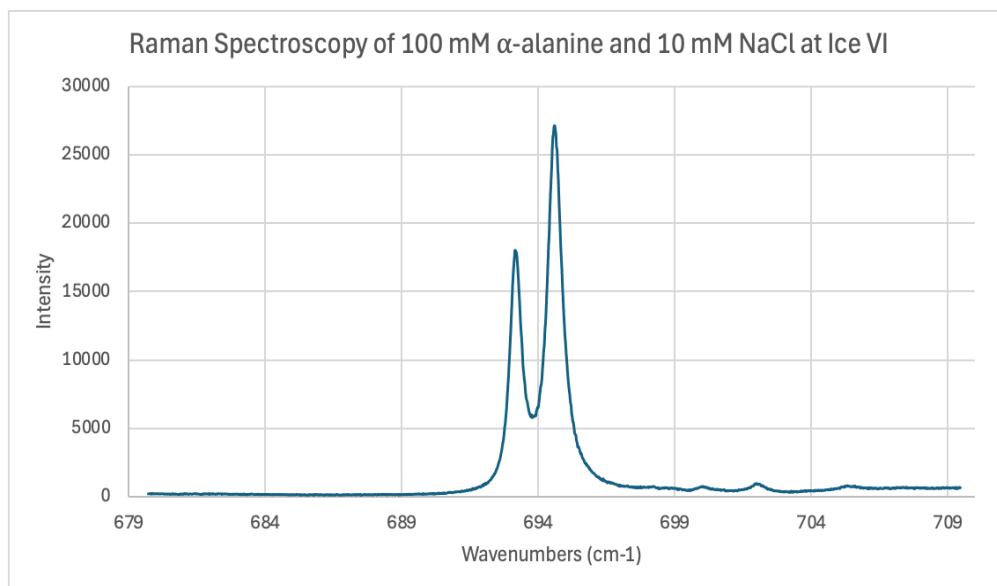


Figure 9. Raman spectroscopy of 100 mM α -alanine and 10 mM NaCl at ice VI

Figure 10 shows the comparison of IRI of ice crystallization in just NaCl solution and both α -alanine and NaCl solutions for ice VI at 30 minutes. The image with just 0.5 M NaCl shows all three types of deformations of ice, which are ice overlapping, transparency, and low contrast. Thus, the image shows that with just 0.5 M NaCl multiple layers are formed throughout the ice crystallization process. In contrast, the IRI activity of Ice VI with 50 mM α -alanine and 10 mM NaCl is much higher. The grains in ice VI with α -alanine have much smaller ice grains compared to ice VI without α -alanine. Comparisons between IRI of water exposed to only NaCl

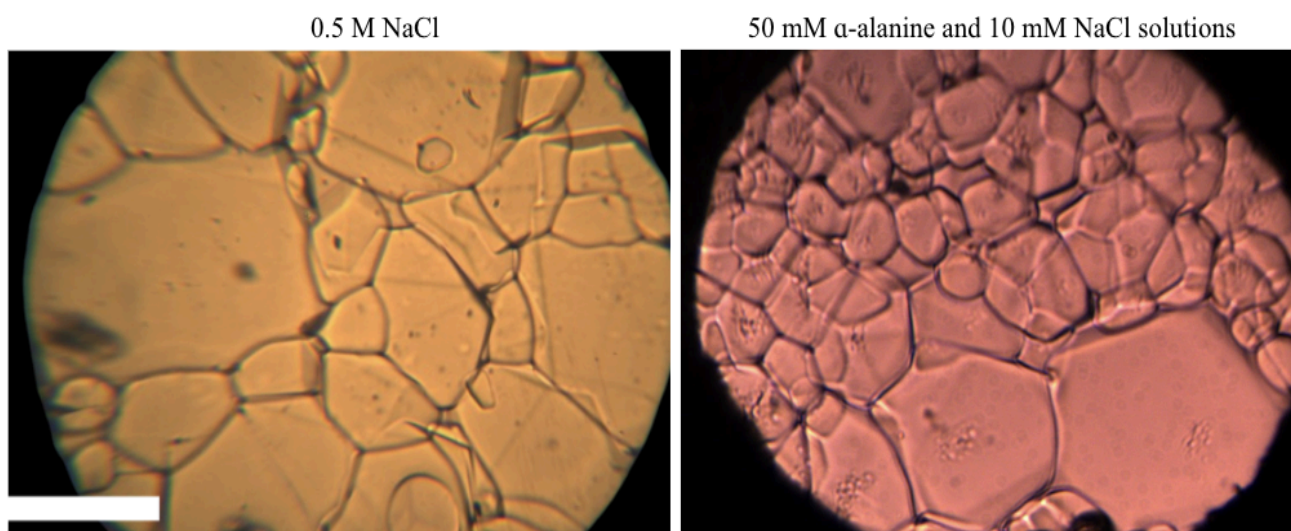


Figure 10. IRI of only 0.5 M NaCl vs. 50 mM α -alanine with 10 mM NaCl. The scale bar is 100 μm and consistent for all micrographs.

solution and both α -alanine and NaCl solutions have shown that α -alanine leads to a much more significant IRI activity.

3.2 Using Spatially-Resolved Chord Length Distribution to Measure Spatial Variation

SR-CLD proved to be accurate because it was able to show high contrast and a visualization of the exact spatial variation of each ice crystal, in addition to ice VI deformation. The heat map also shows the probability of non-uniform ice crystallization. **Figure 10.1, 10.2, 10.3, and 10.4** show the SR-CLD for ice crystallization processes at 5, 10, 30, and 60 minutes. The black lines represent the edge of each ice grain, and the white areas represent the surface of the ice grains. The SR-CLD heat maps show that there are regions in the ice crystal that have chord lengths that are either similar lengths or very different.

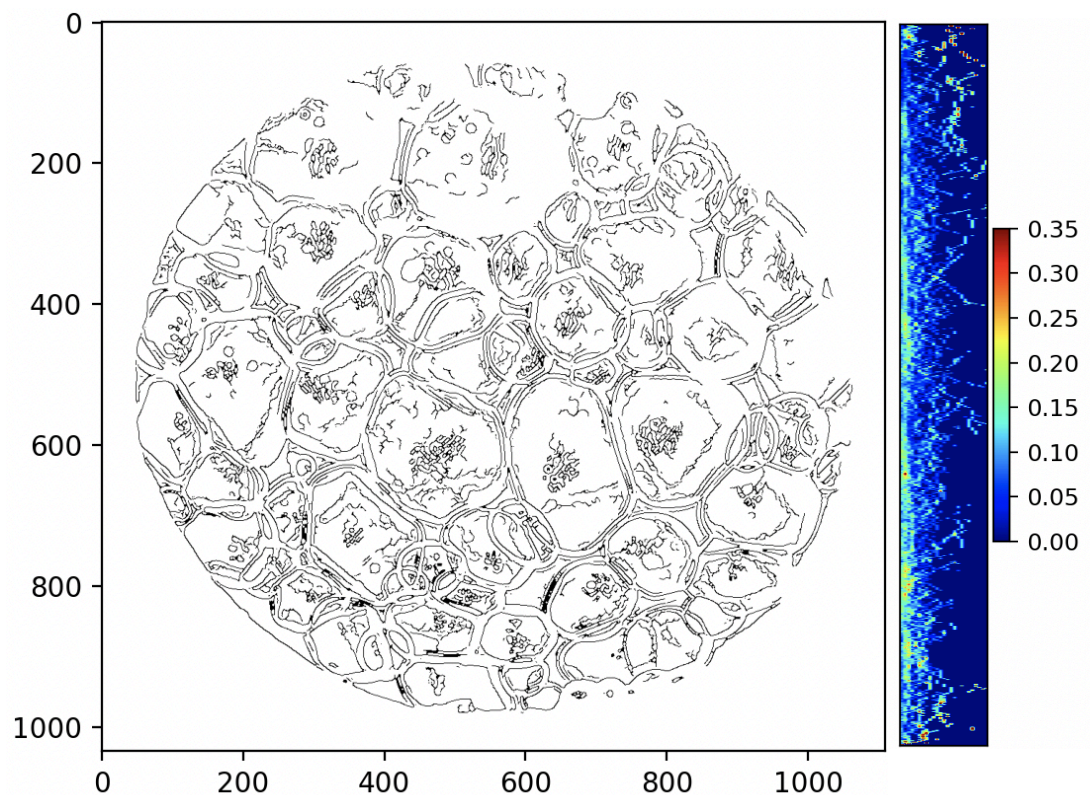


Figure 11.1. SR-CLD results after 5 minutes of ice crystallization.

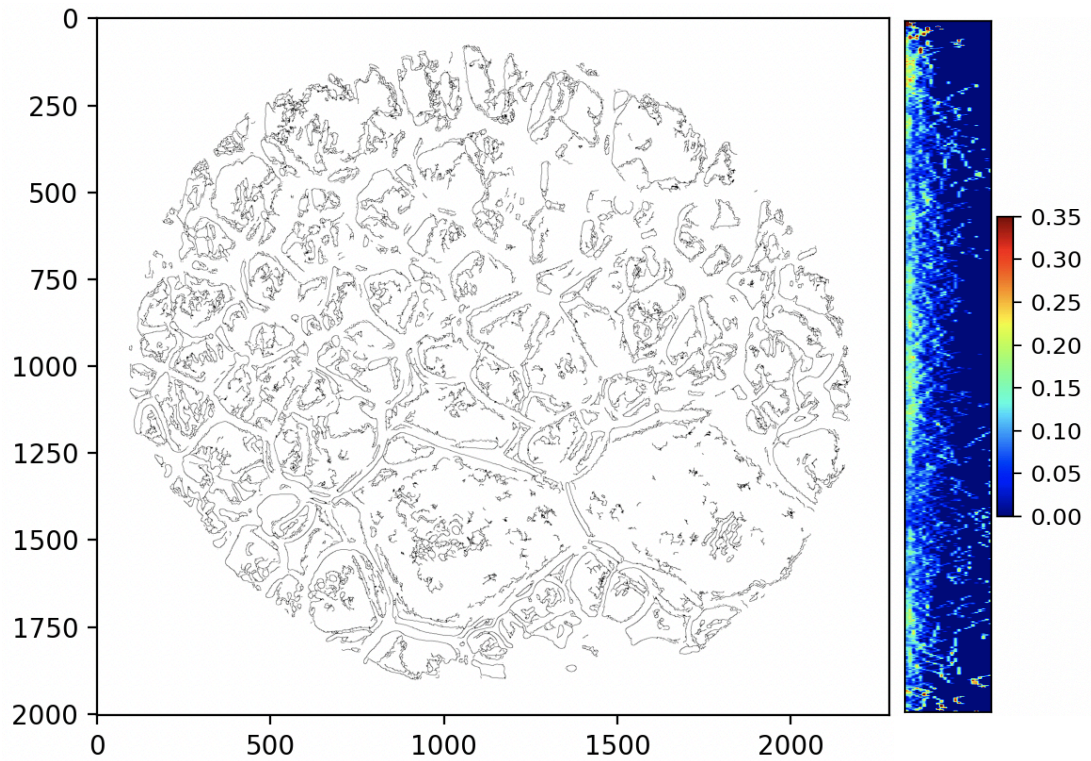


Figure 11.2. SR-CLD results after 10 minutes of ice crystallization.

The spatial variation of each of the images are shown, in which the spatial variation of chord length distributions is measured using arrays for SR-CLD. This can be seen in the heat maps where the x-axis represents each chord length across the image and the y-axis represents the chord length distribution at each scan line. Lastly, the heat maps show stacked chord lengths across the image, which depicts the density of each chord length and is represented by colors. The images and heat maps prove that SR-CLD can accurately measure the spatial variation of the non-uniform microstructures through measuring chord lengths across each row and developing a visualization of the spatial distribution of each image.

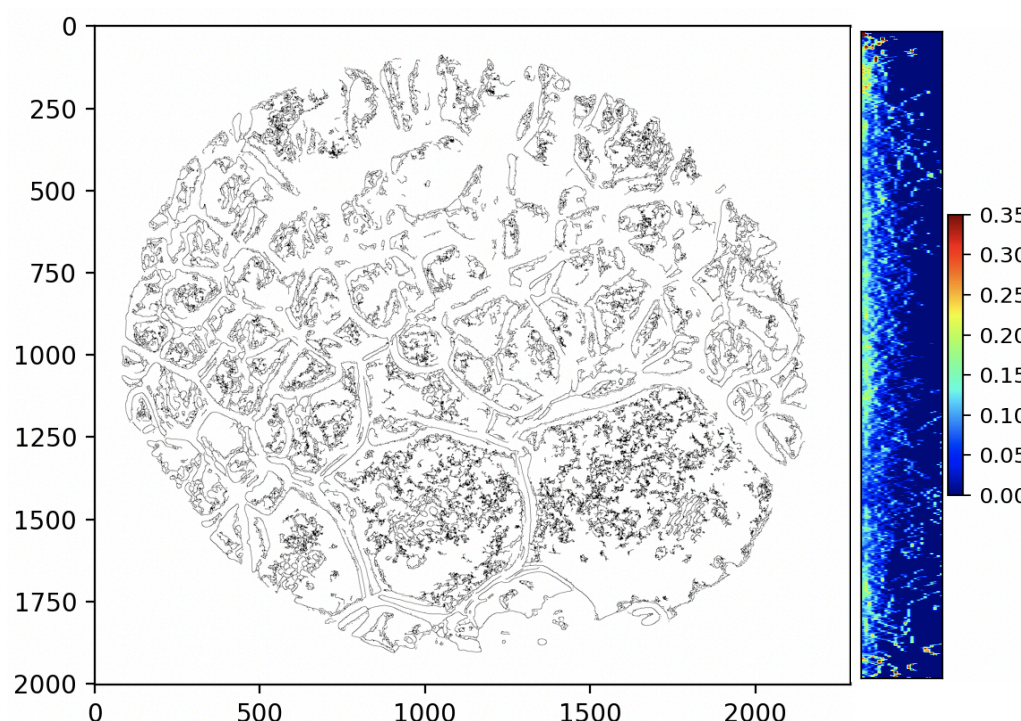


Figure 11.3. SR-CLD results after 30 minutes of ice crystallization.

Both the binary images and heat maps show a trend where the bottom right area of the ice crystal at each time stamp is the main area where there is major ice crystallization. The heat map shows this characterization by showing more concentrated colors that correspond to the more abundant small grains and a large vacant space in the bottom right which corresponds to the less abundant longer grain size. It is hypothesized that this corresponds to higher reaction rate due to higher surface area of water and the lack of α -alanine and NaCl solution when the sample begins nucleation once it is exposed to high pressure ice formation in the MDAC. It is also hypothesized that the smaller black lines and dots depict ice deformations and nucleation.

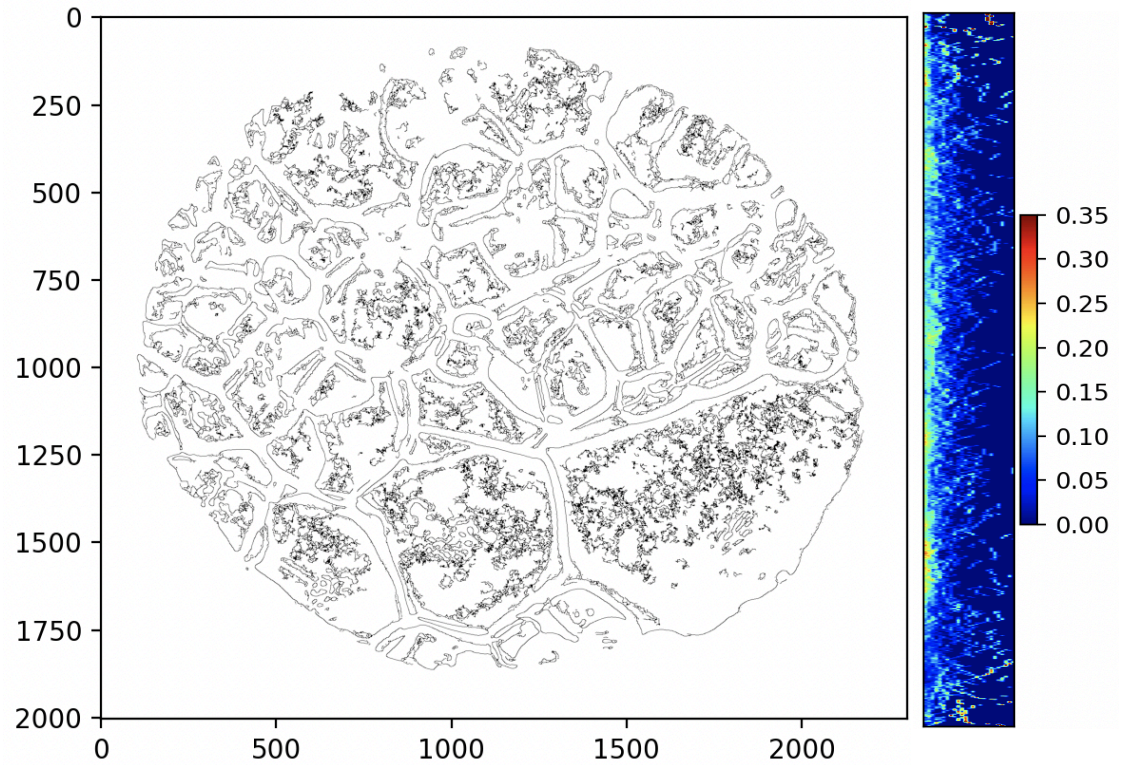


Figure 11.4. SR-CLD results after 60 minutes of ice crystallization.

The three most common reasons why previous automated analytical methods for obtaining quantitative data for ice have failed significantly is due to transparency, low contrast, and overlapping boundaries. This causes programs to detect false ice crystallization areas.

SR-CLD has been shown that one of its main advantages is its ability to analyze even transparent ice crystals by developing images with high contrast. The results from the experiments show that SR-CLD can also help detect overlapping boundaries due to its ability to show deformations of a non-uniform microstructure.

Chapter 4: Conclusions

4.1 Effectiveness of Ice Recrystallization Inhibition Using Amino Acids

The results from this research prove that simple amino acids can be just as effective as peptides in terms of IRI activity. This research shows that salts, such as NaCl, and amino acid based ice recrystallization inhibitors can even be effective at high pressure environments and different crystal structures. This indicates that amino acids also have the potential to be applied to spacecraft and instruments that are used to study planets that have icy environments, where pressure is much higher.

α -alanine is a simple amino acid sequence that can be found in many peptide sequences. This indicates that amino acids, especially α -alanine, have the potential to substitute more complicated peptide sequences. Furthermore, α -alanine may even be used to detect peptides that contain its presence. In terms of practical applications, the results from this research show that amino acids have the potential to be highly effective antifreeze agents. Overall, this research has shown that simple amino acids are just as capable of efficient IRI as complex peptides.

4.2 Spatially-Resolved Chord Length Distribution is a Reliable Quantitative Data Analysis

Understanding crystal structure uniformity is very crucial in order to understand the properties of the crystal being analyzed. A significant method to measure uniformity is to measure the spatial variation of grains within a crystal. Spatial variation can help explain the

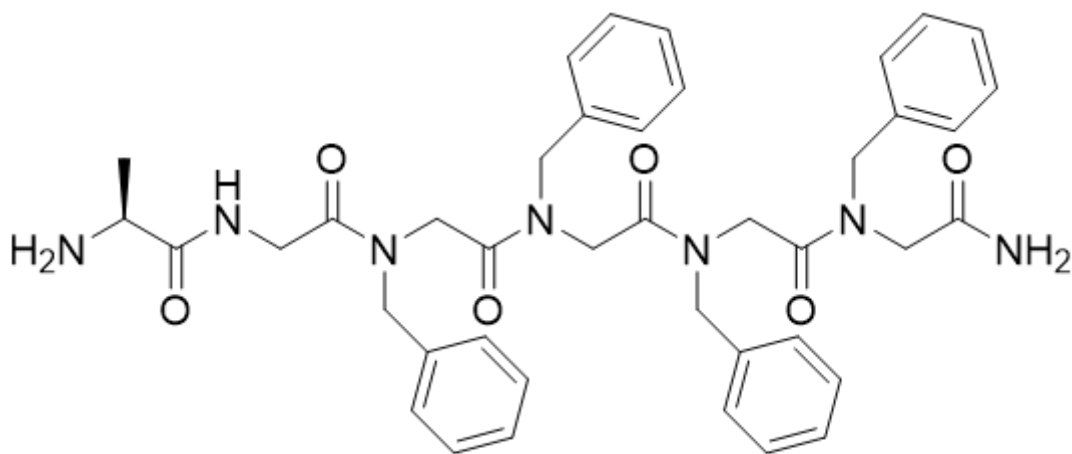
properties of heterogeneous microstructures, such as nucleation occurring within the crystal and pressure conditions of the crystal structures. Until now, manual measurements have still been considered to be more reliable than automated measurements for acquiring quantitative data for non-uniform microstructures. However, SR-CLD is a novel method that has been proven to be capable of calculating exact measurements between grains in an image within a non-uniform crystal structure.

The results from this research show that SR-CLD is an accurate tool that can show spatial variation and can also be useful to predict the ice crystallization patterns. The results from this study prove that SR-CLD is able to accurately measure the spatial variation of non-uniform microstructures compared to previously utilized computational methods. Furthermore, SR-CLD has shown that it is capable of even analyzing complex ice crystal structures.

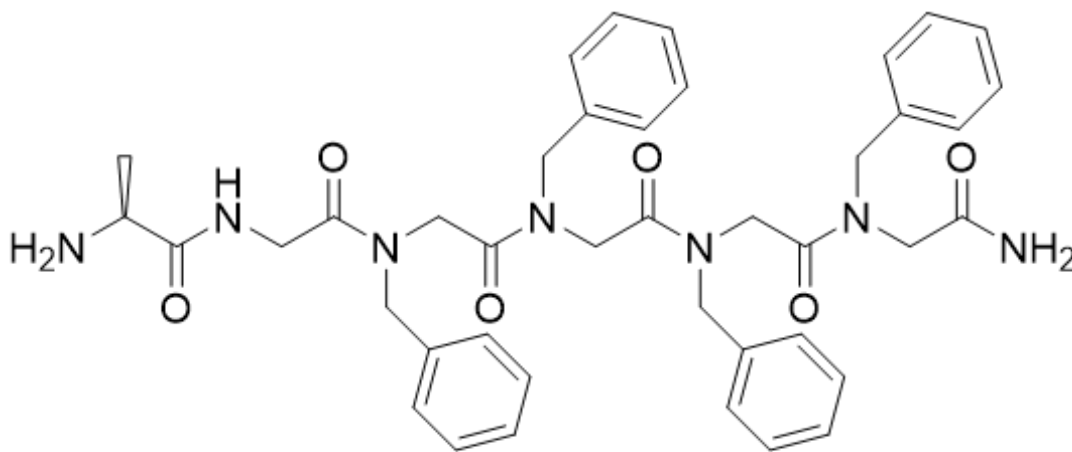
4.3 Potential Future Work

In this study the effects of a simple amino acid on IRI at high pressure was studied. As mentioned earlier in the introduction to peptoids, the efficiency of peptoids at high pressure for different crystal structures has not been studied. However, there have already been analogues of α -alanine that have been synthesized. These peptoids can be used to study and compare the differences between the ice crystallization of amino acids and peptoids. The two peptoid analogues that were synthesized are known as (L-alanine) and (D-alanine). **Figure 11** shows the two types of peptoid analogues for the α -alanine amino acid.

L-alanine



Molecular Weight: 733.87
pKa: 7.329

D-alanine

Molecular Weight: 733.87
pKa: 7.329

Figure 12. Peptoid analogues of α -alanine amino acid

This study focused on the IRI of ice VI. In addition, it has been shown that peptoids have more effective IRI compared to peptides. Thus, it would be interesting to study and observe the IRI results of peptoids and see if peptoids have the ability to enhance IRI of ice VI compared to amino acids.

In general, SR-CLD can be applied to any research that focuses on non-uniform microstructure analysis. There can be further studies on how efficiently SR-CLD is able to obtain quantitative data for mechanical deformation of cryomaterials. The advantage of SR-CLD is how flexible it can be with obtaining quantitative data. This research focused on utilizing a calculation formula known as Doane's Rule. However, SR-CLD has various other quantitative calculation methods, such as the Square Root Rule and Rice's Rule. It would be interesting to see what quantitative data would be acquired by utilizing the different calculation methods that were not used in this research.

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