THE RATIONAL DESIGN OF COILED-COIL PEPTIDES TOWARDS UNDERSTANDING PROTEIN-CRYSTAL INTERACTIONS AND AMORPHOUS-TO-CRYSTALLINE TRANSITIONS

Eric P. Chang

A Dissertation

Submitted to the Graduate College of Bowling Green State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

May 2013

Committee:

Michael Y. Ogawa, Advisor

George S. Bullerjahn

R. Marshall Wilson

John R. Farver, Graduate Faculty Representative
This dissertation reflects efforts to combine biomineralization research and rational peptide design using the coiled-coil peptide motif and calcium hydrogen phosphate dihydrate (CaHPO$_4$ x 2H$_2$O), commonly known as brushite. Coiled-coils were designed to alter the complete growth pathway of brushite beginning with an amorphous precursor and resulting in a modified crystalline state. Both the designed chemical character and the secondary structure of the coiled-coil peptides were found to be important factors in controlling the growth pathway of brushite and the morphology of the final crystalline state.

The impact of peptide secondary structure on the growth of brushite was studied by comparing the effects of a well structured coiled-coil peptide (AQQ5E) to a structurally disordered analog with nearly identical chemical properties (RCA5E) on the final crystal morphology. Typically, brushite crystals formed in the absence of growth modifying agents display {100}, {010}, {10$ar{2}$} and {10$ar{1}$} crystal faces. However, in the presence of AQQ5E the growth of the {10$ar{2}$} faces were selectively inhibited in the final crystal product. Conversely, crystals grown in the presence of RCA5E adopted a wide variety of morphologies without a preferred means of crystal modification. Computational analysis demonstrated how the two peptides may interact with the different crystal faces of brushite and provided insight for explaining this behavior.

In another study, a series of coiled-coil peptides with similar secondary structure yet increasing acidic amino acid content were used to determine the impact of designed peptides on
amorphous-to-crystalline transition of brushite leading up to the final crystalline state. In the absence of peptides, the amorphous-to-crystalline transition of brushite occurred rapidly with the final crystalline state being achieved within several hours. However, the addition of acidic peptides prolonged this process over several days and allowed for the study and characterization of a novel amorphous-crystalline, hybrid phase of brushite adopting a ribbon-like morphology. Studying the formation and stability of the intermediate brushite phase in the presence of structurally similar yet chemically distinctive peptides suggested that both secondary structure and chemical composition impact the manner in which peptides interact with amorphous and crystalline materials.
This dissertation is dedicated to my parents,

Mary Ann and Tom Chang, and to my

loving wife Amber and my son Ethan.
ACKNOWLEDGMENTS

I would like to thank my family for all their love and support over the years. Without them by my side, encouraging me to follow my passions, I would not be where I am today.

I would like to thank my adviser, Dr. Michael Ogawa, for being an excellent mentor during my time at Bowling Green State University. From the moment I joined his group, he has always helped me see the bigger picture, ask the important questions, and continue to study the unknown. From his guidance, I began to view the world of science from a much larger perspective and will always be grateful for the lessons learned.

I would like to thank Dr. George Bullerjahn, Dr. Marshall Wilson, and Dr. John Farver for being members of my dissertation committee and being extremely supportive of my research efforts. Their collective advice and guidance have helped me see the interdisciplinary nature of research that has taught me to reach out to several fields of science to find answers to questions.

I would like to thank the staff at the Instrumentation Center of the University of Toledo, in particular Dr. Pannee Burckel, Dr. Leif Hanson, and Dr. Kristin Kirschbaum, for providing me with training and assisting me with analysis. Their contributions are reflected throughout my work in terms of imaging, X-ray diffraction, and mass spectrometry.

Lastly, I would like to thank all the friends I made over the years at BGSU, in particular, Vasily Morozov, for always being a great lab mate and friend.
# TABLE OF CONTENTS

## CHAPTER I. INTRODUCTION

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>I.2 CRYSTAL GROWTH AND CRYSTALLOGRAPHY</td>
<td>5</td>
</tr>
<tr>
<td>I.3 DESIGNED PEPTIDES AND THE α-HELICAL COILED COIL</td>
<td>14</td>
</tr>
<tr>
<td>I.4 RATIONALE OF STUDY</td>
<td>18</td>
</tr>
</tbody>
</table>

**CHAPTER I REFERENCES** 18

## CHAPTER II. EXPERIMENTAL METHODS

**CHAPTER II REFERENCES** 28

## CHAPTER III. SECONDARY STRUCTURE EFFECTS ON PEPTIDE-CRYSTAL INTERACTIONS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III.1 INTRODUCTION</td>
<td>29</td>
</tr>
<tr>
<td>III.2 RATIONALE OF STUDY</td>
<td>36</td>
</tr>
<tr>
<td>III.3 RESULTS</td>
<td>39</td>
</tr>
<tr>
<td>III.4 DISCUSSION</td>
<td>49</td>
</tr>
</tbody>
</table>

**CHAPTER III REFERENCES** 57

## CHAPTER IV. ELECTROSTATIC EFFECTS ON THE AMORPHOUS TO CRYSTALLINE TRANSITION OF BRUSHITE

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV.1 INTRODUCTION</td>
<td>61</td>
</tr>
<tr>
<td>IV.2 RATIONALE OF STUDY</td>
<td>65</td>
</tr>
<tr>
<td>IV.3 RESULTS</td>
<td>68</td>
</tr>
<tr>
<td>IV.4 DISCUSSION</td>
<td>85</td>
</tr>
</tbody>
</table>

**CHAPTER IV REFERENCES** 89
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.1. (Left) TEM images of hydroxyapatite plates formed during <em>in vitro</em> experiments without the addition of crystal modifying additives. (Right) SEM micrograph of enamel taken from a rodent tooth showing the complex layering of HA ribbon bundles into a cross woven network. (Reprinted with permission from reference 22. Copyright 1984, Elsevier Science B.V.)</td>
<td>4</td>
</tr>
<tr>
<td>I.2. (Left) A geological calcite crystal. (Right) A regenerating sea urchin spine. (Reprinted with permission from reference 23. Copyright 2011, Annual Reviews.)</td>
<td>4</td>
</tr>
<tr>
<td>I.3. Gibbs free energy diagram showing the classical theory of homogeneous nucleation. (Adapted from references 2 and 26.)</td>
<td>8</td>
</tr>
<tr>
<td>I.4. Gibbs free energy diagram showing the direct and sequential pathway for the formation of the same mineral phase beginning from the same dissolved ions. The initial nucleation event for both pathways is defined as the sum of the free energy of nucleation ($\Delta G_N$) and the free energy of phase growth ($\Delta G_g$). As expected on the basis of the fundamentals of CNT, the initial energy requirements for the direct pathway are much greater than the sequential pathway. The transition between states of the sequential pathway is defined as the free energy of transition ($\Delta G_T$) and varies depending on the mechanism of transformation. (Adapted from references 2 and 10.)</td>
<td>10</td>
</tr>
<tr>
<td>I.5. (Left) Representation of two-dimensional, layer-by-layer growth at a planar crystal face. (Right) Representation of spiral growth occurring at a screw dislocation. (Adapted from references 26 and 30.)</td>
<td>12</td>
</tr>
</tbody>
</table>
I.6. (Left) A two-dimensional model of crystal growth with two types of crystal faces (A and B) that grow at different rates. (Adapted from reference 26.) (Right) Picture of a halite crystal.28

I.7. Various levels of structural organization observed in proteins. (Reprinted with permission from reference 34. Copyright 2001, American Chemical Society.)

I.8. (Left) Models of the α-helical, coiled-coil trimer backbone. (Right) Helical wheel diagram of the coiled-coil trimer showing hydrophobic (a,d) and electrostatic (e,g) interactions.

III.1. Diagram of how additives can bind to specific crystal faces to impact growth rates of those faces and the final crystal morphology. (Reprinted with permission from reference 6. Copyright (2008) American Chemical Society.)

III.2. (Left) Typical rhombohedral morphology of calcite observed when grown without additives. (Center) The prismatic morphology observed when calcite is grown in the presence of CBP1 at 3 °C. (Right) The studded morphology observed when calcite is grown in the presence of CBP1 at 25 °C. (Reprinted with permission from reference 24. Copyright (1997) American Chemical Society.)

III.3. CD spectra for AQQ5E and RCA5E in 50 mM acetate buffer (pH 4.75).

III.4. (Top Left) MALDI analysis of crystals grown in the presence of AQQ5E dissolved in 1.0 M acetic acid. The theoretical mass for AQQ5E is 3713 g/mol with both the M + 1 peak, 3714 m/z, and the M + 2 peak, 1858 m/z, observed. (Top Right) MALDI analysis of crystals grown in the presence of RCA5E dissolved in acetic acid. The theoretical mass for RCA5E is 3773 g/mol with only the M + 1 peak, 3774 m/z, observed. (Bottom) Representative spectrum for mature brushite crystals incubated in concentrated AQQ5E
and RCA5E solutions lacking the presence of peptide. (Inserts) Mass spectra of the final rinse solutions used in all individual sample preparations.

III.5. Comparison of UV-Vis absorption spectra of 400 µL of 1.0M HCl solutions containing 1.0 mg of crystals grown in the presence of AQQ5E and in the presence of RCA5E. Background scans were performed on the pure 1.0M HCL solution prior to analysis. The spectrum of 1.0 mg of crystals grown in the absence of peptide dissolved in 400 µL of 1.0 M HCl was subtracted from the data sets to eliminate absorption artifacts produced by dissolving brushite crystals in HCl.

III.6. (Left) Optical image of a nearly intact brushite crystal grown in the absence of peptides used for face indexing shown mounted for analysis. (Right) The same crystal with the experimentally determined Miller indices assigned to their respective faces.

III.7. Representation of an ideal brushite crystal grown in the absence of peptide oriented looking down the b axis. All crystal faces are labeled with their corresponding Miller indices and the relevant interfacial angles are shown.

III.8. (Left) SEM image of a nearly intact brushite crystals grown in the absence of peptide resting ontop of a fractured crystal. Scale bar represents a distance of 100 µM. (Right) Magnified image of the same crystal showing presence of two distinct interfacial angles of 125° and 150° between well-defined crystallographic faces and the corresponding Miller indices.

III.9. (Left) SEM image of a nearly intact brushite crystal grown in the presence of AQQ5E laying adjacent to another crystal. Scale bar represents a distance of 100 µM. (Right) Magnified image of the same crystal showing the presence of two distinct interfacial
angles of 125˚ and 150˚ as well as the corresponding Miller indices, where the size of the (102) face has decreased significantly compared to the control sample.

III.10. (Left) SEM image of a mixture of nearly intact brushite crystals grown in the presence of RCA5E. Scale bar represents a distance of 100 µM. (Right) Magnified image of the same group of crystals showing the presence of two distinct interfacial angles of 125˚ and 150˚, where the size of both the {100} and {102} faces has decreased significantly in different crystals. Miller indices have been omitted for clarity.

III.11. Model of AQQ5E displaying only the coiled coil backbone and solvent exposed glutamic acid residues at b positions. Measurements between adjacent carboxylate groups of 10.3 Å, 11.7 Å, 11.3 Å, and 11.0 Å are shown. Average is 10.9 ± 1.1 Å.

III.12. Computer modeling of the different crystal faces affected by the presence of AQQ5E with the (100) face being the proposed site of AQQ5E interaction. For both modeled surfaces, an area of approximately 20 Å x 20 Å is shown as well as the distances between adjacent calcium ions oriented 90˚ from one another.

III.13. Side-by-side comparison of a 15.2 Å x 15.2 Å area for the (102) and (100) faces of brushite.

III.14. Modeling of the interaction between solvent exposed glutamate residues at b positions of the heptad repeat of AQQ5E with calcium ion channels of the (100) face of brushite.

IV.1. Diagram comparing the classically accepted theory of crystal nucleation and the alternative nucleation and growth pathway.

IV.2. Free energy diagram showing both the direct (A) and sequential (B) pathways for calcium carbonate growth. (Adapted from reference 1.)
IV.3. (Left) CD of all peptides at 25 °C showing that all AQQ peptides display α-helical coiled-coil secondary structure with the θ222 > θ208 and RCA5E showing random coil behavior.
(Right) CD of all peptides at 95 °C showing that all AQQ peptides retain α-helical character upon thermal denaturization......................................................... 69

IV.4. Results obtained from the nucleation turbidity assay performed for AQQ (Top Left), AQQ3E (Top Right), and AQQ5E (Bottom Left) showing that the induction time increases with increasing peptide concentration. The red horizontal lines represent the approximate induction time for each set of trials. Additionally, a direct comparison of average induction times obtained from nucleation turbidity assays containing AQQ, AQQ3E, and AQQ5E at all and concentrations studied is displayed (Bottom Right) showing that increasing the negative charge density delays the initial nucleation....................... 71

IV.5. (Right) SEM image of calcium phosphate phase that initially precipitates. (Left) STEM nanodiffraction pattern showing the amorphous nature of the calcium phosphate initially precipitated................................................................. 72

IV.6. (Right) SEM image of calcium phosphate phase that initially precipitates. (Left) STEM nanodiffraction pattern showing the amorphous nature of the calcium phosphate initially precipitated................................................................. 74

IV.7. (Right) SEM image of calcium phosphate phase that initially precipitates. (Left) STEM nanodiffraction pattern showing the amorphous nature of the calcium phosphate initially precipitated................................................................. 75

IV.8. SEM images of the intermediate phase formed in the presence of 200 µM AQQ....... 76

IV.9. SEM images of the intermediate phase formed in the presence of 200 µM AQQ3E…… 76

IV.10. SEM images of the intermediate phase formed in the presence of 200 µM AQQ5E….. 76
IV.11. SEM images of the ribbon-like particles observed in the polycrystalline intermediate phase formed in the presence of AQQ5E. 77

IV.12. (Left) FT-IR absorbance spectra normalized to the phosphate vibrational peak at 1018 cm$^{-1}$ for all samples extracted during the most stable region of the amorphous-to-crystalline transition (Figure III.3) showing which peptides have the most affinity for the ACP precursor. (Right) Direct comparison of the IR spectra in the region of the peptide amide I (1650 cm$^{-1}$) and amide II (1543 cm$^{-1}$) bands. 78

IV.13. TEM nanodiffraction pattern showing the crystalline nature of the intermediate phase material. 80

IV.14. Powder XRD pattern of the polycrystalline intermediate formed in the presence of AQQ compared to several database powder XRD patterns for brushite. Observed reflections are labeled according to Miller index. 80

IV.15. 200 µM turbidity assays monitoring the transition of the polycrystalline intermediate into brushite crystals beginning 12 hours after the nucleation of the initial amorphous phase. 82

IV.16. FTIR spectra comparing the amorphous phase of brushite formed in the presence of AQQ5E exacted directly after nucleation and one hour after nucleation to the FTIR spectra of the a week-old sample of the intermediate phase stabilized by the presence of AQQ5E. 84

IV.17. SEM images of a week-old sample of the intermediate phase stabilized by the presence of AQQ5E showing that the particles adopt only a blade-like morphology. 84

IV.18. Gibbs free energy diagram showing the progression of the amorphous-to-crystalline transition observed for brushite under the given set of experimental conditions. 86
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III.1. Progression of Primary Sequence Changes of the AQ-Pal14 Peptide</td>
<td>40</td>
</tr>
<tr>
<td>III.2. CONTINLL and CDDSTR Secondary Structure Distribution Analysis</td>
<td>41</td>
</tr>
<tr>
<td>III.3. Summary of Results for Light Scattering Experiments on AQQ5E and RCA5E</td>
<td>42</td>
</tr>
<tr>
<td>III.4. Summation of the chemical species observed within a 15.2 Å x 15.2 Å area of the (102) and (100) faces of brushite</td>
<td>53</td>
</tr>
<tr>
<td>IV.1. The solubility products for different polymorphs of CaCO₃</td>
<td>64</td>
</tr>
<tr>
<td>IV.2. Progression of Primary Sequence Changes of the AQ-Pal14 Peptide</td>
<td>68</td>
</tr>
<tr>
<td>IV.3. Summary of Morphological Properties for the Polycrystalline Intermediate</td>
<td>77</td>
</tr>
</tbody>
</table>
CHAPTER I. INTRODUCTION

1.1 INTRODUCTION

1.1.1 Statement of the Problem

The formation of bones, teeth, shells, and many other robust, natural materials results from the ability of organisms to closely regulate the nucleation and growth of minerals through a complex interaction of organic and inorganic components known as biomineralization.\(^1,2\)

Understanding how such biological materials form may allow for significant advances in medicine and materials science and thus remain an area of significant interest despite the challenges associated with studying these complex mineralization pathways. One of the most difficult aspects of deducing the guiding principles of a biomineralization process is the multitude of factors that impact morphological and structural properties of a naturally mineralized structure. For example, all biomineralization systems must be able to perform the following functions: (1) confine a space for mineralization, (2) control the input of ions and soluble macromolecules, (3) form an insoluble matrix framework for mineral deposition, (4) construct a nucleation site to initiate crystal growth, (5) control the crystal growth and orientation, and (6) terminate the crystal growth process.\(^3\) All of these functions play a vital role in determining the final outcome of a biomineralization pathway. Thus, to simplify research efforts most studies concentrate only on one aspect at a time, with the majority of published studies focusing on the control of crystal growth and orientation.\(^4-8\)

To control crystal growth and orientation, organisms utilize soluble organic additives such as proteins or polypeptides which are thought to either bind directly to the growing crystal
faces or become intercalated within amorphous mineral films that ultimately crystallize.\textsuperscript{9,10} As such, many groups have used synthetic proteins and polypeptides that mimic the chemical properties of those found in natural systems to control crystal nucleation and growth \textit{in vitro}.\textsuperscript{11-13} Since many proteins found within biomineralized tissues contain regions of repeating acidic amino acid residues, most studies utilize simple polypeptide additives such as polyaspartate or polyglutamate as they are readily available and resemble the acidic regions of natural biomineralization proteins. Alternatively, recent developments in directed evolution experiments have developed “biopanning” techniques capable of producing optimized peptide sequences to bind to specific minerals.\textsuperscript{12,14} However, even this complex evolutionary screening method produces relatively short polypeptide sequences typically no more than 10-15 amino acids in length. Thus, while much has been learned concerning the effects of altering primary structure, the effects of the secondary, tertiary, and quaternary protein structure on biomineralization pathways has gone largely unstudied due to the structurally simplistic design of the additives chosen for \textit{in vitro} mineralization studies. This is important because natural biomineralization proteins have much more amino acid diversity and are much longer than simplistic model systems which can allow them to fold into distinct structural conformations and self assemble within mineralizing environments.\textsuperscript{15-17}

Surprisingly, the use of rationally designed peptides has not been extensively employed in studying biomineralization principles. This is interesting since the rational design of peptides allows for specific control over both the chemical and structural properties of the peptide. Thus, peptide analogs that more closely resemble natural proteins can be developed in this manner for specific applications. Over the past 20 years, only a few studies have utilized rationally designed peptides to control the growth and orientation of biominerals.\textsuperscript{5,18,19} Interestingly, all these studies
were conducted by peptide chemists rather than biomineralization researchers which may explain why only a small number of attempts have been made at merging these two fields. Thus, a considerable opportunity exists to expand upon the understanding of biomineralization principles and the developing of synthetic organinc-inorganic hybrid materials using rationally designed peptides.

I.1.2 Biomineralization – Examples and Application

One of the most exquisite examples of nature controlling crystal growth and orientation is the structure of enamel, the hardest mammalian tissue. Enamel is the final product of the simultaneous interaction of several organic components and the calcium phosphate (CaP) mineral hydroxyapatite (Ca$_{10}$(PO$_4$)$_6$ x OH)$_2$. During *in vitro* experiments in the absence of a crystal modifying additive, hydroxyapatite forms nanometer size plates or needles that tend to aggregate in a disordered fashion (Figure I.1). In enamel tissue, hydroxapatite forms micrometer scale ribbons that bundle together to into durable, rope-like subunits. Additionally, the rope bundles further assemble into a cross-woven network of well organized, alternating layers of hydroxyapatite (Figure I.1). Though the mechanisms governing how these components interact with one another to form enamel is still under investigation, the result is a beautiful, hierarchical structure consisting of well-oriented calcium phosphate crystals.

Another fascinating example of biomineralization is the sea urchin spine. Sea urchin spine is composed almost entirely of calcium carbonate in the form of calcite that forms a mesh-like network of porous channels (Figure I.2). On the other hand, calcite formed under geological conditions adopts a well-defined, rhombohedral morphology that reflects the symmetry of its internal atomic arrangement. Interestingly, analysis by X-ray diffraction of the biogenic calcite
reveals that it is highly crystalline even though it has no resemblance to a typical crystalline phase. Thus, structures such as the sea urchin spine demonstrate that nature has developed effective ways to “mold” crystalline materials into complex shapes.

Figure I.1. (Left) TEM images of hydroxyapatite plates formed during *in vitro* experiments without the addition of crystal modifying additives. (Right) SEM micrograph of enamel taken from a rodent tooth showing the complex layering of HA ribbon bundles into a cross woven network. (Reprinted with permission from reference 22. Copyright 1984, Elsevier Science B.V.)

Figure I.2. (Left) A geological calcite crystal. (Right) A regenerating sea urchin spine. (Reprinted with permission from reference 23. Copyright 2011, Annual Reviews.)
Clearly, nature has developed fascinating and inspiring methods for developing hybrid organic-inorganic materials with specific functionality and excellent structural properties. As such, understanding the principle mechanisms that drive their formation and applying those principles to make synthetic systems has become an expansive area of research known as biomimetic mineralization. Many deem the inception of the modern era of both biomineralization and biomimetic mineralization research to a high-profile publication by Addadi and Weiner claiming that nature may use specific, epitaxial-like relationships between acidic proteins and growing crystal to direct crystal morphology. The early work of Addadi and Weiner demonstrated that acidic proteins could influence the growth of crystals in vitro which in turn led them to conclude that nature might use a similar mechanism to control crystal growth. On the basis of this premise which was both popular and controversial at its time of publication, many other groups began “mimicking” the manner in which nature utilizes proteins and other biomolecules to control crystal growth to deduce biomineralization principles and develop new ways of engineering materials. Thus, the field of biomimetic mineralization began with the notion of epitaxial-like matching of soluble proteins to crystallographic surfaces but quickly expanded to include areas such as templating biomineral growth from insoluble scaffolds, controlling amorphous-to-crystalline transitions, and many other topics.

1.2 CRYSTAL GROWTH AND CRYSTALLOGRAPHY

1.2.1 The Chemistry of Crystal Formation

A crystal is a homogeneous solid having an ordered, internal atomic arrangement that repeats over many atomic units in three dimensions. For a crystal to grow, the first criterion that
must be met is its constituent atoms/ions must be available. For example, to form calcite both 
\( \text{Ca}^{2+} \) and \( \text{CO}_3^{2-} \) ions must be present in solution. However, several polymorphs, i.e. different 
structures of a given chemical compound, exist for calcium carbonate besides calcite such as 
vaterite and aragonite. So, what determines which form of calcium carbonate, or any other 
polymorphic mineral, will form when various structures are possible? This question can be 
answered from two different points of view. The first deals with the relationship between 
stability and energy.\(^{26}\) In general, when multiple atomic configurations of a compound are 
possible the one with the lowest energy configuration will be most stable. Thus, higher energy 
states are less stable and if formed will ultimately transform to the most stable, lowest energy 
state. For calcium carbonates, vaterite and aragonite are higher in energy than calcite.

Consequently, calcite is most abundant form of calcium carbonate found in nature.

The second way to rationalize polymorphism is by relating stability and solubility.\(^{2}\) In 
general, the solubility of an inorganic salt is the number of moles of the pure solid that will 
dissolve in one liter of solvent at a given temperature. Since many biominerals do not fully 
dissolve in solution, an equilibrium exists between the bulk solid and its dissolved ions defined 
by an equilibrium constant known as the solubility product (\( K_{sp} \)). For calcium carbonates, the 
general form of the solubility equilibrium expression (1) and the solubility product (2) are 
written as:

\[
\text{(1)} \quad \text{CaCO}_3(\text{s}) \leftrightarrow \text{Ca}^{2+}(\text{aq}) + \text{CO}_3^{2-}(\text{aq})
\]

and

\[
\text{(2)} \quad K_{sp} = [\text{Ca}^{2+}][\text{CO}_3^{2-}]
\]

where \([\text{Ca}^{2+}]\) and \([\text{CO}_3^{2-}]\) are the effective concentrations (activities) of calcium and carbonate 
ions in solution, respectively. Therefore, the solubility product is equivalent to the activity
product (AP) at equilibrium and allows for the determination of the thermodynamic conditions for inorganic precipitation. For example, if the activity product (AP) is greater than the solubility product ($K_{sp}$), then precipitation will occur until AP and $K_{sp}$ are equal. Conversely, if the AP is less than the $K_{sp}$, then more ions from the crystal solid will dissolve into solution until equilibrium is reached. Thus, in a polymorphic system where different mineral states exists the most stable polymorph will have the lowest value of $K_{sp}$ since it is the least likely form to dissolve. For calcium carbonates, this holds true as the solubility products for vaterite and aragonite are greater than the solubility product for calcite.

Another important concept related to equilibrium is supersaturation. Super saturation is a measure of the extent to which a mineral forming solution is out of equilibrium and is defined by the following equations as either the relative supersaturation ($S_R$) or the absolute supersaturation ($S_A$):

$$S_R = \frac{AP}{K_{sp}}$$
$$S_A = \frac{(AP - K_{sp})}{K_{sp}}$$

where AP and $K_{sp}$ represent the activity product and solubility product for a given mineral, respectively. Thus, a mineral forming solution is considered to be supersaturated with respect to a given mineral when AP is greater than $K_{sp}$. In general, increasing the level of supersaturation increases the thermodynamic driving force for precipitation which can have a significant impact on the mechanism by which the crystal grows.

### I.2.2 Crystal Nucleation Pathways

Classically defined, the growth of a crystal in solution begins with the homogeneous nucleation of dissolved ions. In homogeneous nucleation, complimentary ions rapidly collide to
form clusters that either increase in size up to a critical radius or redissolve back into their constituent ions.\(^{26}\) As shown in Figure I.3, the free energy of nucleation ($\Delta G_N$) is described as the sum of the increasing interfacial energy ($\Delta G_I$) associated with the formation of the new solid-liquid interface and the decreasing bulk energy ($\Delta G_B$) associated with formation of bonds within the bulk cluster as related to the increase in cluster size. At the critical radius ($r_c$), the cluster has sufficient energy to overcome the nucleation barrier and form a new crystal nuclei which can grow by the addition of ions at well defined solid surfaces. This notion of homogeneous nucleation, also known as classical nucleation theory (CNT), has little relevance to most biomineralization processes since it is energetically demanding. However, describing this theory provides the foundation for discussion and understanding other crystal growth pathways.\(^3\)

Figure I.3. Gibbs free energy diagram showing the classical theory of homogeneous nucleation. (Adapted from references 2 and 26.)

In contrast to homogeneous nucleation, crystal growth via heterogeneous nucleation takes advantage of existing surfaces in solution such as other crystals or insoluble organic frameworks which act as a nucleus and thus eliminate many of the energetic demands on a growing crystal system. Since all biomineralization systems consist of close interactions between crystals and
organic frameworks, heterogeneous nucleation is likely an important factor in the growth of many biominerals. A specific type of heterogeneous nucleation known as epitaxial nucleation exists when the surface being nucleated onto has a very similar chemical profile to the growing crystal phase in terms of the spatial arrangement and chemical character of atoms at the surface. This is a common phenomenon for geological processes; however, the relevance of epitaxial relationships to biomineralization is not exactly clear. The work of Addadi and Wiener as well as others supported the idea of epitaxial-like interactions between proteins and biominerals. However, current models describing protein-mineral interactions do not require specific epitaxial-like interaction.

For either homogeneous or heterogeneous nucleation to occur, very specific conditions must be met which might not be applicable to all mineralization reactions. Thus, another pathway for crystal growth exists, referred to as alternative nucleation theory (ANT), which describes the manner in which many biominerals are formed much more accurately than CNT. Rather than obtaining a final crystal product directly from the clustering of ions in solution or at solid surfaces, ion clusters may first form highly hydrated liquid precursors known as liquid condensed phases or prenucleation clusters which are lower in free energy than the initial ionic species yet much higher in energy than the final crystalline product. From this point, the liquid precursor phase can dehydrate to from a meta-stable amorphous phase that can transition down a cascade of increasingly stable states (Figure I.4). This model for nucleation and growth applies well to both calcium carbonates and calcium phosphates which have several possible mineral states that form from an amorphous precursor. Thus, ANT has been widely accepted in the biomineralization community as the primary mechanism of biomineral formation.
due in large part to energetic considerations and the observation that many mineralizing organisms have the ability to produce and store amorphous mineral phases.

Figure I.4. Gibbs free energy diagram showing the direct and sequential pathway for the formation of the same mineral phase beginning from the same dissolved ions. The initial nucleation event for both pathways is defined as the sum of the free energy of nucleation ($\Delta G_N$) and the free energy of phase growth ($\Delta G_g$). As expected on the basis of the fundamentals of CNT, the initial energy requirements for the direct pathway are much greater than the sequential pathway. The transition between states of the sequential pathway is defined as the free energy of transition ($\Delta G_T$) and varies depending on the mechanism of transformation. (Adapted from references 2 and 10.)

Additionally, many have also adopted ANT because the ability of amorphous phases to be “molded” into non-equilibrium structures. One of the hallmarks of biomineralization is the ability to make crystal structures with non-equilibrium morphology. For example, in the laboratory the calcium carbonate mineral calcite typically forms well-defined rhombic crystals which reflect the symmetry of its unit cell. However, many organisms such as sea urchins and sea sponges have the ability to mold calcite into a variety of morphologies such as spicules, tabular columns, and mesh-like networks (Figure I.2). Similarly, the calcium phosphate mineral hydroxyapatite typically forms nanometer size needles and plates in the laboratory. However, as
seen in Figure I.1 the interaction of hydroxyapatite with an array of tissues and proteins results in the formation of mineralized ribbon bundles. The mechanisms governing the formation of these non-equilibrium structures are still under investigation and vary dramatically depending on the system in question. Ultimately, understanding how to control amorphous-to-crystalline transitions may allow for the replication of natural structures formed through biomineralization processes in the laboratory.28,29

1.2.3 Principles of Crystal Growth

Classically, after nucleation the growth of a crystal occurs through the attachment of ions to well-defined crystallographic faces. Assuming that crystals are perfect, homogeneous solids with planar surfaces, growth can occur only at the crystal surface in a two-dimensional manner by the attachment of an atom/ion to a crystal face and the diffusion of that atom/ion to an atomic step, if one is present (Figure I.5). This occurs until the face is completed with subsequent layer-by-layer growth continuing in this fashion. On the basis of this model, the most energetically demanding step of crystal growth is the addition of an atom/ion to a new crystal face as it stands alone on the surface and only has the point of contact available for making new chemical bonds. Once a growth step is established, atoms/ions can then add to the growth step where they are stabilized by chemical bonding at multiple points of contact.26,30

Though layer-by-layer growth is the only growth mechanism possible for an ideal crystal, real crystals contain several imperfections such as screw dislocations which provide a much more energetically favorable point for attachment. A screw dislocation is a structural defect where one part of the crystal is displaced relative to the other. This offset produces a spiral growth pattern that continuously provides a location on which to add atoms/ions to the crystal
In the biomineralization literature, various studies have proposed that proteins can bind to growing crystal faces at either idealized, planar surfaces or screw dislocations.\textsuperscript{6,9,31} Though it may not accurately represent how many real crystals grow, most hypotheses regarding how proteins interact with crystal surfaces neglect structural defects such as screw dislocation and model protein binding interactions at idealized, planar surfaces.

![Figure I.5](image)

Figure I.5. (Left) Representation of two-dimensional, layer-by-layer growth at a planar crystal face. (Right) Representation of spiral growth occurring at a screw dislocation. (Adapted from references 26 and 30.)

Understanding the relative rates of growth for the faces of a given crystal is important for determining crystal morphology as the growth may not occur equally in all directions.\textsuperscript{26} Though it seems counterintuitive, the slowest growing crystal faces are typically the most prominent faces observed. Figure I.6 shows a two-dimensional representation of crystal growth occurring for a crystal containing two types of faces that grow at different rates. The crystal initially begins with eight well defined faces with four A faces and four B faces. As the crystal grows, the B faces develop faster than the A faces and thus ultimately grow out of existence, leaving the final crystal with only four A faces. Taking a three-dimensional growth into account, this growth pattern describes the formation of halite, the geological form of sodium chloride, which
only expresses one type of crystal face (Figure I.6). As will be discussed in greater detail in Chapter III, soluble additives can alter the growth rates of specific crystal faces that cause large changes in crystal morphology. These types of interactions have been studied extensively as a means of engineering crystals to adopt specific shapes and understand how proteins interact with crystal surfaces.

![Figure I.6. (Left) A two-dimensional model of crystal growth with two types of crystal faces (A and B) that grow at different rates. (Adapted from reference 26.) (Right) Picture of a halite crystal.](image)

**1.2.1 X-ray Crystallography**

X-ray crystallography is an expansive field of study that focuses on examining the internal atomic arrangement of crystalline solids. This is possible since the atoms within crystalline solids diffract X-rays in distinct patterns which can be used to identify solids and determine the exact arrangement of atoms, i.e. the crystal structure. Structure determination begins with identifying the unit cell of a crystal, which is the smallest repeat unit of atoms that coincides with important symmetry elements in the crystal lattice. Thus, one way to envision a
crystal is as a collection of thousands of contiguous unit cells extending in three dimensions. The repetition of until cells within a crystal results in the formation of well-defined crystal planes or faces.

By convention, crystal faces can be identified by their Miller indices, a numeric designation reported by three numbers \( h, k, \) and \( l \), relating a specific face to the origin and the edges of the unit cell.\(^{33}\) The \( h, k, \) and \( l \) values of a Miller index must be integer values and can be either positive or negative. When referring to a specific crystal face, parentheses are placed around the Miller index such as the \((104)\) face of calcite. Since most crystals contain equivalent crystal faces that are related to one another by symmetry, a family of equivalent faces is referred to by the Miller index of one of the faces placed within braces such as the \(\{104\}\) faces of calcite. When reporting a Miller index containing a negative number, a bar is placed above the negative number such as the \((\bar{1}00)\) face of halite.

### I.3 DESIGNED PEPTIDES AND THE \(\alpha\)-HELICAL COILED COIL

#### I.3.1 De Novo Design of Functionalized Peptides

Proteins are capable of folding into complex molecular structures with specific form and function.\(^{34}\) In general, a host of interactions are responsible for stabilizing the folded state of a protein such as hydrophobic interactions, electrostatic interactions, and covalent bonding. One way to understand how proteins fold is to think the folding process as a hierarchical progression of structural elements beginning with the amino acid sequence of the protein or the primary structure (Figure I.7).\(^{35}\) The arrangements of amino acids in the proper order can result in the
Figure I.7. Various levels of structural organization observed in proteins. (Reprinted with permission from reference 34. Copyright 2001, American Chemical Society.)
formation of secondary structure elements such as α-helices, β-strands, or turns and higher-ordered secondary structural elements such as α-hairpins, β-hairpins, or mixed α-β structures. Additional interactions result in final folded state of the protein, or tertiary structure, which can be proceeded by the assembly of multiple folded proteins into a quaternary structure. Examining how proteins fold using this bottom-up approach suggests that a firm understanding of how to manipulate protein structure and stability at each level can allow for proteins to be designed from scratch. In reality, this is a nontrivial task aided by years of experimental observations and technological advancements.

To simplify the study of proteins, smaller polypeptides are often used that are designed to fold into specific secondary structure motifs. Designed peptides are useful for studying protein structural elements independently outside of their native environment. Once a peptide folding motif is well characterized, it can be utilized to perform a variety of desired functions. An excellent example of this comes from the study and design of the α-helical, coiled-coil peptide motif. Coiled coils are prevalent in nature with one of the most well known examples occurring in the GCN4 luecine zipper protein. Systematic studies on coiled coils have resulted in a wealth of information regarding how to manipulate the chemical and structural properties of this protein folding motif. Consequently, coiled coils have been designed to perform a variety of functions such as the binding of metals ions and the forming of fibrous networks.

I.3.2 Fundamentals of the Coiled Coil

Coiled coils consist of two or more α-helical peptides binding through hydrophobic and electrostatic interactions to form a superhelical assembly. This is generally accomplished through specific hydrophobic and electrostatic interactions based on a heptad repeat of amino
acids designated \( a-g \). Positions \( a \) and \( d \) form the hydrophobic core, positions \( e \) and \( g \) form salt bridges, and the remaining positions interact with the surrounding environment. Solid phase peptide synthesis allows for the strategic manipulation of coiled-coil amino acid sequence. This allows for any number of properties of the coiled-coil system to be controlled such as the oligomerization state, the stability of the coiled coil, and the chemical functionality of the solvent exposed regions. For example, the placement of asparagine, a positively charged amino acid, at a heptad position within the hydrophobic core destabilizes the coiled coil and only allows for the formation of dimeric coiled coils.\(^{45}\) Similarly, different combinations of hydrophobic residues within the hydrophobic core can shift the oligomerization state from dimer to trimer.\(^{40}\) For altering the surface chemistry of the coiled coils, a variety of different hydrophilic residues can be placed at \( b, c, \) or \( f \) positions. For example, the placement of a metal-binding residue at a centrally located \( f \) position caused the trimeric coiled coil AQ-Pal14 to form supramolecular complexes in the presence of transition metals.\(^{43,44}\) Similarly, acidic residues can be placed at solvent exposed positions to increase the negative charge density without significantly impacting the stability of the coiled coil.

Figure I.8. (Left) Models of the \( \alpha \)-helical, coiled-coil trimer backbone. (Right) Helical wheel diagram of the coiled-coil trimer showing hydrophobic \( (a,d) \) and electrostatic \( (e,g) \) interactions.
I.4 RATIONALE OF STUDY

I.4.1 Purpose of Study

The role of protein structure on protein-crystal interactions and amorphous-to-crystalline transitions is poorly understood. Further examination of these important biomineralization processes is needed using soluble additives that provide a high level of control over their structural and chemical properties. Thus, the purpose of this study is to use rationally designed peptides to understand how proteins interact with crystalline and amorphous solids to control crystal growth and orientation. Since the rational design of a peptide allows for control over both the structural and chemical properties of the peptide, experiments are designed to study the relationship between factors such as conformational flexibility and electrostatic character on the growth of biominerals. A detailed understanding of how to properly manipulate these factors may allow for the design of synthetic systems capable of achieving a high level of control over crystal growth and may provide insights into how proteins regulate biomineralization processes.

CHAPTER I REFERENCES


(11) Song, R.-Q.; Coelfen, H. *Crystengcomm* 2011, 13.


(30) Sunagawa, I. *Crystals - Growth, Morphology, and Perfection*; Cambridge University Press: Cambridge, **2005**.


(32) Smithsonian National Museum of Natural History.


(34) Garrett, R. H.; Grisham, C. M. *Biochemistry*; Thomson - Brooks/Cole: Belmont, **2007**.


II.1 Peptide Synthesis

All peptides were synthesized on a 0.10 mmol scale with standard Fmoc chemistry onto 0.66 mmol/gram amide resin on an Applied Biosystems 433A peptide synthesizer using solid-phase methods. Activation was performed by 1-[bis(dimethylamino)methylene]-1H-benzotriazolium hexafluorophosphate 3-oxide (HBTU) and 6-Chloro-1-Hydroxybenzotriazole (6-Cl-HoBt) in DMF. Protection of the N-terminus was achieved through capping with acetic anhydride. A mixture of 88.0% (v/v) trifluoroacetic acid (TFA), 5.0% (v/v) phenol, 5.0% (v/v) triisopropylsilane, and 2.0% (v/v) water was used for deprotection of the amino acid side chains and cleavage from the resin for 2-3 hours at room temperature.

The crude-peptide, cleaved-resin mixture was then filtered through a glass wool, Pasteur pipette filter and precipitated in approximately 50 mL of cold, anhydrous diethyl ether. The crude peptide was separated from the ether through centrifugation, rinsed with clean portions of diethyl ether, and lyophilized until dry. Final purification was performed on a Waters 515 two-pump high performance liquid chromatography (HPLC) system attached to a preparative Vydac C-18 column and a Waters 996 spectrophotometer using an acidic water/acetonitrile gradient as the mobile phase. The purity of all peptide fractions was assessed using matrix-assisted laser deionization time-of-flight mass spectrometry (MALDI-TOF) described further in Section II.7. Peptide were deemed to be approximately 95% pure if no other peptide fractions besides the desired peptide could be observed after multiple MALDI measurements with additional purifications performed as needed.
II.2 Circular Dichroism

Circular dichroism spectroscopy (CD) was performed on an Aviv 202-01 DS circular dichroism spectrometer coupled to a thermoelectric temperature controller. The raw data were converted to mean residue molar ellipticities by the following equation:

\[ [\theta] = \frac{[\theta]_{\text{obs}}}{(10^* l c n)} \]

where \([\theta]\) equals the mean residues molar ellipticity, \([\theta]_{\text{obs}}\) equals the observed ellipticity value in degrees, \(l\) equals the path length of the cell in centimeters, \(c\) equals the molar peptide concentrations, and \(n\) equals the number of amino acid residues within a given peptide sequence. Peptides were dissolved in a 50 mM sodium acetate buffer (pH 4.75) to a final concentration of approximately 50 µM and measured in a 1mm quartz cell. Measurements were performed for the purposes of determining secondary structure distribution for all peptides and determining coiled-coil stability through thermal denaturization.

Scans for determining peptide secondary structure were performed in triplicate on a range of 190-260 nm with an average of five-second readings at 1 nm intervals at 25 or 95°C. Secondary structure distribution analysis for \(\alpha\)-helical, \(\beta\)-strand, and random coil structures was performed using the DICHROWEB online server designed by Wallace and Whitmore for determining protein secondary structure distribution from experimentally obtained circular dichroism data.\(^1\)\(^2\) In scans showing predominantly \(\alpha\)-helical character, the presence of the coiled-coil secondary structure was determined by the following formula:

\[ \frac{[\theta]_{222}}{[\theta]_{208}} \]

where \([\theta]_{222}\) and \([\theta]_{208}\) equal the mean residue molar ellipticities at 222 nm and 208 nm, respectively. Peptides displaying \([\theta]_{222}/[\theta]_{208}\) values greater than or equal to 1.0 were designated as coiled coils on the basis of the work of Hodges.\(^3\)
II.3 Static Light Scattering

Static light scattering (LS) was performed on a Wyatt Technology miniDAWN Tristar detector coupled with a Wyatt Technology Optilab rEX refractive index detector. Peptides were dissolved in a 50 mM sodium acetate/acetic acid buffer (pH 4.75) containing 100 mM NaCl to a final concentration of approximately 400 µM. High performance size exclusion chromatography (HPSEC) was performed on all peptide fractions prior to LS analysis using a Tricorn Superdex 75 10/300 column attached to a Shimadzu LC-10AT HPLC pump. LS data was analyzed using ASTRA 5.3.4 software for determining the polydispersity and oligomerization state for all peptides.

II.4 Mineralization Conditions

Mineralization conditions were adapted from the work of Chi and colleagues to achieve the initial calcium phosphate precipitation within the timescale of a few minutes in control solutions as well to obtain large brushite crystals as the final product to facilitate scanning electron microscopy (SEM) and single-crystal X-ray diffraction analysis. Individual mineralization assays were performed by the rapid mixing of 75 µL of 80 mM aqueous monobasic sodium phosphate (NaH$_2$PO$_4$ x H$_2$O) with 75 µL of 70 mM aqueous calcium acetate (Ca(CH$_3$COO)$_2$ x H$_2$O) and allowing the precipitation reaction to occur without additional stirring or shaking. Peptide was dissolved in the sodium phosphate portion at twice the desired assay concentration prior to mixing. The concentration of peptide in the phosphate stock solution was assessed by measuring absorbance of tyrosine at 276 nm with an extinction coefficient of 1450 M$^{-1}$ cm$^{-1}$ on a Hewlett-Packard 8452A spectrophotometer with a 1cm path length quartz cuvette.
II.5 Turbidity Measurements

Turbidity measurements were conducted on a BioTek ELx808 96-well plate reader at 25 °C in Fisher 96-well polystyrene plates to monitor changes in UV-Vis absorbance measured at 405 nm. All turbidity assays were performed in triplicate and the data are reported with error bars to reflect the variations among different measurements. Induction time for the initial precipitation of calcium phosphate was determined using the following formula:

\[ I_t = \frac{\text{Abs}_{\text{Max}} - \text{Abs}_{\text{min}}}{2} \]

where \( I_t \) equals induction time, \( \text{Abs}_{\text{Max}} \) equals the maximum measured absorbance, and \( \text{Abs}_{\text{min}} \) equals the minimum measured absorbance. This treatment places the induction time approximately halfway between the onset of nucleation and the end of the initial precipitation reaction, similar to the method used by Williams and Sallis for calculating the induction time of calcium phosphate precipitation reactions.  

II.6 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed at the Instrumentation Center of the University of Toledo on a JEOL JSM-7500F field emission scanning electron microscope. SEM images of material produced from mineralization assays were taken with an acceleration voltage of 2.0 kV and emission current of 19.5 µA at working distances ranging from 8.0-9.0 mm. All samples were rinsed with deionized (DI) water then placed onto carbon tape mounted on SEM stubs obtained from Ted Pella, Inc.
II.7 Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry was performed on a Bruker Daltonic OmniFLEX time-of-flight mass spectrometer using reflective positive mode optimized for target masses of approximately 3000 Da for peptide purification. The matrix solution utilized was acetonitrile/H$_2$O/TFA (75:25:0.1 v/v) saturated with 6-aza-2-thiothymine (ATT). For analysis of peptide faction purity, lyophilized peptide was dissolved directly into the ATT matrix and placed directly onto a MALDI target plate. A Bruker UltrafleXtreme time-of-flight mass spectrometer using linear positive mode calibrated with Bruker Peptide Calibration Standard for masses from approximately 1000-4000 Da was utilized for the analysis of acid-digested calcium phosphate crystals containing bound peptide. Calcium phosphate crystals were first rinsed with several aliquots of DI water and placed into a 1.0 M acetic acid solution until completely dissolved. This solution was then mixed with an equal volume of ATT matrix and placed onto the MALDI target plate. This method allowed for the qualitative determination of peptide binding to brushite crystals.

II.8 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) was performed on a Varian FTS4000 spectrometer coupled to a Varian 620-IR imaging microscope. A PIKE MIRacle diamond attenuated total reflectance (ATR) accessory was used for analyzing solid calcium phosphate samples. All calcium phosphate samples were rinsed with DI and lyophilized or air dried prior to analysis.
II.9 STEM

Scanning transmission electron microscopy (STEM) was performed on a HATACHI HD-2300A Ultra-thin Film Evaluation System at an accelerating voltage of 200 kV under high vacuum (> 5.0 x 10^{-5} Pa). Sample images were obtained under high resolution mode with an aperture setting of two and diffraction patterns were obtained under nanodiffraction mode with an aperture setting of four. Samples were prepped by dipping carbon coated copper grids into small aliquots of water containing the desired product for analysis.

II.10 Single Crystal X-ray Diffraction

Single-crystal X-ray diffraction was performed at the Instrumentation Center of the University of Toledo on a Bruker APEX DUO using a molybdenum X-ray source. Diffraction data were analyzed using APEX2 software for unit cell identification and refinement as well as crystal face indexing of brushite crystal obtained from mineralization assays.

II.11 Powder X-ray Diffraction

Powder X-ray diffraction was performed at the Instrumentation Center of the University of Toledo on a PANalytical X'Pert PRO on a zero background holder. Phase identification based on experimentally obtained data was performed using JADE powder diffraction software.

II.12 Crystal and Peptide Modeling

All peptide modeling was performed using the open source software PyMOL version 1.2r1. For modeling of the AQQ peptide series, a symmetry mate of the protein databank
structure 1coi was used for modeling the AQQ peptides backbone for its similarity in structure and stabilizing hydrophobic and electrostatic interactions.\textsuperscript{6} Point mutations were made using the PyMOL mutagenesis tool at solvent exposed positions for modeling the distances of glutamic acid residues relative to one another in AQQ3E and AQQ5E. CrystalMaker version 2.6 was used for building the structure of brushite and modeling the chemical profile of specific crystal faces. Unit cell parameters were experimentally derived using single-crystal X-ray diffraction as described in Section II.11. Atomic coordinates for all atoms within the brushite unit cell were applied from the work of Schofield and colleagues and downloaded from the American Mineralogist Crystal Structure Database.\textsuperscript{7,8}

\textbf{CHAPTER II REFERENCES}


III.1 INTRODUCTION

III.1.1 Experimentally Deduced Pathways of Protein-Crystal Interactions

The formation of natural structures such as bones, teeth, and seashells is the result of a complex interaction of organic and inorganic components known as biomineralization. Though many aspects of biomineralization are poorly understood, a widespread belief is that acidic proteins interact directly with the growing mineral phase and influence structural properties of these materials. To better understand the interaction of proteins and minerals, many studies have investigated how additives such as proteins and protein mimics (peptides, surfactants, low molecular weight organics, etc.) bind to specific crystal faces and alter crystal morphology. A diagram of how additives can alter crystal morphology on the basis of experimentally observed effects is shown in Figure III.1 for a given crystal with two types of faces, A and B. In the absence of a crystal modifying additive, the growth rate of B is greater than the growth rate of A. The difference in growth rates between the two faces is evident since the A faces are larger and grow slower than the B faces which are smaller and grow faster. In the presence of an additive that binds to the B faces, the growth rate of B is suppressed so that the growth rate of A is now much greater than the growth rate of B. This ultimately leads to the disappearance of the A faces since they grow themselves out of existence. For the same crystal system, another possible morphological change caused by the proper additive is the expression of other crystal faces which typically grow too fast to be observed. For example, addition of an additive that binds to the C faces slows the growth of these faces and allows them to be observed in the final crystal.
product. These changes in crystal morphology are easily observed in experimental assays and allow for straightforward assignment of the face of additive binding.

![Diagram of how additives can bind to specific crystal faces to impact growth rates of those faces and the final crystal morphology. (Reprinted with permission from reference 6. Copyright (2008) American Chemical Society.)](figure3)

Though identifying large scale changes in crystal morphology is a relatively easy task, describing the mechanisms by which proteins bind to crystal surfaces is more complex. Over the past two and a half decades, various hypotheses have been proposed to explain how particular proteins bind to a specific crystal face.\(^6,7\) The majority of these explanations model the binding of proteins to ideal, crystallographic faces. More recently, *in situ* atomic force microscopy (AFM) experiments have shown that protein binding may be occurring at the atomic steps of screw dislocations since this relatively small area of the crystal surface is where the crystal growth typically occurs.\(^8-10\) Regardless of which approach may be more accurate for describing protein-crystal interactions, various studies have shown that proteins can recognize and bind to specific crystal faces. For the purposes of this discussion, only mechanisms that deal with the binding of proteins to ideal, crystal faces will be taken into account.
When using a protein as a crystal binding additive, specific factors should be considered when rationalizing how the protein binds to specific crystal faces. First, what residues are located at the crystal binding region of the protein? Typically, acidic residues such as glutamic acid (Glu), aspartic acid (Asp), and phosphoserine (pSer) are associated with binding to crystal faces since the negatively charged character of these residues complements the positively charged ions at the crystal surface. In some cases, positively charged or even neutral amino acid groups have been associated with binding to specific crystal face, though the number of net negatively charged crystal binding proteins is far greater. Regardless, both the number and type of residues present in the crystal binding domain can impact the ability of the protein to bind to specific crystal faces.

Second, does the mineral binding domain of the protein adopt a specific conformation such as a β-sheet, β-strand, α-helix, etc. or does it exist in a predominantly disordered configuration? Various studies have shown that mineral binding domains can consist of ordered or disordered configurations. While the exact role of secondary structure on protein-crystal interactions is still unknown, many studies have recognized that it likely plays an important role in the binding process. Lastly, what is the chemical profile of the face being bound to by the protein? Is the face a high energy face with a net positive, negative, or neutral character? What are the atomic distances between ions at this face? These types of crystal surface characteristics have been used to rationalize why certain proteins may favor one type of crystal face over another as will be discussed in greater detail in the following paragraphs.

To explain how proteins bind to crystal surfaces to slow the growth rate of specific crystal faces and subsequently alter crystal morphology, two rationalizations are currently used. The first, referred to here as the structural model, entails that the mineral binding domain of a
protein must contain a precise, periodic structure such as a β-sheet or α-helix that orients mineral binding residues in a complimentary manner to the crystal surface they bind to in a site specific manner. The second, referred to here as the electrostatic model, states that nonspecific, electrostatic interactions occur between the mineral binding domain of a protein and the target crystal surface which are favored by a structurally disordered protein conformation and a highly charged crystal surface. These opposing ideas are supported by various studies conducted over a wide range of experimental conditions and on numerous protein-mineral systems. Thus, it is difficult to directly compare results from these studies. Rather, it is quite possible that either of these models could apply depending on the system and experimental conditions. Thus, a summary of the structural and electrostatic models of protein-crystal interactions will be provided as they pertain to the binding of proteins to well-defined, crystallographic faces.

III.1.2 The Structural Model

When considering a protein containing negatively charged crystal binding residues interacting with calcium-based biominerals, the structural model for protein-crystal interaction requires the spacing between the negatively charged groups of a protein binding to a specific crystal face to be approximately the same as the spacing between positively charged calcium ions of the target crystal face. This idealized spacing can be achieved by the mineral binding portion of protein adopting a well-defined secondary structure such as β-strand, β-sheet, and α-helix with mineral binding residues oriented on the same side of the polypeptide chain. Various studies, both older and more recent, support this model with one of the most intriguing examples coming from the work of DeOliveria and Laursen who developed a synthetic peptide known as
CBP1 to bind selectively to the \{1\bar{1}0\} faces of calcite when adopting an \(\alpha\)-helical conformation.\(^{24}\)

CBP1 was modeled after antifreeze polypeptide (AFPs) extracted from cold water fish found to prevent the extended growth of ice by binding to the \{20\bar{2}1\} faces of ice crystals. DeOliveria and Laursen noted that several AFPs adopted a predominantly helical structure in cold water solutions (3-5 °C) and concluded that the specific location of certain residues on the helical backbone allowed for the AFP to interact with ice in a site specific manner. Inspired by this, DeOliveria and Laursen designed CBP1 with aspartic acid residues residing on the same side of the helix located approximately 16.5 Å apart to fill vacant carboxylate positions located 17.2 Å apart on the \{1\bar{1}0\} faces of calcite. As designed, applying CBP1 to saturated calcium bicarbonate solutions containing rhombohedral calcite seed crystals at 3 °C hindered the growth at the \{1\bar{1}0\} faces of calcite to produce prismatic rather than rhombohedral crystals (Figure III.2). Conversely, applying CBP1 to saturated calcium bicarbonate solutions containing rhombohedral calcite seed crystals at 25 °C hindered growth of an unidentified family of crystal faces to produce studded calcite crystals, leaving the \{1\bar{1}0\} faces unaffected.

Figure III.2. (Left) Typical rhombohedral morphology of calcite observed when grown without additives. (Center) The prismatic morphology observed when calcite is grown in the presence of CBP1 at 3 °C. (Right) The studded morphology observed when calcite is grown in the presence of CBP1 at 25 °C. (Reprinted with permission from reference 24. Copyright (1997) American Chemical Society.)
To rationalize this effect, DeOliveria and Laursen used circular dichroism spectroscopy to show that CBP1 is approximately 89% helical at 3°C while at 25°C the degree of helicity decreases to approximately 40%. Thus, it was concluded that the designed site-specific interactions of the helical form of CBP1 were required for the peptide to bind to the (110) faces of calcite while the disordered state acted as a general polyanion binding in a nonspecific manner. On the basis of the findings of this study, it can be inferred that the presence of a well-defined secondary structure adds a degree of specificity to a crystal binding peptide system that is not achieved with a more disordered analog. However, as will be discussed in greater detail in Section III.1.4, while a more disordered system may lack a specific structural arrangement it has the advantage of being able to adopt multiple conformations. Thus, though the designed α-helical arrangement was either no longer present or severally distorted in CBP1 at 25 °C, why did DeOliveria and Laursen observe specific binding to the unidentified family of crystal faces to produce only the studded crystal morphology under these conditions? The only explanation offered to account for this behavior was that other similar, poorly helical polyanionic peptides produced the studded morphology as well in unpublished experiments.

Despite the lack of discussion regarding the calcite binding mechanism of CBP1’s disordered state, the successful design of CBP1 clearly demonstrated the principle of orienting mineral binding residues through secondary structure control. Ideally, follow-up studies should have been performed to continue to develop and investigate these types of structure-based interactions. However, the field of biomineralization evolved rapidly and the validity of the structural model came into question since years of studying natural systems revealed that the mineral binding domains of proteins found in many biomineralization systems were highly
disordered.\textsuperscript{25,26} Thus, several questions regarding the role of peptide secondary structure effects on peptide-crystal interactions remain unanswered.

\textbf{III.1.4 The Electrostatic Model}

The electrostatic model has garnered recent attention and widespread acceptance from the field due in large part to the fact that many mineral binding proteins are intrinsically disordered in solution.\textsuperscript{17,18,25,27,28} The hypothesis states that the mineral binding domain of a protein should lack a specific folding behavior and contain several negatively charged residues. This allows for maximum conformational freedom which increases the probability of favorable electrostatic interactions between the anionic residues of the protein and the exposed cations of the crystal surface. Thus, the ability of a disordered protein to recognize and bind to a particular crystal face is governed purely by electrostatics where highly anionic proteins will typically be attracted to the most positively charged crystal surface. Various groups have observed this behavior using both experimental and computational methods for different intrinsically disordered proteins.\textsuperscript{29-32} In one example, Grohe \textit{et al.} prepared three disordered peptides with varying degrees of phosphorylation and added the peptides to mineral growth solutions containing calcium oxalate monohydrate (COM) seed crystals with \{100\}, \{010\}, and \{121\} faces exposed.\textsuperscript{29} Mapping the electrostatic potential of the three faces showed that the (100) face of COM is much more electropositive than the other two. As rationalized by the electrostatic model, all the peptides bound preferentially to the \{100\} faces of COM.

While the biological and experimental evidence in favor of the flexible polyelectrolyte hypothesis have caused it to become widely accepted, it does not account for all protein-crystals interactions. Even as early as the original work of Addadi and Weiner, it has been known that
certain proteins with high β-sheet content bind selectively to specific faces when other, more electropositive surfaces are available. More recently, directed evolution experiments have developed various peptide sequences that bind exceedingly well to crystal surface but often lack a high degree of anionic character. In one such example, Schrier and colleagues computationally developed a peptide (bap4) exhibiting a net positive charge capable of binding to calcite \textit{in vitro} and modifying subsequent crystal growth. To explain how this positively charged peptide could bind to calcite, modeling using the recently developed RosettaSurface program showed that the peptide adopted a helix-turn-helix motif upon binding to the crystal surface. In subsequent experiments, growing calcite in the presence of a sequence-scrambled variant of bap4 produced typical, rhombohedral crystals found in negative controls. Thus, it was concluded that the primary sequence and likely the helix-turn-helix structure were important for determining the peptide-crystal binding. Enhancements in techniques such as solid state NMR and computational methods that model/predict protein-solid surface interactions have demonstrated that specific structural motifs form exclusively at the protein-mineral interface for a variety of systems. Thus, perhaps a new theory in crystal-protein interaction which incorporates aspects of the electrostatic and structural theories will ultimately need to be developed to adequately describe these types of interactions.

III.2 RATIONALE OF STUDY

III.2.1 Statement of the Problem

Understanding the role of protein secondary structure in directing protein-crystal surface interactions is an ongoing effort. This task has only become more difficult as different
hypotheses exist with fairly different criteria concerning how proteins bind to crystal surfaces. In one camp, the belief is that a structural rigidity is needed to guide specific chemical interactions. In the other, the belief is that conformational freedom allows for a higher probability of chemically nonspecific interactions to occur. The debate is only further complicated by several recent examples which show that certain systems that are randomly oriented in solution undergo conformational shifts to structurally ordered states upon binding to crystal surfaces. Thus, perhaps a degree of compromise between the two sides is needed and future studies of protein-crystal interactions should keep both theories in mind when designing experiments and rationalizing results.

III.2.2 Purpose of Study

Rather than attempting to mimic a property observed in a natural biomineralization system or justify a particular protein-crystal interaction theory, the main goal of this study is to elucidate the role of secondary structure in rationally designed systems to direct protein-crystal interactions. Doing so in a careful and controlled manner could potentially show that various pathways for controlling crystal growth are possible within a single, unified study. DeOliveria and Laursen began studying this approach during their investigation of CBP1. However, additional studies emphasizing both the structural and electrostatic models are needed. Thus, the purpose of this study is to investigate how the presence and absence of a well-defined secondary structure affects the binding of a synthetic peptide to specific crystal surfaces.
III.2.3 Hypotheses

On the basis of the principles of the structural and electrostatic models of protein-crystal interactions, it stands to reason that the principles of both theories could be observed within a single study using the same set of experimental conditions. Thus, given two acidic peptides with similar chemical compositions, if one adopts a well-ordered secondary structure while the other has conformational flexibility then differences should be observed in their abilities to affect crystal growth. Specifically, if lattice matching is present between the anionic groups of the peptide adopting a well-ordered secondary structure with the cations of a particular crystal face then a site specific interaction may occur as described by the rules of the structural model. Conversely, if significant differences in electrostatic potentials exist among the expressed crystal faces then a preferred interaction will take place between the most electropositive crystal surface and the peptide with conformation flexibility as described by the electrostatic model. Similarly, if no such lattice matching exists for the well-ordered peptide then binding will also occur at the most electropositive crystal face since the peptide can only act as an inflexible polyelectrolyte.

III.2.4 Experimental Approach

Several peptides adopting the coiled-coil motif were developed with increasing numbers of glutamic acid residues placed at solvent exposed positions. These peptides were added to mineralizing solutions forming hydrogenphosphate dihydrate (CaHPO$_4$ x 2H$_2$O, also known as brushite) until a significant effect on crystal growth was observed using the AQQ5E peptide. Small modifications were then made to AQQ5E that destabilized the coiled coil structure and created a randomly structured yet chemically similar analog named RCA5E. Side-by-side crystal growth experiments were then performed to observe the differences between AQQ5E and
RCA5E. For the crystal growth assays, calcium acetate and monobasic sodium phosphate solutions were prepared at sufficient concentrations to induce the rapid formation of a calcium phosphate precipitate upon mixing. These conditions favored the formation of an amorphous solid initially which transformed into large brushite plates over the course of 1-2 days. Brushite was chosen because it has the advantage of being readily prepared and only requires a pH of less than ~5.5 to obtain a pure phase unlike minerals such as HA and CaCO$_3$ which require strict solution conditions and tight control of pH to prevent the simultaneous growth of different polymorphs. A pH of approximately 4.5-4.8 resulting from the mixing of calcium acetate and monobasic sodium phosphate causes brushite to be a thermodynamically stable product since at this pH it is the least soluble form of calcium phosphate. Additionally, brushite crystals grow up to several hundred microns in length and width making them easy to manipulate and analyze.

III.3 RESULTS

III.3.1 Peptide Design and Characterization

Coiled coils are highly tunable peptide motifs that have been used to the design several functionalized peptides.$^{36-38}$ Thus, a large body of literature is available concerning how to characterized and manipulate their structural and chemical properties in a controlled fashion.$^{39}$ Developed previously by the Ogawa lab for the formation of supramolecular metallo-peptide assemblies, the trimeric coiled coil AQ-Pal14 served as the starting point for mutagenesis in this study.$^{40,41}$ AQ-Pal14 was an ideal candidate as a single point mutation of the 4-pyridal alanine residue (X) at position 14 for glutamine resulted in the formation of AQQ, a coiled coil trimer with no negatively charged or metal binding residues at solvent exposed positions (Table III.1).
The subsequent replacement of uncharged residues for glutamic acids at solvent exposed positions was performed resulting in the design of AQQ3E and AQQ5E. To make RCA5E, the random coil analog of AQQ5E, all leucine residues were replaced with glutamine residues which effectively destabilized the hydrophobic core of the coiled coil.

| Table III.1. Progression of Primary Sequence Changes of the AQ-Pal14 Peptide |
|-----------------------------|---------------------------------|
| Peptide         | Primary Sequence                        |
| AQ-Pal14        | Ac-Q-IAALEQK-IAALEXK-IAALEQK-IAALEQK-GGY-NH₂ |
| AQ             | Ac-Q-IAALEQK-IAALEQK-IAALEQK-IAALEQK-GGY-NH₂ |
| AQQ3E          | Ac-E-IEALEQK-IAALEQK-IAALEQK-IEALEQK-GGY-NH₂ |
| AQQ5E          | Ac-E-IEALEQK- IEALEQK- IEALEQK- IEALEQK-GGY-NH₂ |
| RCA5E          | Ac-E-IEAQEQK-IEAQEQK-IEAQEQK-IEAQEQK-GGY-NH₂ |

Characterization of this designed change in secondary structure was performed with circular dichroism spectroscopy. As seen in Figure III.3, AQQ5E displays a helical nature from the negative elipticity bands centered at 222 nm and 208 nm corresponding to the n-π* and π-π* electronic transitions, respectively. Additionally, the ratio of the signal at 222 nm / 208 nm of approximately 1.0 signifies a further stabilization of the helical structure through helix-helix interactions. In contrast, RCA5E only displays a dominant negative elipticity band centered at 203 nm corresponding to the π-π* transition which typically signifies a predominantly random coil species mixed with other secondary structure elements.

Computational analysis on secondary structure distribution based on the experimentally obtained CD data was performed on the DICHROWeb server using two styles of calculations, CDSSTR and CONTINLL (Table III.2). Both algorithms compare the experimental results to a database of several different proteins with known secondary structure distributions and attempt to reconstruct a theoretical dataset to match the experimental input. The distribution is reported in terms of percentages of α-helix, β-strand, turns, and unordered secondary structures.
as well as the normalized root-mean-square deviation (NRMSD) value showing the quality of the fit between the experimental results and computationally derived dataset. On the basis of the analyses, AQQ5E exists in a predominantly helical form in equilibrium with a disordered state which is typical for similar coiled-coil systems. In contrast, RCA5E exists in a predominantly disordered state in equilibrium with several other secondary structures. Thus, computational analysis confirms that the designed secondary structure elements were expressed for both the AQQ5E and RCA5E peptide.

![Molar Elipticity](Wavelength (nm))

**Figure III.3.** CD spectra for AQQ5E and RCA5E in 50 mM acetate buffer (pH 4.75).

<table>
<thead>
<tr>
<th>Peptide - Analysis</th>
<th>% α-helix</th>
<th>% β-strand</th>
<th>% Turns</th>
<th>% Unordered</th>
<th>NRMSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQQ5E - CDSSTR</td>
<td>70.0</td>
<td>6.0</td>
<td>6.0</td>
<td>18.0</td>
<td>0.009</td>
</tr>
<tr>
<td>AQQ5E - CONTINLL</td>
<td>66.7</td>
<td>3.4</td>
<td>7.5</td>
<td>22.4</td>
<td>0.036</td>
</tr>
<tr>
<td>RCA5E - CDSSTR</td>
<td>15.0</td>
<td>26.0</td>
<td>24.0</td>
<td>35.0</td>
<td>0.028</td>
</tr>
<tr>
<td>RCA5E - CONTINLL</td>
<td>17.6</td>
<td>24.9</td>
<td>21.9</td>
<td>35.6</td>
<td>0.152</td>
</tr>
</tbody>
</table>

Dynamic light scattering experiments were performed to assess the average hydrodynamic radius of AQQ5E and RCA5E to determine their respective oligomerization states.
(Table III.3). The hydronamic radius ($R_h$) was determined using the Astra software program through fitting of the data to correlation functions and the Stokes radius ($R_s$) found through the Stokes-Einstein equation using the experimentally observed diffusion coefficient ($D_{obs}$). Both data treatments give an average hydrodynamic radius of approximately 1.9 nm for AQ5E and 1.4 nm for RCA5E. In a previous characterization of the AQ-Pal14 system using ultracentrifugation a hydrodynamic radius of approximately 1.8 nm was obtained for that trimeric, coiled-coil species. On the basis of the similarities of both the primary sequence and experimentally obtained hydrodynamic radii between AQ-Pal14 and AQ5E, AQ5E appears to adopt a trimeric oligomerization state while RCA5E likely exists predominantly as a monomeric species.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Data Treatment</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQ5E</td>
<td>Stokes-Einstein equation ($D_{obs} = 1.82 \times 10^{-6} \text{ cm}^2\text{s}^{-1}$)</td>
<td>$R_s = 1.9 \text{ nm}$</td>
</tr>
<tr>
<td>AQ5E</td>
<td>Astra Software Prediction</td>
<td>$R_h = 1.9 \pm 0.95 \text{ nm}$</td>
</tr>
<tr>
<td>RCA5E</td>
<td>Stokes-Einstein equation ($D_{obs} = 1.28 \times 10^{-6} \text{ cm}^2\text{s}^{-1}$)</td>
<td>$R_s = 1.38 \text{ nm}$</td>
</tr>
<tr>
<td>RCA5E</td>
<td>Astra Software Prediction</td>
<td>$R_h = 1.4 \pm 0.15 \text{ nm}$</td>
</tr>
</tbody>
</table>

**III.3.2 MALDI Analysis of Acid Digested Crystals.**

Crystal growth assays performed with AQ5E and RCA5E displayed changes to brushite crystal morphology suggesting the peptides may be binding to the crystal. Various experiments were performed to test for the presence of the peptides in the final crystal products with analysis by matrix-assisted laser desorption/ionization (MALDI) of the crystals dissolved in acetic acid providing the clearest results. As seen in Figure III.4, crystals grown in the presence of AQ5E and RCA5E contain their respective peptide with no peptide present in the final rinse solutions used to clean the crystals. This suggests that the peptides are either bound tightly enough to the surface of the crystals to tolerate the rinsing treatment or that the peptides are bound within the
crystals. To test for the affinity of the peptides to mature brushite, crystals grown in the absence of peptides were added to highly concentrated (approximately 1 mM) solutions of AQQ5E and RCA5E separately and allowed to incubate for approximately 24 hours before undergoing the previously described rinsing and analysis protocols. As seen in Figure III.4, peptide is not observed in either the final rinse solution or the acid digested crystals. Thus, MALDI analysis suggests that the peptides are intimately associated with the crystals during the growth phase.

Figure III.4. (Top Left) MALDI analysis of crystals grown in the presence of AQQ5E dissolved in 1.0 M acetic acid. The theoretical mass for AQQ5E is 3713 g/mol with both the M + 1 peak, 3714 m/z, and the M + 2 peak, 1858 m/z, observed. (Top Right) MALDI analysis of crystals grown in the presence of RCA5E dissolved in acetic acid. The theoretical mass for RCA5E is 3773 g/mol with only the M + 1 peak, 3774 m/z, observed. (Bottom) Representative spectrum for mature brushite crystals incubated in concentrated AQQ5E and RCA5E solutions lacking the presence of peptide. (Inserts) Mass spectra of the final rinse solutions used in all individual sample preparations.
III.3.3 UV-Vis Analysis of Acid Digested Crystals

While MALDI analysis can identify the presence of a particular peptide by its specific mass-to-charge ratio, it is not a valid method for estimating the relative amount of each species present. As such, analysis by UV-Vis absorption of the crystals dissolved in hydrochloric acid was used to estimate peptide inclusion by weight. As shown in Figure III.5, the absorption maxima observed at 208 nm and 276 nm in the RCA5E spectrum correspond to absorption of the amide bonds within the peptide backbone and absorption by the terminal tyrosine residue of RCA5E, respectively. Using the molar extinction coefficient for Tyrosine at 280 nm (1280 M\(^{-1}\) cm\(^{-1}\)) and knowing the mass of the crystal dissolved and the total volume of the solution measured, it was estimated that crystals grown in the presence of RCA5E are approximately 1-2% peptide by weight.

![UV-Vis Absorbance spectrum](image)

Figure III.5. Comparison of UV-Vis absorption spectra of 400 µL of 1.0M HCl solutions containing 1.0 mg of crystals grown in the presence of AQQ5E and in the presence of RCA5E. Background scans were performed on the pure 1.0 M HCL solution prior to analysis. The spectrum of 1.0 mg of crystals grown in the absence of peptide dissolved in 400 µL of 1.0 M HCl was subtracted from the data sets to eliminate absorption artifacts produced by dissolving brushite crystals in HCl.
In contrast, the AQQ5E spectrum shows only absorption of the amide bond. Assuming that a linear relationship exists between the absorption signal observed for the amide bond at 208 nm and the concentration of peptide, the ratio of the AQQ5E absorption value to the RCA5E adsorption value produces a crude estimate of approximately 0.2-0.4% peptide by weight for crystals grown in the presence of AQQ5E. This estimate is subject of various assumptions and sources of error and should only be taken as a relative means of comparing AQQ5E and RCA5E to one another. In this regard, UV-Vis analysis of crystals containing peptide dissolved in hydrochloric acid suggests that RCA5E may have a higher affinity for growing brushite crystals than AQQ5E.

III.3.4 Single Crystal X-ray Diffraction and Face Indexing of Brushite

Brushite crystals grown in the absence of peptide were subjected to single-crystal X-ray diffraction for the purposes of face indexing all expressed crystal faces. The unit cell parameters obtained after refinement were $a = 5.81 \text{ Å}$, $b = 15.19 \text{ Å}$, $c = 6.24 \text{ Å}$, $\alpha = 90.01^\circ$, $\beta = 116.38^\circ$, and $\gamma = 89.94^\circ$ which is in agreement with published values for brushite. After achieving the correct unit cell parameters and orientation matrix, face indexing was performed which indentified the $(\bar{1}00)$ lattice plane, the $(10\bar{1})$ lattice plane, the face intersecting the $(\bar{1}00)$ face as the $(\bar{1}01)$ lattice plane, and the plane normal to the shown orientation as the $(0\bar{1}0)$ face (Figure III.6). Since crystal faces parallel to one another are related by symmetry, a model representation of an ideal brushite crystal was developed showing the relationship of specific faces to their interfacial angles (Figure III.7).
Figure III.6. (Left) Optical image of a nearly intact brushite crystal grown in the absence of peptides used for face indexing shown mounted for analysis. (Right) The same crystal with the experimentally determined Miller indices assigned to their respective faces.

Figure III.7. Representation of an ideal brushite crystal grown in the absence of peptide oriented looking down the b axis. All crystal faces are labeled with their corresponding Miller Indices and the relevant interfacial angles are shown.

### III.3.5 SEM Analysis of Crystal Morphology

The morphology of the brushite crystals grown either in the absence of peptides or in the presence of either AQQ5E or RCA5E were analyzed by scanning electron microscopy (SEM). In the absence of peptide, the brushite crystals grew into large, thin plates ranging in thickness from approximately 3-10 µM with an average length-to-width aspect ratio of approximately 3:1.
As seen in Figure III.8, two distinct interfacial angles of 125° and 150° can be observed which are present in all intact brushite crystals grown under these native conditions. This allows for easy visual examination of the changes imparted by the presence of a particular peptide since a simple measurement of the interfacial angle reveals the identity of the adjoining crystal faces based on the Miller index assignments made through single-crystal X-ray diffraction analysis.

As Figure III.9 shows, crystals grown in the presence of AQQ5E strongly favor the expression of the \{100\} faces over the \{\bar{1}02\} faces. Since the dominant crystal faces have the slowest growth rates this suggest that AQQ5E binds to the \{100\} faces and slows the rate of growth.

Additionally, crystals grown with AQQ5E ranged in thickness from 20-50 µM and had an average length-to-width aspect ratio of approximately 1.5:1. This behavior was true for the majority of observed crystals over a range of concentrations with some resembling the crystals grown in the absence of peptide. On the basis of these observations, it was concluded that AQQ5E has a specific effect on the growth of brushite of interacting with the \{100\} face and hindering its growth.

Figure III.8. (Left) SEM image of a nearly intact brushite crystals grown in the absence of peptide resting ontop of a fractured crystal. Scale bar represents a distance of 100 µM. (Right) Magnified image of the same crystal showing presence of two distinct interfacial angles of 125° and 150° between well-defined crystallographic faces and the corresponding Miller indices.
Figure III.9. (Left) SEM image of a nearly intact brushite crystal grown in the presence of AQQ5E laying adjacent to another crystal. Scale bar represents a distance of 100 µM. (Right) Magnified image of the same crystal showing the presence of two distinct interfacial angles of 125° and 150° as well as the corresponding Miller indices, where the size of the (102) face has decreased significantly compared to the control sample.

In contrast to the specific effects caused by the presence of AQQ5E, crystals grown in the presence of RCA5E displayed a variety of morphological changes. Figure III.10 shows the heterogeneous distribution of morphologies with an over expression of both the {100} and {102} faces in different crystals. Also, uneven growth inhibition along the {101} faces may be observed at the crystal faces with jagged edges. As such, both the thickness and aspect ratio of the crystals vary with no discernible averages. However, the crystals are generally thicker than the control crystals and thinner than the crystals grown in the presence of AQQ5E. On the basis of these observations, it was concluded that the flexible nature of RCA5E and its uneven distribution of secondary structure elements allowed for a variety of different peptide-crystal interactions to occur.
III.4 DISCUSSION

III.4.1 Rationalizing the Specific Morphological Changes Caused by AQQ5E

Soluble additives are known to alter crystal morphology by binding to specific faces and slowing their rate of growth. This can cause unaffected crystal faces to grow faster than the affected crystal faces which results in large scale changes of the morphology of the final crystal. In the present study, the coiled coil-peptide AQQ5E was systematically designed to alter the morphology of calcium hydrogen phosphate dihydrate, also known as brushite. During brushite crystal growth assays, AQQ5E caused an over expression of the \{100\} crystal faces while simultaneously decreasing the expression of the \{102\} crystal faces. On the basis of this observed behavior and the known effects of crystal binding additives, the morphological change was likely caused by the binding of AQQ5E to the \{100\} faces. As the \{100\} and \{102\} faces are expressed in similar proportions in brushite crystals grown without AQQ5E, the normal
growth rates of these two faces are also similar. Thus, AQQ5E binding at the \{100\} faces decreases their rate of growth which causes a reduction in the size of the \{\bar{1}02\} faces as they grow faster than the \{100\} faces. Analysis of these morphologically altered crystals dissolved in acid by UV-Vis and MALDI confirms the presence of bound AQQ5E. Unfortunately, these techniques were not able identify if the binding of the peptide was occurring at the \{100\} faces specifically. Future experiments using fluorescently labeled analogs of AQQ5E will be needed to visualize the proposed binding at the \{100\} faces.\textsuperscript{5,24}

Since AQQ5E adopts a well-defined secondary structure and appears to bind selectively to the \{100\} crystal faces of brushite, the structural model for protein-crystal interactions may apply for describing the mechanism by which AQQ5E binds to brushite. As previously discussed, this model supports the hypothesis that the spacing between the negatively charged groups of a protein binding to a specific crystal face is approximately the same as the spacing between positively charged ions of the target crystal face. This idealized spacing can be achieved by the mineral binding portion of the protein adopting a well-defined secondary structure such as the coiled-coil motif. Computer modeling of AQQ5E and the solvent exposed crystal faces of brushite was performed to investigate this possibility. For an ideal α-helix, the spacing for one helical turn is 3.4 residues corresponding to a distance of 5.4 Å.\textsuperscript{48} As shown in Figure III.11, adjacent solvent exposed carboxylate residues of AQQ5E are spaced two helical turns apart which corresponds to a theoretical distance of 10.8 Å. In agreement with this value, the average measured distance between adjacent carboxylates at these positions is 10.9 ± 1.1 Å. This value is based on the coordinates of the modified protein databank file (pdb) for the peptide 1coi, a trimeric coiled coil with similar hydrophobic and electrostatic interactions as AQQ5E. Thus, the use of other computational methods capable of determining the lowest energy
conformation of AQQ5E may produce a slightly different value with a lower standard deviation.\textsuperscript{14} However, as this value is only an estimate and agrees with the theoretical distance for two helical turns it should suffice for the purposes of this discussion.

![Model of AQQ5E](image)

**Figure III.11.** Model of AQQ5E displaying only the coiled coil backbone and solvent exposed glutamic acid residues at \textit{b} positions. Measurements between adjacent carboxylate groups of 10.3 Å, 11.7 Å, 11.3 Å, and 11.0 Å are shown. Average is 10.9 ± 1.1 Å

As shown in Figure III.12, a 20 Å x 20 Å model of the \{02\} and \{100\} faces of brushite shows the presence of calcium ions, hydrogen phosphate ions, and crystallographic water molecules in varying orientations. Modeling of the \{100\} face of brushite shows that adjacent calcium ions oriented parallel to the XZ axis are spaced 6.2 Å apart making the distance between calcium ions spaced one ion apart at this orientation 12.4 Å. While this distance is similar to the distance between adjacent solvent exposed glutamic acids of AQQ5E, it does not fall within the standard deviation of the obtained value. Additionally, the spacing between adjacent calcium ions on the \{100\} face oriented parallel to the XZ axis is 10.5 Å which does fall within the standard deviation of the average calculated distance between the adjacent solvent exposed...
carboxylate residues of AQQ5E. Since the \{102\} faces have more favorable calcium spacing than the \{100\} faces, yet the morphology of brushite crystals grown in the presence of AQQ5E suggest that AQQ5E binds to the \{100\} faces, the structural model fails to explain the specificity of AQQ5E for the \{100\} faces of brushite.

Figure III.12. Computer modeling of the different crystal faces affected by the presence of AQQ5E with the \{100\} face being the proposed site of AQQ5E interaction. For both modeled surfaces, an area of approximately 20 Å x 20 Å is shown as well as the distances between adjacent calcium ions oriented 90° from one another.

Since the structural model fails to explain the specificity of AQQ5E for the \{100\} faces of brushite, the electrostatic model of protein-crystal interactions may rationalize the specific binding of AQQ5E. As previously described, the electrostatic model supports the hypothesis that crystal binding proteins containing negatively charged amino acids will bind to crystal faces with the greatest electropositive character. Thus, on the basis of the electrostatic model the \{100\} faces of brushite would be expected to have a greater density of calcium ions or a larger
ratio of calcium to hydrogenphosphate ions compared to the \{102\} faces for AQQ5E to bind specifically to the \{100\} faces. However, analysis of the electrochemical character of the two faces performed by summing the number of each chemical species within a 15.2 Å x 15.2 Å area for the (\overline{1}02) and (100) faces of brushite show the two faces have a similar chemical profile (Figure III.13). Within the given area of the (\overline{1}02) crystal face four complete calcium ions, three halves of calcium ions, and two quarters of calcium ions are visible summing up to a total of six calcium ions. Similarly, within the given area of the (100) face two complete calcium ions, five halves of calcium ions, and two quarters of calcium ions are visible summing up to a total of five calcium ions.

Figure III.13. Side-by-side comparison of a 15.2 Å x 15.2 Å area for the (\overline{1}02) and (100) faces of brushite.

<table>
<thead>
<tr>
<th>Crystal Face</th>
<th>Sum of Ca(^{2+})</th>
<th>Sum of HPO(_4^{2-})</th>
<th>Sum of H(_2)O</th>
</tr>
</thead>
<tbody>
<tr>
<td>(102)</td>
<td>6</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>(100)</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>
The summation for all chemical species, shown in Table III.4, reveals that the two faces have a calcium to hydrogenphosphate ratio of 1:1 and that the \{\overline{1}02\} faces have a slightly greater density of calcium ions than the \{100\} faces. On the basis of the calcium-to-hydrogenphosphate ratio both faces are charge neutral overall and AQQ5E would not be expected to bind preferentially to either face. Additionally, AQQ5E might be expected to bind more favorably to the \{\overline{1}02\} faces as they have a slightly greater density of calcium ions than the \{100\} faces. Thus, on the basis of the similarity in electrochemical profile of the \{\overline{1}02\} and \{100\} faces, the electrochemical model fails to explain the specificity of AQQ5E for the \{100\} faces of brushite.

Independently, the structural and electrostatic models do not adequately account for the proposed specific binding of AQQ5E to the \{100\} faces of brushite. Thus, using the two models together may be necessary for the present study. For AQQ5E, conformational analysis and computer modeling show that AQQ5E adopts a coiled coil structure with glutamic acid residues oriented towards the solvent approximately two helical turns apart. This spacing causes adjacent glutamate groups on AQQ5E to be oriented in approximately the same manner which could be favorable for binding to solid surfaces. For the surfaces of brushite, the \{100\} faces have calcium ions separated from hydrogenphosphate ions into independent rows while the \{\overline{1}02\} faces have rows of alternating calcium and hydrogenphosphate ions. Thus, while overall both faces have a 1:1 ratio of calcium to hydrogenphosphate the \{100\} faces have localized areas of high calcium density which may act as binding sites for AQQ5E. Docking of AQQ5E onto the \{100\} face of brushite shows that a favorable electrostatic interaction may be possible between the solvent exposed glutamate residues and the rows of calcium ions (Figure III.14). Since this is just cursory examination of this possible interaction, additional computational methods are required to confirm this proposed binding mechanism; however, this analysis shows the
advantages of using principles from both the structural and electrostatic models of protein-crystal interaction to explain the binding of AQ5E to brushite.

Figure III.14. Modeling of the interaction between solvent exposed glutamate residues at $b$ positions of the heptad repeat of AQ5E with calcium ion channels of the (100) face of brushite.

III.4.2 Rationalizing the Nonspecific Binding of RCA5E

Site specific mutations of AQ5E caused a destabilization of the coiled-coil folding motif and resulted in the formation of a structurally disordered yet chemically similar analog of AQ5E known as RCA5E. Unlike AQ5E, RCA5E caused growth inhibition at all solvent exposed crystal faces of brushite except the $\{010\}$ faces which was also unaffected by AQ5E. As the structural analysis of RCA5E determined that it adopts a predominantly disordered configuration in equilibrium with other secondary structure elements, it was not modeled due to the number of possible conformations. To accurately determine the most likely structure of
RCA5E, additional computational techniques such as molecular dynamics would be required which are outside the scope of this study. Thus, for the purposes of analysis and discussion, RCA5E will be treated as a flexible, polyelectrolytic peptide chain capable of binding to crystal surfaces only on the basis of electrostatic interactions. The examination of electrostatic character of the \{\bar{1}02\} and \{100\} faces explains why RCA5E does not bind selectively to either face since no one face has a more electropositive profile than the other. Thus, the observed morphological changes caused by RCA5E and subsequent modeling of the solvent exposed crystal faces of brushite support the electrostatic model.

It may be possible that structural factors are necessary for RCA5E to bind to the specific crystal surfaces. For example, to bind the \{100\} faces RCA5E may have to adopt a slightly different conformation than when binding to the \{\bar{1}02\} faces which is possible due to its flexible nature. In general, the exact means by which proteins bind to specific crystal faces is still unknown. However, it is clear that secondary structure elements impact the growth of particular system, thus making it a possible level of design criteria for nature and for scientists to utilize to enhance the specificity of protein-crystal binding events. Recent improvements in techniques such as solid state NMR and computational modeling of protein-crystal surface interactions suggest that proteins which are disordered in solution adopt ordered structures when bound to a crystal surface. Additionally, various studies have shown that biomineralization proteins undergo conformational transitions in the presence of calcium from a disordered to more ordered state. While the functions of these transitions are still under investigation, it is clear from recent publications and from the results of this study that both structural and electrostatic interactions should be considered when describing the binding of proteins to crystal surfaces.
CHAPTER III REFERENCES


(9) De Yoreo, J. J.; Wierzbicki, A.; Dove, P. M. *Crystengcomm* 2007, 9, 1144.


IV.1 INTRODUCTION

IV.1.1 Statement of the Problem

The amorphous-to-crystalline transition of brushite is a poorly understood mechanism. In order to effectively manipulate this pathway, it must be further characterized. In general, understanding how to control amorphous-to-crystalline transitions using soluble additives can potentially allow for high level of control over the morphology of the final crystal product. In a recent study described in detail in Chapter III, crystals of brushite adopting altered morphologies were produced using peptide additives. The results of that study were rationalized using a model of protein-crystal interactions occurring at well-defined crystal surfaces. However, as these crystals were grown under conditions favoring the formation of an amorphous precursor, i.e. high supersaturation and rapid mixing, the observed changes in morphology may be better understood by studying the amorphous-to-crystalline transition under those conditions.

IV.1.2 Amorphous-to-Crystalline Transitions

For many years, it was widely believed in the field of biomineralization research that crystalline biominerals formed through the interaction of acidic proteins with crystals grown via the classically understood pathway of nucleation and growth.\(^1\) That is, complimentary ions dissolved in solution form clusters that could ultimately reach a critical radius and precipitate out of solution to nucleate the growth of a new solid through ion-by-ion addition to crystal surfaces.\(^2\) As this is an energetically demanding process, it was believed that insoluble organic scaffolds
assisted in the nucleation event with subsequent growth still occurring at solid crystal faces through ion-by-ion addition. This dogma held strong for many years due in large part to the influential work of Addadi and Weiner who were the first to propose that natural biominerals were formed by the modification of crystal morphology by soluble proteins at specific crystal faces. Subsequently, many followed their work and numerous studies were published regarding the modification of crystal growth by soluble additives. While much was learned in these studies, none were able to reproduce the exquisite structures found in nature such as sea urchin spine, the columnar mollusk shell nacre, or mammalian enamel. Thus, it was evident that a mechanism for controlling crystal growth other than the specific modification of crystal faces was present in natural systems to create crystalline biominerals.

Starting in the late 1990s, several groups began investigating the growth of biominerals formed through an amorphous precursor pathway. This pathway deviates from classical nucleation with respect to the stability of ion clusters and the formation of an amorphous phase that precedes the formation of any subsequent crystalline phases (Figure IV.1). First, precritical ion clusters are formed by the aggregation of dissolved ions in solution that are stable chemical species which have been isolated and characterized. In classical nucleation theory, ion clusters that fail to reach the critical radius needed to nucleate the growth of a new crystal are highly unstable and redissolve into their constituent ions. These clusters, shown as stable precritical clusters in Figure IV.1, are a heavily debated area of research. Once the ion clusters form they further aggregate to make an amorphous solid first rather than ordering into a crystal lattice directly. This is the most important distinction from the classical nucleation theory as amorphous solids can be molded into complex structures before transforming into a crystalline matrix. Thus, the growth of biominerals through an amorphous precursor pathway
provides a more logical basis for how nature can create complex crystalline morphologies. As such, the study of how amorphous-to-crystalline transitions occur has become an increasingly important area of research.\textsuperscript{15}

Figure IV.1. Diagram comparing the classically accepted theory of crystal nucleation and the alternative nucleation and growth pathway. (Reprinted with permission from reference 12. Copyright (2008) AAAS. Illustrated by P. Huey of Science.)

The study of biominerals formed through amorphous precursor pathways rather than the adsorption of proteins to well-defined crystal faces caused a paradigm shift in biomineralization research concerning the role of soluble additives. That is, if an amorphous phase initially forms which lacks well-defined crystallographic faces, how do proteins interact with this phase in a controlled manner to regulate growth? To advance the understanding of this topic, various groups have utilized acidic additives to modify the amorphous-to-crystalline transitions of calcium carbonate. In general, these studies demonstrate that additives can impact both the kinetic and thermodynamic properties of a mineralization pathway by either inhibiting or accelerating the formation of a phase or stabilizing a thermodynamically unstable product.
However, much still remains unknown about how altering the kinetic and thermodynamic properties of amorphous-to-crystalline transitions ultimately allows for controlling the morphology of the final crystal product. Controlled studies analyzing the effects of well-characterized soluble additives on the formation and transformation of amorphous precursors will be needed to advance the understanding in this area of research.

While the study of amorphous-to-crystalline transitions of biominerals has only recently become popular, the theory of how such pathways unfold was proposed in the early 1980s by Stephen Mann. In his 1983 paper entitled *Mineralization in Biological Systems*, Mann described the formation of crystals through a stepwise mechanism based on the Ostwald-Lussac rule of stages. This rule states that if a solution is supersaturated with respect to more than one phase, i.e. the concentration of ions in solution is sufficient to precipitate multiple solid phases, the more soluble (least stable) phase will typically form first. This rule perfectly explains amorphous-to-crystalline transitions since amorphous solids are more soluble (less stable) than the crystalline phases they form. For example, as shown in Table IV.1 the solubility product ($K_{sp}$) for the most common polymorphs of calcium carbonate predicts that amorphous calcium carbonate will form first, followed sequentially by vaterite, aragonite, and calcite.

<table>
<thead>
<tr>
<th>CaCO$_3$ Phase</th>
<th>$pK_{sp}$</th>
<th>$K_{sp}$ (moles/liter)$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>6.28</td>
<td>5.24 x 10$^{-7}$</td>
</tr>
<tr>
<td>Vaterite</td>
<td>7.39</td>
<td>4.07 x 10$^{-8}$</td>
</tr>
<tr>
<td>Aragonite</td>
<td>8.22</td>
<td>6.03 x 10$^{-9}$</td>
</tr>
<tr>
<td>Calcite</td>
<td>8.42</td>
<td>3.80 x 10$^{-9}$</td>
</tr>
</tbody>
</table>

This correlates well with experimental observations since calcite is the most commonly obtained form of CaCO$_3$ which is often preceded by either vaterite or aragonite in crystal growth assays. In nature, calcite is also the dominant form of calcium carbonate found; however, many
organisms have developed ways of stabilizing the more soluble forms of calcium carbonate presumably through the use of acidic proteins and insoluble, organic scaffolds. Based on the agreement between the solubility of the different forms of calcium carbonate and the experimentally observed transition of more soluble calcium carbonate polymorphs to less soluble ones, a free energy diagram describing this process has been proposed. This proposed pathway also incorporates the recent discovery of stable prenucleation clusters that precedes amorphous calcium carbonate, though many of the chemical properties of these clusters such as the solubility product are still unknown.

Figure IV.2 Free energy diagram showing both the direct (A) and sequential (B) pathways for calcium carbonate growth. (Adapted from Reference 1.)

**IV.2 RATIONALE OF STUDY**

**IV.2.1 Purpose of Study**

In a recent study described in Chapter III, the systematic modification of peptides was performed to study how secondary structure and electrostatic character impact protein-crystal
interaction. The focus of that study was the analysis and interpretation of the morphological changes observed in brushite crystals grown in the presence of peptides with similar chemical compositions but different secondary structure distributions. While understanding the roles of secondary structure on amorphous-to-crystalline transitions has yet to be explored, other experimental factors must first be understood before attempting to study and rationalize such interactions. Specifically, the amorphous-to-crystalline transition of brushite should first be studied and modified using a variable such as electrostatic character since the basis for the majority of protein-mineral interactions begins with electrostatic interactions. Since a detailed study examining how secondary structure impacts such a transformation would follow this work, the experiment should be designed in a way that secondary structure remains constant while changes are made to the electrostatic character only. This way, the effects of particular results can be more effectively rationalized as either electrostatic or structural in nature. Thus, the purpose of this study is to investigate how structurally similar peptides with different electrostatic character interact with the different phases formed during the amorphous-to-crystalline transition of calcium hydrogenphosphate dehydrate (CaHPO$_4$ x H$_2$O, also known as brushite).

**IV.2.2 Hypothesis**

Previous *in vitro* studies of the amorphous-to-crystalline transitions of different biominerals show that acidic polypeptides can stabilize an amorphous precursor. Similarly, other studies have shown that negative charges improve the ability of a polypeptide to interact with a mineral phase. Thus, if a series of peptides is made with increasing numbers of negatively charged residues then the peptide with the largest negative charge density will have the most
impact on the kinetic, thermodynamic, and morphological properties of the amorphous-to-
crystalline transition of brushite.

IV.2.3 Experimental Approach

A detailed description of the mineralization conditions used to study the amorphous-to-
crystalline transition of brushite can be found in Chapter II, Section 4. In general, the
mineralization conditions caused the rapid precipitation of an amorphous phase of calcium
phosphate that transformed into a polycrystalline intermediate followed by the transformation
into crystals of brushite. In the absence of peptide, the entire process occurred within
approximately 40-48 hours. In the presence of peptide, the duration of the process was extended
with the time between phase transitions varying depending on the peptide used. The transition
between phases was examined with turbidity measurements monitoring changes in absorbance at
405 nm over time. This method was useful for comparing the relative kinetics of phase
transition in the absence and presence of peptide since the three observed phases, i.e. amorphous,
intermediate, and final crystal, displayed different optical properties. All three observed phases
were characterized in terms of morphology, and other experimental methods were used to study
other properties as needed. As the morphology of the final crystalline state of brushite was well
characterized in a previous study as described in Chapter III, the analysis in the present study
culminates with the transition of the intermediate phase to the final crystalline state.
IV.3 RESULTS

IV.3.1 Peptide Design and Conformation Analysis

Coiled coils are highly tunable peptide motifs that have been used to design several functionalized peptides.\textsuperscript{17-19} Thus, a large body of literature is available concerning how to characterized and manipulate their structural and chemical properties in a controlled fashion.\textsuperscript{20} Developed previously by the Ogawa lab for the formation supramolecular metallo-peptide assemblies, the trimeric coiled coil AQ-Pal14 served as the starting point for mutagenesis in this study.\textsuperscript{21,22} AQ-Pal14 was an ideal candidate as a single point mutation of the 4-pyridal alanine residue (X) at position 14 for glutamine resulted in the formation of AQQ, a coiled-coil trimer with no negatively charged or metal binding residues at solvent exposed positions (Table IV.2). The subsequent replacement of uncharged residues for glutamic acids at solvent exposed positions was performed resulting in the design of AQQ3E and AQQ5E. Other coiled-coil peptides were also developed in this manner, but were found to have negligible effects on crystal growth in other mineralization studies. Thus, to study the effects of increasing negative charge density on the amorphous-to-crystalline transition of brushite an uncharged peptide (AQQ), a mediately charged peptide (AQQ3E), and a highly charged peptide (AQQ5E), were developed.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQ-Pal14</td>
<td>Ac-Q-IAALEQK-IAALEXK-IAALEQK-IAALEQK-GGY-NH\textsubscript{2}</td>
</tr>
<tr>
<td>AQQ</td>
<td>Ac-Q-IAALEQK-IAALEQK-IAALEQK-IAALEQK-GGY-NH\textsubscript{2}</td>
</tr>
<tr>
<td>AQQ3E</td>
<td>Ac-\textbf{E}-IAALEQK-IAALEQK-IAALEQK-IEALEQK-GGY-NH\textsubscript{2}</td>
</tr>
<tr>
<td>AQQ5E</td>
<td>Ac-\textbf{E}-IEALEQK-\textbf{IE}-EALEQK-\textbf{IE}-EALEQK-GGY-NH\textsubscript{2}</td>
</tr>
</tbody>
</table>

Circular dichroism spectroscopy was used to characterize the AQQ peptide series (AQQ, AQQ3E, AQQ5E) and determine if the addition of glutamic acid residues to solvent exposed
positions caused any significant changes to the structure and stability of the coiled-coil structure. As Figure IV.3 shows, all AQQ peptides display α-helical structure from the negative elipticity bands centered at 222 nm and 208 nm corresponding to the n-π* and π-π* electronic transitions, respectively. The ratio of the signal at 222 nm / 208 nm of approximately 1.0 for AQQ, AQQ3E and AQQ5E suggests that all peptides in the AQQ series adopt mainly a coiled-coil secondary structure. To determine the relative stability of the coiled-coil peptides, CD measurements were performed at 95 °C to observe the thermally denatured state of the peptides. As seen in Figure IV.3, at 95 °C the negative elipticity bands centered at 222 nm and 208 nm show that the peptides retain an α-helical conformation. This suggests that the coiled coils may not completely unfold upon thermal denaturization and that increasing the number of negative charges at solvent exposed positions does not significantly impact the stability of the coiled coil structure.

Figure IV.3. (Left) CD of all peptides at 25 °C showing that all AQQ peptides display α-helical coiled-coil secondary structure with the 0222 > 0208 and RCA5E showing random coil behavior. (Right) CD of all peptides at 95 °C showing that all AQQ peptides retain α-helical character upon thermal denaturization.

**IV.3.3 Nucleation of the Amorphous Phase**

The nucleation of the amorphous phase was monitored via turbidity assays in the absence and the presence of the AQQ peptides at increasing concentrations (200 µM, 400 µM, and 600
µM). These concentrations were chosen on the basis of several in-house experiments optimizing this procedure to obtain a sufficient difference in turbidity spectra between concentrations. In general, the nucleation of the amorphous phase is attributed to a sharp increase in turbidity caused by the rapidly increasing number of precipitating particles blocking the incident light from reaching the detector. The end of the nucleation event correlates with the leveling off of the turbidity spectra to a maximum absorbance value associated with the solubility of the amorphous phase under the given solution conditions. To relate the differences in time needed for the amorphous phase to nucleate in the absence and presence of peptides, the halfway point between the onset and end of the nucleation event was calculated by the following equation: \[ I_t = \frac{\text{Abs}_{\text{Max}} - \text{Abs}_{\text{Min}}}{2} \], where \( I_t \) equals induction time, \( \text{Abs}_{\text{Max}} \) equals the maximum measured absorbance, and \( \text{Abs}_{\text{Min}} \) equals the minimum measured absorbance.

As shown in Figure IV.4, the average induction time for the nucleation of the amorphous phase in the absence of peptide was approximately 150-175 seconds. The addition of AQQ to the reaction mixture had little effect with the induction time of the highest concentration assayed being approximately 400 seconds. AQQ3E had a slightly greater effect with the induction time for the highest concentration assayed being approximately 700 seconds. In contrast, the induction time for the highest concentration of AQQ5E assayed was approximately 2500 seconds. Since AQQ does not contain any solvent exposed residues, its inability to delay nucleation of the amorphous phase is likely caused by its lack of interaction with the precritical clusters that precede the formation of the amorphous phase. Conversely, the presence of nine solvent exposed Glu residues on AQQ3E and fifteen solvent exposed Glu residues on AQQ5E allows these two peptides to influence the formation and aggregation of the precritical clusters thus increasing induction time. Interestingly, AQQ5E has only 40% more acidic residue content
than AQQ3E yet has more than a threefold increase in induction time at all concentrations. This non-linear increase in induction time with respect to increasing number of acidic residues suggests that either the placement of the acidic residues is an important factor for determining the interaction of proteins with precritical clusters or the increase in induction time scales exponentially with increasing acidic residue content.

Figure IV.4. Results obtained from the nucleation turbidity assay performed for AQQ (Top Left), AQQ3E (Top Right), and AQQ5E (Bottom Left) showing that the induction time increases with increasing peptide concentration. The red horizontal lines represent the approximate induction time for each set of trials. Additionally, a direct comparison of average induction times obtained from nucleation turbidity assays containing AQQ, AQQ3E, and AQQ5E at all and concentrations studied is displayed (Bottom Right) showing that increasing the negative charge density delays the initial nucleation.
Scanning electron microscopy (SEM) analysis of the initially formed phase extracted immediately after the end of the precipitation reaction shows this material forms film-like aggregates lacking a well-defined morphology (Figure IV.5). In general, the presence of peptide did not significantly alter the morphology of the initial phase. Electron dispersive X-ray spectroscopy (EDS) analysis showed that the precipitate is composed primarily of calcium and phosphate with a Ca:P molar ratio of approximately 1.25 (data not shown) which does not correspond to any common mineralized phase of calcium phosphate. Electron diffraction analysis using scanning transmission electron microscopy (STEM) of the initial phase displayed broad rings typically associated with an amorphous phase (Figure IV.5). Based on these observations, it was concluded that the initial phase is an amorphous form of calcium phosphate.

Figure IV.5. (Right) SEM image of calcium phosphate phase that initially precipitates. (Left) STEM nanodiffraction pattern showing the amorphous nature of the calcium phosphate initially precipitated.

IV.3.4 Transition from the Amorphous Phase to the Polycrystalline Intermediate

The transition of the amorphous phase to the intermediate phase was monitored via turbidity assays for the first 12 hours following nucleation in the absence and the presence of the
AQQ peptides at a concentration of 200 µM. This concentration was chosen because it prolonged the lifetime of the intermediate phase when compared to samples containing 400 and 600 µM peptide and thus made for easier analysis. In general, the transition of the amorphous phase to the intermediate phase is attributed to a decrease in turbidity likely caused by a condensing of the amorphous phase into a more ordered state. The end of this transition event correlates with the leveling out of the turbidity spectra associated with the maximum solubility of the intermediate phase under the given solution conditions.

As shown in Figure IV.6, in the absence of peptide and in the presence of AQQ and AQQ3E, the amorphous-to-intermediate transition occurs rapidly with the initial sharp decrease in turbidity occurring within the two hours preceding nucleation. On the other hand, in the presence of AQQ5E the initial amorphous phase persists for several hours after nucleation with the intermediate phase not being fully formed until approximately 12-14 hours after the initial nucleation. The similarity in the transitions between samples without peptide and samples grown with AQQ is expected since AQQ does not contain solvent exposed acidic residues and would not be expected to have a significant impact. However, the similarity of AQQ3E with the control assays is surprising since it contains several solvent exposed acidic residues. Additionally, the extreme difference between AQQ3E and AQQ5E suggests that structural factors such as the exact placement of acidic residues on the coiled-coil backbone rather than just the total number of acidic residues may play an important role in stabilizing the amorphous phase and slowing the rate of transition.
Figure IV.6. 200 µM turbidity assays monitoring the transition of the amorphous phase into the intermediate phase over the initial 12 hours after the formation of the amorphous precursor.

The morphology of the intermediate phase formed in the absence and presence of the AQQ series was analyzed via SEM. Using the software program attached to the SEM, the widths of approximately 20 particles were measured for each sample type providing an estimate of the relative sizes of the particles grown either in the absence of peptide or in the presence of the peptides. The average length of the particles was not determined in this manner but was estimated on the basis of the comparison with the scale bar provided by the SEM software. A summary of the average particle width and the observed morphologies for the intermediate phase formed in the absence and presence of the AQQ peptides is shown in Table IV.3.

In the absence of peptide, the intermediate phase resembles a polycrystalline aggregate of thin blades that grow to approximately a micrometer in length and 164 ± 28nm in width (Figure IV.7). The intermediate phase formed in the presence of AQQ adopts a similar bladed morphology with particles growing to approximately a micrometer in length and 205 ± 32 nm in width (Figure IV.8). In the presence of AQQ3E, the average length of intermediate phase particles increases to approximately 1-2 µm and the average width increases to 243 ± 31 nm.
Additionally, various blade-like particles within the samples containing AQQ3E appear to bend slightly showing that the particles in intermediate phase have a flexible nature that becomes more evident as the particles increase in size. Intermediate phase particles formed in the presence of AQQ5E were the largest particles observed with lengths varying from approximately 2-5 µm and an average width of 293 ± 55 nm (Figure IV.10). Rather than adopting a blade-like morphology, the majority of the particles found in samples containing AQQ5E resembled long ribbons able to adopt unique morphologies such as twists, knots, and helices (Figure IV.11). The flexible nature of these ribbon-like particles suggest that the polycrystalline intermediate formed in the presence of AQQ5E may also possess amorphous qualities since a fully crystalline material would likely fracture or cleave before adopting such morphologies.

Figure IV.7. SEM images of the intermediate phase formed in the absence of peptides.
Figure IV.8. SEM images of the intermediate phase formed in the presence of 200 µM AQQ.

Figure IV.9. SEM images of the intermediate phase formed in the presence of 200 µM AQQ3E.

Figure IV.10. SEM images of the intermediate phase formed in the presence of 200 µM AQQ5E.
Figure IV.11. SEM images of the ribbon-like particles observed in the polycrystalline intermediate phase formed in the presence of AQQ5E.

<table>
<thead>
<tr>
<th>Solution Condition</th>
<th>Average Particle Width</th>
<th>Morphologies Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Peptide</td>
<td>164 ± 28nm</td>
<td>Blades</td>
</tr>
<tr>
<td>200μM AQQ</td>
<td>205 ± 32 nm</td>
<td>Blades</td>
</tr>
<tr>
<td>200μM AQQ3E</td>
<td>243 ± 31 nm</td>
<td>Blades, Bends</td>
</tr>
<tr>
<td>200μM AQQ5E</td>
<td>293 ± 55 nm</td>
<td>Ribbons, Twists, Knots, Helices</td>
</tr>
</tbody>
</table>

Analysis of the intermediate phase was performed with FTIR to determine if peptide was present within the material. Prior to analysis, the material was washed with several aliquots of deionized water and 95% ethanol to remove excess mineralization solution followed by lyophilization to remove all excess rinsing solvents. As the polycrystalline intermediate consisted of calcium phosphate, phosphate vibrational peaks are observed in all samples at 1018 cm\(^{-1}\) and 960 cm\(^{-1}\) as well as a prominent shoulder beginning at 1072 cm\(^{-1}\) (Figure IV.12). This vibrational pattern is unique to this intermediate phase of calcium phosphate as the preceding amorphous phase and the final crystalline states displayed different vibrational spectra. FTIR analysis of the intermediate phase formed in the presence of AQQ does not show the presence of peptide which can be observed if present by the amide I and amide II vibrational bands of the
peptide backbone at 1650 cm\(^{-1}\) and 1543 cm\(^{-1}\), respectively. This shows that AQQ does not have a strong interaction with the polycrystalline intermediate phase and that the rinsing procedure was sufficient to remove excess peptide from the material. The polycrystalline intermediate grown with AQQ3E and AQQ5E clearly shows the presence of peptide by the observation of amide I and amide II vibrational bands. Since experimentally obtained FTIR absorbance spectra were normalized to tallest peak observed, i.e. the 1018 cm\(^{-1}\) phosphate peak, the relative heights of the amide I and amide II bands in the AQQ3E and AQQ5E FTIR spectra suggest that AQQ5E interacts with the intermediate phase to a greater extent than AQQ3E which correlates well with the electrostatic properties of the two peptides and the observed morphological differences between the two peptides.

Figure IV.12. (Left) FT-IR absorbance spectra normalized to the phosphate vibrational peak at 1018 cm\(^{-1}\) for all samples extracted during the most stable region of the amorphous-to-crystalline transition (Figure III.3) showing which peptides have the most affinity for the ACP precursor. (Right) Direct comparison of the IR spectra in the region of the peptide amide I (1650 cm\(^{-1}\)) and amide II (1543 cm\(^{-1}\)) bands.
Electron diffraction of the intermediate phase formed in the absence of peptide shows that it is crystalline by the presence of sharp spots in the diffraction pattern (Figure IV.13). This pattern was observed at the edge of a blade-like particle extracted from a sample grown without peptide; however, analysis of other portions of the same material displayed only broad rings typically associated with an amorphous phase. Electron diffraction analysis of the intermediate phase grown in the presence of the AQQ series peptides displayed a similar mix of diffracting and non-diffracting material. Thus, on the basis of electron diffraction analysis the polycrystalline intermediate appears to be a heterogeneous mixture of both amorphous and polycrystalline precursors of brushite. Powder X-ray diffraction (XRD) was also used to confirm the crystalline nature of this material as well as indentify the crystalline phase. As seen in Figure IV.14, the powder XRD pattern of the intermediate nearly matches the pattern for brushite from database reference 00-011-0293 and contains the prominent (020) reflection that is observed in all three referenced datasets. The lack of additional peaks which are present in database references 01-072-0713 and 98-001-0131 is likely due to the preferred orientation of the (020) crystal plane family. As shown in Chapter III, the dominant faces of fully crystalline brushite are the \{010\} faces which are related by symmetry to the \{020\} faces of brushite. In the polycrystalline intermediate, a bladed or ribbon-like morphology was observed that also contained a dominantly expressed face, i.e. the flat surface of the blade or ribbon. Thus, on the basis of the electron and X-ray diffraction methods it was concluded that the intermediate phase is a heterogeneous mixture of both amorphous and poorly crystalline form of brushite.
Figure IV.13. STEM nanodiffraction pattern showing the crystalline nature of the intermediate phase material.

Figure IV.14. Powder XRD pattern of the polycrystalline intermediate formed in the presence of AQQ compared to several database powder XRD patterns for brushite. Observed reflections are labeled according to Miller index.
IV.3.5 Transition from the Polycrystalline Intermediate to Brushite Crystals

The transition of the polycrystalline intermediate phase to the final crystalline phase of brushite was monitored via turbidity assays for 60 hours following the formation of the intermediate phase in the absence and the presence of the AQQ peptide series. A peptide concentration of 200µM was used for all assays because it prolonged the lifetime of the intermediate phase when compared to samples containing 400 µM and 600 µM peptide. In general, the transition of the intermediate phase to the final crystal phase is attributed to a decrease in turbidity likely caused by a dissolution-recrystallization mechanism of the small brushite precursor particles into large, well-ordered brushite crystals as described by Ostwald ripening. The end of this transition event correlates with turbidity spectra approaching the baseline absorbance value of approximately 0.3-0.4 since brushite crystal do not effectively absorb light at 405 nm.

In the absence of peptide, the intermediate phase transforms into the final crystalline state within 40-48 hours after the nucleation of initial amorphous phase (Figure IV.15). The addition of either AQQ or AQQ3E slightly prolonged the transition to approximately 50-60 hours. In the presence of AQQ5E, the intermediate phase was stabilized well beyond the measured 72 hour time period. The large error bars shown for AQQ5E show that stability of the intermediate phase varied significantly starting at approximately 24 hours. These variations between samples with respect to the stability of the intermediate phase were further shown as the majority of samples containing AQQ5E completed the final transition within 4-7 days while some remained stable in the intermediate form for approximately 1-2 weeks. Though it is unclear why AQQ5E caused such a wide distribution in the stability of the intermediate phase, overall the electrostatic
character of AQQ5E and possibly the structural placement of its negatively charged residues allow it to interact effectively with all phases of the amorphous-to-crystalline transition of brushite.

![UV-Vis Absorbance (405 nm) against Time (Hours) graph](image)

Figure IV.15. 200 µM turbidity assays monitoring the transition of the polycrystalline intermediate into brushite crystals beginning 12 hours after the nucleation of the initial amorphous phase.

**IV.3.6 The Role of AQQ5E on the Amorphous-to-Crystalline Transition of Brushite**

To better understand how AQQ5E effects the overall amorphous-to-crystalline transition of brushite, FTIR analysis was performed on a week old sample of the intermediate phase grown in the presence of AQQ5E and compared to the FTIR spectra of the amorphous phase formed in the presence of AQQ5E extracted directly after nucleation and one hour after nucleation. As shown in Figure IV.16, the amorphous phase extracted directly after nucleation contains the prominent 1018 cm$^{-1}$ peak of phosphate but lacks definition in the 960 cm$^{-1}$ band and does not
contain the 1072 cm$^{-1}$ vibrational band associated with the intermediate phase. Additionally, the presence of peptide directly after nucleation is observed by the amide I and amide II vibrational peaks at approximately 1650 cm$^{-1}$ and 1543 cm$^{-1}$, respectively. After one hour, the amorphous phase displays more prominent amide vibration bands relative to the phosphate vibrational peak at 1018 cm$^{-1}$ suggesting that as the amorphous phase matures AQQ5E interacts with the phase to a greater extent. Also, the phosphate vibrational band at 960 cm$^{-1}$ is clearly observed after one hour of growth as well as a new vibrational band at 1102 cm$^{-1}$. This vibrational band may correspond to the transient form of calcium phosphate present during the amorphous-to-intermediate transition; however, further characterization of this transitional event is necessary.

After one week, the calcium phosphate precipitate formed in the presence of AQQ5E displays the three phosphate vibrational peaks of 1072 cm$^{-1}$, 1018 cm$^{-1}$, and 960 cm$^{-1}$ associated with the intermediate phase. However, the heights of the amide vibrational bands relative to the 1018 cm$^{-1}$ phosphate vibrational band decreased suggesting that as the intermediate phase matures to a more crystalline state the peptide is expelled from the material. SEM analysis of the week-old sample of the intermediate phase formed in the presence of AQQ5E shows that particles no longer adopt the ribbon-like morphology observed at approximately 12-24 hours after nucleation (Figure IV.17). Instead, the particles display a rigid, blade-like morphology similar to samples prepared in the absence of peptide and in the presence of AQQ. The shift in morphology from flexible, ribbon-like particles formed approximately 12-24 hours after nucleation to rigid, blade-like particles observed approximately one week after nucleation demonstrates that AQQ5E slows the rate the amorphous-to-crystalline transition by stabilizing the formation of an intermediate phase containing both amorphous and crystalline properties.
Figure IV.16. FTIR spectra comparing the amorphous phase of brushite formed in the presence of AQQ5E exacted directly after nucleation and one hour after nucleation to the FTIR spectra of the a week-old sample of the intermediate phase stabilized by the presence of AQQ5E.

Figure IV.17. SEM images of a week-old sample of the intermediate phase stabilized by the presence of AQQ5E showing that the particles adopt only a blade-like morphology.
IV.4 DISCUSSION

IV.4.1 The Amorphous-to-Crystalline Transition of Brushite

Previously uncharacterized, examination of the amorphous-to-crystalline transition of brushite demonstrates the existence of amorphous and intermediate phases that precede the formation of crystalline brushite. As shown by the addition of AQQ5E to this mineralization pathway, the presence of acidic peptides containing sufficient negative charge density can slow the rate of transformation between amorphous and intermediate phases to stabilize the formation of calcium phosphate ribbons possessing both amorphous and crystalline properties. On the basis of the observations of this study, a complete description of the growth of brushite through an amorphous precursor pathway can now be proposed in terms of a descending free energy diagram (Figure IV.18). The process begins with dissolved ions in solution aggregating to form precritical clusters followed by the transformation of the clusters into an amorphous phase. Precritical clusters were not directly observed in this study; however, in-house titration experiments similar to those performed by Colfen et al. who originally proposed the existence of precritical clusters suggest they may have formed briefly under the mineralization conditions used in this study (data not shown). Additionally, various publications have confirmed that precritical clusters form prior to other forms of amorphous calcium phosphate. Thus, they have been included on the proposed pathway for the growth of brushite formed through an amorphous precursor. The amorphous phase is followed by the formation of an intermediate phase that has both amorphous and crystalline properties. The polycrystalline intermediate then transforms into crystals of brushite that are thermodynamically stable under the given experimental conditions. All the listed phases and transitions for the sequential pathway are observed in either the absence
or presence of peptide; however, the addition of a peptide such as AQQ5E slows the rate of transformation by temporarily stabilizing the amorphous and intermediate phases.

Figure IV.18. Gibbs free energy diagram showing the progression of the amorphous-to-crystalline transition observed for brushite under the given set of experimental conditions. *Indicates that this part of the pathway was not studied in depth.

IV.4.2 Structural Comparison of AQQ3E and AQQ5E

The differences observed between the ability of AQQ3E and AQQ5E to interact with amorphous and intermediate precursors of brushite still remain unexplained. As previously described, the trimeric forms of AQQ3E and AQQ5E contain nine and fifteen solvent exposed glutamic acid residues, respectively. On the basis of the ability of AQQ5E to impact the kinetic and morphological properties of the amorphous-to-crystalline transition of brushite, AQQ3E would be expected to have a comparable effect since AQQ5E only has approximately 66% more acidic character than AQQ3E. However, at all stages of the mineralization pathway studied the samples containing AQQ3E resembled control (no peptides) and negative control samples (AQQ). This observed behavior begs the question, do acidic peptides merely require a sufficient
negative charge density to effectively interact with amorphous and poorly crystalline phases or do structural factors such as the orientation and conformational flexibility of negatively charged residues impact these interactions? Answering this question will be important for the rational design of proteins towards the design of biomimetic materials and may ultimately lead to a higher level of understanding regarding natural biomineralization mechanisms.

The analysis of the specific effects of AQQ5E on the morphology of brushite crystals suggested that a structural-electrostatic interaction may allow AQQ5E to bind to the \{100\} faces of brushite. Although this interaction was modeled and described from the point of view of a peptide binding to a well-defined surface, the same atomic configurations present at the \{100\} faces of brushite may also be present in the amorphous and intermediate phases of brushite, though not in an ordered, long-range crystalline state. However, even a short- or medium-range ordered atomic configuration resembling the \{100\} faces may allow for a specific structural-electrostatic interaction to occur between AQQ5E and the precursors of crystalline brushite. This may explain why AQQ5E displayed a more significant difference on the growth pathway of brushite than AQQ3E though both peptides contained several solvent-exposed, negatively charged residues.

Assuming that the structural arrangement and not just the total number of negatively charged amino acids is important for how proteins interact with amorphous and crystalline solids, a direct comparison of the structures of AQQ3E and AQQ5E may allow for indentifying these structural factors. Figure V.1 shows a side-by-side comparison of AQQ3E and AQQ5E displaying the relative placements of solvent-exposed glutamate (Glu) residues. Each helix of AQQ3E contains three solvent-exposed Glu residues at sequences positions 1, 3, and 24, which places these residues on approximately different sides of the helix. The same is true for AQQ5E;
however, each helix of AQQ5E also contains two additional solvent-exposed Glu residues at positions 10 and 17. This places the solvent exposed acidic residues approximately two helical turns apart along the length of the helix, which not only increases the negative charge density but also allows for 2-3 solvent-exposed Glu residues to be facing in approximately the same direction.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQQ3E</td>
<td>Ac-EIEALEQK-IAALEQK-IAALEQK-IEALEQK-GGY-NH₂</td>
</tr>
<tr>
<td>AQQ5E</td>
<td>Ac-EIEALEQK-IEALEQK-IEALEQK-IEALEQK-GGY-NH₂</td>
</tr>
<tr>
<td></td>
<td>Glu(1) Glu(3) Glu(10) Glu(17) Glu(24)</td>
</tr>
</tbody>
</table>

Figure V.1 Computer modeling comparison of AQQ3E (blue) and AQQ5E (green) with solvent exposed Glu residues labeled by sequence number on one of the three coils for each peptide.

This analysis suggests that modifying AQQ3E such that its solvent exposed Glu residues are at sequence positions 3, 10, and 17, or at sequence positions 10, 17, and 24, may allow for AQQ3E to have a stronger interaction with the amorphous and crystalline states of brushite. However, addition experiments will be required to confirm this hypothesis. Also, it cannot be ruled out that a threshold exists in terms of negative charge density which may cause AQQ5E to have a more significant impact on the growth of brushite than AQQ3E. Testing of this hypothesis could be achieved by developing an AQQ4E analog with careful consideration as to
the placement of the solvent exposed Glu residues so that they are not allowed to orient on the same side of a helix.

CHAPTER IV REFERENCES


(9) Colfen, H. Macromolecular Rapid Communications 2001, 22.


CHAPTER V. DISSERTATION CONCLUSIONS

An excellent opportunity exists to merge the fields of rational peptide design and biomineralization research. As shown in this body of work and others, rational peptide design allows for the control the structural and chemical properties of model peptide systems.\textsuperscript{1,2} These peptides can be used to understand the fundamentals of how proteins interact with amorphous and crystalline solids which could contribute to fields such as medicine and materials science. While rationally designed peptides have been used in this manner before on a limited basis,\textsuperscript{3,4} it seems that now more than ever this approach is needed since simplistic polypeptide sequences often used in biomineralization research have not been able to provide detailed information regarding how protein structure impacts protein-biomineral interactions.

Several recent studies have shown that protein structure is likely an important aspect of how proteins interact with amorphous and crystalline solids since natural biomineralization proteins undergo disordered-to-ordered structural rearrangements when in the presence of a mineralizing environment.\textsuperscript{5} Similarly, other studies now suggest that the quaternary structure that results from the assembly of multiple proteins – and not just the secondary or tertiary structural elements of a single protein – may have significant impacts of biomineralization processes.\textsuperscript{6} Regarding how to study these two areas with rationally designed peptides, years of research have gone into developing peptides capable of undergoing conformational switches in the presence of environmental stresses such as changes in pH or ionic strength.\textsuperscript{7} Additionally, various self-assembling peptide constructs have been developed which can be used to mimic the quaternary structural properties of self assembling proteins.\textsuperscript{8} While several other lines of
investigation are likely possible, designing peptides that self assemble or undergo conformational transitions are important examples of how current issues within biomineralization research can be studied using rationally designed peptides.

CHAPTER V. REFERENCES


(2) Gribbon, C.; Channon, K. J.; Zhang, W.; Banwell, E. F.; Bromley, E. H. C.; Chaudhuri, J. B.; Oreffo, R. O. C.; Woolfson, D. N. Biochemistry 2008, 47, 10365.


(8) Woolfson, D. N. Biopolymers 2010, 94, 118.