

Chemical Controls on the Formation of Amorphous and Crystalline Calcium Phosphates

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ABSTRACT

Transformation of amorphous calcium phosphate (ACP) and brushite into hydroxylapatite, an important biomineral, has been documented. The relationships between synthesis conditions and the formation and transformation of these phases are not comprehensively understood. The metastable nature of ACP has made it historically challenging to investigate, and many analyses attempt to stabilize the phase through drying or including additional ions or proteins in the reaction. *In situ* investigations provide an incisive approach to examining the structure and transformation of ACP and brushite as a function of synthesis conditions.

The first project develops a new method for *in situ* analyses of the structure of ACP and brushite shortly after reagent mixing, without chemical stabilization. This method was used in the second project to examine how the initial Ca/P affects ACP structure and transformation. Our results identify the first structural differences in types of ACP, controlled by the initial Ca/P. At ratio 0.2 the Ca – P bonding geometry is primarily monodentate, ratio 5.0 produces a coordination that is primarily bidentate, and there is a mix of monodentate and bidentate coordinates at intermediate ratios between the two. These results are independent of system pH between the examined range of 6-11. Further *ex situ* transformation experiments identified that at ratio 0.2, ACP transformed directly into hydroxylapatite, but at higher ratios the transformation product is brushite. This is a promising mechanism for direct ACP to hydroxylapatite conversion at a biologically relevant pH. In the final project, the statistically significant synthesis parameters (age, pH, temperature, supersaturation, and initial ion ratio) for ACP, brushite, and hydroxylapatite formation are evaluated. Binary logistic regression analysis and nonlinear continuous logistic regression analysis are applied to a dataset compiled from the literature. Equations were developed that predict the percentage of ACP and

brushite that will form. The equations and significant variables seem to depend on the transformation pathway of brushite and ACP. The current analysis did not comprehensively describe hydroxylapatite formation when trying to concurrently evaluate the ACP to hydroxylapatite and brushite to hydroxylapatite pathways.

Taken together, these studies provide new ways to study and interpret calcium phosphate phases as they form and transform. Experiments identified new relationships between the chemistry and structure of ACP. The new *in situ* experimental method and the equations we developed can be used to improve future experimental designs towards a comprehensive understanding of the calcium phosphate system.

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GENERAL AUDIENCE ABSTRACT

Hydroxylapatite is a mineral made of calcium and phosphate, that is similar to the mineral components of bones and teeth in humans and other mammals and fish. Hydroxylapatite and other calcium phosphate phases can form when solutions, rich in calcium and phosphate, are mixed. Phases without long-range crystal structure, are amorphous calcium phosphates (ACP). Additional calcium phosphate minerals, like brushite can also form. If brushite and ACP are left in solution, they will transform into hydroxylapatite over time. Major questions include the need to learn the short-range atomic structure of ACP and how ACP and brushite transform into hydroxylapatite

In this dissertation, I investigate how chemistry and other variables such as age and time impact the calcium phosphate phase to form and how it transforms with aging. The first project develops a new method to study the structure of ACP and brushite without drying the study materials or adding additional chemicals or proteins to prevent them from transforming. The sample forms in a solution and flows directly through an X-ray beam for structural analysis.

This method is used in project two to examine how the ratio of calcium and phosphate in the beginning of the reaction affected the structure of ACP and how it transformed. The results identify the first structural differences in types of ACP, controlled by the initial Ca/P. At a ratio of 0.2 a calcium and phosphorus atom mostly share only one oxygen between them, but at $\text{Ca/P} = 5.0$, they mostly share two oxygens. At ratios in between 0.2 and 5.0 they share a mix of one and two oxygens. The results are independent of pH. Additionally, at ratio 0.2, ACP transformed directly into hydroxylapatite, but at all other ratios it transformed to brushite. Investigations of direct ACP to hydroxylapatite transformation are usually performed at a pH above that found in humans, but the transformation at low ratio occurred at a biologically relevant pH.

In the final project statistical analysis was used to identify what synthesis conditions (out of age, pH, temperature, supersaturation, and initial ion ratio) had a significant impact on the formation of ACP, brushite, and hydroxylapatite. Equations were developed that can be used to predict the percentage of ACP and brushite that form based on the statistically significant variables. The current analysis did not fully describe hydroxylapatite formation. Results suggest separate equations are needed for hydroxylapatite forming directly from ACP and directly from brushite.

Combined, these studies have created new ways to study calcium phosphate phases as they form and transform. This work experimentally identified new relationships between the chemistry and structure of ACP. Both the method and equations will help researchers improve their future experimental designs so investigations can be more directly compared to create a comprehensive understanding of calcium phosphates.

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GENERAL

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CHAPTER 2

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CHAPTER 3

This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357. Beam time for the data collection presented in this paper was allocated under GUPs 55036, 57625, and 62246. FMM gratefully acknowledges financial support provided by the National Science Foundation through EAR-1451996 and CAREER-1652237, as well as the Virginia Tech National Center for Earth and Environmental Nanotechnology Infrastructure (NSF Cooperative Agreement 1542100). ICP-AES analysis was completed through Athena Tilley and the Virginia Tech Soil Testing Lab. The authors also acknowledge J. Donald Rimstidt for assistance with the experimental design of sample synthesis conditions.

CHAPTER 4

The authors acknowledge Brady Ziegler for assistance with supersaturation calculations.

ATTRIBUTIONS

AUTHOR CONTRIBUTIONS TO CHAPTER 2

SM and FMM designed and fabricated the MFRs. AH, SM, and FMM worked together to design the experiment and collect and process the data. OJB provided instrumentation support during data collection and wrote the instrumentation specifications for the paper. AH designed the figures and wrote the rest of the document with input from SM, OJB, PMD, and FMM.

AUTHOR CONTRIBUTIONS TO CHAPTER 3

The experimental design was put together by AH and FMM. AH, SM, and FMM worked together to collect the PDF data with instrumentation support provided by OJB. The data was processed by AH. AH and FMM worked together to collect the SAXS data. FMM processed the SAXS data (with contributing discussion from SM), ran the LCF and Rietveld analysis, designed the SAXS figures/tables, and wrote the SAXS, LCF, and Rietveld related methods and results. The ICP and XRD data was prepared and analyzed by AH. AH designed the remaining figures and wrote the rest of the document with input from SM, OJB, and FMM.

AUTHOR CONTRIBUTIONS TO CHAPTER 4

The literature search and data compilation were completed by AH. The statistical methods were chosen by RSM and the analyses were completed by AH and RSM with input from FMM. AH designed the figures and wrote the document with input from RSM and FMM.

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CHAPTER 1: INTRODUCTION

IMPORTANCE OF APATITE

The apatite group minerals are the most prominent source of phosphorus in the natural environment. They are the primary ore mineral of phosphate that is used as fertilizers (Hughes & Rakovan, 2002, 2015). Apatites are a common, stable phase that can form under a variety of conditions in igneous, metamorphic, sedimentary, hydrothermal, biological, and extraterrestrial environments. The structure and chemistry of apatite allows for half of the periodic table to be incorporated as substitutions, making them a useful geochronometer and thermochronometer (Chew & Spikings, 2015; Harlov, 2015). Apatites also have industrial applications, notably being used in fluorescent lighting and can be deployed to assist with environmental remediation of heavy metals (Rakovan & Pasteris, 2015; Ewing & Wang, 2002; Magalhães & Williams, 2007). The most common apatite species form a calcium phosphate ternary anion solid solution with the end members defined as fluorapatite, chlorapatite, and hydroxylapatite.

Hydroxylapatite (also commonly called hydroxyapatite) is structurally and chemically similar to the carbonated calcium phosphate mineral phase found in the bones and teeth of humans and other animals (Wopenka & Pasteris, 2005; Pasteris *et al.*, 2014). The inorganic mineral component makes up 60-70 weight % of human bone and over 90 weight % of mature enamel (Elliott, 2002; Schwarcz, 2015). Apatite is thus considered an important biomineral and the growth and structural properties of hydroxylapatite are investigated across disciplines. Early investigations focused on examining the growth mechanisms of hydroxylapatite as a proxy to understand the growth and development of bones (Albee & Morrison, 1920; Eanes *et al.*, 1965). There is also considerable interest in using hydroxylapatite and other calcium phosphate phases to develop biocompatible prosthetics (Levitt *et al.*, 1969; Driskell, 1994; Daculsi, 1998). There are several excellent review papers that cover the relationship between hydroxylapatite and bone mineral and the history and milestones of investigations and developments that are beyond the scale of this introduction (Gross & Berndt, 2002; Elliott, 2002; Glimcher, 2006; Habraken *et al.*, 2016).

AMORPHOUS AND CRYSTALLINE CALCIUM PHOSPHATES

It was discovered that hydroxylapatite synthesized at low temperature, and supersaturated aqueous solution conditions begins with the formation of an amorphous phase, commonly called

amorphous calcium phosphate (ACP). This material forms before crystalline materials can be detected (Eanes *et al.*, 1965; Eanes & Posner, 1965). ACP is hypothesized to be an aggregation of calcium phosphate nanoclusters, commonly called Posner's clusters, the existence and structure of which are a topic of some debate (Posner & Betts, 1975; Yin & Stott, 2003a; Habraken *et al.*, 2013). The formation and subsequent transformation of ACP into hydroxylapatite was initially proposed to be a step in the process of bone formation and development (Termine & Posner, 1967). Later studies do not find evidence for ACP in bones, even at the earliest stages of formation (Grynpas *et al.*, 1984; Roberts *et al.*, 1992), and the presence of ACP in bones is still a topic of some controversy (Combes & Rey, 2010).

The presence of ACP precipitated in solution has been seen before the formation of other crystalline calcium phosphates such as brushite and octacalcium phosphate (OCP) (Meyer & Eanes, 1978; Kibalczyk *et al.*, 1990; Borkiewicz *et al.*, 2010). If these crystalline calcium phosphates are left in solution they will transform into hydroxylapatite overtime (Borkiewicz *et al.*, 2010; Ito *et al.*, 2014; Shamrai *et al.*, 2015). This shows that growth of synthetic hydroxylapatite in supersaturated solutions is not limited to the classical growth theory of terrace, step, and kink surface propagation. The aggregation of the proposed calcium phosphate nanoclusters to form ACP and its subsequent transformation into hydroxylapatite or other crystalline calcium phosphates (**Figure 1.1**) aligns with the recent paradigm of nonclassical pathways to crystal nucleation and growth (De Yoreo *et al.*, 2015).

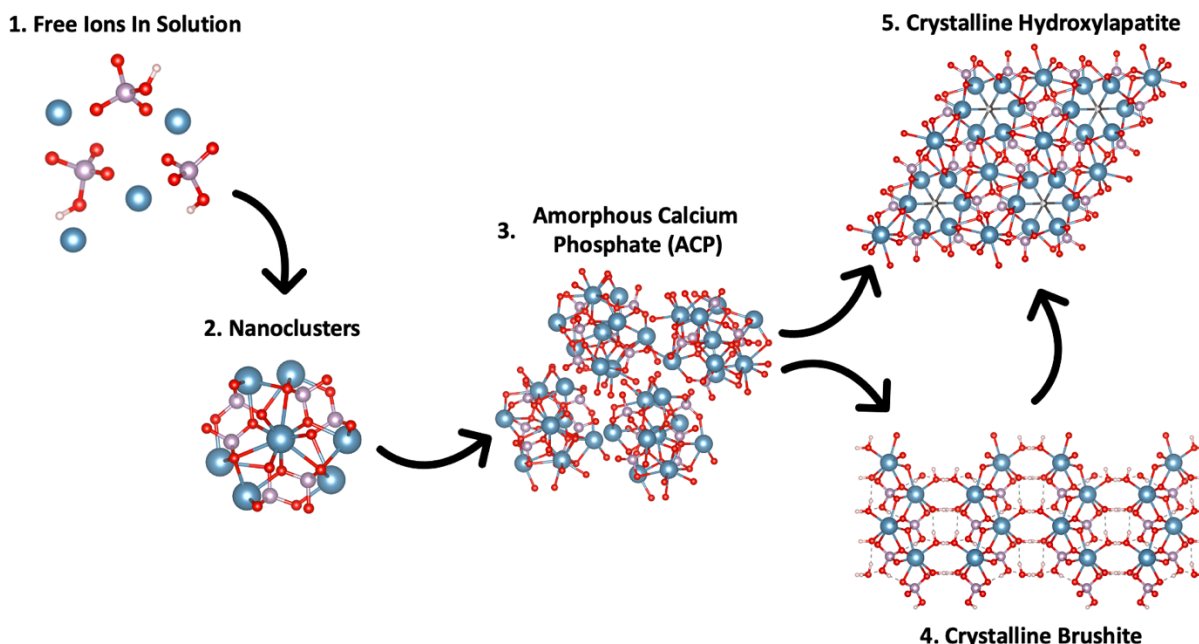


Figure 1.1 Illustration of the nonclassical crystallization pathway of hydroxylapatite in supersaturated solutions. Free calcium and phosphate ions (**1.**) form calcium phosphate nanoclusters (**2.**), which aggregate to form ACP (**3.**). Depending on variations in the synthesis conditions, ACP transforms directly into crystalline hydroxylapatite (**5.**) or can first transform into another crystalline calcium phosphate such as brushite (**4.**), which subsequently transforms into hydroxylapatite if it remains in solution.

SYNTHESIZING AND STUDYING ACP

The role of ACP as a precursor phase for a variety of crystalline calcium phosphates has driven numerous investigations into the synthesis and characterization of this amorphous phase. A wide range of methods have been developed to prepare ACP at a variety of temperatures and solution chemistries (Combes & Rey, 2010). Due to its metastable nature, ACP has often been dried, both to preserve the amorphous nature and for easier analysis (Eanes *et al.*, 1973a; Meyer & Eanes, 1978; Stämmeier *et al.*, 2018). Adjustments to the system chemistry have been found to prolong the stability of ACP, the most common of which involve raising the pH of the solution to above 9 and the addition of magnesium cations, though other ions and proteins can have similar effects (Termine *et al.*, 1970b; Blumenthal *et al.*, 1977; Kibalczyk *et al.*, 1990; Liu *et al.*, 2001; Othman *et al.*, 2016).

A major focus of previous ACP investigations was to define or constrain the short-range order present in ACP. However, the amorphous definition of the material makes traditional X-ray diffraction structure determination impossible. The first proposed structure (Posner's cluster) was a subset of the hydroxylapatite structure, that was chosen based on a combination of structural coherency (9 Å) extracted from radial distribution function analysis, and a Ca/P of 1.5, commonly seen in ACP precipitates (Posner & Betts, 1975). Later studies applied *ab initio* modeling and molecular dynamics to this proposed structure to try and refine the atom positions for something more practical for an isolated particle (Treboux *et al.*, 2000; Yin & Stott, 2003a; Mancardi *et al.*, 2017). Other studies have proposed ion association complexes smaller than Posner's cluster as the nucleation state for calcium phosphates (Habraken *et al.*, 2013; Mancardi *et al.*, 2016). Relatively few studies have placed constraints on the short-range order of ACP using experimental techniques due to the difficulty of investigating the short-range order of metastable amorphous phases (Harries *et al.*, 1987; Du *et al.*, 2013; Hoeher, 2015; Habraken *et al.*, 2016).

There is a growing interest in studying ACP *in situ* to eliminate any structural or chemical alterations that may result from the drying process (Eichert *et al.*, 2003). Several studies analyze aliquots of the precipitate in solution suspension (Onuma, 2006; Borkiewicz *et al.*, 2010; Du *et al.*, 2013; Zhang *et al.*, 2015). This method that requires minimal special equipment, but may not be suitable for all circumstances, depending upon the goals of the study. Metastable ACP will continue to transform when left in solution and ACP structure and chemistry of the aliquot supersaturation may change during extended durations of data collection. Alternative experimental set-ups allow *in situ* ACP to be analyzed at an isolated time point (Bremholm *et al.*, 2008; Ibsen & Birkedal, 2010, 2012; Ibsen *et al.*, 2016, 2018). These set-ups have primarily been utilized at synchrotrons, where the suspension flows through the X-ray beam. As long as the flow rate remains continuous, the age of the sample at the point of analysis will remain constant (discussed in more detail in Chapter 2). While many laboratory analyses are not compatible with a flow-through experiment, this method allows for a greater degree of control of the sample and is ideal when possible.

The biomedical applications of calcium phosphate phases like ACP and hydroxylapatite make them a topic of interdisciplinary interest. Synthetic calcium phosphate phases are investigated by biologists, chemists, geologists, material scientists, and engineers. This has

resulted in an extensive literature base, but also in complications with comparing the results of studies that were performed under different disciplines. Chemists use different nomenclature to describe structural symmetry in crystalline materials from geologists. Also, the required reporting of methods and materials to ascribe to a general level of knowledge changes across disciplines. In addition, synthetic calcium phosphates can be synthesized at a variety of temperatures, pH, supersaturations and ion ratios. The system chemistry also varies, with different researchers commonly using $\text{Ca}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, or $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ as a calcium source and Na_2HPO_4 , $(\text{NH}_4)_2\text{HPO}_4$, or K_2HPO_4 as a phosphate source and the reaction stoichiometry has been shown to impact the phase yield of the reaction (Gross & Berndt, 2002). The use of buffers, the addition of magnesium or proteins for ACP stabilization, or use of simulated body fluids as a reaction medium are all common alterations to the synthesis and investigation of ACP and crystalline calcium phosphates. While the impact of some variations, such as pH, are generally understood, others are uninvestigated, or have only been examined in small scale studies (Blumenthal *et al.*, 1972a; Kim *et al.*, 2004; Ibsen *et al.*, 2018). Morphological variations in ACP have been identified, but they have not been correlated with variations in synthesis (Christoffersen *et al.*, 1989). Significantly more work needs to be done to evaluate the different impacts of initial synthesis conditions on the formation of calcium phosphates.

RESEARCH OBJECTIVES

This dissertation is comprised of three core research objectives, which are all linked to investigating the impacts of synthesis conditions on ACP and crystalline calcium phosphates.

CREATING AN *IN SITU* TECHNIQUE COMPATIBLE WITH STRUCTURAL ANALYSIS

Pair distribution function (PDF) analysis is a total X-ray scattering technique that can be used to analyze the structure of materials with long-range, mid-range, or short-range order. As ACP is a metastable material that can transform from an amorphous to crystalline material in minutes under the right conditions, an analysis technique that could examine diverse materials was critical. The goal of Chapter 2 was to create an *in situ* flow through set-up, compatible with synchrotron total X-ray scattering that was able to examine the structure of ACP and crystalline calcium phosphates. In addition, the technique needed to allow for a rigorous control of the system chemistry and allow data collection to occur at distinct time points. Chapter 2 discusses

the details of the method and the testing process we performed, as well as provides examples of the quality of data that we obtained. A version of this chapter was published in *Acta Crystallographica Section A Foundations*.

THE IMPACTS OF CA/P ON THE STRUCTURE AND EVOLUTION OF CALCIUM PHOSPHATES

Utilizing the method developed in Chapter 2, the goal of Chapter 3 was to analyze the impact of varying initial ion ratios on calcium phosphates. *In situ* PDF analysis identified systematic structural variations in calcium phosphates at a circumneutral pH five minutes after synthesis. There is a change in the monodentate-bidentate bonding geometry between calcium and phosphate that can be controlled by the ion ratio. This is the first reported evidence of structural differences in ACP and is directly linked to the initial system chemistry. Subsequent *ex situ* experiments indicated the variation in ion ratio impacted the transformation pathway of ACP, with samples at low ratios (0.2) and only bidentate geometries evolve directly to hydroxylapatite at a circumneutral pH. Higher ratios (5.0 and 1.0) with only monodentate or mixed monodentate and bidentate geometries saw evidence of brushite formation as well as hydroxylapatite formation. This chapter identifies the critical role of the initial solution chemistry on the structure and evolution of synthetic calcium phosphates and a version is in advanced preparation to be submitted to the journal of *Crystal Growth and Design*.

STATISTICAL ANALYSIS OF VARIOUS SYNTHESIS CONDITIONS AND THEIR IMPACTS ON CALCIUM PHOSPHATE PHASE DEVELOPMENT

A literature search was conducted to build a data set of previous calcium phosphate synthesis studies. The dataset focuses on initial synthesis conditions including chemicals, concentrations, initial ion ratio, temperature, pH, age, and phase identification. Binary logistic regression analysis and nonlinear continuous logistic regression analysis are used to evaluate which synthesis parameters have a significant impact on the formation of ACP, brushite, and hydroxylapatite. Our analysis indicates age, pH, temperature, and supersaturation have a significant role in the formation of ACP. Brushite formation is controlled by age, pH and temperature while hydroxylapatite formation is impacted by age, temperature, and supersaturation. This analysis yielded equations that can be used to comprehensively predict the formation of ACP and brushite across a range of synthesis conditions. Results suggest that the regression analysis is pathway dependent and the appearance of hydroxylapatite follows two

different pathways. An expanded database is required to evaluate the separate critical synthesis conditions for hydroxylapatite forming after ACP and brushite.

STRUCTURE OF THIS DISSERTATION

The dissertation is divided into three research chapters, plus the introduction and conclusion. Each major chapter discusses one of the three research objectives outlined above and has its own abstract, introduction, methods, results, discussion, and conclusions. Each major chapter also has its own appendix (A, B, and C) that include supplementary information for the research and can be found at the end of the dissertation. The reference list is continuous for the entirety of the dissertation and follows Chapter 5, before the appendices. The figures and tables in the main chapters have two digits in their numeration, the first referencing the chapter and the second, the order of the figure within that chapter. For example, Figure 2.1 is the first figure in the second chapter. In contrast, figures and tables in the appendices contain a letter and a number in their name, the letter indicating which appendix the figure belongs in, and the number giving the order within the appendix. For example, Figure A-2 is the second figure in Appendix A. Abbreviations and notations are defined in each chapter.

CHAPTER 2: A NEW METHOD FOR *IN SITU* STRUCTURAL INVESTIGATIONS OF NANO-SIZED AMORPHOUS AND CRYSTALLINE MATERIALS USING MIXED-FLOW REACTORS

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ABSTRACT

Structural investigations of amorphous and nanocrystalline phases forming in solution are historically challenging. Few methods are capable of *in situ* atomic structural analysis and rigorous control of the system. We use a mixed-flow reactor (MFR) for total x-ray scattering experiments to examine the short- and long-range structure of phases *in situ* with pair distribution function (PDF) analysis. The adaptable experimental set-up enables data collection for a range of different system chemistries, initial supersaturations, and residence times. The age of the sample during analysis is controlled by adjusting the flow rate. Faster rates allow for younger samples to be examined, but if flow is too fast, not enough data is acquired to average out excess signal noise. Slower flow rates form older samples, but at very slow speeds, particles settle and block flow, clogging the system. Proper background collection and subtraction is critical for data optimization. Overall, this MFR method is an ideal scheme for analyzing the *in situ* structures of phases that form during crystal growth in solution. As proof of concept, high resolution total x-ray scattering data of amorphous and crystalline calcium phosphates and amorphous calcium carbonate were collected for PDF analysis.

INTRODUCTION

Investigations of mineral growth in solution have identified a new paradigm of crystallization that occurs through reactive intermediates (Niederberger & Cölfen, 2006; Gebauer & Cölfen,

2011; De Yoreo *et al.*, 2015). Of particular interest are the amorphous materials and nanoparticles that can dissolve and reprecipitate or aggregate to form crystalline structures. Recent attempts to establish the short-range structure and evolution pathways of these phases are challenging due to their metastable nature (Goodwin *et al.*, 2010; Reeder & Michel, 2013; Schmidt *et al.*, 2014; Posner & Betts, 1975; Yin & Stott, 2003a; Du *et al.*, 2013; Habraken *et al.*, 2013; Reeder *et al.*, 2013). Initial efforts physically stabilized samples, for example, by isolating the sample from solution by freeze-drying (Posner & Betts, 1975; Meyer & Eanes, 1978). This approach inhibits the crystallization process long enough for analysis and enables investigative techniques that are not compatible with solutions. Drying a sample, however, can cause structural alterations (Eichert *et al.*, 2003), and does not easily allow for further investigations of sample evolution over time. Additionally, sublimation of the water can cause counter-ions to precipitate additional phases that interfere with the analysis of the metastable intermediate. In response to these limitations, there are an increasing number of *in situ* structural investigations of amorphous phases, primarily by examining aliquots (Du *et al.*, 2013; Zhang *et al.*, 2015; Borkiewicz *et al.*, 2010). These often small-scale experiments lack rigorous control of the solution conditions and the potential for samples to structurally evolve during data collection. To address these challenges, we adapted the mixed-flow reactor (MFR) design documented in Blue *et al.* (2013) to conduct structural investigations of amorphous and crystalline materials. The MFR approach allows us to control solution chemistries and supersaturations, precipitate samples under steady-state conditions, create highly reproducible samples, and maintain precise control of the sample age during data collection (Blue *et al.*, 2017).

Here we present a powerful method for *in situ* structural analysis that is easily adapted to various systems. Using the calcium phosphate and calcium carbonate systems as a proof of concept, we focus on collecting synchrotron total x-ray scattering data for pair distribution function (PDF) analysis. This includes both Bragg and elastic diffuse scattering, allowing us to examine the structure of materials that only have short-range order (e.g. nanosized and/or amorphous substances) and materials with long-range order (e.g. micro- and macro-crystalline minerals) using the same analytical technique. Suspensions of crystalline and amorphous materials that are synthesized in the MFR proceed directly through the x-ray beam to collect and compare structures of the samples. We present the steps performed to optimize data extraction for high quality *in situ* structural data of both well- and poorly-ordered materials. Examples of

data collected in the calcium phosphate and calcium carbonate system are included. Our approach also offers the potential to examine a series of different residence times and capture structural transformations that occur throughout the crystallization process. This adaptable procedure provides a new approach for collecting high quality, reproducible, structural information from *in situ* crystallization experiments.

MIXED-FLOW REACTORS FOR TOTAL X-RAY SCATTERING

The basic design of the MFR method is presented in Blue *et al.* (2013, 2017). A high precision syringe pump (Harvard Apparatus PHD ULTRA™ 4400) is used to continually pump the reactant solutions (Sigma-Aldrich ACS reagent CaCl₂ and Na₂HPO₄ in 18 MΩ ultra-pure water) into the MFR. A stir bar placed within a well at the base of the reactor, provides steady mixing (800 RPM). As mixing proceeds, the solution within the reactor becomes supersaturated and precipitation occurs continuously for the duration of mixing. As the MFR internal volume is filled, the well-mixed suspension flows out a single opening at the top and through the scattering window, a Kapton® capillary (3 mm diameter with 0.05 mm wall thickness), which intersects the path of the x-ray beam (**Figure 2.1**).

The experiment design is the same for all of the samples collected. The pump creates a precise flow rate of 3 mL/min for a 5-minute residence time (see discussion below). The Monoject™ 140cc piston syringes are modified with custom Teflon plunger caps and connect to the reactor with FEP-lined Tygon® SE-200 tubing (Cole-Palmer, 1/8" I.D.) via two ports at the base of the reactor. Additional tubing connects the reactor output to the Kapton scattering window, where x-ray scatter from the capillary is collected by the detector. The effluent then proceeds to a waste container. The reactors are manufactured from a cast acrylic rod and contain an internal volume of ~ 25 mL (**Figure 2.2**). Similar reactors with modified designs have been produced by desktop stereolithography 3D printing, providing a rapid and cost-effective alternative to creating the reactors via traditional machining (Michel *et al.*, 2018).

The age of the sample when it passes through the beam is controlled by the residence time, the average time from initial mixing, until the mixed solution suspension flows into the x-ray scattering window. Hydraulic residence time (τ) is calculated

$$\tau = \frac{\text{reactor volume (mL)}}{\text{flow rate } \left(\frac{\text{mL}}{\text{min}}\right)} + \frac{\text{tubing volume (mL)}}{\text{flow rate } \left(\frac{\text{mL}}{\text{min}}\right)} \quad (1)$$

and is adjusted by changing flow rate. As long as the solution is still flowing, and the sample remains suspended, the residence time and the age of the sample passing through the x-ray beam remains constant. Once the solution exits the reactor and flows through the tubing and scattering window, it behaves as a plug-flow reactor. Minimizing the tubing length helps to control the residence time and reduce the duration of time spent in the plug-flow system.

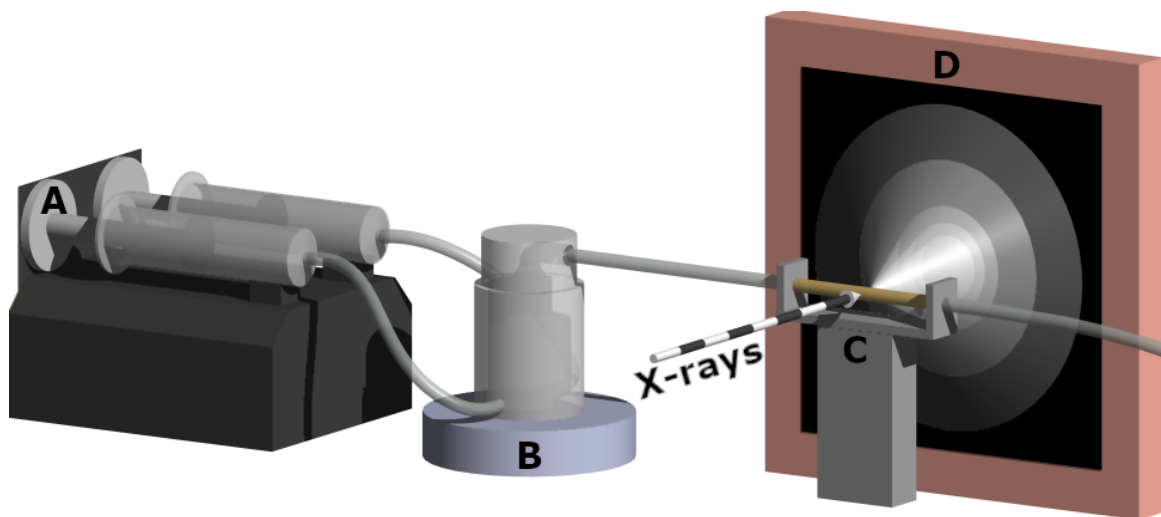


Figure 2.1 A schematic of the experimental set up used at Sector 11-ID-B of the APS. **A** syringe pump with two syringes, **B** mixed flow reactor on a stir plate, **C** x-ray scattering window, and **D** 2D detector.

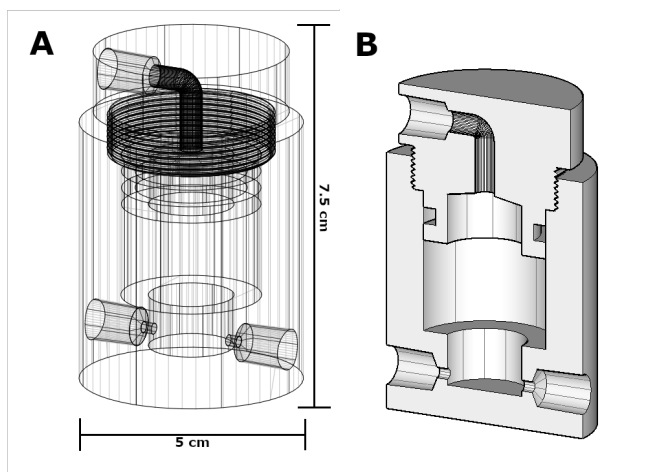


Figure 2.2 Schematics of the mixed-flow reactor shown as (A) a wire frame of the entire reactor and (B) a rendered cross-section. Solutions flow into the reactor through the two inlets at the bottom (tubing connections not shown). A small stir bar in the well at the bottom of the reactor, mixes the solutions as they enter the reactor and exit through the outlet at the top. The reactor has an internal volume of about 25 mL.

DATA ACQUISITION

BEAMLINE SPECIFICATIONS

Collection of total x-ray scattering data for PDF analysis took place at Sector 11 of the Advanced Photon Source, Argonne National Laboratory. In-depth explanations of the PDF technique are found elsewhere (Proffen et al., 2003, 2005; Reeder & Michel, 2013; Egami & Billinge, 2003). In its current configuration, 11-ID operates three individual end-stations simultaneously for 100% of the time. X-rays are delivered through a combination of two in-line undulators. One, a 3.3 cm device, delivers x-rays to the main-branch and end station 11-ID-D. The second, a 2.3 cm device, is used for the side-branch, plus two end-stations: 11-ID-B and 11-ID-C. A double-crystal Si (111), vertical-bounce monochromator is used for the main branch to deliver x-rays in the energy range of 6-30 keV. Two individual, in-line, bent-Laue brilliance-preserving monochromators are used to distribute x-rays to 11-ID-B and 11-ID-C. A symmetrically-cut Si crystal is used to generate energies of 58.6 keV (Si 311, 5th harmonic of the undulator) and 85.6 keV (Si 422) at 11-ID-B and an asymmetrically-cut Si crystal is used at 11-ID-C to deliver x-rays of 105.6 keV (Si 311, 9th harmonic).

All experiments were performed at 11-ID-B. At 11-ID-B, an amorphous silicon-based area detector (2048 x 2048 pixel Perkin-Elmer, Chupas et al., 2007) was positioned about 16 cm from the samples to collect total scattering intensity data. A cerium dioxide standard (CeO₂, NIST diffraction intensity standard set 674a) mixed with glassy carbon (ratio ~1:25 CeO₂ to carbon) was used to calibrate the sample-detector distance, beam energy, and beam center and non-orthogonality. Data were primarily collected during top-up mode and results were highly reproducible using this technique, regardless of mode (top-up or non top-up).

DATA OPTIMIZATION

A crucial part of data collection involves minimizing noise from the detector. The signal for *in situ* samples, especially for syntheses involving low-z elements, such as carbon, oxygen, calcium, and phosphorous, are quite weak and consist mostly of parasitic background scattering. The detector poses the highest potential risk of error in data collection for these experiments. A common problem is the overexcitation of pixels, which results in a false positive signal from the detector and can affect sequential data scans (Skinner *et al.*, 2012).

To minimize overexcitation, the exposure times are kept short, minimizing detector saturation. A single scan is an average of 200 frames, each frame based on 0.3 seconds of beam exposure, which amounts to 1-minute total, per scan. Every 5 scans, we collect a dark current scan, in which the beam is turned off for 1 minute. This allows the detector to rest, removes residual intensity, and prevents overexcitation. The dark currents are subtracted from the raw patterns automatically within the QXRD program (Jennings, 2010). Intense scattering signals that occur for crystalline materials with high electron density are most likely to produce overexcited pixels in the detector. The duration of beam exposure is shorter for well-crystalline samples than for amorphous or poorly crystalline samples. To reduce the chance that overexcited pixels will create false intensities in sequential experiments, total scattering data is first collected for all amorphous, then poorly-crystalline, then well-crystalline samples.

After data collection, the FIT2D program (Hammersley *et al.*, 1996) is used to average scans and convert the data from 2D TIF images to 1D CHI-files. The masks used to remove dead pixels and the beamstop from data integration and the effects of different masks on signal noise is outlined in **Appendix A**. xPDFsuite (Yang, Juhas *et al.*, 2014) is then used to subtract the background for the samples and to perform Fourier transformation of the total scattering data for PDF analysis. Typical Qmax values achieved in our experiments are between 18 – 22 Å⁻¹. To optimize the quality of the data collected using the MFR, background subtraction is the most critical stage of data processing. For this type of system, the background to sample ratio is substantially higher than for traditional *ex situ* analysis methods. The contribution of amorphous or crystalline precipitate is often only between 1-3% of the total x-ray scattering signal (**Figure 2.3**). A background subtraction with precision in the third or fourth decimal place is commonly needed to eliminate under or over subtraction errors for amorphous samples.

Over or under-subtraction can result in a PDF profile consistent with that of water, instead of the signal from the sample (**Figure 2.4**). Because the necessary precision in background subtraction is so high, each data set needs to be closely evaluated and adjusted individually. A universal background subtraction value for a suite of samples under our conditions is not possible. It could be considered under different conditions where a sample produces a higher signal to background ratio. Under ideal conditions, i.e. using top-up mode and identical exposure times for the sample and blank, the multiplication factor between the sample and background equals one. In most cases, however, there is deviation from this value due to minor variations in beam intensity, even during top-up mode. If top-up mode is not available, normalizing the sample and background data at high Q values helps to determine an appropriate scaling and background subtraction.

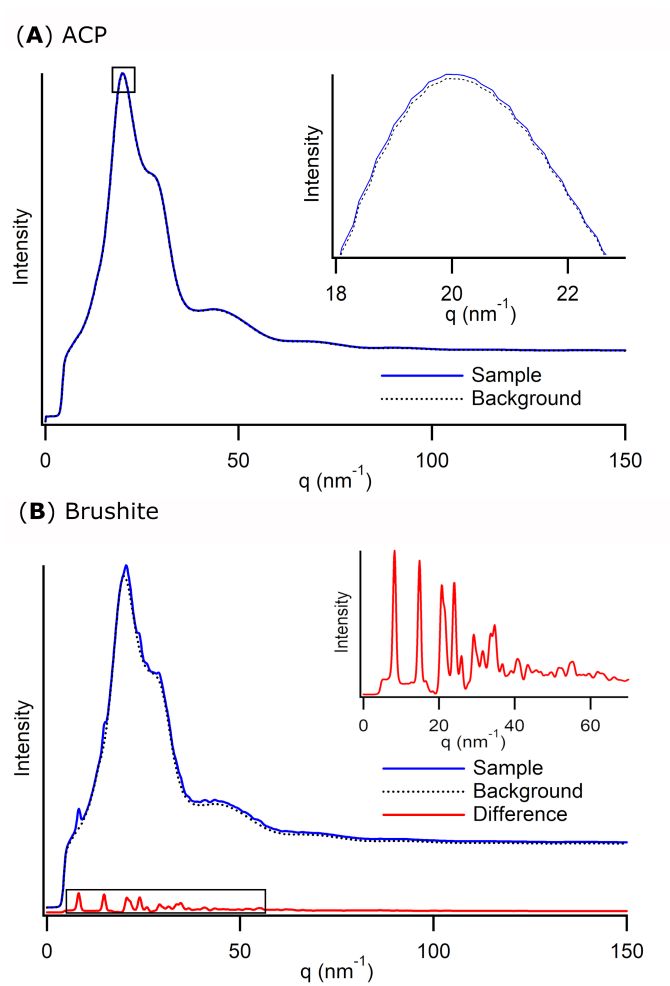


Figure 2.3 Integrated scattering data and the water background that is subtracted from the data presented for (A) an ACP profile with 97% of the total scattering signal attributed to background signal. An enlargement of the profile at its maximum intensity (inset) shows the minimal difference between the sample and background signal. In B, 97.9% of the total intensity of a brushite profile is background. The red profile (inset) is the difference between the two profiles, which produces indexable peaks.

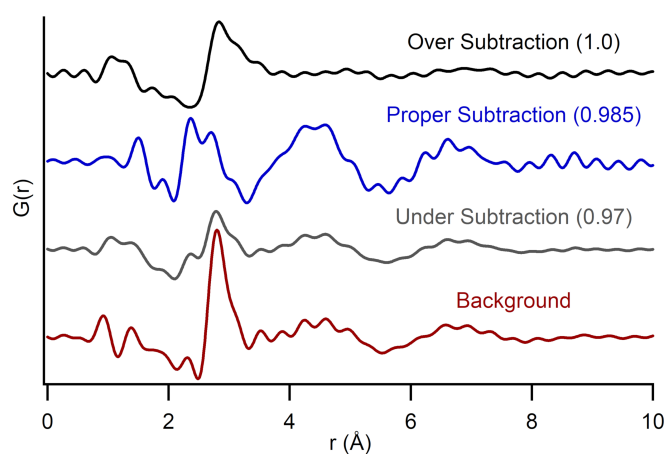


Figure 2.4 A series of PDF profiles show the consequences of over or under-subtraction of an ACP data set. In this example, subtraction that is off by only ± 0.015 results in a profile more similar to the water background and eliminates some of the major structural features.

Choosing an appropriate background for subtraction is critical for data optimization with *in situ* experiments. Different potential backgrounds are considered, including a capillary of the same size filled with deionized water and a capillary filled with either of the reactant solutions (**Figure 2.5**). Because solution concentrations of ions from the low solubility calcium phosphate and calcium carbonate phases are below 50 mM, we find the signal from deionized water most suitable for background subtraction. If this technique is used on samples far from steady-state or samples with higher solubility, using a reactant solution as a background signal may be necessary. For each sample, background and *in situ* signals are collected using the same number of scans. The comparison of PDF profiles of reagent solution to those of the precipitate samples

is also an important proof of concept to evaluate data quality and to check for inherited structural elements (**Figure 2.5**).

It should be noted that all background subtractions are performed in reciprocal space because the focus of this study is the total structure of our samples and all atom pairs. It is possible to perform background subtractions in real space; however, this is typically done for studies of differential PDFs focusing on a specific chemical species. Examples of this are antimonate and arsenate speciation, as outlined in van Genuchten & Peña (2016) and absorbed species on ferrihydrite in Harrington *et al.* (2010) and Zhu *et al.* (2014) Detailed methods and considerations for this type of processing can be found in Wurden *et al.* (2010).

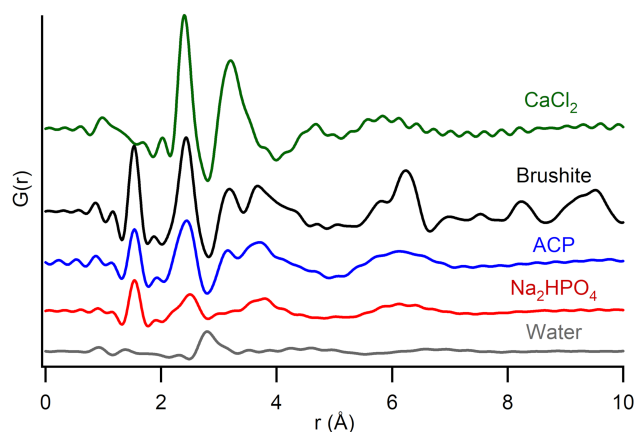


Figure 2.5 Comparison of the PDF data collected for ACP and brushite, and different considered backgrounds: CaCl₂, Na₂HPO₄, and deionized water. The first two peaks in the ACP and brushite data represent mainly the P-O and Ca-O distances, with some O-O contributions. The PDF profiles for CaCl₂·H₂O and Na₂HPO₄ have similar locations for these initial peaks, but ACP and brushite also contain structure that is not inherited from the reactant solutions. The water PDF does not have a peak in the same location as peaks in ACP and brushite, ensuring that no signal from the sample is accidentally subtracted.

The user input compositions within xPDFsuite have little impact on the final structure compared to other data processing software, such as PDFgetX2 (Qiu *et al.*, 2004). This is advantageous for materials without a well-defined chemical composition, such as the amorphous

intermediates in this study. Despite this input flexibility, assigning a composition with elements or compounds not found in your sample produces a PDF profile with features that are not real. For ACP materials, a composition of $\text{Ca}_9(\text{PO}_4)_6$ is used. This is a widely applied composition for calcium phosphate materials with the short-range order seen in ACP (Posner & Betts, 1975; Betts *et al.*, 1975). For crystalline phases their theoretical chemical compositions are used, which is $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ for brushite. The limited variables available in xPDFsuite make data extraction straight forward once a suitable background subtraction value is achieved.

SPECIAL CONSIDERATIONS FOR MFR SAMPLES

Further optimization of PDF profiles can be performed by carefully evaluating the time-scales needed for each experiment and adapting x-ray exposures accordingly. For example, simple mixing theory shows that it takes approximately three residence times for the MFR system to reach steady state (Jensen, 2001). This can vary for different experiments depending upon the concentration of particles produced and the density of the solids (Jensen, 2001). **Figure 2.6** shows PDF data for ACP (Fig. 7A) and brushite (Fig. 7B) that were both synthesized with $\tau = 5$ minutes. The data are presented as a series of 5-minute averages. The major peak locations are visible in all the profiles, but data from the first 5-minutes of data collection show a much lower intensity. Sequential groups of averaged time-points present no significant differences, which means we achieve steady state between one and two residence times. It also provides evidence that the structure is not evolving during data collection and no features are being averaged out. All subsequent data were collected with a minimum data collection time equivalent of 2τ (at least 10 minutes for data shown here, but variable depending on flow rate).

Materials with low scattering intensity require further data optimization by averaging multiple scans from a single sample to minimize Fourier noise. **Figure 2.7** shows a significant reduction in noise between a single scan and an average of 5 scans for the ACP example. Further noise reduction by averaging 20 scans is not significant when compared to the 5 scan averages. Use of more than 20 scans to produce one average does not significantly improve the overall quality of the data.

Note that the data collection times and number of scans per sample depend upon residence time. Data are collected while the sample flows through the x-ray window at constant speed. Short residence times (faster flow rates) produce few scans, potentially too few to yield a

signal of sufficient quality. There is also an upper limit to the maximum practical residence times. Low pumping speeds ($\tau > 25$ minutes) cause solids to settle both in the reactor and the tubing, even at high rates of stirring (800 rpm). This blocks the solution flow at the connector transition between the tubing and the scattering window. Settling also leads to variable age in the sample. The supersaturation of the system and the amount of precipitate can vary widely and have a significant impact on the upper range of residence time for an experiment. Varying experimental design by altering the initial volume of reactant, the reactor volume, and tubing inner diameter expands the accessible range of residence times.

To test the limitations of the device, we performed experiments for $\tau = 2 - 25$ minutes. We found a residence of 5 minutes, (~ 25 scans and 5τ) was suitable for investigating the structures that dominate early stages of crystal growth. At this residence time we captured ACC as well as ACP and brushite. The presence of ACP or brushite could be controlled by altering the initial system conditions (*e.g.* concentration and pH).

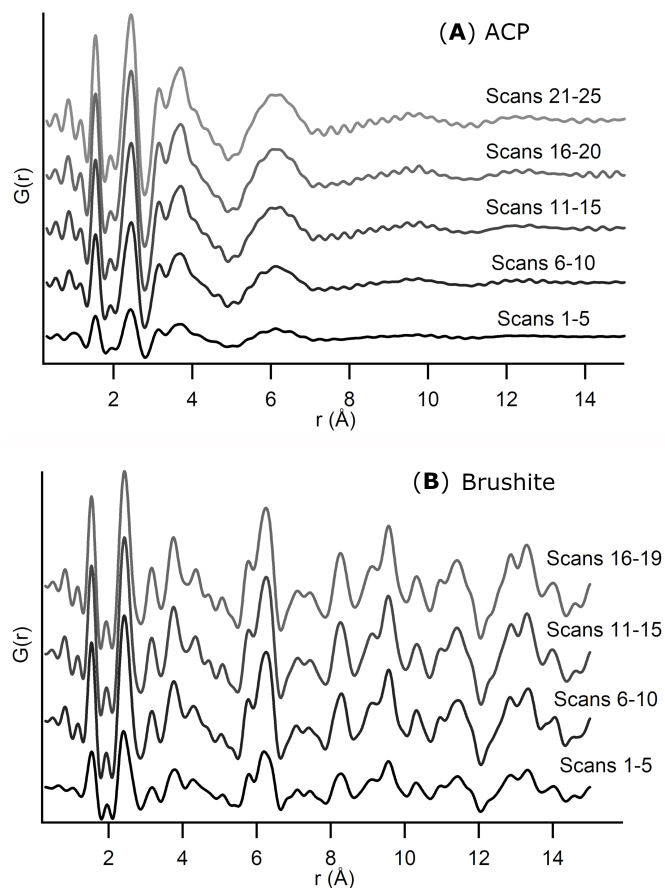


Figure 2.6 To demonstrate that samples do not evolve during data collection, the PDF data for a sample of (A) ACP and (B) brushite are shown as sequential averages of 5 scans. The structure remains consistent across the scans, but there is an increase in overall intensity for both samples after the first five scans. During these first 5 scans (the first five minutes of data collection), the solution inside the MFR is still approaching steady-state and there is a lower particle leading to a less intense scattering signal.

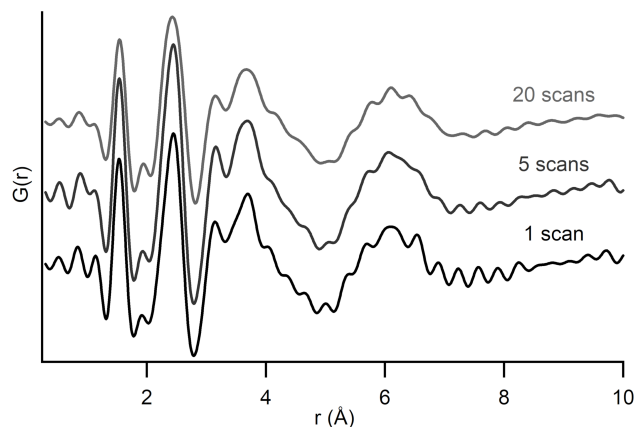


Figure 2.7 Averaging multiple scans of data contributes to PDF profiles that have a reduced amount of noise. A single sample of ACP is presented as a single scan, an average of 5, and an average of 20 scans. The noise in the data is significantly reduced between 1 and 5 scans. There is a slight reduction in noise between 5 and 20 scans. Averages beyond 20 scans did not have a significant reduction in noise. Each scan represents one minute of data collection, indicating noise reduction is optimized with at least 20 minutes of collection time.

PDF ANALYSIS OF *IN SITU* SAMPLES

Our adaptation of the MFR design allows us to obtain high quality total x-ray scattering data for PDF analysis of *in situ* crystalline and amorphous phases. To identify and structurally refine the collected phases a theoretical PDF is calculated from a published structure file using PDFgui (Farrow *et al.*, 2007). The crystalline calcium phosphate phase was identified as brushite through comparison with a structure published by Sainz-Díaz *et al.* (2004). We refined unit cell dimensions, linear atomic correlation factor, phase scale factor, thermal parameters, atom positions, and instrument dampening (**Appendix A**). **Figure 2.8** compares the PDF of the collected data with the refined calculated PDF. There is a 30% residual misfit between these two data sets. While this would be considered a fairly high residual for a well-crystalline powder, we justify this residual on the intricacies of the data extraction and large opportunities for error in background subtraction. The fit accounts for all major peaks and features in the profile. A concentrated contribution of misfit between the sample and the calculated PDF originates in the Fourier noise at the beginning of the profile, which likely does not correspond to real features. The first structural peak for the calcium phosphate system is the P-O peak at 1.56 Å. If the profiles are compared without considering any signal below 1.3 Å, the residual drops to 25%.

A previous study (Proffen *et al.*, 2005) identified concurrent amorphous and crystalline phases in the same sample by examining the difference profile after fitting the crystalline phase. It is possible that there is ACP mixed with brushite in this sample, we don't find evidence for it in the difference profile. The misfit appears random and centered around zero. With the low signal to noise ratio for these samples, a degree of noise is expected and also contributes to the overall error.

The MFR is also used to collect *in situ* PDF data for amorphous calcium carbonate (ACC), demonstrating the adaptability of the MFR to address questions in various chemical systems. A separate reactor is used for all calcium carbonate experiments to avoid cross contamination. The synthesis of ACC involves mixing CaCl_2 and MgCl_2 solutions with a pH adjusted NaHCO_3 solution in the MFR (Sigma-Aldrich ACS reagent, pH = 9.5, Mg/Ca = 1.93 in solution). The process for data collection and extraction developed for ACP can be applied to ACC samples and **Figure 2.9** compares the quality of PDF profiles produced. A notable difference between the two profiles is the location of the first peak, at $\sim 1.3 \text{ \AA}$, due to the shorter average bond length between C – O in carbonate than is seen in the average P – O length of phosphate. This integrated area of these peaks is also different, due to differences in coordination number. In contrast the second peak for both ACC and ACP is at $\sim 2.4 \text{ \AA}$ because the Ca – O bond distance is the same for both phases. The profile contributions beyond the first two peaks are more convoluted, but certain structural influences can still be interpreted. Monodentate or bidentate bonding geometries, especially between Ca – P in ACP and Ca – C in ACC can impact the structure and be reflected in peak positions. The C – C distances in structure with trigonal planar CO_3 will differ from the P – P distances with PO_4 tetrahedra. These predictable contributions can help further interpret complex profiles.

Comparing the PDF profiles of ACP and ACC in **Figure 2.9** with examples of ACP from Posner and Betts (1975) and ACC in Michel *et al.*, (2008) shows the Ca – O, P – O, and C – O peak locations are consistent between our samples and the literature. Both *in situ* PDF profiles suggest a shorter range of structural coherency than is described in the *ex situ* literature. The Q_{max} for our ACP sample is $20 \text{ \AA}^{-1}$, while Posner and Betts (1975) terminated their data at $13 \text{ \AA}^{-1}$, resulting in significant differences between the samples. Our ACC profile is very similar to the one presented in Michel *et al.* (2008). There are some differences between 3 and $5 \text{ \AA}$, potentially from magnesium in the synthesis and contributions from associated water.

The strength of this method is that we can examine the structures of *in situ* precipitates for a wide variety of different chemical systems simply by minor variations in the initial set up. By changing our initial solutions, we examined the structural variations between ACC and ACP. Similarly, experiments could be designed to investigate the structural impacts of other influences on ACC and ACP such as pH, supersaturation, and ion substitution. Combining MFRs with total x-ray scattering allows us to investigate a range of structure and evolution questions that are difficult, if not impossible, to answer using other techniques.

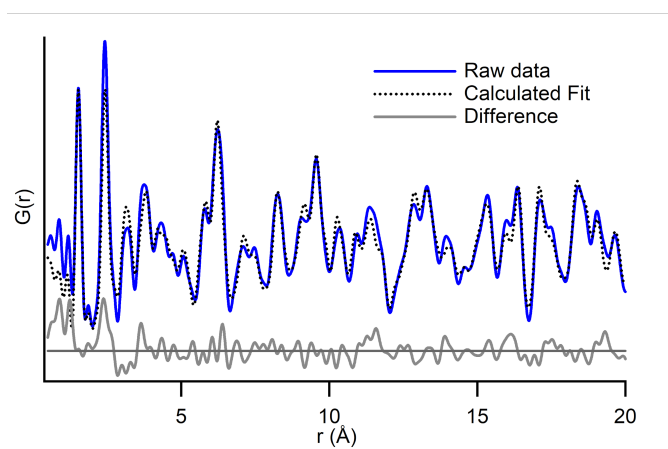


Figure 2.8 PDF data of an *in situ* brushite sample, modeled against a published brushite structure. The fit was determined by calculating a theoretical PDF from the published structure, allowing for some parameter refinement, and comparing it with the observed PDF data. The difference between the observed and the fitted data is random and mostly oscillates near zero. At low r (x-axis), where the signal contains artifacts of the Fourier transform, the errors in peak positions and intensities are high.

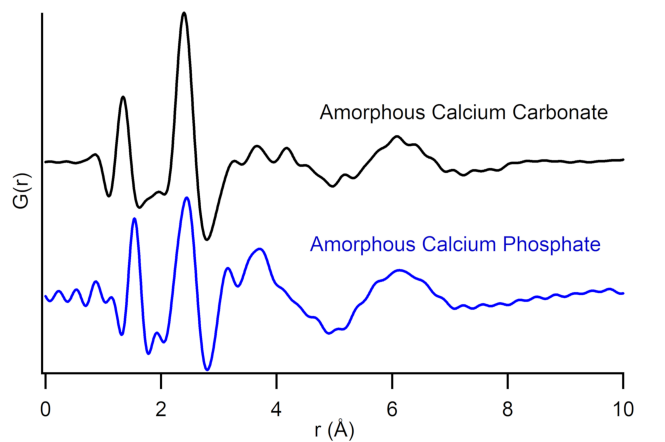


Figure 2.9 *In situ* PDF data from ACC and ACP synthesized in the MFR. There are distinct differences between the phases, most notably in the position of the first peak at 1.6 Å for the P-O distances in ACP and at 1.3 Å for the C-O distances in ACC.

CONCLUSIONS

We use an MFR-based experimental design that can be combined with x-ray total scattering techniques for *in situ* investigations of crystal growth pathways. The reactor allows precise control of system chemistry and residence times and is appropriate for investigations of various chemical systems where the reaction time of interest is between 2 and 25 minutes. This technique enables structural examination of samples in suspension, without the sample chemistry or structure evolving during data collection. Data are collected soon after initial mixing and sample precipitation, allowing investigations into initial phase formations and structural developments that occur in the crystallization process. Optimizing data collection and processing alongside collecting proper background data and careful background subtraction is noted as a key step.

We have collected structural information on crystalline and amorphous phases *in situ* for both the calcium carbonate and calcium phosphate systems using the MFR. An *in situ* crystalline sample was successfully modeled in real space against a published structure for brushite. The amorphous profiles showed similarities with their crystalline counterparts, which allowed us to infer short-range structural information for these phases, without performing modeling. MFRs will allow us to investigate the structural impacts of different initial variables on these materials that have previously proven challenging to examine.

This new technique presents an opportunity to improve data collection on the structures of amorphous and poorly crystalline phases and lead to more robust structural models. Results from MFR experiments will need to be combined with other techniques before a comprehensive picture of the structure of these complex amorphous materials can be reached. Future efforts will also focus on expanding the range of residence times that are possible and adding adaptations such as those that will allow us to monitor pH or ion concentrations during the reaction.

CHAPTER 3: IMPACTS OF INITIAL CA/P ON AMORPHOUS CALCIUM PHOSPHATE

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ABSTRACT

Amorphous calcium phosphate (ACP) is a metastable phase in the crystallization pathway of calcium phosphates. Understanding its chemical and structural properties could provide critical insights into biomineralization mechanisms. *In situ* pair distribution function analysis allows us to examine the impact of variations in the initial Ca/P, on the structure of ACP. The influence of Ca/P on the size, precipitate chemistry, and transformation of ACP is also examined. At five minutes after mixing, the Ca–P bonding geometry varies from predominantly monodentate, to mixed monodentate and bidentate, then to predominantly bidentate as Ca/P increases from 0.2 to 5.0. This relationship is consistent across a pH range of 6–11. Samples with only monodentate Ca–P geometries transform directly to hydroxylapatite, while samples with mixed monodentate and bidentate geometries form brushite. Regardless of initial ion ratio, the Ca/P of precipitates is close to 1.0. *In situ* small-angle X-ray scattering shows that particle size increases with increasing Ca/P. This is the first evidence of structural variations in ACP and is directly linked to variations in system chemistry. Synthesis of ACP with monodentate Ca–P geometries is a potential method to control the pathway of crystallization and form hydroxylapatite at circumneutral pH without stabilizing ions.

INTRODUCTION

Amorphous calcium phosphate (ACP) can form as a metastable intermediate during the synthesis of calcium phosphate minerals such as brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) and hydroxylapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$). Hydroxylapatite is a close crystalline analog of bone mineral and is used as a

proxy for investigations of bone growth and considered for biomedical applications with prosthetic development (Veis & Dorvee, 2013; Cazalbou *et al.*, 2004; Habraken *et al.*, 2016). The role of ACP as a precursor to hydroxylapatite, as well as the nature and structure of ACP, are topics of considerable focus in the literature (Termine *et al.*, 1970b; Posner & Betts, 1975; Heughebaert & Montel, 1982; Treboux *et al.*, 2000; Yin & Stott, 2003b; Du *et al.*, 2013; Habraken *et al.*, 2013). However, the transitory nature of amorphous intermediates like ACP makes them difficult to investigate.

To examine the properties of ACP, transformation is commonly delayed by raising the pH of the system using buffers or hydroxide salts (e.g. NaOH) (Termine & Posner, 1970; Blumenthal *et al.*, 1972b). Adding magnesium, proteins, and other organic ligands also stabilizes ACP (Termine & Posner, 1970; Blumenthal *et al.*, 1977; Kibalczyk *et al.*, 1990). ACP is commonly dried prior to examination, which both prevents transformation, and creates a form that is easily analyzed (Eanes *et al.*, 1973b; Meyer & Eanes, 1978; Stammeier *et al.*, 2018). Investigations that adjust the system pH commonly raise it to 9 or higher, above what is biologically relevant (Liu *et al.*, 2001; Othman *et al.*, 2016). While the addition of magnesium, proteins, and other ions creates a more realistic biologic environment for an experiment, it is unknown what effect they have due to the challenges of stabilizing ACP without them.

There are numerous techniques to target the synthesis of different calcium phosphate phases (Koutsopoulos, 2002; Combes & Rey, 2010), but precipitation from supersaturated solution is common because it is relatively simple and inexpensive. A range of initial calcium and phosphate salt solutions are used including CaCl_2 , $\text{Ca}(\text{NO}_3)_2$, $\text{Ca}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$, $(\text{NH}_4)_2\text{HPO}_4$, NaH_2PO_4 , and KPO_4 (Christoffersen *et al.*, 1989; Oyane *et al.*, 1999; Kim *et al.*, 2004; Borkiewicz *et al.*, 2010; Zhang *et al.*, 2016). Thus, reported results of ACP investigations often span a wide variety of chemical conditions which may not be directly comparable.

Major questions remain about the impacts of solution chemistry on ACP formation and transformation. For example, the impact of the initial Ca/P on the crystallization pathway of hydroxylapatite has only been examined by a handful of studies (Füredi-Milhofer *et al.*, 1971; Oyane *et al.*, 1999; Kim *et al.*, 2004, 2005; Zhang *et al.*, 2016). Oyane *et al.* (1999) and Kim *et al.* (2004, 2005) observe variations in calcium phosphate crystallization rates at different Ca/P.

Zhang *et al.*, (2016) suggest that systems with low Ca/P are less reactive, potentially due to bidentate geometry complexes between calcium and phosphate.

Using the mixed-flow reactor (MFR) method described in Hoeher *et al.*, (2019), chemistry and sample age of experiments in this study are precisely controlled during data collection. In addition, the use of a MFR enables *in situ* measurements without drying or chemically stabilizing ACP. X-ray total scattering data for pair distribution function (PDF) analysis is used to examine the impact of variable ion ratios and pH on the structure of amorphous and crystalline calcium phosphate phases at a set time point.

In addition to determining ACP structure, we quantify the primary particle size of the samples using *in situ* small-angle x-ray scattering (SAXS). A commonly discussed primary particle size for ACP is 9 Å, which is the size proposed for Posner's cluster, a theoretical building block of the hydroxylapatite structure (Posner & Betts, 1975). Both Blumenthal *et al.*, (1972) and Kim *et al.*, (2004) suggest particle size impacts the rate of ACP transformation. Blumenthal *et al.*, (1972) show that higher pH and supersaturation form smaller particles that slow transformation under these conditions. In contrast Kim *et al.*, (2004) noticed that as they increase the Ca/P from 1.5 to 2.0, the average particle size decreases from 39 (± 9) to 24 (± 4) nm, but the induction time for crystallization is shorter. The method for *in situ* PDF analysis is applied to SAXS, producing complementary size and structure information to help analyze literature discrepancies and obtain a complete picture of the impact of varying Ca/P.

While the SAXS and PDF analysis allows us to examine the impact of system chemistry *in situ*, we also perform *ex situ* powder X-ray diffraction (XRD) for Rietveld analysis and inductively coupled plasma-atomic emission spectroscopy (ICP-AES) on dried samples. One limitation of the current MFR experimental set-up is that it cannot analyze residence times greater than 20 minutes after initial mixing due to particle settling at lower flow rates (Hoeher *et al.*, 2019). Filtering and drying the samples at longer time scales, before examining them with XRD allows us to evaluate how the initial solution conditions impact the transformation pathways of ACP into crystalline phases. Filtering and drying a separate set of samples for ICP-AES allows us to investigate how the initial system chemistry impacts the chemistry of the precipitate that forms.

In this study we examine samples across an initial Ca/P range of 0.2-5.0. We also investigate two ranges of solution pH, low (6-7) and high (10-11), which are common ranges for calcium phosphate synthesis in the literature. Results show that a higher Ca/P ratio produces dominantly bidentate Ca – P bonding geometries, intermediate ratios form a combination of bidentate and monodentate bonding geometries, and the low ratios dominantly have monodentate geometries. These results are consistently ratio dependent, regardless of pH. At the lowest ratio of 0.2 and in the low pH range, that produces dominantly monodentate bonding geometries, ACP transforms directly to hydroxylapatite without any brushite formation. Intermediate to high ratios all produce a mix of brushite and hydroxylapatite during evolution. Despite a wide range of initial Ca/P, the Ca/P of the precipitate reported with ICP-AES is similar for all samples and close to 1.0. The SAXS results indicate that the most common particle radius for Ca/P 0.2, 1.0, and 5.0 are 0.4, 1.0, and 2.0 respectively. There does not appear to be a direct correlation between particle size and rate of ACP transformation in these samples.

METHODS

IN SITU SAMPLE SYNTHESIS AND PDF ANALYSIS

The initial calcium and phosphate solutions are created using Sigma-Aldrich ACS reagents $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and Na_2HPO_4 in 18.2 M $\Omega \cdot \text{cm}$ ultra-pure water. 2 mL of a 1M NaOH solution is added to Na_2HPO_4 to raise the pH for select samples. **Table 3.1** details solution conditions for each sample and all syntheses are performed at room temperature. 100 mL of the initial solutions are loaded into syringes and a high precision syringe pump (Harvard Apparatus PHD ULTRATM 4400) is used to continually pump them through a 25-mL mixed-flow reactor (MFR) and through the x-ray scattering cell. A stirring rate of 800 rpm is maintained in the reactor. The residence time is 5 minutes for all samples, controlled by the pumping rate. The total flow rate of both syringes is 6 mL/min and it takes approximately 45 seconds for the effluent to travel from the reactor to the scattering cell. The process of *in situ* x-ray total scattering data collection and refinement for PDF analysis using a MFR is outlined in detail in Hoehner *et al.*, (2019). All x-ray scattering data is collected during top-up mode at Sector 11-ID-B of the Advanced Photon Source at Argonne National Laboratory. FIT2D (Hammersley *et al.*, 1996) and xPDFsuite (Juhás *et al.*, 2013; Yang, Juhás *et al.*, 2014) are used for data processing and refinement. The assumed chemical composition for normalization during data processing is $\text{Ca}_9(\text{PO}_4)_6$ for all samples

except ratios 1.0 and 1.6 with the pH unadjusted, where $\text{CaHPO}_4 \cdot 2(\text{H}_2\text{O})$ is used instead. Average coherent scattering domain (CSD) size estimates correspond with the PDF signal reducing to statistical noise. Fractions of ACP and brushite are estimated by linear combination fitting (LCF) using the software WinXAS v.3.0. LCF is performed on sample PDFs between 0-15 Å using synthetic references for ACP and brushite (see Supplemental for additional information)

Table 3.1 Synthesis details for *in situ* PDF analysis samples. The initial Ca/P, initial solution concentrations, initial solution pH's before mixing (* indicates NaOH is added to this sample), supersaturation relative to brushite (Ω_{Br}) and hydroxylapatite (Ω_{Hap}) (details on the supersaturation calculations can be found in the Supplemental information), and pH after mixing are reported. The mixed pH would drift slightly during data collection, so the median value collected during the experiment is reported.

Sample	Ca/P	[Ca ²⁺] mM	[PO ₄ ³⁻] mM	Ca initial pH	P initial pH	Ω_{Br}	Ω_{Hap}	Mixed pH
1	0.2	9	44	5.81	9.16	1.87	67.6	~7.8
2	0.6	15.4	25.6	5.57	9.37	1.95	66.8	~7.4
3	1.0	20	20	5.61	9.37	1.96	64.3	~6.7
4	1.6	25.6	15.4	5.61	9.34	1.93	62.8	~6.3
5	5.0	44	9	5.61	9.23	1.84	61.7	~6.0
6	0.2	9	44	5.35	11.0*	1.86	80.3	~11
7	1.0	20	20	5.52	10.5*	2.05	79.7	~10.5
8	5.0	44	9	5.46	10.3*	1.87	78.9	~10.3
9	0.2	9	44	5.14	9.23	1.91	63.4	~6.7
10	1.0	20	20	5.55	9.49	1.96	64.7	~6.8
11	5.0	44	9	5.53	9.54	1.84	62.5	~6.2

In-line pH measurements are collected using a custom flow-through cell fabricated using inverse stereolithography 3D printing (Michel *et al.*, 2018; Kletetschka *et al.*, 2018) (**Figure 3.1**). The pH cell (Oakton PC 2700 pH meter, instrument precision ± 0.01) is positioned downstream from the scattering cell and pH is measured with a delay of about 10 seconds after the samples flow through the scattering cell for structural analysis. During extended data collections at the synchrotron, the meter was calibrated about every 24 hours using Thermo Scientific buffer solutions (4.01, 7.00 and 10.01). The calibration slope between pH's 4 – 7 and 7

– 10 ranged between 98.7 – 100.7% and 95.5 – 96.6% respectively. The pH was recorded on a computer every 3 seconds for the duration of the experiment using the software CyberComm 2700 (Version 1.12) and the average pH was taken from the drift curves (See Appendix B, **Figure B-1**). Once the suspension exited the holder it flowed into a waste container and is discarded.

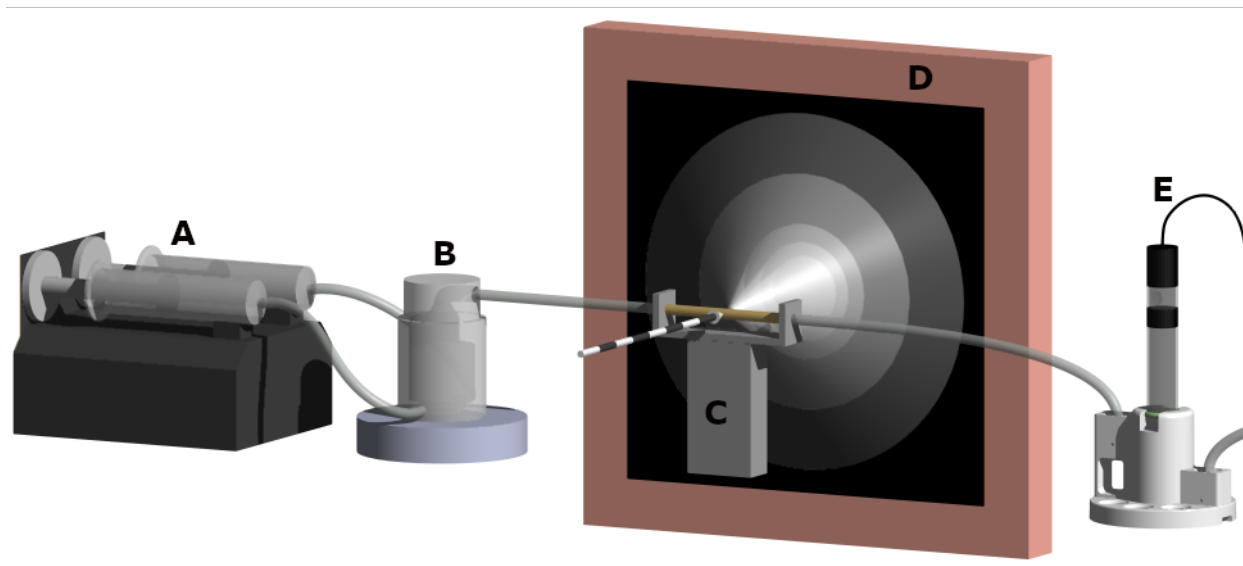


Figure 3.1 A schematic of the experimental set up for *in situ* PDF (modified from (Hoehler *et al.*, 2019)) and pH measurements includes (A) a syringe pump and two syringes, (B) mixed flow reactor on a stir plate, (C) X-ray scattering window, (D) 2D detector, (E) pH meter in-line flow cell, and arrow indicating the X-ray path.

SAXS ANALYSIS

The pump and mixed flow reactor setup shown in **Figure 1** is also used for *in situ* laboratory SAXS measurements. The experiments are performed for select Ca/P ratios of 0.2, 1.0, and 5.0, and only for the low pH conditions (**Table 1**, samples 9-11). Similar to the synchrotron experiments, the residence time is maintained at 5 minutes for all samples by controlling the pumping rate. SAXS intensities are collected using an Empyrean Nano Edition diffractometer system (Malvern-Panalytical) equipped with a Cu source ($\lambda_{K\alpha} = 1.5406 \text{ \AA}$). The incident optic is a W/Si elliptical focusing mirror with a $1/32^\circ$ fixed slit. The x-ray scattering cell for the experiments is a custom 3D-printed flow cell (U.S. Patent Application No.: 62/576,928)

that allows nanoparticle suspensions to be measured under vacuum (~30 Torr) in the ScatterX78 sample stage (Malvern-Panalytical). Scattered sample intensities from a silica glass capillary (2 mm O.D.) in the cell are measured using a GaliPIX^{3D} area detector system outfitted with an anti-scatter shield. Data are collected from -0.15 to 6° 2 θ at 0.014° increments and exposure time of ~108 s/step. The individual scan time was approximately 3.75 min and six scans are collected per experiment. SAXS experiments are replicated two to four times at the different ion ratios in order to improve sample signal to background. The first scans of each run are discarded and only the final two or three scans are averaged and used for further analysis. Parasitic scattering from the same flow cell loaded with DI water is measured and subtracted directly from each sample data using EasySAXS software (Malvern-Panalytical) with appropriate scaling factors applied. Volume-weighted size distribution (Dv(R)) analysis of the background-subtracted SAXS is performed using the same software. The low-q limit of the fitting window varied from 0.1 to 0.25 nm⁻¹ for the different samples. The high-q limit is 4.25 nm⁻¹ in all fits. Volume weighted size distributions are calculated up to 20 nm.

ICP-AES ANALYSIS

Samples with initial ion ratios of 0.2, 1.0, and 5.0 are synthesized in triplicate to analyze the Ca and P concentration of the precipitate via ICP-AES. The synthesis followed a similar procedure as the *in situ* analyses by using the syringe pump to mix the calcium and phosphate solutions in the MFR. Instead of the sample flowing through a scattering cell for analysis, it is instead collected, and the precipitate is separated by centrifugal ultrafiltration using Corning Spin-X[®] UF 20 poly-ether sulfonate (PES) filters (Corning, Corning, NY USA) at 10,000 x g for 40 minutes. Samples are acidified with 10% Nitric acid (exact synthesis specifications for each sample can be found in Appendix B **Table B-1**) and ICP-AES analysis is performed at the Virginia Tech Soil Testing Laboratory using a Spectro[®] ARCOS SOP instrument and a Ca and P detection limit of 25 and 21 µg/L, respectively and a measurement precision of ± 0.1 mg/L.

POWDER XRD ANALYSIS

To examine the impact of initial ion ratio on the transformation of calcium phosphates, samples with initial ion ratios of 0.2, 1.0, and 5.0 are synthesized in a batch reactor. 100 mL each of the calcium and phosphate solutions (with concentrations identical to those reported for the ratios in **Table 3.1**) are rapidly added to the reactor and stirred (800 rpm) for a set duration. The pH for

each sample is unadjusted and circumneutral. 30 minutes and 24 hours after initial mixing samples are filtered (polyethersulfone membrane 0.22 μm filter Stericup[®], Millipore Sigma) and washed with water and acetone before being left to air dry. The dried sample is then ground in an agate mortar and pestle to a fine powder, before loading it onto a silica zero-background pack mount. Samples are analyzed with powder XRD using a Rigaku Miniflex II with a Cu K- α emission. XRD data is collected between 5 and 60 2θ with a step width of 0.02° and a dwell time of 2 seconds per step. Rietveld analysis is performed using the General Structural Analysis System II (GSAS-II, version 4489 (Toby & Von Dreele, 2013)). Initial structural parameters for brushite were taken from Schofield *et al.*, (2004) and for hydroxylapatite from Hughes *et al.*, (1989). Backgrounds were fit using a cosine function with 15-20 coefficients and ~ 20 anchor points that are placed manually. The instrumental weight function (profile parameters U, V, W, X and Y) is determined using NIST silicon standard 640d. These parameters are carried over to the sample refinements and left fixed. The unit cell of each phase is the only structural parameter allowed to refine. Peak broadening for hydroxylapatite is fit using a uniaxial domain size model and microstrain models, both with the [002] as the unique axis. An isotropic microstrain model and spherical harmonics preferred orientation model (6 – 8 orders) is used for brushite. Rietveld refinement is used to determine phase fractions of (nano)crystalline hydroxylapatite and brushite in the mixed-phase samples as a function of aging time. The fraction of ACP in the samples is not refined.

RESULTS

PDF ANALYSIS

There are notable structural differences between the circumneutral pH samples (Table 1, samples 1-5), which only varied in the initial Ca/P (**Figure 3.2**). The relative intensities of the peaks corresponding to the P-O and Ca-O first shell distances (peak A and peak B respectively) change in correspondence to the initial Ca/P. As Ca/P increases there is also development of peak C and a corresponding decrease in intensity of peak D. The atom pair contributions and intensity variations between peaks C and D are discussed in detail in the next section. The PDF profiles for ratios of 0.2, 0.6, and 5.0 appear to show only short-range order, with structural coherency limited to less than 8 Å. In contrast ratios 1.0 and 1.6 show structural peaks beyond 8 Å. A Ca/P of 1.0 and 1.6 produces precipitates with discernable peaks in the I(q) scattering data (**Figure**

3.3) that correspond to crystalline brushite, in contrast to the broad amorphous humps for Ca/P of 0.2 and 0.6. Despite an apparent short limit of coherence in the PDF of the 5.0 ratio, the $I(q)$ shows small peaks, consistent with those developing in the intermediate ratios. LCF analysis indicates that the fraction of brushite varies from 0 to 36% ($\pm 5\%$) in the samples formed at ratios 0.6 to 5.0 (See Appendix B, **Table B-3**).

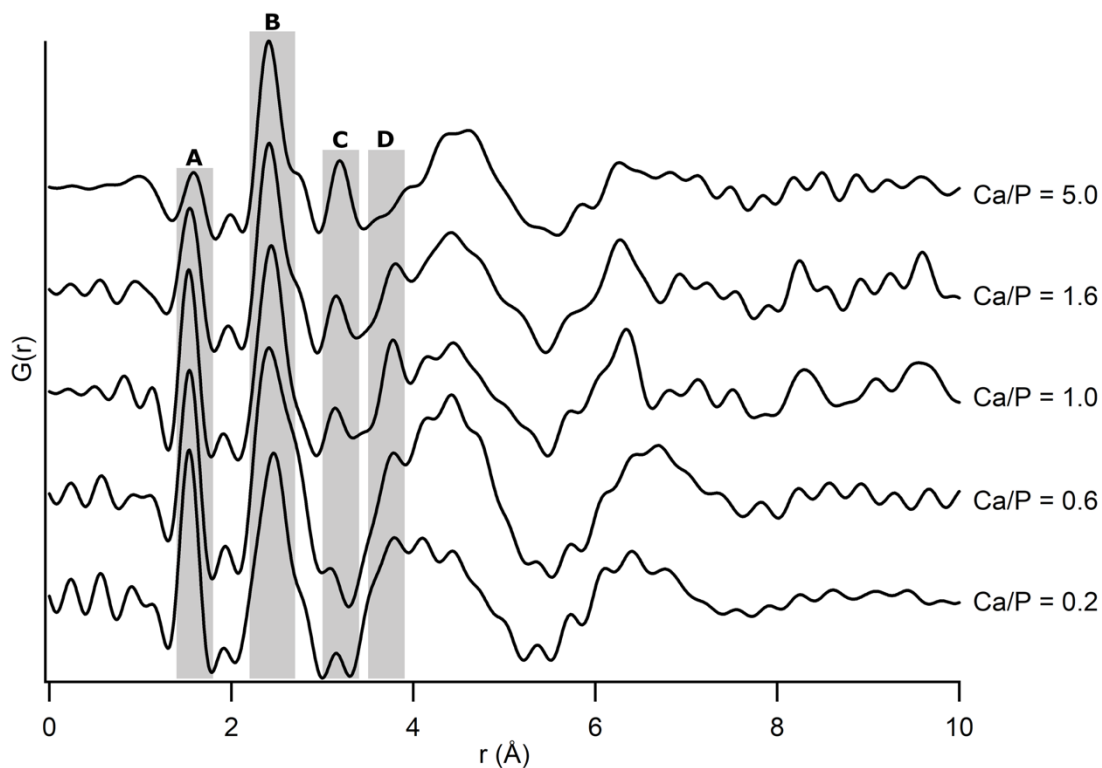


Figure 3.2 Normalized PDF profiles of samples as a function of initial Ca/P. The pH for these samples is between 6 and 8. The relative intensity of peak A decreases in intensity relative to peak B as the ion ratio increases. As the Ca/P increases, peak C increases in intensity, while peak D decreases. Finally, the ion ratios of 1.0 and 1.6 uniquely show a structural signal beyond 8 Å.

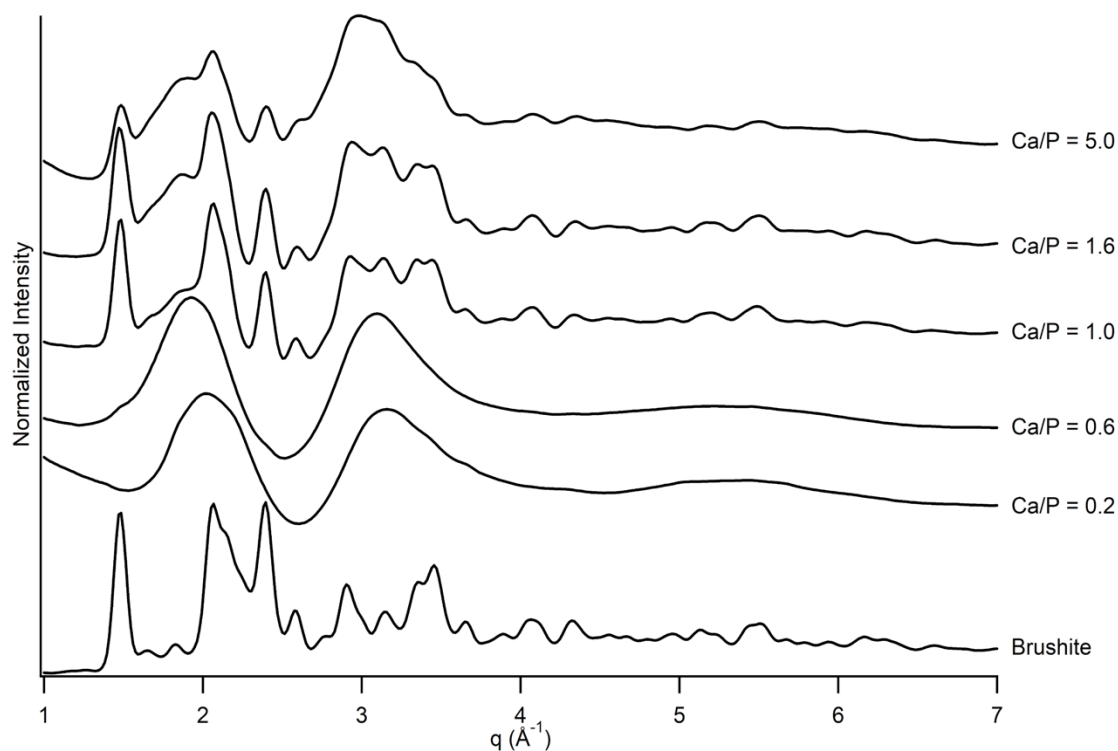


Figure 3.3 Normalized $I(q)$ profiles of samples synthesized with different initial Ca/P of 0.2, 0.6, 1.0, 1.6, and 5.0. The 0.2 and 0.6 samples show broad amorphous features. The 1.0, 1.6, and 5.0 samples show distinct peaks developing, though in 5.0 the peaks are not as pronounced as in the other two samples. These peaks indicate brushite growth, though likely in a disordered state.

When Ca/P is altered without controlling the initial pH, the amount of phosphate in solution buffers the pH of the system and there is a natural variation in the pH of the mixed solution at different Ca/P (See Appendix B, **Figure B-1** and **Table 3.1**). Within the pH range of 6-7.8 in these samples, the dominant phosphate speciation in solution changes from H_2PO_4^- to HPO_4^{2-} . To differentiate the structural impacts of Ca/P from this variation in speciation we repeated the experiments for ratios 0.2, 1.0, and 5.0. In one data set the pH is between 6-7 where the phosphate speciation is a mix of H_2PO_4^- to HPO_4^{2-} , but with H_2PO_4^- the more dominate. The second set of measurements use samples with pH values between 10-11, where the speciation is almost completely HPO_4^{2-} , and there is a negligible amount of H_2PO_4^- present in solution (See Appendix B, **Figure B-2**). Raising the pH alters the supersaturation index relative to hydroxylapatite, Ω_{HAP} , but it has been documented in the literature that ACP is more stable and

slower to transform into hydroxylapatite at a pH above 9 (Termine & Posner, 1970; Blumenthal *et al.*, 1972b; Boskey & Posner, 1973).

PDF profiles indicate few structural differences between the samples with different pH values (**Figure 3.4**). The relative intensity contributions for A, B, C, and D do not vary significantly between samples synthesized at low and high pH, but there is a systematic variation of A/B and C/D with an increase in Ca/P. While ratio 1.0 is partially crystalline at the lower pH range, the sample synthesized in the high pH range is amorphous, as expected, and shows no evidence of crystallinity in the $I(q)$ (See Appendix B, **Figure B-3**). All of the ratios only show short-range order at high pH, and Ca/P 5.0 and 0.2 show no significant differences from their lower pH counterparts.

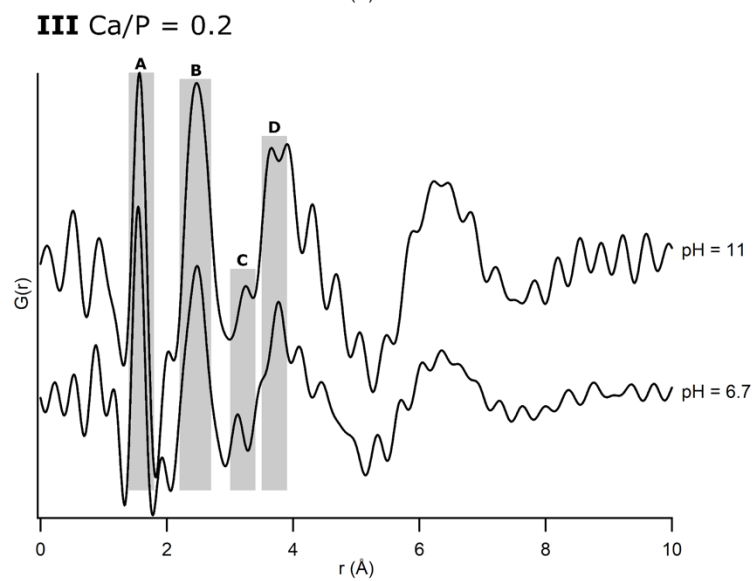
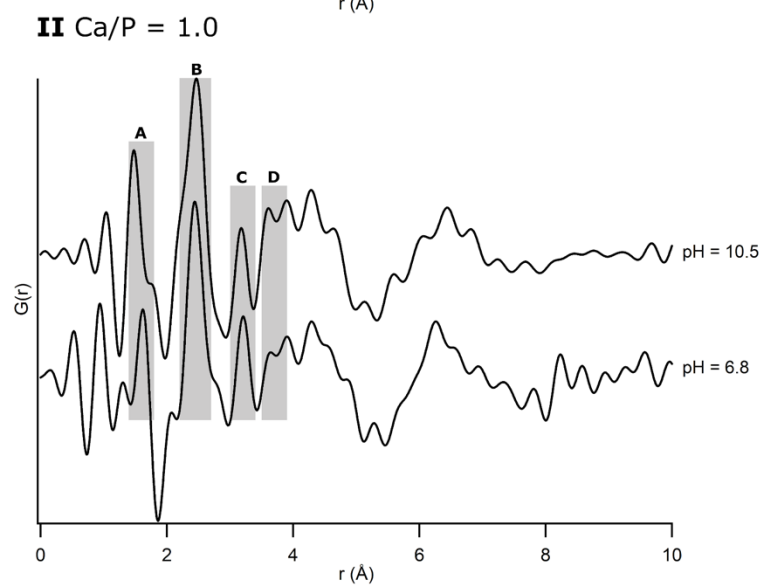
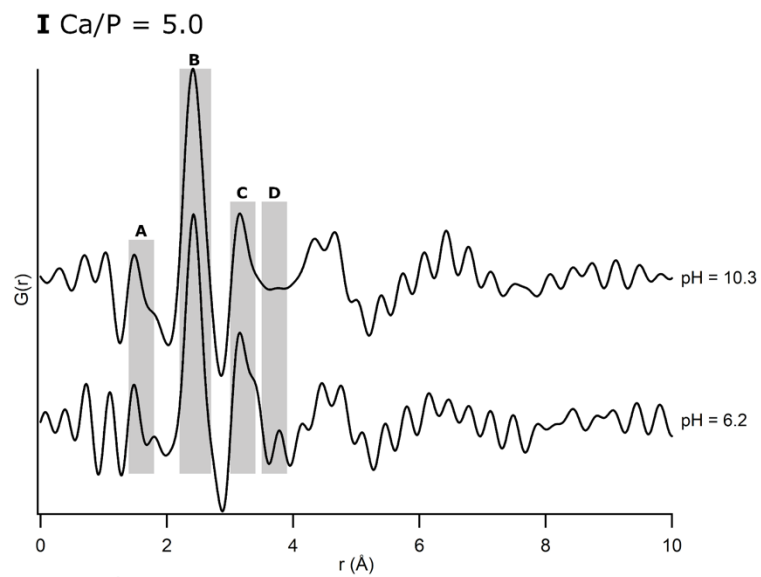


Figure 3.4 The PDF profiles of initial Ca/P 5.0, 1.0, and 0.2 are shown in I, II, and III. The structure of the solid at each initial ratio is measured at two different pH values. Samples synthesized at the same Ca/P, but different pH are structurally equivalent. The relative intensities of peaks A, B, C, and D do not change as a function of varying pH. Similar to what is seen in Figure 2, as the Ca/P increases, peak A and peak D decrease in intensity, while peak C increases.

SAXS ANALYSIS

Volume-weighted size distribution ($D_v(R)$) analysis of the background-subtracted SAXS profiles indicate differences with Ca/P (**Figure 3.5**). The most notable trend is the corresponding increase between the Ca/P and both the average and most frequent particle radius. The most intense feature in the $D_v(R)$ histograms corresponds with the most frequent particle size for each sample. As shown in **Table 3.2**, the most frequent size increases approximately two-fold from ratio 0.2 to 1.0. This trend is observed again between ratios 1.0 and 5.0. The average particle radius shows an even higher-fold increase when the Ca/P is raised. The variations in average sizes for the two higher ion ratios (1.0 and 5.0) is correlated with changes in the overall particle size distribution. For example, the $D_v(R)$ histogram for the 1.0 ratio shows additional minor populations of particles with radii of approximately 4 and 8 nm. Similarly, the histogram for the 5.0 ratio has a main feature that is more lognormal, as well as larger two populations of 8 and 15 nm particles (**Figure 3.5**, full range not shown). This overall increase in the particle size distribution with Ca/P is also evident in the cumulative undersize distributions for ratios 1.0 and 5.0 shown in **Figure 3.5** and in **Table 3.2**. In contrast, the average and most frequent sizes observed for the 0.2 sample are almost identical, which indicates a highly monodisperse population of particles. SAXS experiments at ratios 1.0 and 5.0 are expected to have some proportion of brushite in addition to ACP. LCF of *in situ* PDF data indicates that the proportion of brushite is ~36% in ratio 1.0. Although some variation in the brushite fraction would be expected in repeat experiments, its presence may have some impact on ACP size estimates as certain Miller indices (e.g., (020) and (011)) for brushite have d-spacings that are within the range of sizes estimated from SAXS.

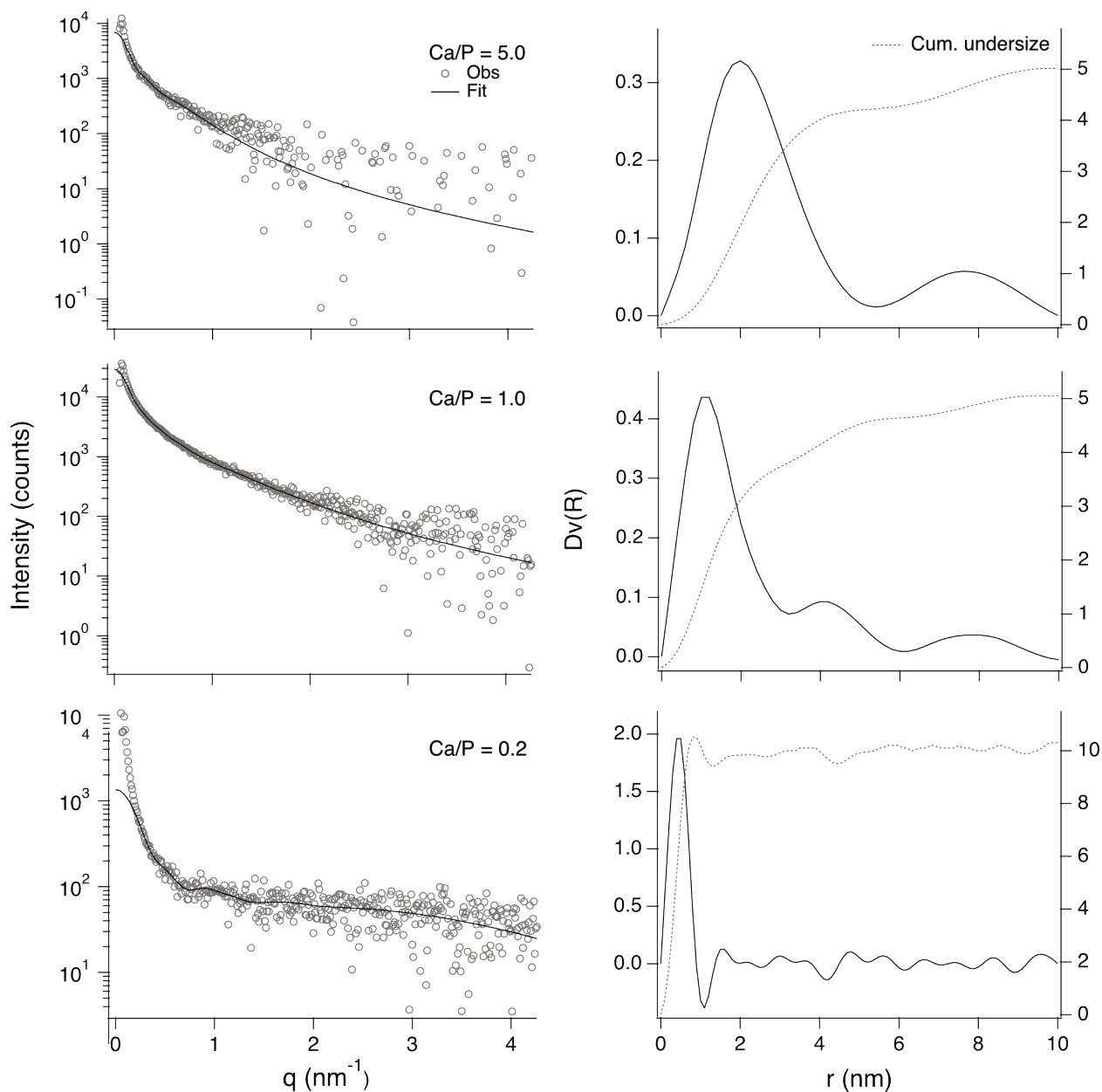


Figure 3.5 (Left) Background-subtracted SAXS profiles (open circles) and corresponding $Dv(R)$ fits (solid lines) for experiments conducted at Ca/P ion ratios of 5.0, 1.0, and 0.2. (Right) Calculated volume-weighted size distribution histograms (solid lines) and cumulative undersize distributions (dashed lines).

Table 3.2 The average radius and most frequent radius values for all three Ca/P examined with SAXS. There is a corresponding increase between the Ca/P and the particle average and most frequent radius. Also reported is the radius where 20, 50, and 80% (R20, R50, and R80 respectively) of the particles are of that size or smaller.

Ca/P	0.2	1.0	5.0
Average Radius (nm)	0.45	2.44	3.15
Most Frequent Radius (nm)	0.4	1.0	2.0
R20 (nm)	0.3	0.9	1.5
R50 (nm)	0.4	1.7	2.5
R80 (nm)	0.6	3.9	4.1

ICP-AES ANALYSIS

The Ca/P of the sample precipitate is calculated from the ion concentrations collected through ICP-AES (**Supplemental Table B-1**) and averaged together across the three samples analyzed at each initial ratio. A Ca/P of 0.98 (± 0.08), 1.1 (± 0.001), and 1.2 (± 0.04) is reported for the precipitate of initial ion ratios 0.2, 1.0, and 5.0 respectively (standard error reported).

POWDER XRD ANALYSIS OF TRANSFORMATION PRODUCTS

After 30 mins to 24 hrs, ACP transformation to crystalline calcium phosphate minerals was observed. The XRD profiles of the Ca/P 0.2, 1.0, and 5.0 syntheses vary significantly from one another, both at 30 minutes and 24 hrs after initial synthesis (See Appendix B, **Figure B-4**). Experiments at Ca/P = 0.2 form a poorly crystalline hydroxylapatite, while ratios 1.0 and 5.0 show a mix of disordered hydroxylapatite and crystalline brushite (**Table 3.3**).

Rietveld analysis shows how the phase fractions for hydroxylapatite and brushite change for these samples during aging (See Appendix B, **Figure B-5**). As shown in **Table 3.3**, brushite is the dominant phase for ratio 1.0 in the early stages of aging but becomes less abundant with time as the fraction of hydroxylapatite increases. In contrast, hydroxylapatite is initially the dominant phase in ratio 5.0 and the fraction of brushite increases with aging. The average hydroxylapatite size for all samples is 8 (± 1) nm by 27 (± 3) nm (error is one standard deviation).

Table 3.3 Phase fractions of hydroxylapatite and brushite from Rietveld analysis. Brushite is not added as a phase for sample 0.2 and is assumed to be zero.

Ca/P	Age (hours)	Hydroxylapatite (%)	Brushite (%)
0.2	0.5	100	-
0.2	24	100	-
1.0	0.5	34	66
1.0	24	62	38
5.0	0.5	97	3
5.0	24	83	17

DISCUSSION

This study provides the first structural evidence for variations in ACP structure based on initial solution chemistry, which are identified through the variations in PDF profiles in **Figure 3.2**. The changing Ca/P is reflected in the varying relative intensities of peaks A and B. Peak A is centered at about 1.5 Å, which is the same as the P-O bond distance. Peak B, at 2.4 Å, correlates with the average Ca-O bond distance. As the initial Ca²⁺ concentration increases relative to the overall amount of phosphate in the system, there is a corresponding decrease in the intensity of the P-O peak.

To identify the ion pair contributions that are associated with the peaks C and D, we examined the partial PDF contributions for an *in situ* crystalline brushite we synthesized (**Figure 3.6**). There are two different contributions to peak C from O-O and Ca-P pairs. Peak D has several different ion pair contributions, but also has a contributing intensity from Ca-P pairs. Because we are varying the Ca/P we interpret the variations in intensity for peaks C and D to be a function of Ca-P pair contributions. This is a direct link between ion ratio and the structural information presented in the PDF profile. While changes in peaks A and B can be attributed to system chemistry and the total amount of calcium and phosphate in the system, variations in C and D represent structural changes in the precipitate.

Peak C represents the Ca-P pair distance between the ions when they share two oxygens in a bidentate bonding geometry. A monodentate bonding geometry has a longer pair distance that corresponds to peak D in both **Figure 3.2** and **Figure 3.6**. This means that at the lowest Ca/P the Ca – P bonding geometry is predominantly monodentate. As the ratio increases there is a mixture of both monodentate and bidentate, but at the highest ratio, the bonding geometry is

predominantly bidentate. The PDF profile beyond peak D in **Figure 3.2** shows broad peaks and artifacts of noise from the Fourier transform. Due to the multiple contributions of different ion pairs and the noise, we limit our structural interpretations to these four peaks.

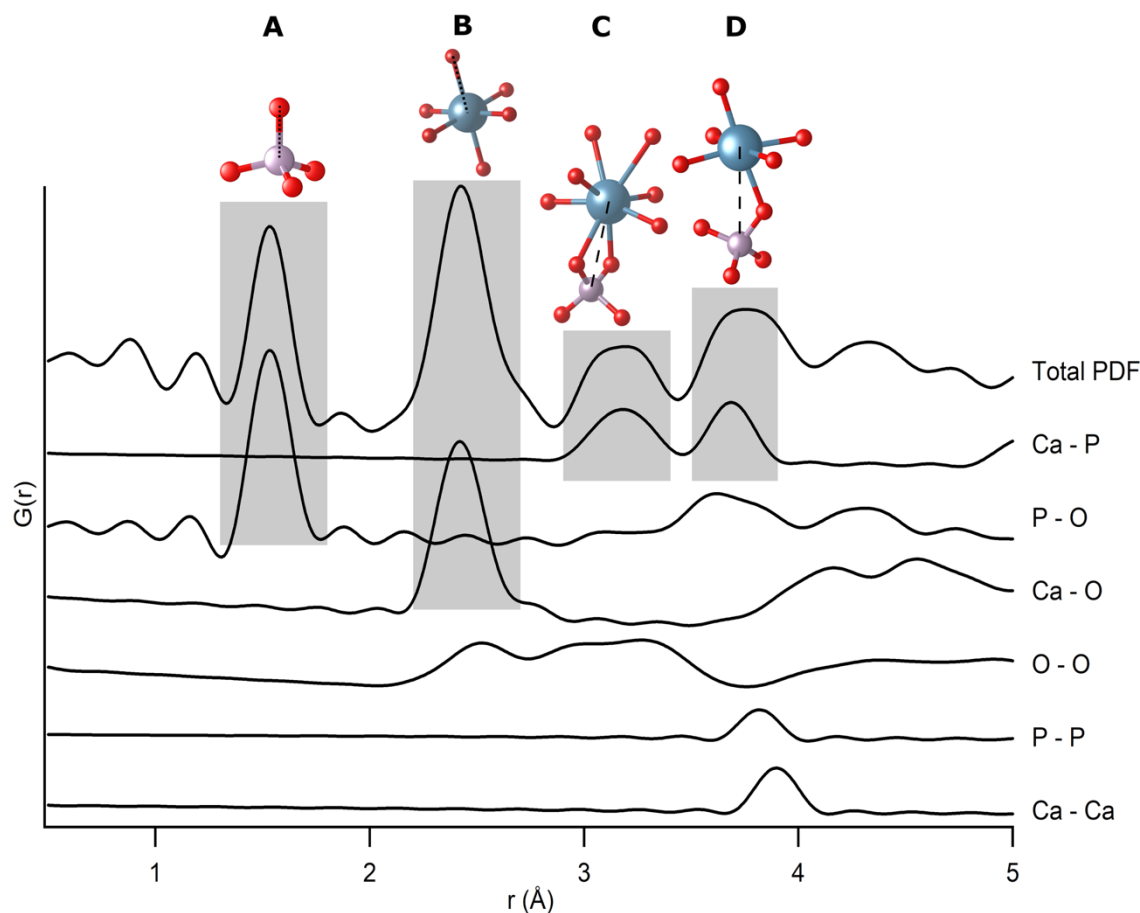


Figure 3.6 The total PDF of a crystalline brushite synthesized *in situ*, is shown along with partial PDFs of contributing ion pairs in the structure. Peak A represents the P – O bond distance and Peak B the Ca – O bond distance. Peaks C and D are dominantly Ca – P distances associated with bidentate and monodentate bonding geometries respectively. Peaks A, B, C, and D are the same locations as in Figures 2 and 3, therefore we interpret the structure of ACP in this range with the atom pair contributions seen here in brushite.

At a circumneutral pH, samples synthesized with intermediate ratios of 1.0 and 1.6 are the only samples to have visible structure beyond the first 8 $\text{\AA}$ in the PDF, though the $I(q)$ shows

the sample at 5.0 is partially brushite. Recall that for all samples, there is a five-minute residence time between the initial mixing of the calcium and phosphate solutions and the sample precipitate flowing through the X-ray beam for data collection. Residence time is rigidly controlled by the reactor volume and pumping speed. Thus, the increase in brushite at higher Ca/P must be the result of a more rapid rate of transformation. Multiple studies for various minerals show that when a wide range of ion ratios are compared, rates of crystallization are most rapid at or near to 1.0 (Bijma *et al.*, 2007; Lin & Singer, 2005; Stack & Grantham, 2010; Bracco *et al.*, 2016). While these references examine growth on preexisting mineral surfaces, the rate of calcium phosphate precipitation in solutions inferred by our structural data support this hypothesis. As brushite is forming at Ca/P = 5.0, but not at Ca/P = 0.2, the calcium limited system appears more effective for preventing crystallization.

In **Figure 3.4** the extended structure past 8 Å for ratio 1.0 is not present at a higher pH, but that is an anticipated result of slower transformations at higher pH as discussed above. Samples synthesized at a higher pH show no significant alteration of the monodentate – bidentate relationship when compared to the lower pH counterpart. When the system contains excess phosphate, the Ca-PO₄ bonding geometry is predominantly monodentate. The average Ca-O peak position for Ca/P = 0.2 is 2.47 Å, compared to 2.41 Å for Ca/P = 5.0. The longer average bond distance at lower Ca/P is expected for bidentate geometries. In contrast, excess calcium produces bidentate bonding geometries. Zhang *et al.* (2016) suggests that low Ca/P preferentially form bidentate geometries when comparing results with Ca/P = 0.5, 1.0, and 1.5. Note that the two studies are not directly comparable, as Zhang *et al.* (2016) use both a fixed calcium with variable phosphate concentration and a fixed phosphate and variable calcium concentration to obtain their initial ratios. This produces a greater range in supersaturation variation across their ratios. They evaluate the reaction rates at each ratio as a function of the volume of precipitate and note that the least precipitate forms at the lowest ratios. They hypothesize that bidentate geometries are less reactive than monodentate, and thus there would be more bidentate geometries at the lower ratio, with the slower rate. In contrast we note more rapid transformation at intermediate ratios where we see a mix of monodentate and bidentate geometries.

Both hydroxylapatite and brushite have only one phosphate environment in their structure. There are both bidentate and monodentate geometries associated with that environment for both crystalline phases. The phosphate environment in brushite, however, has a bidentate

geometry with two separate calcium ions, while hydroxylapatite has a bidentate geometry with a single calcium ion. In contrast only one of the phosphate oxygens in brushite has a monodentate geometry with respect to Ca, while phosphate oxygens in hydroxylapatite are shared between several Ca (See Appendix B, **Figure B-6**). The PDF samples at ratios 1.0 and above show the strongest contributions of bidentate geometries and already exhibit the development of brushite in the I(q) (**Figure 3.3**). The Rietveld analysis shows the brushite beyond the first 5 minutes, with it still being present after 24 hours for both ratios 1.0 and 5.0. Brushite left in solution will transform over time into hydroxylapatite, which is clearly seen in Rietveld results for ratio 1.0 with development of more apatite at 24 hours than at 30 minutes, but a complete transformation can take days (Borkiewicz *et al.*, 2010). As the reverse transformation is not thermodynamically favored, the relative increase in the fraction of brushite from 0.5 to 24 h in ratio 5.0 is likely caused by the transformation of residual ACP to brushite, which will also eventually transform to hydroxylapatite. Further experiments are required to determine the transformation behavior. Ratio 0.2 shows no evidence of brushite formation and the profile only exhibits contributions from poorly crystalline hydroxylapatite.

The lowest Ca/P of 0.2 dominantly has monodentate Ca – P bonding geometries and evolves directly to hydroxylapatite, without any evidence of brushite seen in the PDF or XRD profiles. Higher ratios, where additional bidentate geometries are identified in the PDF, form brushite first, which of the two crystalline phases, has the greater number of bidentate geometries. Altering the initial ion ratios adjusts not just the short-range order of the initial precipitates and the rate of crystallization, it also impacts the transformation pathway of the sample. Lowering the initial ratio allows ACP to transform directly to hydroxylapatite at a biologically relevant pH range, without the addition of stabilizing ions like magnesium.

While our initial ion ratios had a wide variation, the ion ratio of the precipitate was close to 1.0, as reported in **Table 3.3**. Brushite has a Ca/P = 1.0, so this was an expected result for the initial 1.0 ratio. Literature reports of Ca/P for ACP precipitates are commonly ~1.5, though samples synthesized at lower pH ranges with more HPO_4^{2-} than PO_4^{3-} have been reported with lower ratios (Combes & Rey, 2010). The precipitate ratio of 1.2 for the initial ratio of 5.0 is slightly closer to the standard reports for ACP and may be a factor of the mix of brushite and ACP identified in the I(q) and/or the phosphate speciation. The initial ratio of 0.2 has the greatest mismatch to its correlated precipitate ratio of 0.98, despite no evidence of brushite. The slower

rate of transformation at this initial ratio may correlate with the sample being less developed and having a lower Ca/P in the precipitate. The precipitate composition for all three initial conditions differ significantly from Ca/P measured in the mineral components of bone and enamel, which is typically ~1.6 and close to that of hydroxylapatite (Legros *et al.*, 1987; Bocchi & Valdre, 1993; Gross & Berndt, 2002).

Precipitates at ratios 0.2, 1.0, and 5.0 differ in terms of size and size distribution. We interpret the most frequent particle size (**Table 3.2**) as corresponding with the average physical dimensions of the primary particles in each sample. Assuming a spherical shape, the diameters of the primary particles in samples 0.2, 1.0, and 5.0 are 0.8, 2.0, and 4.0 nm, respectively. These primary particle sizes can be compared to the CSD size, which is the maximum range of correlations observed in the PDF. The CSD size is an indication of the degree of short- to intermediate-range atomic structural order (Reeder & Michel, 2013). The CSD size of the 0.2 and 5.0 ratios are similar at ~0.8 nm (**Figure 3.2**). Interestingly, the CSD size and the primary particle size of the 0.2 ratio are approximately identical (within respective error estimates). This suggests that, on average, the primary particles are structurally ordered. In contrast, the primary particle size of the 5.0 ratio is more than four times larger than the CSD size. This decoupling between the average physical dimension of the particles and CSD size may indicate increased structural disorder or an aggregated network of structured primary particles, approximately four particles in diameter. Sample 1.0 also shows a discrepancy, although it is difficult to compare the CSD size and primary size directly because this sample is partially crystallized, as evidenced by higher *r*-correlations in the PDF (**Figure 3.2**) and Bragg peaks corresponding with brushite in the *I*(*Q*) data (**Figure 3.3**).

The majority of studies investigating the size of primary calcium phosphate particles utilize either transmission electron microscopy (TEM) or dynamic light scattering (DLS) and primary particle sizes of 1-2 nm have been reported using both techniques (Oyane *et al.*, 2003; Habraken *et al.*, 2013). These align reasonably well with the most frequent particle sizes from the SAXS analysis reported here. Larger sizes between 20 and 100 nm are reported and commonly seen in TEM with disordered aggregates of spheroids (Christoffersen *et al.*, 1989; Kim *et al.*, 2004). The secondary particle size distributions we see in the volume-weighted size distribution histograms (**Figure 3.5**) is likely due to aggregation of the primary spheroids as seen in some TEM images, which generates larger, but still disordered, particles.

As mentioned above, previous studies have suggested that particle size is linked to rate of ACP transformation (Blumenthal *et al.*, 1972b; Kim *et al.*, 2004). We see an increase in particle size with ratio, but the samples at intermediate ratios are transforming into crystalline products more rapidly than at Ca/P = 0.2 or 5.0. Our results currently have no correlation between rate and size and cannot be compared with the proposed theories in the literature.

ACP and crystalline calcium phosphates can synthesize under a wide range of supersaturations, ion ratios, and source chemicals for the calcium and phosphate components (Koutsopoulos, 2002; Combes & Rey, 2010), yet results are often compared indiscriminately. This is counterintuitive to previous suggestions that there may be different types of ACP. Christoffersen *et al.* (1989) shows two forms of ACP, differentiating based on morphological differences identified with transmission electron microscopy images, despite both phases appearing amorphous in electron diffraction. Due to the amorphous signal obtained with X-ray diffraction, amorphous calcium phosphates are commonly treated as equivalent, regardless of the synthesis method. This study shows that small variations in the system chemistry can produce materials with different structures.

This paper identifies that varying the ratio of the initial solution can be used to adjust the kinetic rate and pathway of transformation for a sample. Raising or lowering the initial ion ratio can be used to synthesize ACP without the rapid crystallization seen at intermediate ratios. It also controls the pathway of transformation, with ACP transforming directly to hydroxylapatite at a pH of 6-7 and low ion ratio of 0.2. Previous literature that directly investigates ACP or examines ACP to hydroxylapatite transformation has commonly done so by adding stabilizing ions and/or raising the pH above 9 (Termine & Posner, 1970; Boskey & Posner, 1973; Blumenthal *et al.*, 1972b, 1977). Our results suggest that lowering the Ca/P has a similar effect on the system and does not raise the pH above biologically relevant levels. Additional studies to evaluate the impact of varying concentrations, temperature, and sources of calcium and phosphate ions on the structure of metastable calcium phosphates would be valuable.

CONCLUSIONS

Variations in the bonding geometry between calcium and phosphate in ACP is a structural biproduct of the initial Ca/P when samples are precipitated out of a supersaturated solution. Across the range of ratios investigated, samples went from having primarily

monodentate bonding geometries at low ratios, to primarily bidentate bonding geometries at high ratios, with a mixture in the middle. The initial Ca/P also influences the rate of crystallization and the precipitate particle size but has little impact on the Ca/P of the precipitate. When ACP shows only monodentate bonding geometries, it transforms directly to hydroxylapatite, without any brushite formation. Samples with bidentate geometries form brushite as a first crystalline phase, with hydroxylapatite developing over time.

We present the first structural variations in ACP linked to the initial synthesis conditions and demonstrate the impact of variations on the role of ACP as a precursor to crystalline calcium phosphates. Adjusting the initial ion ratio to 0.2 slowed the transformation of ACP to hydroxylapatite and shows no evidence of the formation of brushite, despite precipitation occurring at a circumneutral pH. This manipulation could be an ideal technique for further investigations of the direct relationship between ACP and hydroxylapatite without the addition of stabilizing ions or raising the pH above biologically relevant conditions.

The literature provides numerous methods to synthesize ACP and crystalline calcium phosphates, but the potential impacts of variations in system chemistry in many cases is not fully explored. Further work, especially *in situ* structural analysis, to examine the effects of temperature, supersaturation, additional ions, and other variables is needed to provide a comprehensive view of controls on the calcium phosphate system. The current lack of understanding combined with wide variety of methods makes it difficult to directly compare results in the literature. Greater insight into the controls of system chemistry on the synthesis and transformation of ACP will hopefully improve our understanding of biomineralization and assist in the design of biocompatible materials for prosthetic development.

CHAPTER 4: STATISTICAL INSIGHT INTO KEY VARIABLES FOR CALCIUM PHOSPHATE SYNTHESIS

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ABSTRACT

Amorphous and crystalline calcium phosphates can form under a wide range of synthesis conditions. Some chemistry – phase properties, such as brushite formation at pH 6-7, are well documented in literature. However, a comprehensive understanding of critical variables and their relationship with amorphous calcium phosphate (ACP), brushite, and hydroxylapatite formation have yet to be established. Binary logistic regression analysis and nonlinear continuous logistic regression analysis are used to evaluate which synthesis parameters have a significant impact on a database compiled from literature. The critical variables for ACP are age, pH, temperature, and supersaturation; for brushite are age, pH, and temperature; and for hydroxylapatite are age, temperature, and supersaturation. Equations for each regression analysis predict the presence of a precipitated phase (binary) or phase percentage (continuous). When compared with the recorded results, the continuous analysis equations predicted 93% of the variance for ACP, 83% for brushite and 58% for hydroxylapatite. In the database, hydroxylapatite is a secondary phase, forming as a transformation product of either ACP or brushite. We hypothesize that the impact of the independent variables is pathway dependent and additional databases should be developed in future work to develop pathway specific equations for hydroxylapatite and evaluate other calcium phosphate phases. The equations presented here for ACP and brushite will allow researchers to predict the amount of calcium phosphate phase to form for a specific set of synthesis conditions, allowing for more experimental control.

INTRODUCTION

Studies of hydroxylapatite formation in solution, originally performed as proxies for bone growth, demonstrate that other calcium phosphate phases such as amorphous calcium phosphate

(ACP) or brushite can precipitate first and transform into hydroxylapatite over time (Eanes *et al.*, 1965; Eanes & Posner, 1965; Meyer & Eanes, 1978; Borkiewicz *et al.*, 2010; Shamrai *et al.*, 2015; Ito *et al.*, 2014). This has inspired numerous investigations into different pathways of calcium phosphate synthesis and mechanisms to control the formation and transformation of specific phases. Natural and synthetic hydroxylapatite is similar in structure and composition to bone mineral, although a separate and distinct chemistry for bone has been defined (Pasteris *et al.*, 2014). Investigations of synthetic calcium phosphate minerals and their applications for understanding and stimulating bone growth are a major research focus in the past century (Albee & Morrison, 1920).

For some calcium phosphates, relationships between synthesis conditions and phase formation are established. When synthesis occurs in a low temperature, supersaturated, aqueous solution, ACP can form before any crystalline phases are detected (Eanes *et al.*, 1965; Eanes & Posner, 1965). ACP will transform into a crystalline calcium phosphate unless stabilized, commonly achieved by drying, raising the pH above 9, or adding magnesium ions (Termine & Posner, 1970; Blumenthal *et al.*, 1977; Kibalczyk *et al.*, 1990; Liu *et al.*, 2001; Othman *et al.*, 2016). It is also established that brushite precipitates readily in a supersaturated aqueous solution with a circumneutral pH (Lopez-Valero *et al.*, 1992; Borkiewicz *et al.*, 2010; Shamrai *et al.*, 2015). While the impact of pH is well understood for both of these examples and is likely related to variations in solubility in calcium phosphates phases (De Rooij *et al.*, 1984), other synthesis conditions such as initial ion ratio have yet to be fully examined (see Chapter 3).

The impacts of synthesis conditions on phase formation and transformation are primarily experimentally determined. Studies typically investigate one or two variables at a time in relation to specific calcium phosphate phases. Additionally a significant number of studies examine the effects of adding proteins or additional ions of interest (Termine *et al.*, 1970a; Blumenthal *et al.*, 1977; Lopez-Valero *et al.*, 1992; Boanini *et al.*, 2010; Zhao *et al.*, 2016). Without a fundamental understanding of the controls on the base system it can be difficult to compare the results of these experiments that sometimes vary widely in their initial synthesis conditions.

This research identifies key variables that control the formation of ACP, brushite, and hydroxylapatite. Through a comprehensive literature review I quantitatively evaluate the impacts of five primary parameters on the formation of calcium phosphates with multivariate logistic

analysis. I hypothesize that sample age, mixing pH, initial ion ratio, temperature, and supersaturation control calcium phosphate formation. The addition of organic molecules or other additives are not considered. Experimentally established relationships between variables and phases, such as the link between brushite and pH, are used to judge the validity of the models.

The analysis shows ACP formation is controlled by sample age, pH, temperature, and supersaturation, and brushite formation is controlled by sample age, pH, and temperature. Within the database parameters, ACP formation is favored at higher pH and lower temperature, sample age, and supersaturation. Brushite formation is favored at lower pH, temperature, and sample age. Predictive equations explain 93% of the variance between the model and the database results for ACP and 83% for brushite (92% when only considering temperatures below 80 °C). Age, temperature, and supersaturation all have a significant impact on hydroxylapatite, but only explained 60% of the variance. In this database hydroxylapatite is a secondary phase that formed after the initial precipitation of either ACP or brushite. The evolution pathway impacts the regression analysis and separate analyses are needed for ACP to hydroxylapatite and brushite to hydroxylapatite models.

METHODS

DATABASE CRITERIA

Various synthesis methods for crystalline and amorphous calcium phosphate phases were identified in the literature. The five chosen synthesis parameters, sample age, mixing pH, initial ion ratio, temperature, and supersaturation, are the most commonly investigated and reported variables in mineral growth experiments. A database of 139 samples was compiled from four sources that quantified the phase distribution of calcium phosphate phases and reported the solution volume of their reactants or provided supersaturation information. (**Appendix C, Table C-1**). When supersaturation was not provided, this was determined using Geochemist's Workbench (Aqueous Solutions LLC, Champaign, IL USA) to calculate the ionic strength of each solution. Most of the ionic strength values fell within the range valid for the Davies equation, which was used to calculate the ion activity coefficients for Ca^{2+} and HPO_4^{2-} (Langmuir, 1997). The dielectric constant of water (79.9) was calculated using equation 4.29 on page 128 of Langmuir (1997) and then used to determine the constant A (0.506) in the Davies equation (assuming a negligible temperature effect on the density of water in the range of studied

conditions because the actual range in density is between 1.0 and 0.96 g/cm³). Ion activity products for brushite and hydroxylapatite were taken from Jaynes *et al.* (1999) and amorphous calcium phosphate were taken from Meyer & Eanes (1978) and used to calculate supersaturation indices. The contributions of solutions used to adjust the pH were not included in the supersaturation calculation because the additional volume of these solutions was commonly not reported. **Table C-2 in Appendix C** reports the calculated ionic strengths, activity coefficients, activities, and ion activity products for each sample. Phase percentage quantifications were extracted by hand from figures in both Borkiewicz *et al.* (2010) and Boskey & Posner (1973). The error is $\pm 2.5\%$ for Borkiewicz *et al.* (2010) and $\pm 1\%$ for Boskey & Posner (1973) based on the grid spacing overlain on the figures to extract the data values. We limited our statistical analysis to three major phases formed at the tested conditions: amorphous calcium phosphate, brushite and hydroxylapatite.

STATISTICAL ANALYSES

JMP Pro 15.0.0 (© SAS Institute Inc.) was used to perform multivariate logistic analysis for amorphous calcium phosphate, brushite, and hydroxylapatite. We performed two types of statistical data analysis: 1) binary, which aimed at understanding whether a certain product formed ('1') or did not form ('0') at each set of conditions, and 2) continuous, which aimed at quantifying the effects of the different variables on the percentage of each product observed for each sample. For binary analysis, a phase was only considered to have formed if its product fraction was $> 5\%$, due to the error in percentage determination discussed above and confidence uncertainties for Rietveld analysis (Toby, 2008). In both cases, we evaluated the contributions of supersaturation, mixing pH, temperature (°C), sample age (min) and initial Ca/P to explain the formation of each reaction product at a level of confidence of 95% (unless otherwise noted). For both binary and continuous analysis, a logistic function (equation 1) was used. The function creates a prediction (P) about the presence (binary) or quantified percentage (continuous) of product observed at a given set of conditions (Reed & Berkson, 1929).

$$P = \frac{1}{1 + e^{-x}} \quad (1)$$

In the case of multiple independent variables equation 1 acquires the form of equation 2, where the effect of one single variable is replaced by the sum of the independent effects of each tested variable (Freedman, 2009).

$$P = \frac{100}{1 + e^{-b_0 + b_1 \cdot Age + b_2 \cdot pH + b_3 \cdot Temp + b_4 \cdot \Omega + b_5 \cdot Ca/P}} \quad (2)$$

This equation has one dependent variable (P), five independent variables (age, pH, Temperature, supersaturation and Ca/P), and six regressed coefficients b_0 , b_1 , b_2 , b_3 , b_4 , and b_5 .

For all three phases we initially included all five independent variables in the regression analysis. Variables that did not have a statistically significant effect (p-value >0.05) were removed from the model. Variables were removed one at a time and if multiple variables were not statistically significant, the one with the larger p-value was removed first and the regression was rerun. This was repeated until only statistically significant independent variables remained.

RESULTS

BINARY LOGISTIC REGRESSION ANALYSIS

The calculated coefficients and their associated p-values for the binary logistic analysis are reported in **Table 4.1**. Age and temperature were statistically significant for all three phases. Brushite formation is also controlled by pH, hydroxylapatite is also controlled by supersaturation, and ACP is also controlled by pH and supersaturation. The initial ion ratio did not have a statistically significant effect on any of the phases. Reformatting equation 2 to include only the significant independent variables and their calculated coefficients produces equation 3 (ACP), equation 4 (brushite), and equation 5 (hydroxylapatite). These new equations are used to calculate the probability (P) that each phase formed for each set of tested conditions. In the binary predictions, if $P > 0.5$ it is a positive outcome and the phase is considered to be present. **Table 4.2** compares predicted values from equations 3-5 with recorded values in the dataset. The binary logistic analysis describes 81% of the variance between the predicted and recorded values for ACP, 84% for brushite, and 60% for hydroxylapatite.

Table 4.1 The results of the regression equations for binary logistic analysis with contributions of different independent variables for ACP, brushite (Br), and hydroxylapatite (HAP). P-values >0.05 are identified in red. Variables were removed from the regression equation individually until only statistically significant parameters remained. The bold parameters are used in equations 3-5.

Variables Considered in Model		b_0	p-value	b_1	p-value	b_2	p-value	b_3	p-value	b_4	p-value	b_5	p-value
<i>Binary ACP</i>													
1	Age, pH, Temp, Ω , Ca/P	38 ± 16	0.01	-0.03 ± 0.01	0.001	12 ± 3.9	0.002	-0.17 ± 0.07	0.01	-3.8 ± 1.4	0.005	-1.1 ± 1.8	0.55
2	Age, pH, Temp, Ω	39 ± 15	0.01	-0.03 ± 0.01	0.001	12 ± 3.8	0.002	-0.18 ± 0.07	0.01	-3.9 ± 1.3	0.004	-	-
<i>Binary Br</i>													
1	Age, pH, Temp, Ω , Ca/P	53 ± 60	0.37	-0.05 ± 0.02	0.02	-14 ± 8.7	0.10	-0.30 ± 0.12	0.01	20 ± 18	0.28	-	0.80
2	Age, pH, Temp, Ω	88 ± 93	0.34	-0.05 ± 0.02	0.01	-17 ± 11	0.10	-0.31 ± 0.12	0.008	20 ± 15	0.19	-	-
3	Age, pH, Temp	290 ± 130	0.02	-0.05 ± 0.02	0.01	-40 ± 18	0.02	-0.35 ± 0.13	0.009	-	-	-	-
<i>Binary HAP</i>													
1	Age, pH, Temp, Ω , Ca/P	27 ± 18	0.13	0.05 ± 0.01	<0.0001	0.81 ± 1.7	0.64	0.04 ± 0.02	0.06	-0.53 ± 0.44	0.23	0.48 ± 1.2	0.68
2	Age, pH, Temp, Ω	23 ± 14	0.12	0.05 ± 0.01	<0.0001	0.35 ± 1.3	0.78	0.04 ± 0.02	0.04	-0.41 ± 0.33	0.21	-	-
3	Age, Temp, Ω	20 ± 8.4	0.02	0.05 ± 0.01	<0.0001	-	-	0.04 ± 0.02	0.03	-0.33 ± 0.12	0.007	-	-

$$P_{ACP \text{ Binary}} = \frac{100}{1 + e^{-39 - 0.03 \cdot Age + 12 \cdot pH - 0.18 \cdot Temp - 3.9 \cdot \Omega}} \quad (3)$$

$$P_{Br \text{ Binary}} = \frac{100}{1 + e^{-290 - 0.05 \cdot Age - 40 \cdot pH - 0.35 \cdot Temp}} \quad (4)$$

$$P_{HAP \text{ Binary}} = \frac{100}{1 + e^{-20 + 0.05 \cdot Age + 0.04 \cdot Temp - 0.33 \cdot \Omega}} \quad (5)$$

Table 4.2. A comparison of the predicted and experimentally determined formation of ACP, brushite, and hydroxylapatite in the binary dataset, based on equations 3-5.

		Predicted	Determined Experimentally	Error %	R ²
ACP	Formed	58	60	3.3	0.81
	Did not form	77	79	2.5	
Brushite	Formed	39	41	4.9	0.84
	Did not form	97	98	1.0	
Hydroxylapatite	Formed	106	108	1.9	0.6
	Did not form	24	31	23	

Equation 3 demonstrates that within the ranges examined, ACP formation is preferable at lower temperatures, supersaturations and age ranges, but at higher pH. Results suggest that optimal conditions for ACP formation are at temperatures below 26 °C, a pH above 8, age less than 250 minutes, and a supersaturation below 30.7 (**Figure C-1**). Equation 4 indicates that brushite formation is preferable at low pH, temperature, and age, optimally at a pH below 6.7, temperatures below 27 °C, and an age less than 100 minutes (**Figure C-2**). Equation 5 shows that hydroxylapatite formation (via precursor) is preferable at a lower supersaturation, but higher temperature and age. Optimal conditions for hydroxylapatite formation are at supersaturation below 70, a temperature above 28 °C, and an age greater than 55 minutes (**Figure C-3**).

NONLINEAR CONTINUOUS LOGISTIC REGRESSION ANALYSIS

The calculated coefficients and their associated p-values for the nonlinear continuous logistic analysis are reported in **Table 4.3**. The statistically significant independent variables for each phase are the same as in the binary analysis. Equations 6-8 are used to calculate the predicted percentage of ACP, brushite, and hydroxylapatite formation based on the key parameters. **Figure 4.1** compares the predicted percentages with the recorded percentages in the dataset. The continuous logistic analysis describes 94% of the variance between the predicted and recorded values for ACP, 83% for brushite, and 58% for hydroxylapatite.

Table 4.3 The results of the regression equations for continuous logistic analysis with contributions of different independent variables for ACP, brushite (Br), and hydroxylapatite (HAP). P-values >0.05 are identified in red. Variables were removed from the regression equation individually until only statistically significant parameters remained. The bold parameters are used in equations 6-8.

Variables Considered in Model		b_0	p-value	b_1	p-value	b_2	p-value	b_3	p-value	b_4	p-value	b_5	p-value
<i>Continuous ACP</i>													
1	Age, pH, Temp, Ω , Ca/P	2.3 $\pm$ 2.6	0.38	-0.03 $\pm$ 0.003	< 0.0001	5.3 $\pm$ 0.60	< 0.0001	-0.10 $\pm$ 0.02	< 0.0001	-1.2 $\pm$ 0.22	< 0.0001	-0.59 $\pm$ 0.45	0.19
2	Age, pH, Temp, Ω	3.5 $\pm$ 2.6	0.18	-0.03 $\pm$ 0.003	< 0.0001	5.3 $\pm$ 0.62	< 0.0001	-0.10 $\pm$ 0.02	< 0.0001	-1.3 $\pm$ 0.22	< 0.0001	-	-
<i>Continuous Br</i>													
1	Age, pH, Temp, Ω , Ca/P	33 $\pm$ 21	0.12	-0.02 $\pm$ 0.002	< 0.0001	-4.9 $\pm$ 2.2	0.03	-0.07 $\pm$ 0.01	< 0.0001	1.3 $\pm$ 2.0	0.52	0.97 $\pm$ 1.5	0.53
2	Age, pH, Temp, Ω	44 $\pm$ 14	0.002	-0.02 $\pm$ 0.002	< 0.0001	-6.1 $\pm$ 1.5	0.0001	-0.07 $\pm$ 0.01	< 0.0001	0.58 $\pm$ 1.5	0.70	-	-
3	Age, pH, Temp	49 $\pm$ 6.7	< 0.0001	-0.02 $\pm$ 0.002	< 0.0001	-6.6 $\pm$ 0.96	< 0.0001	-0.07 $\pm$ 0.01	< 0.0001	-	-	-	-
<i>Continuous HAP</i>													
1	Age, pH, Temp, Ω , Ca/P	18 $\pm$ 7.6	0.02	0.01 $\pm$ 0.001	< 0.0001	1.1 $\pm$ 0.74	0.14	-0.02 $\pm$ 0.009	0.03	-0.39 $\pm$ 0.19	0.04	0.005 $\pm$ 0.46	0.99
2	Age, pH, Temp, Ω	18 $\pm$ 6.4	0.005	0.01 $\pm$ 0.001	< 0.0001	1.1 $\pm$ 0.58	0.06	-0.02 $\pm$ 0.008	0.01	-0.39 $\pm$ 0.15	0.01	-	-
3	Age, Temp, Ω	10 $\pm$ 3.1	0.002	0.01 $\pm$ 0.001	< 0.0001	-	-	-0.04 $\pm$ 0.008	< 0.0001	-0.15 $\pm$ 0.04	0.0002	-	-

$$P_{ACP\ Continuous} = \frac{100}{1 + e^{-3.5 - 0.03 \cdot Age + 5.3 \cdot pH - 0.10 \cdot Temp - 1.3 \cdot \Omega}} \quad (6)$$

$$P_{Br\ Continuous} = \frac{100}{1 + e^{-49 - 0.02 \cdot Age - 6.6 \cdot pH - 0.07 \cdot Temp}} \quad (7)$$

$$P_{HAP\ Continuous} = \frac{100}{1 + e^{-10 + 0.01 \cdot Age - 0.04 \cdot Temp - 0.15 \cdot \Omega}} \quad (8)$$

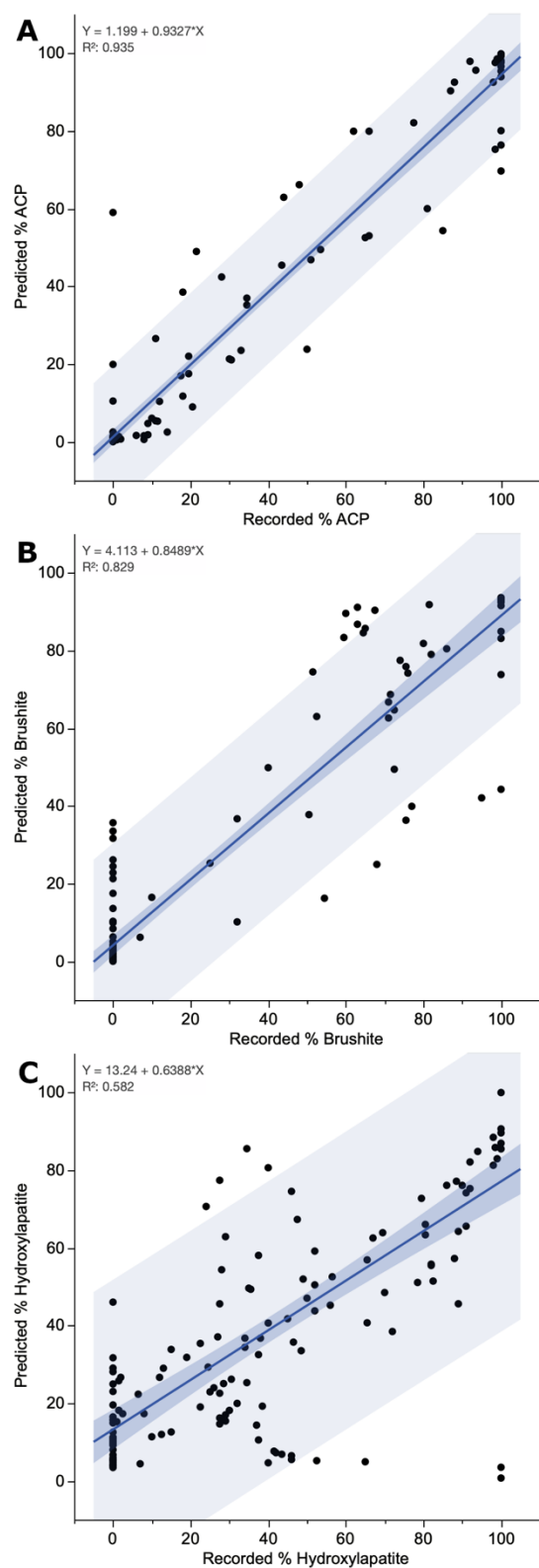


Figure 4.1 Comparisons of the predicted phase percent based on nonlinear regression analysis with the recorded phase percent in the dataset for ACP (**A**), brushite (**B**), and hydroxylapatite

(C). A linear fit is applied to each comparison and the darker shaded region represents the standard error, while the lighter shaded region indicates the standard deviation of each fit. Equations 6-8 were used to calculate the predicted percentages.

The ACP continuous analysis corresponds with the binary analysis and demonstrates that more ACP forms at lower temperatures, supersaturations and age ranges, but higher pH (equation 6). To demonstrate the median range of phase formation, at a temperature of 26 °C, a pH of 9, supersaturation of 30.5, and an age of 300 minutes the predicted amount of ACP in the precipitate is 52% (**Figure C-4**). The brushite continuous and binary analyses also align and there is a greater amount of brushite at low temperature, age, and pH (equation 7). At 29 °C, pH 6.8, and 100 minutes, the predicted percentage of brushite is 52% (**Figure C-5**). Hydroxylapatite binary analysis (equation 5) and continuous analysis (equation 8) vary in the effect of temperature. Binary analysis suggests hydroxylapatite forms at higher temperatures, while continuous analysis demonstrates greater percentages at lower temperatures. Both regression analyses indicate that lower supersaturations and higher ages are optimal. At a supersaturation of 70, 28 °C, and 100 minutes, the predicted percentage of hydroxylapatite is 52% (**Figure C-6**).

DISCUSSION

The relationships between the significant independent variables and ACP and brushite formation demonstrated in both the binary and continuous equations are consistent with the literature (Termine & Posner, 1970; Termine *et al.*, 1970a; Lopez-Valero *et al.*, 1992; Shamrai *et al.*, 2015). Under the examined conditions, ACP and brushite are both transforming into hydroxylapatite over time because it is the most thermodynamically stable phase (Elliott, 1994). This correlates with age being the most significant variable in predicting hydroxylapatite in both the binary and continuous analyses (equations 5 and 8, **Figures C-3** and **C-6**). Kinetics also play a significant role in the reaction. Boskey & Posner, (1976) found direct hydroxylapatite precipitation at low ranges of supersaturation, while at higher supersaturations hydroxylapatite formation is shown to form via a precursor phase (Nancollas & Tomažič, 1974; Feenstra & De Bruyn, 1979). The conditions for hydroxylapatite in our database fall into the higher supersaturation range described by these papers which is consistent with the observed precursor phases. The preference of hydroxylapatite in equation 5 and 8 to synthesize at the lower range of

supersaturation may correlate with the preference of direct hydroxylapatite formation at lower supersaturation described in literature. There is a discrepancy in the influence of temperature on hydroxylapatite formation between the binary and continuous logistic regression analyses. Synthesis at high temperatures ($>90\text{ }^{\circ}\text{C}$) instead of room temperature also produces a more well-crystalline hydroxylapatite (Gross & Berndt, 2002). Kumar *et al.*, (2004) interpreted an increase in crystallinity between $40\text{--}100\text{ }^{\circ}\text{C}$ as an increase in particle size and saw a linear correlation between particle size and temperature, suggesting that hydroxylapatite was forming faster at higher temperatures. Because brushite and ACP transform to hydroxylapatite more rapidly at higher temperatures, the literature overall suggests that higher temperatures favor hydroxylapatite formation (Termine & Posner, 1970; Termine *et al.*, 1970a; Borkiewicz *et al.*, 2010; Shamrai *et al.*, 2015), which contradicts the relationship suggested in equation 8.

When comparing the regression predictions for hydroxylapatite with the recorded values, neither the binary nor continuous regression analysis explain more than 60% of the variance. This suggests that there are additional variables that impact hydroxylapatite formation. Particle size may be a critical parameter, especially if it relates to and resolves the temperature discrepancy discussed above. Additionally, in this dataset hydroxylapatite is a secondary phase that forms through transformation of a primary phase, either brushite or ACP. The reaction kinetics and the identity of the primary phase may be a critical variable for predicting hydroxylapatite formation. Different mechanisms of transformation have been suggested for hydroxylapatite based on the precursor phase (Francis & Webb, 1970; Nancollas & Tomažič, 1974; Christoffersen *et al.*, 1989; Tao *et al.*, 2008). Additional datasets that examine hydroxylapatite formation only through ACP or brushite, or as a primary phase could be used to address this theory.

The regression equations for both ACP and brushite have good prediction capabilities. The nonlinear continuous logistic analysis for ACP explains 93% of the variance between the predicted and recorded phase percentages. Equation 6 is a strong tool for future experimental designs that want to ensure ACP formation. While ACP growth and transformation are a major focus in literature, its metastable nature can make investigations challenging. Utilizing equation 6 to determine the reaction outcome for a set of synthesis conditions will allow for greater experimental control and comparability of results. For this purpose, the continuous regression is more suitable than the binary because it allows quantification of the amount of ACP that a

particular synthesis reaction should form. This can ensure that ACP is a dominant phase in appropriate experiments.

For brushite, 83% of the variance between the predicted and recorded phase percentages is explained by equation 7. It is noteworthy that the regression model for brushite predicted the formation of up to 40% for a number of samples for which brushite was experimentally not detected. Brushite has the largest number of samples in the dataset that have no brushite present: 99 of the 139 samples. From the 99 samples where brushite did not form, 17 were synthesized at a high temperature above 80 °C and the predicted value of 59% of these fell outside of the standard error of the fit. All 17 high temperature samples were taken from Borkiewicz *et al.* (2010a), which recorded a larger quantities of monetite (CaHPO_4) forming above 80 °C than in correlating experiments at lower temperatures. The transformation pathway of brushite is altered at the higher temperature and results in different phase developments than are seen at lower temperatures. This correlates with the idea proposed above for hydroxylapatite, that the transformation pathway impacts the regression analysis. The dataset contains 21 samples that are synthesized at a temperature >80 °C. If these samples are removed from the continuous regression analysis the variance between the predicted and recorded phase percentages improves to 92% (**Figure C-7**).

CONCLUSIONS

This research has provided key insights into the relationship between synthesis chemistry and calcium phosphate phase formation. We have generated equations that can be used to predict the amount of ACP or brushite that will form under a given set of solution conditions. Our statistical analyses correlate with experimental observations of the roll the critical independent variables for both ACP and brushite. ACP formation is favored at a lower supersaturation, temperature and age, but higher pH. Brushite formation is favored at lower temperatures, pH, and age ranges within the conditions investigated. The brushite model does not perform well for samples synthesized above 80 °C, where the transformation pathway is different. Our results did not produce a comprehensive understanding of hydroxylapatite formation. Sample age, temperature, and supersaturation were all statistically significant, but only explained up to 60% of the variance between the predicted and recorded values. Alternative variables than those examined here, such particle size, may be needed to comprehensively understand hydroxylapatite

formation. Additionally, hydroxylapatite synthesis in this database follows two different pathways, through either ACP or brushite. New databases for ACP to hydroxylapatite and brushite to hydroxylapatite specific pathways are needed to evaluate if the equation is pathway dependent.

While understanding the hydroxylapatite system is still under development, this method could also be applied to other calcium phosphates that form as precursors to hydroxylapatite. Octacalcium phosphate, tricalcium phosphate, and α - and β -tetracalcium phosphate have been associated with hydroxylapatite formation (Crane *et al.*, 2006; Borkiewicz *et al.*, 2010; Combes & Rey, 2010; Castro *et al.*, 2012; Habraken *et al.*, 2013). Databases similar to the one compiled here could be used to quantitatively evaluate the impact of initial solution condition on their formation. This would provide a more comprehensive view of chemical controls on calcium phosphates than has previously been compiled.

CHAPTER 5: CONCLUSIONS

The family of crystalline and amorphous calcium phosphate phases related to biomineralization represents a highly complex system. Despite many years of study, the intricate relationships between system chemistry, phase formation, and the mechanisms of transformation are not comprehensively understood. The work presented, has developed a new method to investigate these system dynamics, has identified important chemical – structural relationships in ACP, and has quantified the impact of synthesis conditions with equations that can predict the formation of certain phases. These are critical innovations that have expanded our understanding of calcium phosphate formation, but are also a launching point to develop a comprehensive framework for investigating aqueous calcium phosphate formation in the future.

Chapter 2 introduces a method of *in situ* analysis that furthers investigations of both amorphous and crystalline materials. Utilizing a mixed-flow reactor and flowing the precipitate directly through the beam to collect total X-ray scattering for PDF analysis, enables structural investigations of both crystalline and amorphous phases. This set-up produces a rigorous control of system chemistry and residence time, facilitating correlations between synthesis conditions and phase structure at specific time points. The optimal experimental conditions, including limitations, and guidelines for data extraction are described in detail. The experimental design is optimal for examining ages under 20 minutes and careful collection and precise subtraction of the background is critical. High quality PDF profiles of ACP, amorphous calcium carbonate, and brushite demonstrate the versatility and potential applications of this new method.

Chapter 3 utilizes the method developed in Chapter 2 to examine the influence of initial ion ratio and pH on the structure of calcium phosphate precipitates. The results demonstrate the first identified structural variations in ACP related to system chemistry. The Ca – P bonding geometry changes from predominantly monodentate, to mixed monodentate and bidentate, to mostly bidentate as Ca/P increases from 0.2 to 5.0. This link between structure and ion ratio is independent of system pH between 6 and 11. Adapting the method in Chapter 2 for SAXS analysis shows that as the Ca/P increases, the particle size also increases. Sample transformation occurs most rapidly at intermediate ratios of 1.0 and 1.6 and *ex situ* XRD analysis shows different evolution pathways for calcium phosphate at different ratios and circumneutral pH. Rietveld analysis identified brushite and poorly crystalline hydroxylapatite at ratios 1.0 and 5.0

at both 0.5 and 24 hours after initial mixing. At ratio 0.2 only hydroxylapatite was seen in the XRD pattern. Controlling the ion ratio alters the structure of ACP and also controls the transformation pathway as either $\text{ACP} \rightarrow \text{hydroxylapatite}$ or $\text{ACP} \rightarrow \text{brushite} \rightarrow \text{hydroxylapatite}$. This provides a novel mechanism to study direct $\text{ACP} \rightarrow \text{hydroxylapatite}$ formation at a biologically relevant pH and identifies the first structural variations in ACP.

Chapter 4 provides a different approach to analyze the impacts of various synthesis conditions on calcium phosphate formation. Experimental data from the literature was mined to create a database for statistical analysis. Both binary logistic regression analysis and nonlinear continuous logistic regression analysis are used to evaluate which synthesis parameters have a significant impact on the formation of ACP, brushite, and hydroxylapatite. Within the range of conditions examined ACP formation is favored at low temperature, supersaturation, and age, but high pH, while brushite is favored at low temperature, age, and pH. Brushite formation is not influenced by the supersaturation and neither phase showed any relationship with ion ratio. Age, temperature, and supersaturation are determined to be significant parameters for hydroxylapatite formation, but the established dataset was not successful in developing a comprehensive picture for this phase. Results suggest that the regression parameters are pathway dependent. Hydroxylapatite is a secondary phase following two different formation pathways in the dataset, and thus is not well described. The ability to quantify the predicted phase abundance of synthesis products is a significant step forward towards a comprehensive understanding and control over calcium phosphate synthesis.

While Chapter 3 provides evidence that the initial ion ratio impacts the structure and evolution of amorphous calcium phosphate, our statistical analysis in Chapter 4 does not demonstrate a relationship between Ca/P and ACP formation. The continuous analysis evaluated the impact of the independent variables on whether or not the phase would form and how much of the phase formed. It did not evaluate how the phase transformed and what secondary phase developed. Chapter 3 does show that the rate of transformation is more rapid at Ca/P of 1.0 and 1.6, which we expected to have some control over the phase percentage, correlated with sample age. However, 128 of the 139 samples in our dataset have a Ca/P between 1.0 and 1.67. If the initial ratio outside of this range has a statistically significant impact on ACP formation, the dataset here was not adequately able to represent it.

Calcium phosphates are a major focus of biomineral investigations, and the ease of synthesis and expansive literature base make them a topic of interdisciplinary interest. The results presented here highlight the critical, but sometimes overlooked, role of the sample synthesis conditions on the resulting phases. Attempts to evaluate the influence of extraneous ions or proteins will continue to be incomplete until we understand the fundamental controls of the system in lieu of those additions. The method presented in Chapter 2 is optimal to identify chemical – structure property relationships for ACP beyond those explored in Chapter 3. It can also be adapted to investigate similar questions for other minerals and metastable phases such as amorphous calcium carbonate and ferrihydrite. The data collected in Chapter 3 can be utilized to develop improved models of short-range structures in ACP in the future. Additionally, the new control of direct ACP to hydroxylapatite transformation at circumneutral pH provides an optimal environment for future biomineralization studies. Finally, the equations developed in Chapter 4 are a comprehensive picture of the collaborative impacts of critical synthesis variables. These are a significant step forward for methods development and a template for future experimental studies that try to synthesize ACP or brushite. A next step is to continue these statistical analyses for other members of the calcium phosphate system. With the framework put in place within this document, future work can develop a comprehensive understanding of controls on the formation, structure, and evolution of aqueous calcium phosphates.

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APPENDIX A: SUPPLEMENTAL INFORMATION FOR CHAPTER 2

MASKING

Masking the beam stop, major dead zones, and the edge of the detector (where the angle of the beam is very shallow) are typical practices (**Figure A-1**). With the extremely low signal to noise ratio present in our amorphous samples we created a more extensive mask than normal in an attempt to reduce the amount of noise seen in the raw data at higher momentum transfer values. Typical Q_{max} values achieved in our experiments are between 18 – 22 Å. As can be seen in **Figure A-2**, the $F(q)$ of amorphous samples shows significantly reduced noise when the extensive mask is applied. The decrease in noise does not have a significant effect on the resolution or observed structure of our samples after undergoing a Fourier transform to obtain the PDF ($G(r)$). In addition, for a crystalline brushite sample, there appears to be no difference in the noise, resolution, or structure of the samples before or after the Fourier transform, regardless of which mask is used. Minimizing excess noise is important for accurate data analysis, but this comparison shows that the excess noise from the unmasked dead pixels is only observable in amorphous samples and doesn't impact the final PDF profile of a sample.

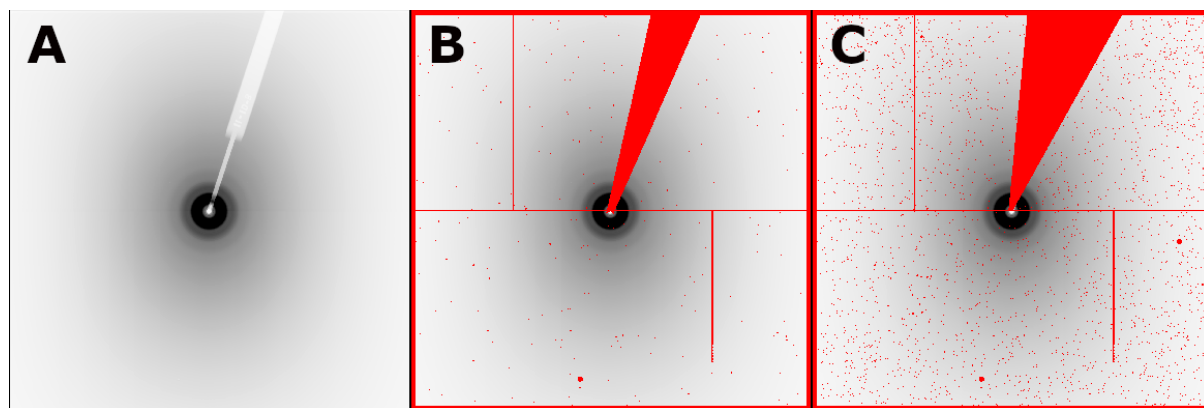


Figure A-1 (A) The raw signal collected on the 2D detector includes a shadow of the beam stop which in (B) and (C) is overlain with a red mask that covers unwanted signals and prevents their inclusion in data integration. Unwanted signals include the beam stop, dead pixels or zones, and the edge of the detector (where the angle at which the beam interacts with the detector is shallow). The mask in (B) is typically used at Sector 11 ID-B and covers the most significant and obvious dead zones and pixels on the detector. The mask in (C) is more expansive, blocking out a significantly greater number of dead and anomalous

pixels. Additionally, for both (B) and (C) intensity data collected in the corners of the detector are not used because the radial integration in these regions is incomplete.

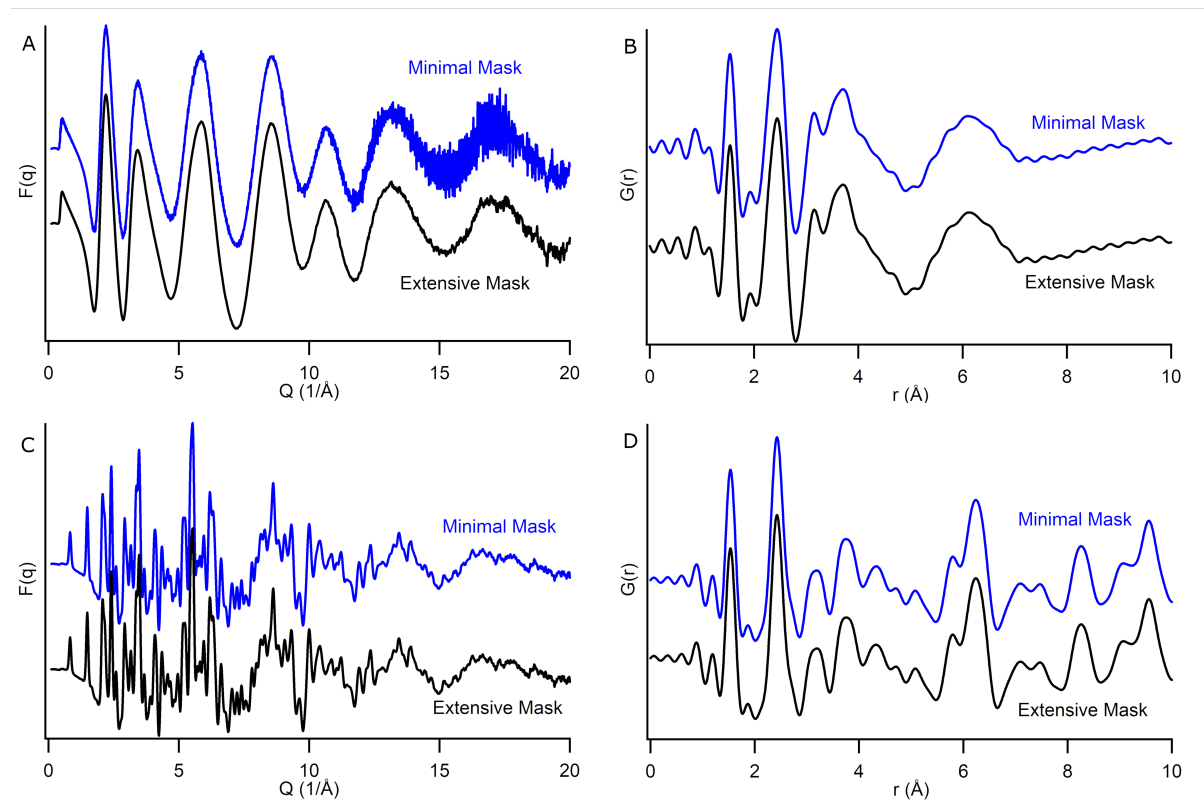


Figure A-2 Using a more extensive mask with amorphous samples (A and B) greatly reduces the amount of noise seen at high Q^{-1} in the $F(q)$ (A), but has no impact on the PDF profile after the data undergoes a Fourier transform (B). For a sample of crystalline brushite (C and D) there is no significant difference in the data processed with a minimal or extensive mask applied.

BRUSHITE MODELING REFINEMENTS

The refinement outputs for the *in situ* brushite modeling in PDFgui, presented in this paper are listed here. Atom position refinements (**Table 1**), anisotropic temperature factors (**Table 2**), unit cell parameters, scale factor, and quadratic correlation parameters (**Table 3**) are all listed below.

Table A-1 A list of refined atom positions.

Atom	Position		
	X	Y	Z
Ca	0.49	0.67	0.75
Ca	0.49	0.33	0.25
Ca	0.99	0.17	0.75
Ca	0.99	0.83	0.25
P	0.01	0.68	0.77
P	0.01	0.32	0.27
P	0.51	0.18	0.77
P	0.51	0.82	0.27
O	0.76	0.64	0.53
O	0.76	0.36	0.03
O	0.26	0.14	0.53
O	0.26	0.86	0.03
O	0.91	0.72	0.93
O	0.91	0.28	0.43
O	0.41	0.22	0.93
O	0.41	0.78	0.43
O	0.20	0.59	0.84
O	0.20	0.41	0.34
O	0.70	0.09	0.84
O	0.70	0.91	0.34
O	0.12	0.74	0.65
O	0.12	0.26	0.15
O	0.62	0.24	0.65
O	0.62	0.76	0.15
O	0.27	0.57	0.40
O	0.27	0.43	0.90
O	0.77	0.07	0.40
O	0.77	0.93	0.90
O	0.69	0.55	0.01
O	0.69	0.45	0.51
O	0.19	0.05	0.01
O	0.19	0.95	0.51
H	0.71	0.67	0.37
H	0.71	0.33	0.87
H	0.21	0.17	0.37
H	0.21	0.83	0.87
H	0.25	0.51	0.39
H	0.25	0.49	0.89
H	0.75	0.01	0.39
H	0.75	0.99	0.89
H	0.25	0.59	0.21
H	0.25	0.41	0.71
H	0.75	0.09	0.21
H	0.75	0.91	0.71
H	0.72	0.49	1.00

H	0.72	0.51	0.50
H	0.22	0.99	1.00
H	0.22	0.01	0.50
H	0.89	0.57	0.15
H	0.89	0.43	0.65
H	0.39	0.07	0.15
H	0.39	0.93	0.65

Table A-2 The anisotropic temperature factors used for modeling atoms in general positions

Atom	Anisotropic Temperature factor
Ca	0.006
P	0.004
O	0.015
H	0.010

Table A-3 The unit cell dimensions and other factors used to refine the brushite model.

Scale factor	0.119
Linear correlation factor	0
Quadratic correlation factor	1.49
a	6.37
b	15.2
c	5.80
α	90
β	119
γ	90

APPENDIX B: SUPPLEMENTAL INFORMATION FOR CHAPTER 3

ADDITIONAL PH INFORMATION

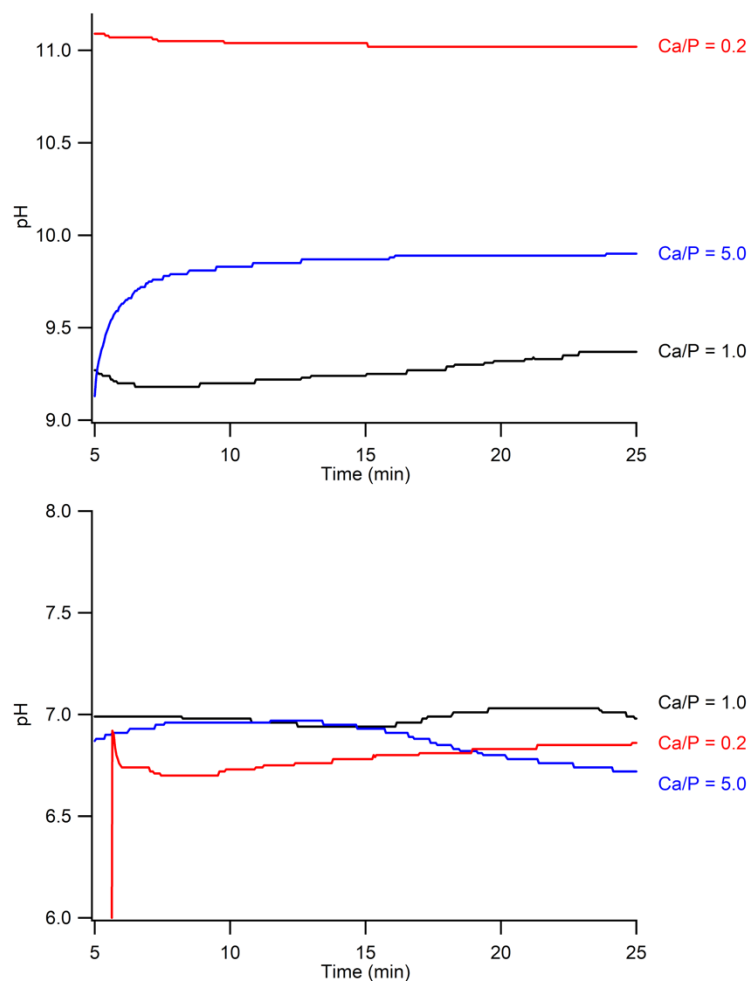


Figure B-1 Examples of pH drift curves for Ca/P = 0.2 (red), 1.0 (black), and 5.0 (blue) at the high pH range (top) and low pH range (bottom). The curves show minimal drift (usually less than 0.5 pH) during the course of the experiment. These curves were collected during replicate data collections of the Ca/P ratios and are not representative of the exact pH for the samples discussed in the main text. In the lower pH range, ratios 1.0 and 5.0 have slightly higher pH than 0.2 for the majority of data collection, due to the excess amount of phosphate in the system at the higher ratios. Overall though, the pH of samples in the lower pH range is relatively similar. There are greater variations in the pH in the higher pH range, caused by variations in the amount of NaOH added to each sample to elevate the pH of the system.

The pH of the experiments we performed range from 6 to 11. There is a change of phosphate speciation in this range, as can be seen in **Figure B-2**. At the low end of our functional pH range there is a mix of H_2PO_4^- and HPO_4^{2-} , but as we go up in pH, there is an increase in the amount of HPO_4^{2-} until it is the dominant species.

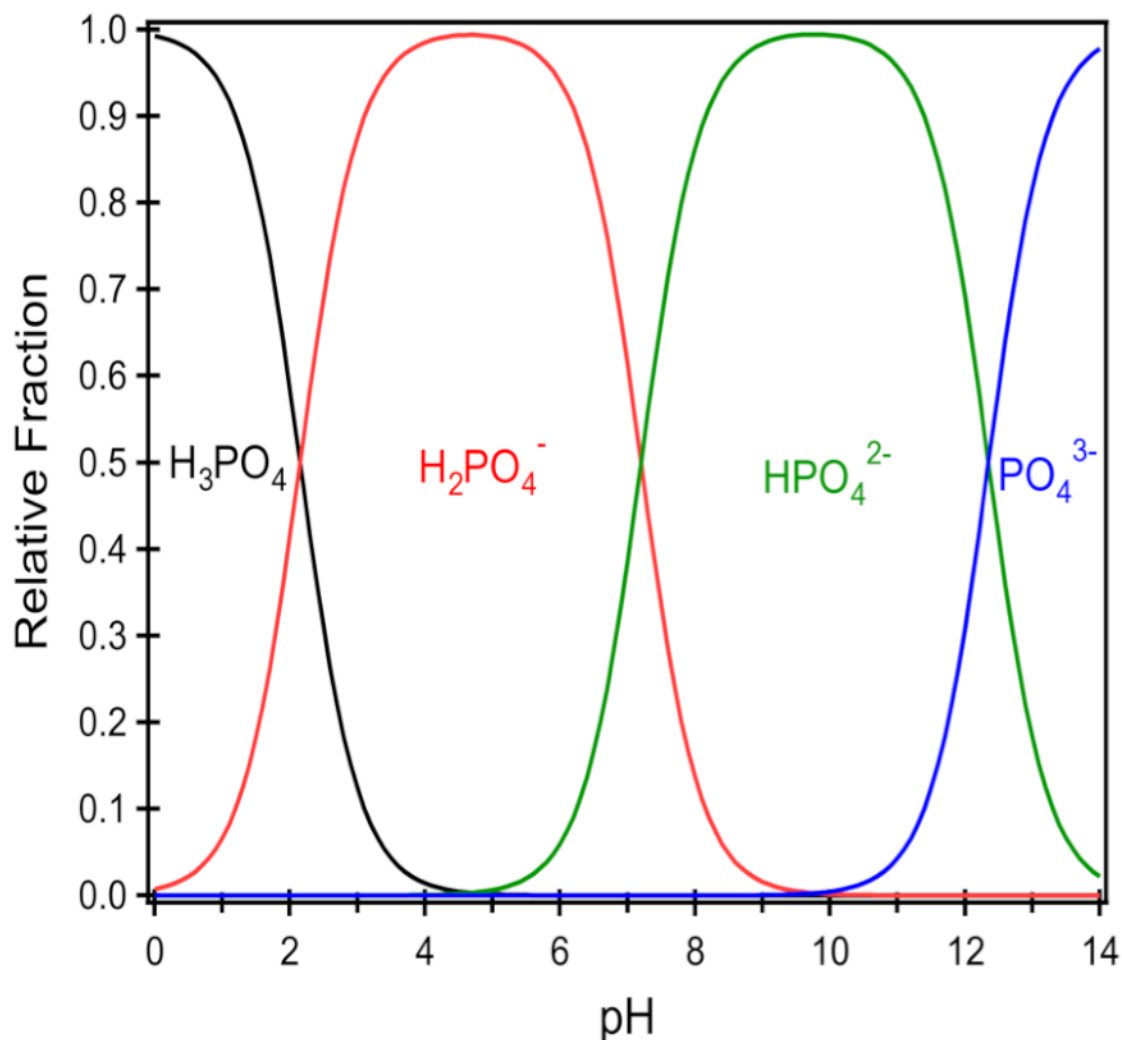


Figure B-2 A diagram expressing the relative fraction of different species of phosphate in solution as a function of the pH of the system.

It is commonly reported in the literature that synthesizing calcium phosphates in solution above a pH of 9 helps stabilize amorphous materials and prevent rapid crystallization (Termine & Posner, 1970; Blumenthal *et al.*, 1972b). It can be seen in **Figure B-3** that our results coincide with the

literature. Two samples were synthesized that were identical, except that one had the addition of NaOH to raising the pH of the system during mixing to above 10. The sample synthesized at a high pH shows no evidence of crystallinity, while the corresponding sample at a lower pH below 7, indicates a crystalline brushite is present.

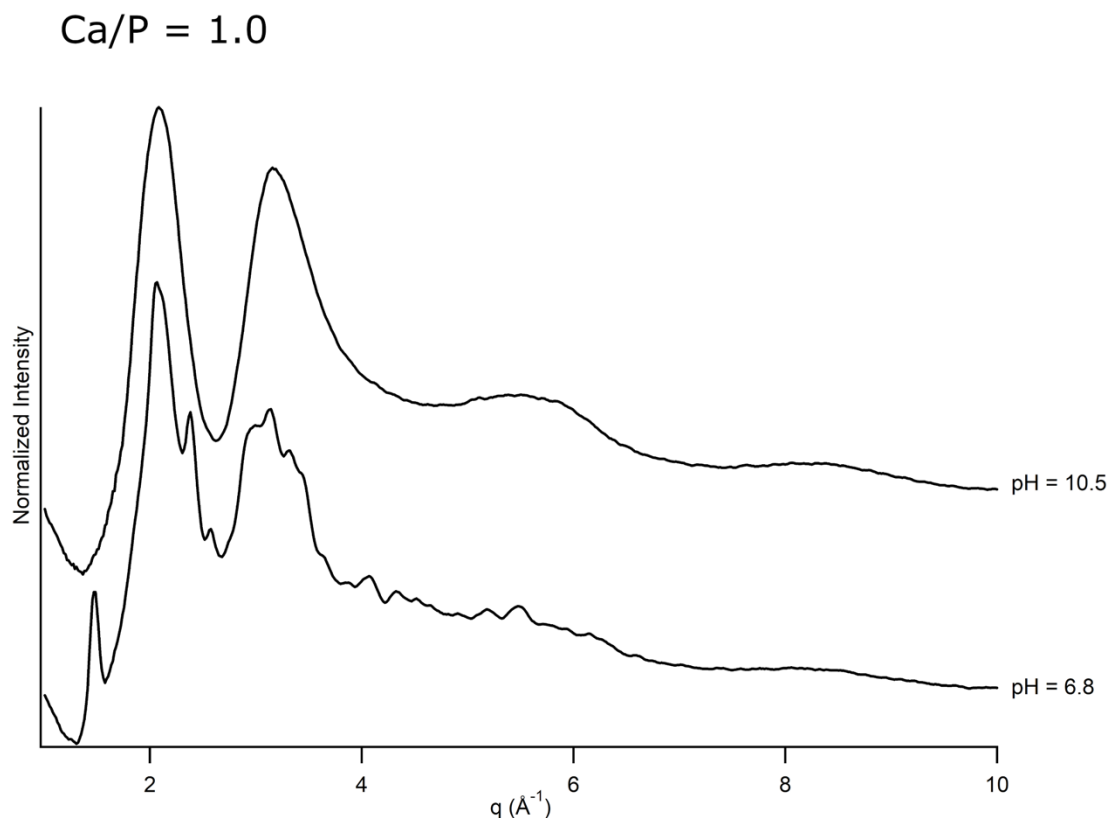


Figure B-3 A comparison of the normalized $I(q)$ profiles for two samples both synthesized with a Ca/P = 1.0, but varying pH. The pH of the sample in the top profile was adjusted to about 10.5 by adding NaOH, in contrast to the pH of 6.8 in a sample without NaOH. Both samples have a residence time of five minutes. Peaks present in the sample at low pH show the development of brushite, but with an elevated pH, the sample remains amorphous and no crystallinity is apparent.

POWDER XRD AND RIETVELD ANALYSIS

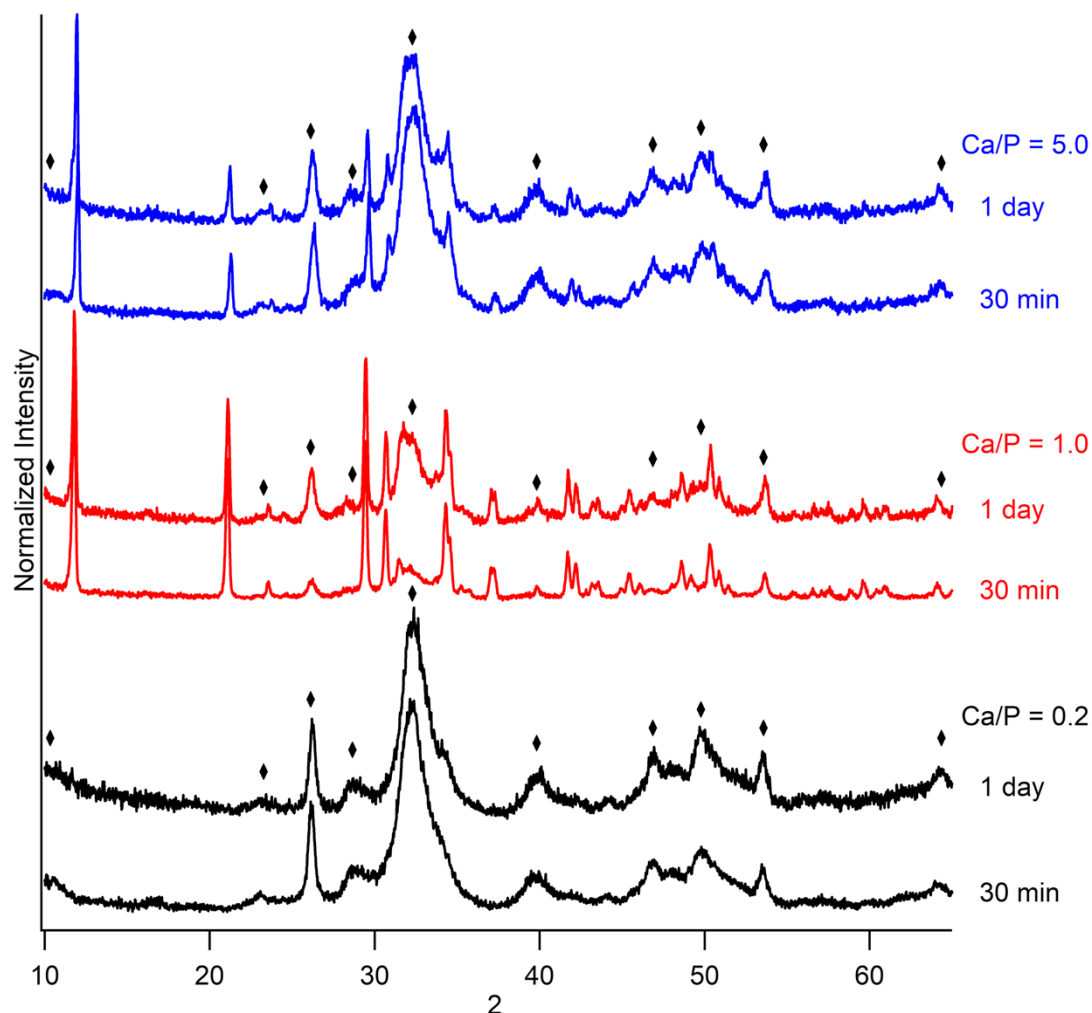


Figure B-4 XRD profiles of the initial Ca/P of 0.2 (black, bottom), 1.0 (red, middle), and 5.0 (blue, top) that were filtered and dried 30 minutes and 1 day after initial mixing. The profiles for Ca/P 0.2 at 30 min and 1 day only show the peaks of poorly crystalline hydroxylapatite, indicated with ◆. The same ◆ identifies the major peak locations and contribution of hydroxylapatite to the XRD profiles of the 1.0 and 5.0 ratios. All other peaks seen in ratios 1.0 and 5.0 that do not align with the hydroxylapatite contributions were indexed as brushite.

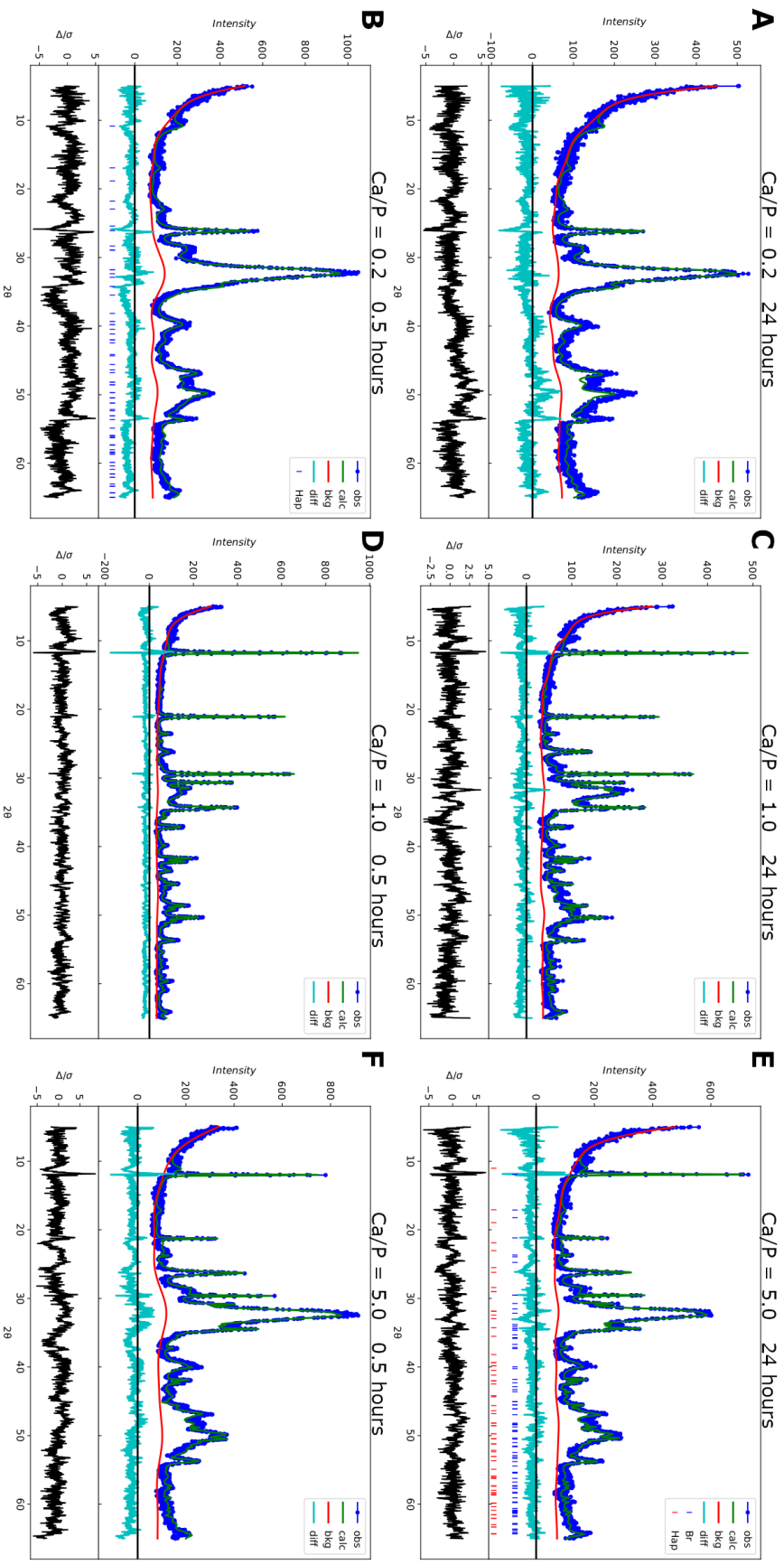


Figure B-5 The Rietveld fits for initial Ca/P = 0.2, 1.0, and 5.0 twenty-four hours after mixing (A, C, and E) and half an hour after mixing (B, D, and F). The dark blue is the original diffraction pattern, while the green is the calculated pattern. The red line is the subtracted background and the light blue is the difference between the original and calculated diffraction patterns.

ICP-AES

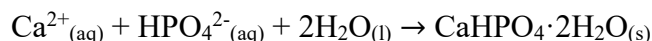
Table B-1 Synthesis specifications and results for all samples prepared for ICP-AES analysis.

$[Ca^{2+}]_i$ and $[PO_4^{3-}]_i$ are the initial ion concentrations, while $[Ca^{2+}]_f$ and $[PO_4^{3-}]_f$ are the measured concentrations of the precipitate. Sample names first list the initial Ca/P as either 0.2, 1.0, or 5.0. All ratios were run in triplicate, indicated by 1, 2, or 3. The ion ratios were averaged across the triplicate samples for each ratio (presented in **Table 3**).

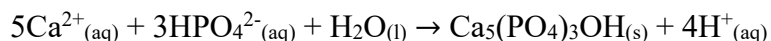
Sample Name	$[Ca^{2+}]_i$ mM	$[PO_4^{3-}]_i$ mM	10% HNO ₃ (mL)	Total Volume (mL)	$[Ca^{2+}]_f$ mM	$[PO_4^{3-}]_f$ mM	Ca/P
0.2-1	9	44	1.5	30	1.47	1.53	0.96
0.2-2	9	44	1.5	30	1.17	1.01	1.15
0.2-3	9	44	1.5	30	1.45	1.75	0.83
1.0-1	20	20	1.5	30	2.99	2.79	1.07
1.0-2	20	20	1.5	30	2.91	2.70	1.08
1.0-3	20	20	5 mL of 10% and 10 mL of 1% HNO ₃	25	9.11	8.48	1.07
5.0-1	44	9	1.5	30	1.61	1.28	1.26
5.0-2	44	9	1.5	30	1.35	1.17	1.15
5.0-3	44	9	1.5	30	1.53	1.18	1.30

SUPERSATURATION CALCULATIONS

The Geochemist Workbench (Aqueous Solutions LLC, Champaign, IL USA) was used to calculate the ionic strength of each solution. The Davies equation was used to calculate the ion activity coefficients for Ca^{2+} and HPO_4^{2-} (Langmuir, 1997). The dielectric constant of water (79.9) was calculated using equation 4.29 on page 128 of Langmuir (1997) and then used to determine the constant A (0.506) in the Davies equation (with the assumption that temperature change does not alter the density of water). Standard ion activity products for brushite ($\log K_{sp} = -6.60$) and hydroxylapatite ($\log K_{sp} = -56.02$) were taken from Jaynes *et al.* (1999). Using the following chemical reactions for brushite



And hydroxylapatite



Ion activity products relative to brushite (Br) and hydroxylapatite (HAP) were calculated for each sample using the equations

$$IAP \text{ for Br} = [Ca^{2+}] \cdot [HPO_4^{2-}]$$

$$IAP \text{ for HAP} = \frac{[Ca^{2+}]^5 \cdot [HPO_4^{2-}]^3}{[H^+]^4}$$

Where [] denotes activity and the supersaturation indices relative to brushite and hydroxylapatite are calculated for each sample using the equations

$$\Omega_{Br} = \log \frac{IAP \text{ for Br}}{K_{sp Br}}$$

$$\Omega_{HAP} = \log \frac{IAP \text{ for HAP}}{K_{sp HAP}}$$

The results of these calculations are reported in **Table B-2**

Table B-2 The ionic strength, Ca^{2+} and HPO_4^{2-} activity coefficients (γ), Ca^{2+} and HPO_4^{2-} activity calculated using the Davies equation, ion activity products for brushite (Br) and hydroxylapatite (HAP), and saturation indices for brushite and hydroxylapatite (Ω) are presented above.

Sample ID	Ionic strength	$Ca^{2+} \gamma$	$HPO_4^{2-} \gamma$	Ca^{2+} activity	HPO_4^{2-} activity	IAP for Br	IAP for HAP	Ω Br	Ω HAP
Ratio 0.2 pH low	0.060	0.435	0.435	0.002	0.010	1.88E-05	4.02E+11	1.87	67.6
Ratio 0.6 pH low	0.043	0.478	0.478	0.004	0.006	2.26E-05	6.20E+10	1.95	66.8
Ratio 1.0 pH low	0.042	0.480	0.480	0.005	0.005	2.30E-05	1.77E+08	1.96	64.3
Ratio 1.6 pH low	0.047	0.466	0.466	0.006	0.004	2.14E-05	5.55E+06	1.93	62.8
Ratio 5.0 pH low	0.068	0.420	0.420	0.009	0.002	1.75E-05	4.54E+05	1.84	61.7
Ratio 0.2 pH low-2	0.051	0.455	0.455	0.002	0.010	2.05E-05	2.28E+07	1.91	63.4
Ratio 1.0 pH low-2	0.042	0.480	0.480	0.005	0.005	2.30E-05	4.46E+08	1.96	64.7
Ratio 5.0 pH low-2	0.067	0.421	0.421	0.009	0.002	1.75E-05	2.91E+06	1.84	62.5
Ratio 0.2 pH high	0.067	0.421	0.421	0.002	0.009	1.75E-05	1.93E+24	1.84	80.3
Ratio 1.0 pH high	0.033	0.511	0.511	0.005	0.005	2.62E-05	4.68E+23	2.02	79.7
Ratio 5.0 pH high	0.066	0.424	0.424	0.009	0.002	1.78E-05	7.71E+22	1.85	78.9

LINEAR COMBINATION FITTING

Linear Combination Fitting (LCF) was completed on the PDFs of the samples that did not have a modified pH, to examine how much ACP and brushite was in each sample. A PDF of a synthetic brushite was used as the brushite end member and the PDF for the initial Ca/P = 0.2 was chosen as the reference for ACP. As demonstrated in the main text, the structure of ACP varies with

Ca/P and thus the 0.2 PDF may not be the ideal end member for all samples. We were confident from the $I(q)$ that Ca/P was the purest ACP in the dataset, so it was chosen as the end member, despite the structural discrepancies. We believe the LCF still provides reasonable estimates in the quantities of brushite and ACP per sample.

Table B-3 The results of LCF show that within an error of $\pm 5\%$, the initial Ca/P of 0.6 doesn't contain any brushite, but higher ratios all show significant amounts.

Ca/P	ACP (%)	Brushite (%)
0.6	100	-
1	64	36
1.6	68	32
5	77	23

PHOSPHATE ENVIRONMENT

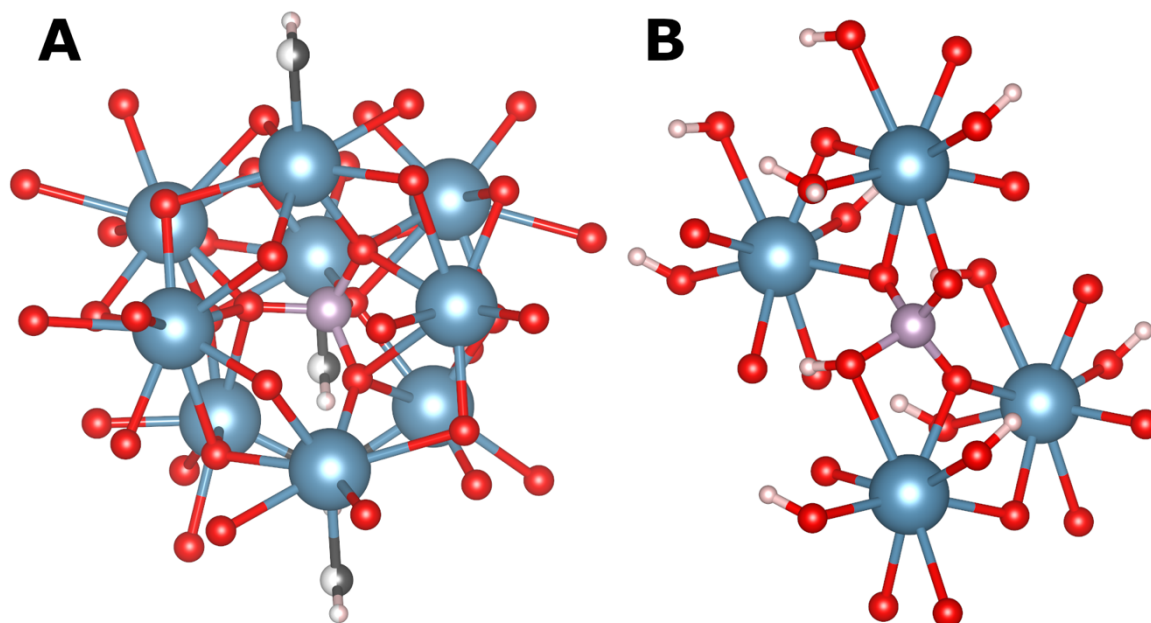


Figure B-6 Trimmed structures demonstrating the local phosphate environment in hydroxylapatite (A) and brushite (B). Calcium atoms are blue, phosphorus are purple, oxygen are red, hydrogen are white, and the oxygens in a hydroxyl group in hydroxylapatite are half black and half white.

APPENDIX C: SUPPLEMENTAL INFORMATION FOR CHAPTER 4

SAMPLE DATABASE

Table C-1 The calcium and phosphate source solutions, supersaturation (Ω) relative to ACP, brushite (Br), and hydroxylapatite (HAP), Initial Ca/P, temperature, pH, age, phase percentage, and binary phase attribution for all the samples examined in this analysis.

Paper	Sample ID	Ca source	P source	Ω for ACP	Ω for Br	Ω for HAP	Initial Ca/P	Temp °C	Mixing pH	Age min
Borkiewicz et al., 2010	ES-6	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.46	2.65	66.43	1.67	90	6.5	5
Borkiewicz et al., 2010	ES-10	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	33.69	2.74	76.80	1.67	90	9	5
Borkiewicz et al., 2010	IS-2.1	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	0
Borkiewicz et al., 2010	IS-2.2	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	5
Borkiewicz et al., 2010	IS-2.3	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	10
Borkiewicz et al., 2010	IS-2.4	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	15
Borkiewicz et al., 2010	IS-2.5	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	20
Borkiewicz et al., 2010	IS-2.6	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	25
Borkiewicz et al., 2010	IS-2.7	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	30
Borkiewicz et al., 2010	IS-2.8	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	45
Borkiewicz et al., 2010	IS-2.9	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	50
Borkiewicz et al., 2010	IS-2.10	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	55
Borkiewicz et al., 2010	IS-2.11	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	60
Borkiewicz et al., 2010	IS-2.12	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	90
Borkiewicz et al., 2010	IS-2.13	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	120
Borkiewicz et al., 2010	IS-2.14	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	150
Borkiewicz et al., 2010	IS-2.15	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	180
Borkiewicz et al., 2010	IS-2.16	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	210
Borkiewicz et al., 2010	IS-2.17	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	240
Borkiewicz et al., 2010	IS-2.18	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	270
Borkiewicz et al., 2010	IS-2.19	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.74	2.76	66.88	1.67	45	6.5	300
Borkiewicz et al., 2010	IS-3.1	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	0
Borkiewicz et al., 2010	IS-3.2	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	5
Borkiewicz et al., 2010	IS-3.3	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	10
Borkiewicz et al., 2010	IS-3.4	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	15
Borkiewicz et al., 2010	IS-3.5	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	20
Borkiewicz et al., 2010	IS-3.6	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	25
Borkiewicz et al., 2010	IS-3.7	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	30
Borkiewicz et al., 2010	IS-3.8	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	45
Borkiewicz et al., 2010	IS-3.9	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	50
Borkiewicz et al., 2010	IS-3.10	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	55
Borkiewicz et al., 2010	IS-3.11	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	60
Borkiewicz et al., 2010	IS-3.12	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	90
Borkiewicz et al., 2010	IS-3.13	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	120
Borkiewicz et al., 2010	IS-3.14	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	150
Borkiewicz et al., 2010	IS-3.15	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	180
Borkiewicz et al., 2010	IS-3.16	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	210
Borkiewicz et al., 2010	IS-3.17	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	240

Sample ID	Freeze dried	pH adjuster	Percent ACP	Binary ACP	Percent HAP	Binary HAP	Percent Br	Binary Br	Percent OCP	Binary OCP	Percent Monetite
ES-6	No	None	0	0	100	1	0	0	0	0	0
ES-10	No	NH4OH	0	0	100	1	0	0	0	0	0
IS-2.1	No	None	0	0	0	0	100	1	0	0	0
IS-2.2	No	None	0	0	0	0	100	1	0	0	0
IS-2.3	No	None	0	0	0	0	100	1	0	0	0
IS-2.4	No	None	0	0	2.5	1	81.5	1	16	0	0
IS-2.5	No	None	0	0	30	1	63	1	7	0	0
IS-2.6	No	None	0	0	22.5	1	67.5	1	10	0	0
IS-2.7	No	None	0	0	32	1	60	1	8	0	0
IS-2.8	No	None	0	0	25	1	63	1	12	0	0
IS-2.9	No	None	0	0	26	1	65	1	9	0	0
IS-2.10	No	None	0	0	28.5	1	64.5	1	7	0	0
IS-2.11	No	None	0	0	30.5	1	59.5	1	10	0	0
IS-2.12	No	None	0	0	48.5	1	51.5	1	0	0	0
IS-2.13	No	None	0	0	45	1	52.5	1	0	2.5	0
IS-2.14	No	None	0	0	52	1	40	1	0	8	0
IS-2.15	No	None	0	0	52	1	32	1	0	16	0
IS-2.16	No	None	0	0	47.5	1	25	1	0	27.5	0
IS-2.17	No	None	0	0	46	1	10	1	0	44	0
IS-2.18	No	None	0	0	40	1	0	0	0	60	0
IS-2.19	No	None	0	0	34.5	1	0	0	0	65.5	0
IS-3.1	No	None	0	0	0	0	100	1	0	0	0
IS-3.2	No	None	0	0	0	0	80	1	20	0	0
IS-3.3	No	None	0	0	0	0	86	1	14	0	0
IS-3.4	No	None	0	0	0	0	82	1	18	0	0
IS-3.5	No	None	0	0	10	1	74	1	16	0	0
IS-3.6	No	None	0	0	12.5	1	75.5	1	12	0	0
IS-3.7	No	None	0	0	15	1	76	1	9	0	0
IS-3.8	No	None	0	0	27.5	1	71.5	1	1	0	0
IS-3.9	No	None	0	0	29	1	71	1	0	0	0
IS-3.10	No	None	0	0	27.5	1	72.5	1	0	0	0
IS-3.11	No	None	0	0	29	1	71	1	0	0	0
IS-3.12	No	None	0	0	27.5	1	72.5	1	0	0	0
IS-3.13	No	None	0	0	24.5	1	75.5	1	0	0	0
IS-3.14	No	None	0	0	27	1	68	1	0	5	0
IS-3.15	No	None	0	0	27.5	1	54.5	1	0	18	0
IS-3.16	No	None	0	0	28	1	32	1	0	40	0
IS-3.17	No	None	0	0	29	1	7	1	0	64	0

Paper	Sample ID	Ca source	P source	Ω for ACP	Ω for Br	Ω for HAP	Initial Ca/P	Temp °C	Mixing pH	Age min
Borkiewicz et al., 2010	IS-3.18	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	270
Borkiewicz et al., 2010	IS-3.19	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.66	2.73	66.75	1.67	60	6.5	300
Borkiewicz et al., 2010	IS-4.1	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	0
Borkiewicz et al., 2010	IS-4.2	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	5
Borkiewicz et al., 2010	IS-4.3	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	10
Borkiewicz et al., 2010	IS-4.4	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	15
Borkiewicz et al., 2010	IS-4.5	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	20
Borkiewicz et al., 2010	IS-4.6	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	25
Borkiewicz et al., 2010	IS-4.7	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	30
Borkiewicz et al., 2010	IS-4.8	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	45
Borkiewicz et al., 2010	IS-4.9	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	50
Borkiewicz et al., 2010	IS-4.10	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	55
Borkiewicz et al., 2010	IS-4.11	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	60
Borkiewicz et al., 2010	IS-4.12	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	90
Borkiewicz et al., 2010	IS-4.13	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	120
Borkiewicz et al., 2010	IS-4.14	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	150
Borkiewicz et al., 2010	IS-4.15	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	180
Borkiewicz et al., 2010	IS-4.16	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	210
Borkiewicz et al., 2010	IS-4.17	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	240
Borkiewicz et al., 2010	IS-4.18	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	270
Borkiewicz et al., 2010	IS-4.19	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.50	2.66	66.49	1.67	85	6.5	300
Boskey and Posner, 1973	Sup 1	CaCl ₂	(NH ₄) ₂ HPO ₄	28.20	2.37	66.20	1.11	26	6.8	240
Boskey and Posner, 1973	Sup 2	CaCl ₂	(NH ₄) ₂ HPO ₄	28.60	2.37	67.00	1.11	26	7	240
Boskey and Posner, 1973	Sup 3	CaCl ₂	(NH ₄) ₂ HPO ₄	29.60	2.36	69.00	1.11	26	7.5	240
Boskey and Posner, 1973	Sup 4	CaCl ₂	(NH ₄) ₂ HPO ₄	30.62	2.37	71.02	1.11	26	8	240
Boskey and Posner, 1973	Sup 5	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	240
Boskey and Posner, 1973	Sup 6	CaCl ₂	(NH ₄) ₂ HPO ₄	35.00	2.52	79.63	1.11	26	10	240
Boskey and Posner, 1973	Sup 7	CaCl ₂	(NH ₄) ₂ HPO ₄	28.20	2.37	66.20	1.11	26	6.8	1440
Boskey and Posner, 1973	Sup 8	CaCl ₂	(NH ₄) ₂ HPO ₄	28.60	2.37	67.00	1.11	26	7	1440
Boskey and Posner, 1973	Sup 9	CaCl ₂	(NH ₄) ₂ HPO ₄	29.60	2.36	69.00	1.11	26	7.5	1440
Boskey and Posner, 1973	Sup 10	CaCl ₂	(NH ₄) ₂ HPO ₄	30.62	2.37	71.02	1.11	26	8	1440
Boskey and Posner, 1973	Sup 11	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	1440
Boskey and Posner, 1973	Sup 12	CaCl ₂	(NH ₄) ₂ HPO ₄	35.00	2.52	79.63	1.11	26	10	1440
Boskey and Posner, 1973	a1	CaCl ₂	K ₂ HPO ₄	29.61	2.37	69.01	1.11	26	7.5	35
Boskey and Posner, 1973	a2	CaCl ₂	K ₂ HPO ₄	29.61	2.37	69.01	1.11	26	7.5	50
Boskey and Posner, 1973	a3	CaCl ₂	K ₂ HPO ₄	29.61	2.37	69.01	1.11	26	7.5	60
Boskey and Posner, 1973	a4	CaCl ₂	K ₂ HPO ₄	29.61	2.37	69.01	1.11	26	7.5	65
Boskey and Posner, 1973	a5	CaCl ₂	K ₂ HPO ₄	29.61	2.37	69.01	1.11	26	7.5	75

Sample ID	Freeze dried	pH adjuster	Percent ACP	Binary ACP	Percent HAP	Binary HAP	Percent Br	Binary Br	Percent OCP	Percent Monetite
IS-3.18	No	None	0	0	24	1	0	0	0	76
IS-3.19	No	None	0	0	27.5	1	0	0	0	72.5
IS-4.1	No	None	0	0	0	0	100	1	0	0
IS-4.2	No	None	0	0	0	0	95	1	5	0
IS-4.3	No	None	0	0	7	1	77	1	16	0
IS-4.4	No	None	0	0	40	1	50.5	1	9.5	0
IS-4.5	No	None	0	0	65	1	0	0	0	35
IS-4.6	No	None	0	0	52.5	1	0	0	0	47.5
IS-4.7	No	None	0	0	46	1	0	0	0	54
IS-4.8	No	None	0	0	46	1	0	0	0	54
IS-4.9	No	None	0	0	43.5	1	0	0	0	56.5
IS-4.10	No	None	0	0	42	1	0	0	0	58
IS-4.11	No	None	0	0	41.5	1	0	0	0	58.5
IS-4.12	No	None	0	0	37.5	1	0	0	0	62.5
IS-4.13	No	None	0	0	37	1	0	0	0	63
IS-4.14	No	None	0	0	38.5	1	0	0	0	61.5
IS-4.15	No	None	0	0	34.5	1	0	0	0	65.5
IS-4.16	No	None	0	0	37.5	1	0	0	0	62.5
IS-4.17	No	None	0	0	40	1	0	0	0	60
IS-4.18	No	None	0	0	35.5	1	0	0	0	64.5
IS-4.19	No	None	0	0	37.5	1	0	0	0	62.5
Sup 1	No	Tris buffer	0	0	100	1	0	0	0	0
Sup 2	No	Tris buffer	0	0	100	1	0	0	0	0
Sup 3	No	Tris buffer	98	1	2	0	0	0	0	0
Sup 4	No	Tris buffer	86	1	14	1	0	0	0	0
Sup 5	No	NH4OH NH4Cl	67	1	33	1	0	0	0	0
Sup 6	No	NH4OH NH4Cl	100	1	0	0	0	0	0	0
Sup 7	No	Tris buffer	0	0	100	1	0	0	0	0
Sup 8	No	Tris buffer	0	0	100	1	0	0	0	0
Sup 9	No	Tris buffer	0	0	100	1	0	0	0	0
Sup 10	No	Tris buffer	0	0	100	1	0	0	0	0
Sup 11	No	NH4OH NH4Cl	0	0	100	1	0	0	0	0
Sup 12	No	NH4OH NH4Cl	0	0	100	1	0	0	0	0
a1	No	Tris buffer	100	1	0	0	0	0	0	0
a2	No	Tris buffer	81	1	19	1	0	0	0	0
a3	No	Tris buffer	66	1	34	1	0	0	0	0
a4	No	Tris buffer	53.5	1	46.5	1	0	0	0	0
a5	No	Tris buffer	28	1	72	1	0	0	0	0

Paper	Sample ID	Ca source	P source	Ω for ACP	Ω for Br	Ω for HAP	Initial Ca/P	Temp °C	Mixing pH	Age min
Boskey and Posner, 1973	a6	CaCl ₂	K ₂ HPO ₄	29.61	2.37	69.01	1.11	26	7.5	83
Boskey and Posner, 1973	a7	CaCl ₂	K ₂ HPO ₄	29.61	2.37	69.01	1.11	26	7.5	100
Boskey and Posner, 1973	a8	CaCl ₂	K ₂ HPO ₄	29.61	2.37	69.01	1.11	26	7.5	120
Boskey and Posner, 1973	a9	CaCl ₂	K ₂ HPO ₄	29.61	2.37	69.01	1.11	26	7.5	140
Boskey and Posner, 1973	a10	CaCl ₂	K ₂ HPO ₄	29.61	2.37	69.01	1.11	26	7.5	165
Boskey and Posner, 1973	a11	CaCl ₂	K ₂ HPO ₄	29.61	2.37	69.01	1.11	26	7.5	170
Boskey and Posner, 1973	a12	CaCl ₂	K ₂ HPO ₄	29.61	2.37	69.01	1.11	26	7.5	205
Boskey and Posner, 1973	a13	CaCl ₂	K ₂ HPO ₄	29.61	2.37	69.01	1.11	26	7.5	245
Boskey and Posner, 1973	b1	CaCl ₂	Na ₂ HPO ₄	29.61	2.37	69.02	1.11	26	7.5	25
Boskey and Posner, 1973	b2	CaCl ₂	Na ₂ HPO ₄	29.61	2.37	69.02	1.11	26	7.5	58
Boskey and Posner, 1973	b3	CaCl ₂	Na ₂ HPO ₄	29.61	2.37	69.02	1.11	26	7.5	105
Boskey and Posner, 1973	b4	CaCl ₂	Na ₂ HPO ₄	29.61	2.37	69.02	1.11	26	7.5	110
Boskey and Posner, 1973	b5	CaCl ₂	Na ₂ HPO ₄	29.61	2.37	69.02	1.11	26	7.5	135
Boskey and Posner, 1973	b6	CaCl ₂	Na ₂ HPO ₄	29.61	2.37	69.02	1.11	26	7.5	210
Boskey and Posner, 1973	b7	CaCl ₂	Na ₂ HPO ₄	29.61	2.37	69.02	1.11	26	7.5	250
Boskey and Posner, 1973	e1	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	90
Boskey and Posner, 1973	e2	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	110
Boskey and Posner, 1973	e3	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	145
Boskey and Posner, 1973	e4	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	150
Boskey and Posner, 1973	e5	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	175
Boskey and Posner, 1973	e6	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	200
Boskey and Posner, 1973	e7	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	215
Boskey and Posner, 1973	e8	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	243
Boskey and Posner, 1973	e9	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	295
Boskey and Posner, 1973	f1	Ca(NO ₃) ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	63
Boskey and Posner, 1973	f2	Ca(NO ₃) ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	110
Boskey and Posner, 1973	f3	Ca(NO ₃) ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	120
Boskey and Posner, 1973	f4	Ca(NO ₃) ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	195
Boskey and Posner, 1973	f5	Ca(NO ₃) ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	180
Boskey and Posner, 1973	f6	Ca(NO ₃) ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	205
Boskey and Posner, 1973	f7	Ca(NO ₃) ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	245
Boskey and Posner, 1973	f8	Ca(NO ₃) ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	350
Boskey and Posner, 1973	f9	Ca(NO ₃) ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	380
Boskey and Posner, 1973	f10	Ca(NO ₃) ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	390
Boskey and Posner, 1973	g1	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	50
Boskey and Posner, 1973	g2	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	68
Boskey and Posner, 1973	g3	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	150
Boskey and Posner, 1973	g4	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	203

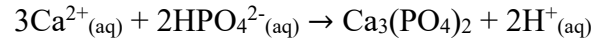
Sample ID	Freeze dried	pH adjuster	Percent ACP	Binary ACP	Percent HAP	Binary HAP	Percent Br	Binary Br	Percent OCP	Percent Monetite
a6	No	Tris buffer	34.5	1	65.5	1	0	0	0	0
a7	No	Tris buffer	11	1	89	1	0	0	0	0
a8	No	Tris buffer	17.5	1	82.5	1	0	0	0	0
a9	No	Tris buffer	12	1	88	1	0	0	0	0
a10	No	Tris buffer	11	1	89	1	0	0	0	0
a11	No	Tris buffer	9	1	91	1	0	0	0	0
a12	No	Tris buffer	9	1	91	1	0	0	0	0
a13	No	Tris buffer	8	1	92	1	0	0	0	0
b1	No	Tris buffer	98.5	1	1.5	0	0	0	0	0
b2	No	Tris buffer	85	1	15	1	0	0	0	0
b3	No	Tris buffer	50	1	50	1	0	0	0	0
b4	No	Tris buffer	30	1	70	1	0	0	0	0
b5	No	Tris buffer	18	1	82	1	0	0	0	0
b6	No	Tris buffer	8	1	92	1	0	0	0	0
b7	No	Tris buffer	1	0	99	1	0	0	0	0
e1	No	Tris buffer	93.5	1	6.5	1	0	0	0	0
e2	No	Tris buffer	98	1	2	0	0	0	0	0
e3	No	Tris buffer	77.5	1	22.5	1	0	0	0	0
e4	No	Tris buffer	62	1	38	1	0	0	0	0
e5	No	Tris buffer	48	1	52	1	0	0	0	0
e6	No	Tris buffer	21.5	1	78.5	1	0	0	0	0
e7	No	Tris buffer	18	1	82	1	0	0	0	0
e8	No	Tris buffer	19.5	1	80.5	1	0	0	0	0
e9	No	Tris buffer	10	1	90	1	0	0	0	0
f1	No	Tris buffer	92	1	8	1	0	0	0	0
f2	No	Tris buffer	88	1	12	1	0	0	0	0
f3	No	Tris buffer	87	1	13	1	0	0	0	0
f4	No	Tris buffer	65	1	35	1	0	0	0	0
f5	No	Tris buffer	44	1	56	1	0	0	0	0
f6	No	Tris buffer	43.5	1	56.5	1	0	0	0	0
f7	No	Tris buffer	30.5	1	69.5	1	0	0	0	0
f8	No	Tris buffer	1.5	0	98.5	1	0	0	0	0
f9	No	Tris buffer	0	0	100	1	0	0	0	0
f10	No	Tris buffer	0	0	100	1	0	0	0	0
g1	No	NH4OH NH4Cl	99	1	1	0	0	0	0	0
g2	No	NH4OH NH4Cl	98.5	1	1.5	0	0	0	0	0
g3	No	NH4OH NH4Cl	66	1	34	1	0	0	0	0
g4	No	NH4OH NH4Cl	51	1	49	1	0	0	0	0

Paper	Sample ID	Ca source	P source	Ω for ACP	Ω for Br	Ω for HAP	Initial Ca/P	Temp °C	Mixing pH	Age min
Boskey and Posner, 1973	g5	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	220
Boskey and Posner, 1973	g6	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	253
Boskey and Posner, 1973	g7	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	280
Boskey and Posner, 1973	g8	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	300
Boskey and Posner, 1973	g9	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	343
Boskey and Posner, 1973	g10	CaCl ₂	(NH ₄) ₂ HPO ₄	32.75	2.43	75.24	1.11	26	9	370
Heughebaert and Montel, 1982	1	Ca(NO ₃) ₂ * 4H ₂ O	H(NH ₄) ₂ PO ₄	37.49	3.59	83.56	2.31	20	9.9	30
Heughebaert and Montel, 1982	2	Ca(NO ₃) ₂ * 4H ₂ O	H(NH ₄) ₂ PO ₄	37.49	3.59	83.56	2.31	20	9.9	120
Heughebaert and Montel, 1982	12	Ca(NO ₃) ₂ * 4H ₂ O	H(NH ₄) ₂ PO ₄	37.49	3.59	83.56	2.31	20	9.9	3000
Hoehner samples	M-ACP 5	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	34.81	2.87	78.91	1.67	21	9.4	20
Hoehner samples	HA1	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	36.08	2.89	81.42	1.67	21	10	20160
Hoehner samples	HA2	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	33.96	2.85	77.23	1.67	21	9	27360
Hoehner samples	ACP 22	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	33.96	2.85	77.23	1.67	21	9	30
Hoehner samples	ACP 23	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	33.96	2.85	77.23	1.67	21	9	30
Hoehner samples	Ratio 0.2 pH low	CaCl ₂ * 2H ₂ O	Na ₂ HPO ₄	28.68	1.88	67.64	0.2	21	7.8	5
Hoehner samples	Ratio 0.6 pH low	CaCl ₂ * 2H ₂ O	Na ₂ HPO ₄	28.31	1.96	66.83	0.6	21	7.4	5
Hoehner samples	Ratio 0.2 pH high	CaCl ₂ * 2H ₂ O	Na ₂ HPO ₄	35.04	1.86	80.38	0.2	21	11	5
Hoehner samples	Ratio 1.0 pH high	CaCl ₂ * 2H ₂ O	Na ₂ HPO ₄	34.87	2.06	79.85	1	21	10.5	5
Hoehner samples	Ratio 5.0 pH high	CaCl ₂ * 2H ₂ O	Na ₂ HPO ₄	34.36	1.87	79.00	4.9	21	10.3	5
Hoehner samples	S10CLNA-DuTrue-2	CaCl ₂ * 2H ₂ O	Na ₂ HPO ₄	29.67	1.80	69.70	0.2	21	8.39	5
Hoehner samples	S10CLNA-3	CaCl ₂ * 2H ₂ O	Na ₂ HPO ₄	36.06	3.61	80.67	0.67	21	9.2	5
Hoehner samples	S10CLNA-Du-4	CaCl ₂ * 2H ₂ O	Na ₂ HPO ₄	37.69	3.95	83.59	0.33	21	9.66	5
Hoehner samples	S10CLNA-Du	CaCl ₂ * 2H ₂ O	Na ₂ HPO ₄	29.92	2.99	69.01	0.67	21	6.9	5
Hoehner samples	S10CLNA-Du-2	CaCl ₂ * 2H ₂ O	Na ₂ HPO ₄	31.25	3.60	71.05	0.67	21	6.8	5
Hoehner samples	S7ACENH4-2	Ca(CH ₃ COO) ₂ * H ₂ O	(NH ₄) ₂ HPO ₄	28.94	2.24	67.80	1.67	21	7	7

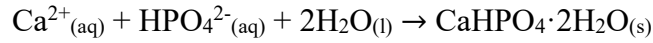
Sample ID	Freeze dried	pH adjuster	Percent ACP	Binary ACP	Percent HAP	Binary HAP	Percent OCP	Binary Br	Percent OCP	Percent Monetite
g5	No	NH4OH NH4Cl	34.5	1	65.5	1	0	0	0	0
g6	No	NH4OH NH4Cl	19.5	1	80.5	1	0	0	0	0
g7	No	NH4OH NH4Cl	20.5	1	79.5	1	0	0	0	0
g8	No	NH4OH NH4Cl	11.5	1	88.5	1	0	0	0	0
g9	No	NH4OH NH4Cl	6	1	94	1	0	0	0	0
g10	No	NH4OH NH4Cl	2	0	98	1	0	0	0	0
1	Yes	NH4OH	100	1	0	0	0	0	0	0
2	Yes	NH4OH	100	1	0	0	0	0	0	0
12	Yes	NH4OH	0	0	100	1	0	0	0	0
M-ACP 5	Yes	KOH	100	1	0	0	0	0	0	0
HA1	No	KOH	0	0	100	1	0	0	0	0
HA2	No	NH4OH	0	0	100	1	0	0	0	0
ACP 22	Yes	KOH	100	1	0	0	0	0	0	0
ACP 23	Yes	NH4OH	100	1	0	0	0	0	0	0
Ratio 0.2 pH low	No	None	100	1	0	0	0	0	0	0
Ratio 0.6 pH low	No	None	100	1	0	0	0	0	0	0
Ratio 0.2 pH high	No	NaOH	100	1	0	0	0	0	0	0
Ratio 1.0 pH high	No	NaOH	100	1	0	0	0	0	0	0
Ratio 5.0 pH high	No	NaOH	100	1	0	0	0	0	0	0
S10CLNA-DuTrue-2	No	NaOH	100	1	0	0	0	0	0	0
S10CLNA-3	No	NaOH	100	1	0	0	0	0	0	0
S10CLNA-Du-4	No	NaOH	100	1	0	0	0	0	0	0
S10CLNA-Du	No	NaOH	0	0	0	0	100	1	0	0
S10CLNA-Du-2	No	NaOH	0	0	0	0	100	1	0	0
S7ACENH4-2	No	None	0	0	0	0	100	1	0	0

SUPERSATURATION CALCULATION PARAMETERS

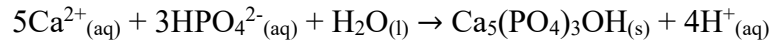
Using the following chemical reactions for ACP



Brushite (Br)



And hydroxylapatite (HAP)



Ion activity products were calculated for each sample using the equations

$$IAP \text{ for ACP} = \frac{[\text{Ca}^{2+}]^3 \cdot [\text{HPO}_4^{2-}]^2}{[\text{H}^+]^2}$$

$$IAP \text{ for Br} = [\text{Ca}^{2+}] \cdot [\text{HPO}_4^{2-}]$$

$$IAP \text{ for HAP} = \frac{[\text{Ca}^{2+}]^5 \cdot [\text{HPO}_4^{2-}]^3}{[\text{H}^+]^4}$$

Where [] denotes activity. The supersaturation indices relative to ACP, brushite, and hydroxylapatite are calculated for each sample using the equations

$$\Omega_{ACP} = \log \frac{IAP \text{ for ACP}}{K_{sp \text{ ACP}}}$$

$$\Omega_{Br} = \log \frac{IAP \text{ for Br}}{K_{sp \text{ Br}}}$$

$$\Omega_{HAP} = \log \frac{IAP \text{ for HAP}}{K_{sp \text{ HAP}}}$$

Where standard ion activity products for brushite ($\log K_{sp} = -6.60$) and hydroxylapatite ($\log K_{sp} = -56.02$, Jaynes *et al.*, 1999) and ACP ($\log K_{sp} = -25.23$ Meyer & Eanes, 1978)

Table C-2 The ionic strength, Ca^{2+} and HPO_4^{2-} activity coefficients (γ), Ca^{2+} and HPO_4^{2-} activity calculated using the Davies equation, ion activity products for ACP, brushite (Br) and hydroxylapatite (HAP), and saturation indices for ACP, brushite, and hydroxylapatite (Ω).

Sample ID	Ionic strength	$\text{Ca}^{2+} \gamma$	$\text{HPO}_4^{2-} \gamma$	Ca^{2+} activity	HPO_4^{2-} activity	IAP for ACP	IAP for Br	IAP for HAP	Ω ACP	Ω Br	Ω HAP
ES-6	0.157	0.273	0.273	0.014	0.008	1.70E+03	1.12E-04	2.59E+10	28.5	2.65	66.4
ES-10	0.113	0.303	0.303	0.015	0.009	2.89E+08	1.38E-04	6.06E+20	33.7	2.74	76.8
IS-2.1	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.2	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.3	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.4	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.5	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.6	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.7	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.8	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.9	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.10	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.11	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.12	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.13	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.14	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.15	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.16	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.17	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.18	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-2.19	0.166	0.310	0.310	0.015	0.009	3.21E+03	1.44E-04	7.16E+10	28.7	2.76	66.9
IS-3.1	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.2	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.3	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.4	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.5	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.6	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.7	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.8	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.9	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.10	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.11	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.12	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.13	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.14	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.15	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.16	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.17	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.18	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-3.19	0.163	0.299	0.299	0.015	0.009	2.68E+03	1.34E-04	5.35E+10	28.7	2.73	66.7
IS-4.1	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.2	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.3	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.4	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.5	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.6	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5

IS-4.7	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.8	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.9	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.10	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.11	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.12	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.13	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.14	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.15	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.16	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.17	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.18	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
IS-4.19	0.158	0.277	0.277	0.014	0.008	1.85E+03	1.15E-04	2.95E+10	28.5	2.66	66.5
Sup 1	0.074	0.407	0.407	0.007	0.008	9.43E+02	5.83E-05	1.53E+10	28.2	2.37	66.2
Sup 2	0.074	0.407	0.407	0.007	0.008	2.37E+03	5.83E-05	9.62E+10	28.6	2.37	67.0
Sup 3	0.074	0.406	0.406	0.007	0.008	2.36E+04	5.82E-05	9.57E+12	29.6	2.36	69.0
Sup 4	0.072	0.409	0.409	0.007	0.008	2.43E+05	5.89E-05	1.00E+15	30.6	2.37	71.0
Sup 5	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
Sup 6	0.039	0.487	0.487	0.008	0.010	5.85E+09	8.37E-05	4.09E+23	35.0	2.52	79.6
Sup 7	0.074	0.407	0.407	0.007	0.008	9.43E+02	5.83E-05	1.53E+10	28.2	2.37	66.2
Sup 8	0.074	0.407	0.407	0.007	0.008	2.37E+03	5.83E-05	9.62E+10	28.6	2.37	67.0
Sup 9	0.074	0.406	0.406	0.007	0.008	2.36E+04	5.82E-05	9.57E+12	29.6	2.36	69.0
Sup 10	0.072	0.409	0.409	0.007	0.008	2.43E+05	5.89E-05	1.00E+15	30.6	2.37	71.0
Sup 11	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
Sup 12	0.039	0.487	0.487	0.008	0.010	5.85E+09	8.37E-05	4.09E+23	35.0	2.52	79.6
a1	0.073	0.408	0.408	0.007	0.008	2.40E+04	5.86E-05	9.85E+12	29.6	2.37	69.0
a2	0.073	0.408	0.408	0.007	0.008	2.40E+04	5.86E-05	9.85E+12	29.6	2.37	69.0
a3	0.073	0.408	0.408	0.007	0.008	2.40E+04	5.86E-05	9.85E+12	29.6	2.37	69.0
a4	0.073	0.408	0.408	0.007	0.008	2.40E+04	5.86E-05	9.85E+12	29.6	2.37	69.0
a5	0.073	0.408	0.408	0.007	0.008	2.40E+04	5.86E-05	9.85E+12	29.6	2.37	69.0
a6	0.073	0.408	0.408	0.007	0.008	2.40E+04	5.86E-05	9.85E+12	29.6	2.37	69.0
a7	0.073	0.408	0.408	0.007	0.008	2.40E+04	5.86E-05	9.85E+12	29.6	2.37	69.0
a8	0.073	0.408	0.408	0.007	0.008	2.40E+04	5.86E-05	9.85E+12	29.6	2.37	69.0
a9	0.073	0.408	0.408	0.007	0.008	2.40E+04	5.86E-05	9.85E+12	29.6	2.37	69.0
a10	0.073	0.408	0.408	0.007	0.008	2.40E+04	5.86E-05	9.85E+12	29.6	2.37	69.0
a11	0.073	0.408	0.408	0.007	0.008	2.40E+04	5.86E-05	9.85E+12	29.6	2.37	69.0
a12	0.073	0.408	0.408	0.007	0.008	2.40E+04	5.86E-05	9.85E+12	29.6	2.37	69.0
a13	0.073	0.408	0.408	0.007	0.008	2.40E+04	5.86E-05	9.85E+12	29.6	2.37	69.0
b1	0.073	0.408	0.408	0.007	0.008	2.42E+04	5.88E-05	9.96E+12	29.6	2.37	69.0
b2	0.073	0.408	0.408	0.007	0.008	2.42E+04	5.88E-05	9.96E+12	29.6	2.37	69.0
b3	0.073	0.408	0.408	0.007	0.008	2.42E+04	5.88E-05	9.96E+12	29.6	2.37	69.0
b4	0.073	0.408	0.408	0.007	0.008	2.42E+04	5.88E-05	9.96E+12	29.6	2.37	69.0
b5	0.073	0.408	0.408	0.007	0.008	2.42E+04	5.88E-05	9.96E+12	29.6	2.37	69.0
b6	0.073	0.408	0.408	0.007	0.008	2.42E+04	5.88E-05	9.96E+12	29.6	2.37	69.0
b7	0.073	0.408	0.408	0.007	0.008	2.42E+04	5.88E-05	9.96E+12	29.6	2.37	69.0
e1	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
e2	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2

e3	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
e4	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
e5	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
e6	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
e7	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
e8	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
e9	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
f1	0.058	0.435	0.435	0.007	0.009	3.33E+07	6.68E-05	1.66E+19	32.8	2.43	75.2
f2	0.058	0.435	0.435	0.007	0.009	3.33E+07	6.68E-05	1.66E+19	32.8	2.43	75.2
f3	0.058	0.435	0.435	0.007	0.009	3.33E+07	6.68E-05	1.66E+19	32.8	2.43	75.2
f4	0.058	0.435	0.435	0.007	0.009	3.33E+07	6.68E-05	1.66E+19	32.8	2.43	75.2
f5	0.058	0.435	0.435	0.007	0.009	3.33E+07	6.68E-05	1.66E+19	32.8	2.43	75.2
f6	0.058	0.435	0.435	0.007	0.009	3.33E+07	6.68E-05	1.66E+19	32.8	2.43	75.2
f7	0.058	0.435	0.435	0.007	0.009	3.33E+07	6.68E-05	1.66E+19	32.8	2.43	75.2
f8	0.058	0.435	0.435	0.007	0.009	3.33E+07	6.68E-05	1.66E+19	32.8	2.43	75.2
f9	0.058	0.435	0.435	0.007	0.009	3.33E+07	6.68E-05	1.66E+19	32.8	2.43	75.2
f10	0.058	0.435	0.435	0.007	0.009	3.33E+07	6.68E-05	1.66E+19	32.8	2.43	75.2
g1	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
g2	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
g3	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
g4	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
g5	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
g6	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
g7	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
g8	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
g9	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
g10	0.058	0.435	0.435	0.007	0.009	3.34E+07	6.69E-05	1.66E+19	32.8	2.43	75.2
1	0.214	0.310	0.310	0.031	0.032	1.84E+12	9.72E-04	3.48E+27	37.5	3.59	83.6
2	0.214	0.310	0.310	0.031	0.032	1.84E+12	9.72E-04	3.48E+27	37.5	3.59	83.6
12	0.214	0.310	0.310	0.031	0.032	1.84E+12	9.72E-04	3.48E+27	37.5	3.59	83.6
M-ACP 5	0.127	0.351	0.351	0.018	0.011	3.78E+09	1.85E-04	7.72E+22	34.8	2.87	78.9
HA1	0.113	0.362	0.362	0.018	0.011	7.03E+10	1.97E-04	2.51E+25	36.1	2.89	81.4
HA2	0.139	0.343	0.343	0.017	0.010	5.33E+08	1.76E-04	1.61E+21	34.0	2.85	77.2
ACP 22	0.139	0.343	0.343	0.017	0.010	5.33E+08	1.76E-04	1.61E+21	34.0	2.85	77.2
ACP 23	0.139	0.343	0.343	0.017	0.010	5.33E+08	1.76E-04	1.61E+21	34.0	2.85	77.2
Ratio 0.2 pH low	0.060	0.435	0.435	0.002	0.010	2.75E+03	1.88E-05	4.02E+11	28.7	1.87	67.6
Ratio 0.6 pH low	0.043	0.478	0.478	0.004	0.006	1.18E+03	2.26E-05	6.20E+10	28.3	1.95	66.8
Ratio 0.2 pH high	0.067	0.421	0.421	0.002	0.009	5.81E+09	1.75E-05	1.93E+24	35.0	1.84	80.3
Ratio 1.0 pH high	0.033	0.511	0.511	0.005	0.005	3.50E+09	2.62E-05	4.68E+23	34.8	2.02	79.7
Ratio 5.0 pH high	0.066	0.424	0.424	0.009	0.002	1.17E+09	1.78E-05	7.71E+22	34.3	1.85	78.9
S10CLNA-DuTrue-2	0.055	0.445	0.445	0.002	0.009	2.70E+04	1.59E-05	4.59E+13	29.7	1.80	69.7
S10CLNA-3	0.350	0.289	0.289	0.026	0.039	6.69E+10	1.01E-03	4.42E+24	36.1	3.61	80.7
S10CLNA-Du-4	0.607	0.304	0.304	0.027	0.082	2.87E+12	2.24E-03	3.67E+27	37.7	3.95	83.6
S10CLNA-Du	0.182	0.320	0.320	0.013	0.019	4.90E+04	2.46E-04	9.75E+12	29.9	2.99	69.0
S10CLNA-Du-2	0.391	0.288	0.288	0.026	0.039	1.04E+06	1.01E-03	1.08E+15	31.2	3.60	71.1
S7ACENH4-2	0.270	0.297	0.297	0.027	0.002	5.12E+03	4.36E-05	6.02E+11	28.9	2.24	67.8

REGRESSION EQUATION CURVES

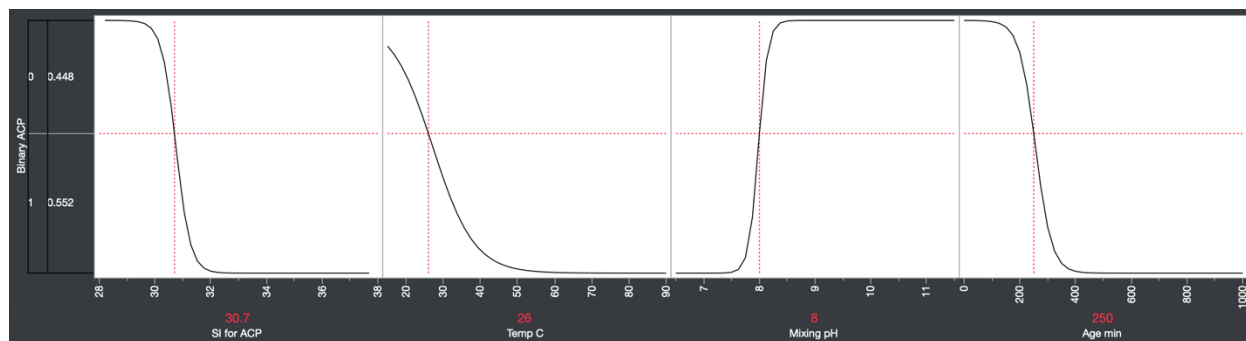


Figure C-1 The profile curves based on the ACP binary logistic equation (equation 3 in Chapter 4) demonstrate that at a supersaturation of 30.7, a temperature of 26 °C, pH of 8, and age of 250 minutes, the probability function is 0.552. In this evaluation probabilities >0.5 are considered a positive outcome that predict the presence of ACP. The overall formation of ACP is preferred at lower supersaturation, temperature, and age, but higher pH.

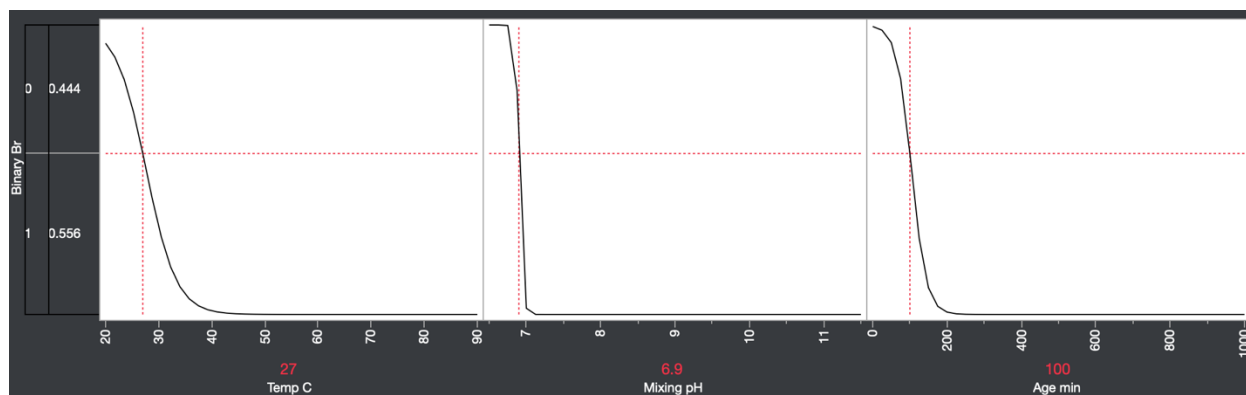


Figure C-2 The profile curves based on the brushite binary logistic equation (equation 4 in Chapter 4) demonstrate that at a temperature of 27 °C, pH of 6.9, and age of 100 minutes, the probability function is 0.556. In this evaluation probabilities >0.5 are considered a positive outcome that predict the presence of brushite. The overall formation of brushite is preferred at lower temperature, pH, and age.

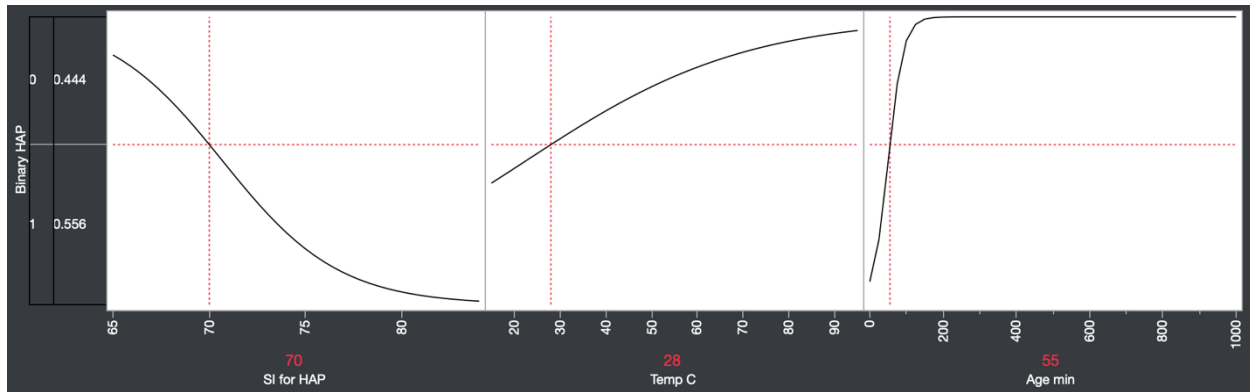


Figure C-3 The profile curves based on the hydroxylapatite binary logistic equation (equation 5 in Chapter 4) demonstrate that at a temperature of 28 °C, supersaturation of 70, and age of 55 minutes, the probability function is 0.556. In this evaluation probabilities >0.5 are considered a positive outcome that predict the presence of hydroxylapatite. The overall formation of hydroxylapatite is preferred at lower supersaturation, but higher temperature, and age.

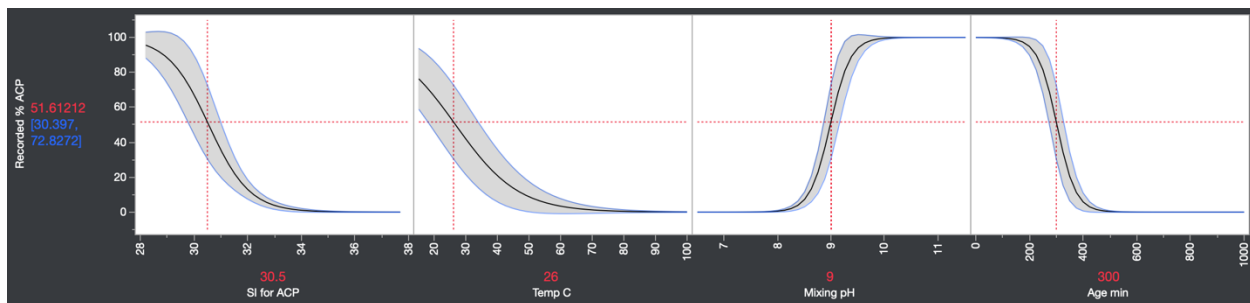


Figure C-4 The profile curves based on the ACP nonlinear continuous logistic equation (equation 6 in Chapter 4) demonstrate that at a supersaturation of 30.5, a temperature of 26 °C, pH of 9, and age of 300 minutes, the predicted percentage of ACP is 52%. The overall formation of ACP is preferred at lower supersaturation, temperature, and age, but higher pH. The shaded grey region represents the standard error of the curve.

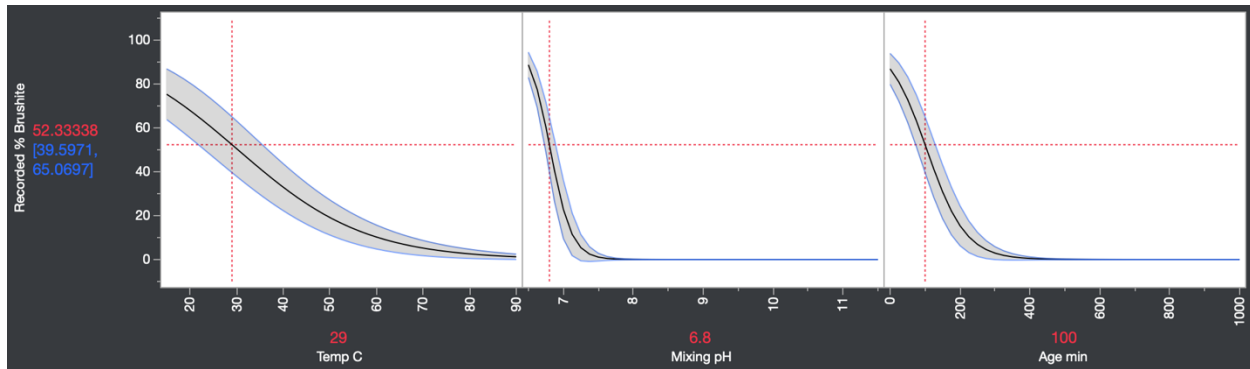


Figure C-5 The profile curves based on the brushite nonlinear continuous logistic equation (equation 7 in Chapter 4) demonstrate that at a temperature of 29 °C, pH of 6.8, and age of 100 minutes, the predicted percentage of brushite is 52%. The overall formation of brushite is preferred at lower temperature, pH, and age. The shaded grey region represents the standard error of the curve.

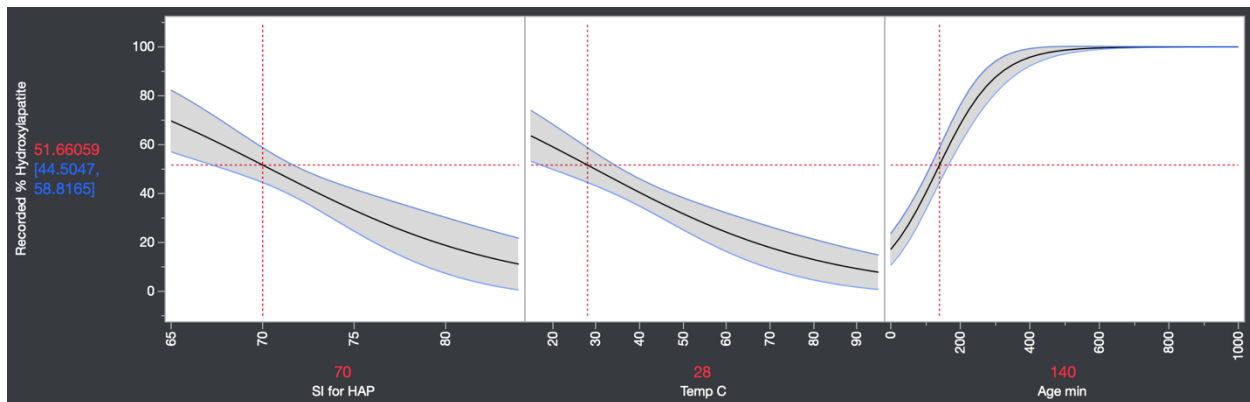


Figure C-6 The profile curves based on the hydroxylapatite nonlinear continuous logistic equation (equation 8 in Chapter 4) demonstrate that at a temperature of 28 °C, supersaturation of 70, and age of 140 minutes, the predicted percentage of hydroxylapatite is 52%. The overall formation of hydroxylapatite is preferred at lower supersaturation, but higher temperature, and age. The shaded grey region represents the standard error of the curve.

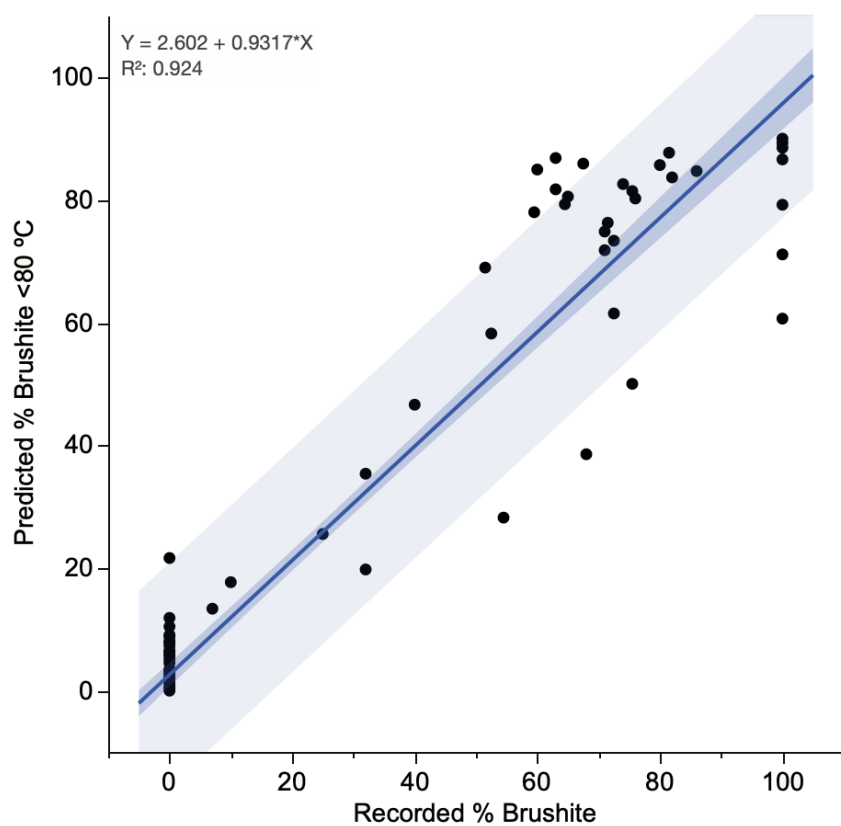


Figure C-7 The linear regression between the predicted percent of brushite and the recorded percent of brushite when the nonlinear continuous logistic regression analysis is reported for samples synthesized at temperatures below 80 °C.