THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOMEDICAL ENGINEERING

THE DEVELOPMENT OF CALCIUM DOPED CITRATE BOUND BIOMATERIALS FOR MULTIFUNCTIONAL ORTHOPEDIC TISSUE ENGINEERING APPLICATIONS

JOHN FADEL
SPRING 2017

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biomedical Engineering
with honors in Biomedical Engineering

Reviewed and approved* by the following:

Jian Yang,
Principal Investigator of
Transformative Biomaterials and Biotechnology Lab
& Professor of Biomedical Engineering,
Thesis Supervisor

William Hancock,
Professor of Biomedical Engineering,
Honors Advisor

Justin Brown,
Associate Professor of Biomedical Engineering,
Faculty Reader

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

Bone has become the second most transplanted tissue, only behind blood, with more than $30 billion spent annually in orthopedic related medical costs [1]. Previous research has confirmed the presence of strongly bound citrate-rich molecules that serve to stabilize the apatite nanocrystals within natural bone. The regulation of apatite nanostructure and the formation of apatitic calcium phosphate crystals impart natural bone with its mechanical properties and citrate is now thought to be a critical component in bone metabolism [2]. A citrate based, biodegradable elastomer, poly (1,8-octanediol-co-citric acid) (POC), has been developed, displaying excelled *in vitro* and *in vivo* biocompatibility; however, pure POC materials display insufficient mechanical properties in hydrated conditions, rapid degradation, and insufficient osteoconductivity [3]. Multiple ions, including calcium, are present throughout the body fluid and tissues, with roles in diverse biological processes including bone regeneration [4]. Citric acid, a polycarboxylic acid present in POC, readily chelates with cationic species and thus the addition of ionic species to POC results in the formation of a secondary, reversible ionic network, capable of improving mechanical strength while maintaining elasticity. Furthermore, the addition of calcium ions to POC is capable of imparting the bioactivity of the incorporated species into the resulting material. In this study, the development of a secondary ionic network was the focus as well as expanding the versatility of POC and its functional capabilities. Through various synthesis doping modifications, ionic doping sources, and a plethora of studies to characterize and test the formulated materials, this innovative design has solidified an enhanced polymer material for orthopedic applications. Calcium doping of POC has displayed improved mechanical strength in both dry and hydrated conditions with the maintenance of elastomeric character, shortened

degradation rate, decreased swelling, minimal soluble content, improved cytocompatibility, a high degree of homogeneity, and a wide versatility in scaffold fabrication and molar concentration variability.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

Foremost, I would like to thank Dr. Jian Yang for his commitment to the Transformative Biomaterials and Biotechnology Lab and to the educational growth of those interested in the biomedical engineering field. His guidance and leadership have helped me develop my project and allowed me to be successful in my research pursuit.

Secondly, I would like to thank Ethan Gerhard for his unconditional support throughout the length of my project. Ethan's motivation and brilliance are key factors in what has propelled me to the position I am in today in completing this thesis and doing so with enjoyment.

I would also like to thank the rest of my thesis committee: Dr. William Hancock and Dr. Justin Brown. They have been extremely insightful and encouraging with their comments, edits, and overall supervision of my project.

Lastly, I would like to thank my family. My parents, Rania and Michel Fadel, for without them, I would not be here, and that surely deserves gratitude. My siblings, George, Chris, and Nicole, who did not contribute to the thesis at all, but deserve some love too.

Chapter 1

Introduction

Orthopedic Engineering Background

With more than $30 billion spent annually in orthopedic related medical costs, bone has become the second most transplanted tissue, only behind blood [1]. The field of orthopedic engineering focuses on the biological aspects of bone and relates it to how the osseous tissue and implants work together. From considering tissue engineering applications, in cell differentiation, proliferation, and seeding, biomechanics, in replicating the material properties of native bone, material science, in formulating a wide array of polymers, and organic chemistry, in following the reaction mechanisms of organic molecules, orthopedic engineering is inclusive of various subsets of the sciences. Orthopedic engineering focuses on the use of osteoprogenitors, or osteogenic stem cells, for biomedical applications of bone fracture healing and general skeletal reconstruction [5].

There are immense possibilities for the advancement of healthcare and medicine through the investment in orthopedic biomedical engineering. Orthopedic medicine affects tens of millions of patients each year, with procedures ranging from fracture fixation to total joint replacement. Knee, hip, and other joint replacements, as well as fracture fixations are just some of the many current orthopedic medical issues that research has taken a large focus in. The discoveries in the functioning or failure to function in the orthopedic discipline, as well as the

fabrication of novel techniques, treatments, and tools have the opportunity to shape the future of orthopedic medicine.

Orthopedic Surgery Implant Sources

Metal Implants

Metal implants such as titanium or stainless steel screws and plates possess significant and desirable mechanical properties to reinforce bone during healing. However, due to the larger mechanical modulus of metal versus native bone, this material implant choice usually results in bone loss around the implant [6]. Additionally, the potential for patient immune response, allergic reactions, and possible systemic toxicity resulting from released metal fragments and metal oxidation *in vivo* is far too prevalent for this implant material to be an effective choice for the future of orthopedic repairs [6]. Another unfortunate characteristic of metal implants is there lack of degradability. These implants offer no possibility of bone ingrowth and in many cases, require a secondary surgery or the permanent placement of the metal piece in the body [6]. Current research takes a strong focus in the formulation of synthetic, biomimetic biomaterials to develop orthopedic implants for diseased and fractured bones.

Bone Grafts

Marking the potential to improve the field of reconstructive orthopedic surgery includes research into the generation of viable and functional bone grafts that replicate the mechanical and osteogenic bioactivity of native bone. Success in obtaining the ability to readily tailor the

physical and bioactive properties of these synthetic, biomimetic biomaterials is highly sought after. The various biomedical applications that would come from this control would lead to exciting advancements in orthopedic healthcare. The success in designing functional bone grafts with maximal efficiency that replicate the viscoelastic and antifatigue properties as well as the bioactivity of physiological bone tissue is also critical for the repair and reconstruction of congenital defects, cancer resections, and trauma related injury.

The transplantation of bone tissue, or bone grafting, is a common surgical procedure with the mission to fix damaged bone from trauma, problem joints, or natural deterioration. It is also beneficial for bone growth around an implanted device by its principal of covering the voids where there is an absence of bone, resulting in assisting the structural stability of the location. Regulation of the regenerative potential of bone grafts include three key biological mechanisms: osteoinduction, osteoconduction, and osteogenesis. Growth factors mediate the osteoinduction, or the activation of host mesenchymal stem cells that differentiate into bone-forming cells. The three-dimensional structure of the graft imposes factors on the osteoconduction potential, which is the capability of providing structural frameworks for host cells to develop Haversian systems. Osteogenic precursors mediate the capacity of the formation of bone, otherwise known as osteogenesis [7].

The most prevalent forms of bone grafts include allografts and autografts. While allografts utilize bone tissue stored in a tissue bank originating from a deceased donor, autografts originate from bone tissue within the patient's own body [8]. The prevalence of autografts and allografts in reconstructive orthopedic surgery has been substantial, but not without their setbacks. The significant limitations of these techniques come from the difficulty in harvesting material and of three-dimensional contouring to match the original tissue geometry to be

replaced [5]. Allografts might be functional in quantity, but there are continued limitations in their risk of infections and disease transmission. Allografts restrictions in not having osteogenic properties is also a major setback for this grafting technique [5,9]. Due to the preparation process, allografts do not have the potential of osteogenesis, even though having osteoconduction and osteoinduction capacity [7].

While autografts remain the clinical standard for orthopedic repair, there are many reservations to this protocol. For one, the limited supply and potential donor site complications restrict their clinical use. Though autografts maintain the biological properties of interest, this grafting technique is limited by the morbidity of the donor site and may be restricted in volume [9,10]. Autographs come from either the cancellous, the spongier bone, cortical, the more compact form of bone, or corticocancellous, a mixture of the two. While the cancellous bone in the human body has the potential for osteogenesis, osteoinduction, and osteoconduction, the limitations for this source of autograft is due to the poor mechanical strength. On the other hand, cortical bone has the stronger mechanical properties that cancellous bone lacked, but due to the lack of osteoblasts and stem cells, cortical bone has poor osteogenesis and osteoconductive potential [11].

Allografts on the other hand might be functional in quantity, but there are continued limitations in their risk of infections and disease transmission. Allograft's restrictions in not having osteogenic properties is also a major setback for this grafting technique [5,9]. Due to the preparation process, allografts do not have the potential of osteogenesis, even though having osteoconduction and osteoinduction capacity [7].

There have been alternative methods that are promising in that they eliminate donor site morbidity and minimize the risk to the patient from disease and immune response. This method

involves the use of decellularized bone matrices, and even with those benefits, there are disadvantages that persist. The use of the decellularized bone still maintains dependency on the harvesting and shaping of bone as well as the effect of completely denuding the specimen of native cells [8]. Decellularized bone matrix provides the field of synthetic bone substitutes with osteoconduction, flexibility with porosity, crystalline composition, and mechanical resistance. However, the lack of osteoinduction and osteogenesis activity is far too great of a limiting factor for the ceramic-based substitutes [12].

Polymer Based Scaffolds

Another bone construction method that has gained appeal is using polymer based scaffolds. Though the need of harvesting organic tissue is eliminated, many polymers continue to display limited usefulness in terms of incompatible mechanical properties and harmful degradation product generation *in vivo*. The ideal aspects of polymers that are the reason for continued research in this field of polymer engineering for orthopedic reconstruction is their ability to exhibit versatile physical properties within the construction of complex geometries [8].

Treatment based from obtaining osteoblasts, chondrocytes, and mesenchymal stem cells from the patient's own soft and hard tissues help many of the limitations of pathogen transfer, donor site scarcity, and immune rejection [13]. This treatment strives on the culturing and seeding of these obtained cells onto some sort of scaffold that then show specific degradation and resorption properties *in vitro* and/or *in vivo* when grown in the tissue structures [14]. With polymer scaffolds, there has to be a focus on selecting a material that is highly biocompatible and does not have the potential of an immunological or clinically detectable foreign body

reaction [15]. Also, while the attachment of the specific tissue cells is seeded into the construct, the material should simultaneously be able to resorb and degrade at a controlled rate. The life of this implant and the degradation rate factors in the amount of time it takes for the implant to serve its purpose in cell proliferation to the target tissues, matching the rate of tissue regeneration by the cell of interest [16].

To successfully pursue the target applications of a three-dimensional scaffold for cell growth and differentiation, the scaffold should ideally be biocompatible, biodegradable, facilitate cell proliferation, as well as differentiation, replicate physical and mechanical properties of the target area, and host adequate cell loading [14,17]. Synthetic biodegradable polymers have been a growing focus in bone tissue engineering research with the goal of bone reconstruction. Advantages of these include the reproducibility of their mechanical and physical properties, and a control in their material impurities. With the development of relatively simple monomers, the risk of toxicity and infections can be limited [18].

Saturated aliphatic polyesters are some of the most utilized three-dimensional scaffolds within tissue engineering research of biodegradable synthetic polymers. These include the poly-α-hydroxy esters: poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly (lactic-*co*-glycolide) (PLGA) copolymers [18,19]. The degradation of these polyesters is through the uptake of water followed by the hydrolysis of ester bonds. Other factors of these polymers' degradation rates include polydispersity, chemical composition, configurational structure, molar mass, porosity, hydrophobicity, and stress and strain [20]. Once these polymers are dissolved, the monomers can exit the body through natural pathways. PLA and PGA have been US Food and Drug Administration (FDA) approved for these reasons and have been used in the medical field as a biodegradable material [21].

PGA is characterized as having a rapid degradation, inevitably resulting in the loss of mechanical strength, paired with an insolubility in many common solvents, and a significant local production of glycolic acid, which is linked to strong, unfavorable inflammatory responses. PGA has many setbacks as a biomaterial that limit its biomedical applications past acting as short-term tissue engineering scaffolds [22]. Many of the other current materials, such as PLA and its chiral molecules, PLGA, and PGA, fall short in meeting the increased demands of regenerative engineering applications. These polymers are limited by the total amount of bioceramic that can be incorporated into the composite before becoming too brittle, in turn, limiting their osteogenic potential in load bearing applications, and cannot meet sufficient mechanical property mimicking of native bone [23].

Background on POC and its Calcium Doping

POC Background

The readily available intermediate in the Kreb's cycle, citric acid, is an inexpensive, multifunctional monomer that has three carboxyl groups and one hydroxyl group [23]. The characteristics and structure of this monomer supplies strong advantages for citrate based biomaterials and for biodegradable elastomers in general. For one, the ability of citric acid to be used with diol monomers, like poly (1,8-octanediol-co-citric acid) (POC), in an effective thermal polycondensation reaction allows for the previously mentioned advantages of a polyester and its hydrolytic degradable characteristics. Also, this combination is beneficial for the inherent antioxidant, antimicrobial, and adhesive properties, which are huge benefits for reconstructive orthopedic materials. POC is a previously developed, citrate based, biodegradable elastomer, and

as citrate is a critical component in bone metabolism [20], POC displays excellent *in vitro* and *in vivo* biocompatibility [2].

Findings have created a special interest in the role of citrate, the ionic form of citric acid, as a significant role in bone physiology and development. These findings show that citrate may be closely related with early stage bone markers, alkaline phosphatase and osterix, as well as mid to late stage bone markers, osteopontin and osteocalcin, all in turn showing its association with the gene expression for bone [24].

Purpose and Goals

Pure POC, as a material generalized for a wider variety of applications, displays insufficient mechanical properties in hydrated conditions, limited osteoconductivity [3]. The potential for this polymer is still of large interest for further research though. For one, POC hosts rich carboxylic groups that display its ability to chelate with calcium containing hydroxyapatite. The interactions facilitated between the polymer and the hydroxyapatite are similar to that of the natural formation and interaction of citrate bound apatite nanocrystal in native bone [25]. This interaction allows for increased bioactivity, degradation, and mechanical properties, however, this compositing with an inorganic filler results in diminished elastomeric behavior. Thus, the goal is to develop a material that allows for the improvement of the bioactivity, mechanical properties, and degradation rate of POC, while maintaining the elastomer character [26].

There has been a plethora of polymer systems where the incorporation of ions has been studied. The goal of the ion incorporation is to be able to increase the mechanical properties and derive applications of the resulting elasticity properties [27]. As a polycarboxylic acid, citric acid

can readily chelate with cationic species, such as calcium. Citric acid is often used as a carrier agent for oral administration of such cationic species. This ionic-doped POC is hypothesized to lead to the improvement of mechanical strength, with the maintenance of elasticity in comparison to regular POC. This is due to the creation of a secondary, reversible ionic network between the divalent cation and the polymer.

In bone, citric acid binds to phases of hydroxyapatite to halt it from growing and thus creates controlled layers of hydroxyapatite and citrate. The usefulness of this comes with the inhibition of forming large crystals, thus preventing brittleness and leading to better mechanical characteristics. This also creates for the potential of carboxyl groups and calcium ions to bind [27]. This natural event inspired the desire to dope the citrate-based polymer with calcium. The focus is to take advantage of the ionic interactions while still preserving the advantages of the original polymer, such as its ability to form composites with ceramics (i.e. hydroxyapatite), form films, and remain biocompatible.

The calcium doped POC is also hypothesized to form an osteoconductive, high strength elastomer based on the combination of the citrate polymer and the ion species. There is the possibility for the enhancement of osteoblast maturation and growth, osteoinduction and osteoconduction. This enhancement of osteopotential is predicted to be in line with the simultaneous improvement of the physical properties as well.

The novelty comes with the material choice in using an established polymer in POC and doping it with calcium to adjust the osteoconductivity and mechanical properties to create and enhance more multifunctional applications. Research in the methodology for formulation and characterization of the new material is of high importance to better understand the material features. The bioactivity of the ionic doping on top of the mechanical upside for the polymer

material is the factor that fuels this biomaterial research. Creating a more developed and multifunctional polymer in general to impart active properties for orthopedic tissue engineering is the overall goal. These applications could range from developing hemostat and antibacterial potential to adjusting enzyme healing pathways to forming orthopedic surgical implants for membranes such as the periosteum.

Following this current chapter, Introduction, this study is reported with sequential chapters on Methods, Results, Discussion, and Conclusion. Methods will involve the set up and procedure to synthesize, fabricate, and test the materials. Results will involve the reported data and findings on the testing of the materials. Discussion will take a more in depth analysis of how the results connect and how the results were interpreted. Lastly, the conclusion will summarize the results and discussions to conclude on this study.

Chapter 2

Methods

Material Synthesis

POC Synthesis

The synthesis of poly (1,8-octanediol-co-citric acid) (POC) was focused around the two monomers, citric acid and octanediol, in a one-to-one molar ratio. All chemicals used for the material syntheses were obtained from Sigma Aldrich or Fisher Scientific. This polycondensation reaction was started with the melting of the monomer solutions at 160°C and stirring at a rate of 360 rotations per minute (rpm) for roughly ten minutes. The temperature was then dropped to 140°C, which was then the set temperature for the remainder of the reaction. The rotation speed was also dropped to 300 rpm once the solution was being heated at 140°C. The polymer solution was watched and over time, the solution became more viscous. Once the stir bar began to twitch and could not make a full rotation, the stir speed was dropped to 200 rpm. Once the stir bar twitched again when rotating at 200 rpm, the speed was dropped once more to 100 rpm. Once the stir bar twitched when rotating at 100 rpm, roughly 70 mL of 1,4-dioxane was poured into the pre-polymer solution and the solution was manually stirred using a stirring apparatus. The pre-polymer was removed from the heating source and left sitting overnight being stirred at 200 rpm.

The synthesized pre-polymer was to be purified in water by slowly mixing in the pre-polymer solution into a beaker of deionized water. Using a stirring apparatus, the purified pre-polymer was extracted into a separate tinted bottle. The bottle with the purified pre-polymer was filled with deionized (DI) water and left to sit out overnight. Then the DI water was emptied and the solution in the bottle was left in a -80°C freezer for a few hours or until frozen. The frozen solution is then put into a freeze dryer for lyophilization for twenty-four hours. The weight of the pre-polymer was obtained by taking the current weight with the bottle and subtracting it by the original weight of the bottle. This weight was used to dissolve the pre-polymer into a 30% polymer solution with 70% ethanol solvent.

POC Soaking in Ionic Solutions

Pure POC film samples were soaked in calcium chloride solutions of various concentrations as well as in 0.1M phosphate buffered saline. This soaking was to compare the effects of how calcium was introduced to the POC polymer to further understand the best mechanism to produce calcium induced POC material. These samples were tested under either hydrated conditions after two weeks, after being lyophilized, or after being air dried. Figure 1 shows the general scheme for POC synthesis, crosslinking, and the soaking of POC in an ionic solution.

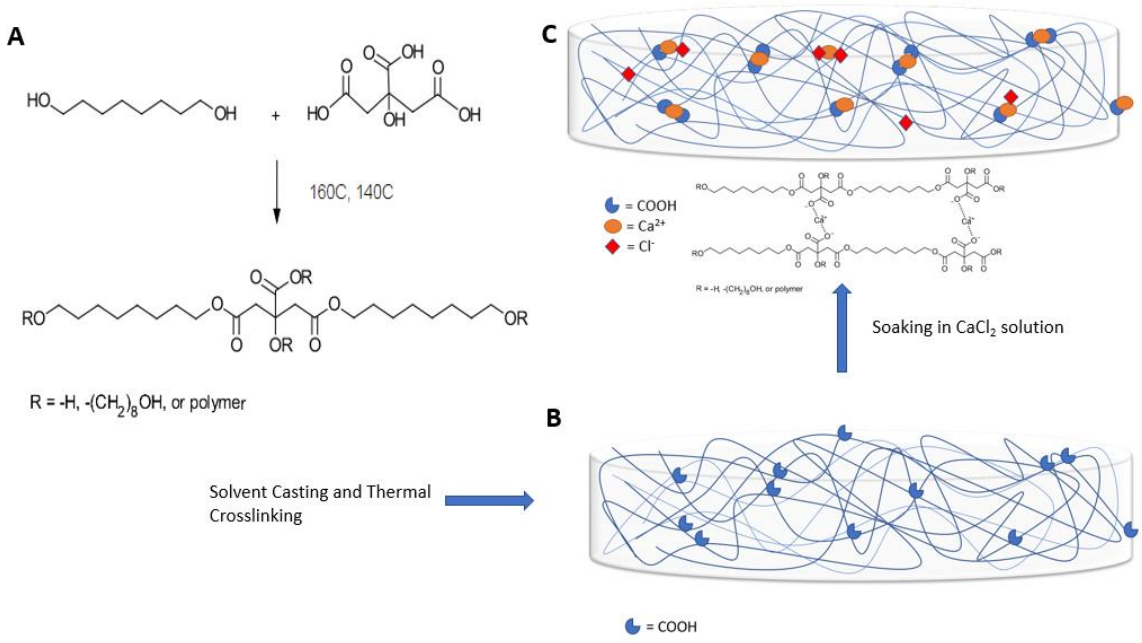

Figure 1: Synthesis scheme for calcium soaked POC. A) POC synthesis, B) Crosslinked POC film showing remaining free carboxyl side groups, & C) Chelation of calcium with carboxyls, resulting in ionic crosslinks

Synthesis of Ion Doped POC

The addition of ions can impart bioactivities and these specific ions that have been doped with POC include monovalent, divalent, trivalent, and tetravalent cations. POC was doped with various ion chloride salts including $CaCl_2$, $MgCl_2$, $SrCl_2$, $ZnCl_2$, and others. These additions to the original POC polymer are done as these ion chloride salts act as water soluble ion sources.

A family of various calcium ion species were used to dope POC in a similar synthesis process as previously explained under POC synthesis. The doped species included both water soluble and water insoluble calcium sources. The water-soluble calcium sources included POC doped with $CaCl_2$, CaI_2, $CaBr_2$, and $Ca(NO_3)_2$, while the water insoluble calcium sources included calcium citrate and calcium carbonate.

The synthesis strategy for the water-soluble calcium sources doping with POC match the POC synthesis except for the addition of $CaCl_2$ dissolved in 10 mL of DI water to the polymer solution when the reaction temperature is dropped to 140°C. The synthesis strategy for the water insoluble calcium sources doping with POC match the POC synthesis except for the initial ingredients do not only include citric acid and octanediol, but also include the water insoluble calcium species. All the various polymer syntheses also had various formulations associated with different molarity doping of the added ions. Figure 2 and Figure 3 expand on the proposed synthesis schematic for the calcium doping of POC with calcium chloride and calcium citrate, respectively. Table 1 shows example molarity formulations of the calcium doped POC with both the water insoluble and water soluble species, POC Calcium Citrate and POC Calcium Chloride, respectively.

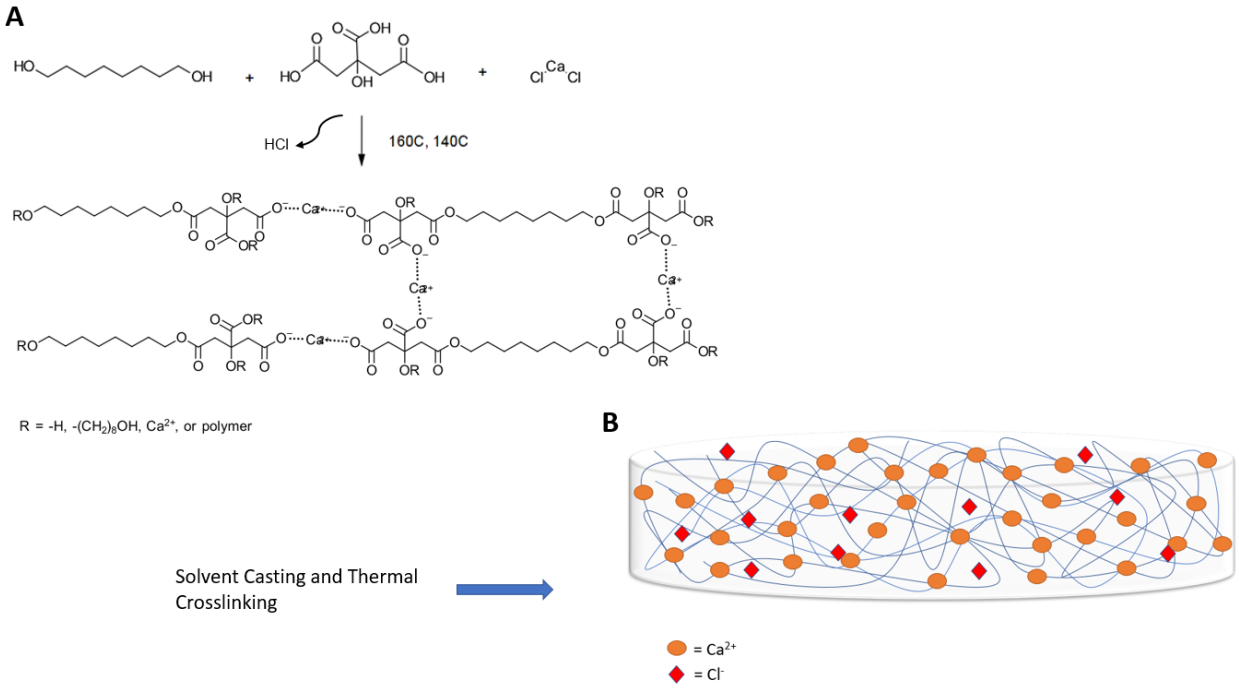

Figure 2: Synthesis scheme for calcium doped POC. A) $CaCl_2$ POC synthesis, Ca^{2+} ions are present as side groups as well as in the main polymer chains, unlike in soaked films, B) Crosslinked $CaCl_2$ POC film showing formation of calcium secondary ionic network

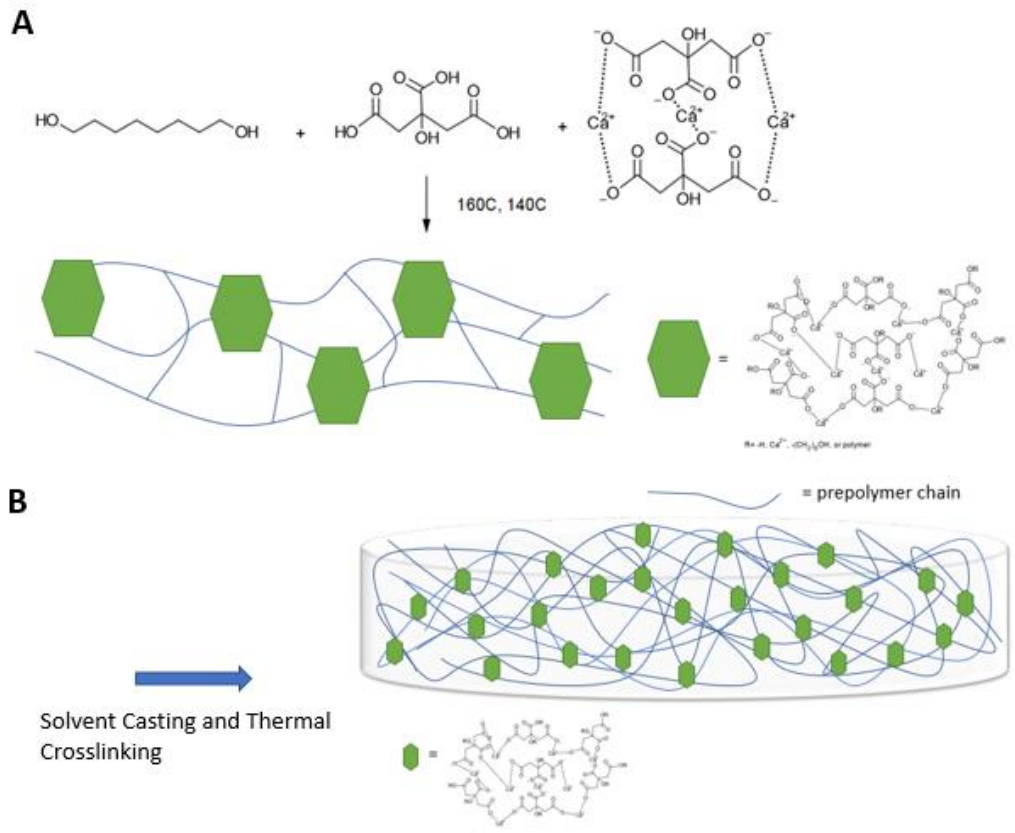

Figure 3: Synthesis scheme for CaCitrate doped POC films. A) Formation of distributed microparticles of CaCitrate connected to the polymer chains via chelation & B) Film with distributed CaCitrate microparticles bound to matrix

Table 1: Ion Doped POC Example Formulations with Molar Values

Formulation	Calcium Citrate	Citric Acid	Octanediol	Formulation	$CaCl_2$	Citric Acid	Octanediol
POC CaCitrate 0.005	0.005	0.095	0.1	POC $CaCl_2$ 0.005	0.005	0.1	0.1
POC CaCitrate 0.01	0.01	0.09	0.1	POC $CaCl_2$ 0.01	0.01	0.1	0.1
POC CaCitrate 0.02	0.02	0.08	0.1	POC $CaCl_2$ 0.02	0.02	0.1	0.1
POC CaCitrate 0.03	0.03	0.07	0.1	POC $CaCl_2$ 0.04	0.04	0.1	0.1
POC CaCitrate 0.04	0.04	0.06	0.1	POC $CaCl_2$ 0.06	0.06	0.1	0.1

Modification of Synthesis Reactions

To best understand the mechanism of the different POC ion doping reactions, as well as to find the most efficient and productive polymer materials for the orthopedic applications, different modifications to the polymer synthesis were conducted. These different modifications would be later tested to see the different applications they could hold based on their results.

The reaction involving the water-soluble calcium sources was modified in the time the dissolved chemicals were added to the reaction. With no exact way to relate time elapsed to the progress of the reaction, the water-soluble calcium source was varied by adding it at the times when the stir speed was changed to 300, 200, or 100 rpm. Other modifications include varying the crosslinking times of the pre-polymers, for example, 3 days at 80°C and then 3 days at 120°C, or 5 days at 80°C, and many others including some with vacuum and some crosslinking conditions without. The last modification was made to the basic monomer relationship of POC. Citric acid and octanediol were varied from molar ratios of 1.5:1 to 1:1.5.

Material Fabrication

The various polymer formulations were fabricated into different forms to test the versatility of the material make up and the results that could be obtained. Polymer films were made by pouring either 3.5 grams or 7 grams of the pre-polymer into a plastic sonication tube for ultrasonication. The sonicated pre-polymer was then vortexed for roughly one minute and then poured into a Teflon dish. The pre-polymer in the Teflon dish was left out on a steady and leveled surface for roughly five days and then crosslinked; crosslinking conditions could vary, but the standard condition was 3 days at 80°C, followed by 3 days at 120°C.

Porous scaffolds were also fabricated. Various porosity levels could be fabricated, but the standard scaffold fabrication was 80% porosity with a pore size roughly between 250 and 425 μm. The pre-polymer was poured into a Teflon disk and mixed in with sodium chloride salt. The final solution had 80% weight salt, or whatever percentage weight salt to fabricate the desired porosity level. The solution was mixed until a smoothened paste formed. The paste was left to dry overnight and then followed by oven crosslinking. Crosslinking conditions can be varied, but again, standard conditions were used. Following crosslinking, the scaffold was soaked in water to remove the salt.

Composites were also fabricated to evaluate the polymer materials with a ceramic mixture. The pre-polymer was poured into a Teflon disk and mixed with 60% weight hydroxyapatite. This solution was stirred until a clay like paste formed. Once this was dry enough to be physically handled, the mixture was flattened using a pasta press until it reached a thickness of 1 mm. With metal molds capping Teflon tubes, the polymer hydroxyapatite material was pieced into the Teflon tube to measure at a height of 12 mm and a diameter of 6 mm with a weight of 0.81 grams per cylinder. These molds were then compressed through locking in a clamp for roughly a day before being crosslinked. Crosslinked included setting the compressed molds with clamps for 3 days at 80°C, followed by taking the samples out of the mold to then crosslink for 3 days at 120°C.

One of the applications of the material is using it in the design of vasculature microchannels. This fabrication can be fitted into many applications, one specifically is for the periosteum membrane. The channels were designed using SolidWorks and 3D printed using polyvinyl alcohol (PVA). These printed channels were laid into the uncrosslinked pre-polymer

solution and then crosslinked together. The PVA was then leached out in 40°C warm water for roughly three days.

Physical Characterization and Testing of Calcium Doped POC

Mechanical Testing

Mechanical tensile testing was conducted on all polymer formulations in their film form under both dry and hydrated conditions. Hydrated conditions were created by leaving the film samples (n=8) in 10 mL of DI water for two weeks with refilling of the DI water every two days. Both the dry and hydrated tensile tests were done using the INSTRON Universal Testing System. Tensile samples were shaped as rectangular strips and the width and thickness of those samples were inputted into the INSTRON program. Typically, samples have a width of 6 mm, thickness of 1 mm, and a length of 45 mm. A crosshead speed of 500 mm/min.

The INSTRON Universal Testing System was also used for the mechanical testing of the porous samples and composites. Tensile machining was used for the porous samples, and thus a similar protocol was followed. Composites were tested through the compression settings of the fabricated material cylinders.

Swelling Study

Swelling tests were conducted on the various biomaterial formulations to see how the materials would react when in an environment matching the osmolarity and ion concentration of the isotonic solutions of the human body. To accomplish this, 7 mm diameter, 1-1.5 mm

thickness, film disk samples (n=8) were soaked in 10 mL of phosphate-buffered saline (PBS) each. Mass recordings were taken at 1, 3, 5, 7, 10, and 14-day time points, and those mass changes were compared to the original sample weights to calculate percent mass changes. PBS was changed at every time point.

Soluble Content Study

Soluble content tests were conducted on all biomaterial formulations through both soaking in DI water (n=8) and 1,4-dioxane (n=8). Time points were at 1-day, 7-days, and 14-days. The soaked samples were then lyophilized for five days and then reweighed to measure the percent mass change. This measure is to find the percent of soluble content remaining after synthesis and crosslinking of the materials.

Degradation Study

Film disk-shaped samples (7 mm diameter, 1-1.5 mm thickness) were set in test tubes with 10 mL of PBS (pH 7.4) and incubated at 37°C. Time points included 1, 2, 4, 6, 8, 10, 12, and 16 weeks and at each time point, samples (n=8) were washed with DI water and freeze-dried for one week. At each time point, PBS was changed for the remaining time point samples. The masses of each sample were measured after freeze-drying and compared to its original dry mass to calculate percent mass loss.

pH Study

7 mm diameter, 1-1.5 mm thickness film disk-shaped samples (n=8) were set in test tubes with 10 mL of PBS (pH 7.4) and incubated at 37°C. Time points included 1-day, 3-day, 5-day, 1 week, and every week thereafter until 16 weeks. At each time point, the PBS was pipetted into a separate tube to have the pH measured using a pH meter. PBS refilling of test tubes holding the samples was done after pH was measured at each time point.

Contact Angle

Rectangular film samples of desired material formulations were cut and placed on a stable, leveled surface. A high-definition camera was set up orthogonal to the sample to obtain a straight on view of the sample for photo capturing. A dark backdrop was set up behind the sample and as the background of the picture to be taken by the camera. 8 μL droplets of DI water (n=5) were micropipetted on the film samples and a photo was captured of the droplets on each sample. Using imageJ, the contact angle between the surface and the water droplet was measured. The angle was taken from the center of the droplet to both the left and right contacts with the surface, then averaged.

Cytotoxicity

For the leached cytotoxicity study, twenty-four well plate size polymer disks were incubated in 1.33 mL of DMEM culture media for twenty-four hours at 37°C. The media containing the leached products was then removed and filtered through a 0.2 μm filter to

sterilize. The media was then diluted using sterile PBS to obtain desired concentrations. MG63 cells were seeded in a 96 well plate (n=6) and incubated for twenty-four hours in 100 μL pure DMEM to allow cell attachment. 10 μL of the leached product dilutions were added to the desired wells and then incubated for twenty-four hours. 10 μL of CCK-8 was then added to each well and incubated for two hours. Absorbance was measured with a plate reader at 425 nm. The results were normalized by dividing the experimental absorbance values by the absorbance value of the untreated cells to obtain a relative viability.

For the degraded products cytotoxicity study, polymer samples were fully degraded in 2M NaOH (1g per 10mL), leaving 2 mLs for pH solutions. HCl and NaOH were used to pH the degraded polymer solutions to 7.4 and PBS was then added to make each sample exactly 10 mLs. Solutions were filtered through a 0.2 μm filter to sterilize. The rest of the procedure matches the procedure of the leached cytotoxicity from the media dilution with sterile PBS to normalizing values to obtain relative viability.

For the film cytotoxicity study, 96 well plate size polymer disks were sterilized in 70% ethanol for three hours and under UV for thirty minutes. Samples were then leached in DMEM until media no longer changed color. The disks were plated in a 96 well plate. MG63 cells were seeded in the well plate (n=6) and incubated for twenty-four hours in 100 μL of pure DMEM. 10 μL of CCK-8 were then added to each well and incubated for two hours. Media was removed to a new plate and the absorbance was measured with a plate reader at 425 nm. The results were normalized by dividing the experimental absorbance values by the absorbance value of the untreated cells to obtain a relative viability.

Chapter 3

Results

Ion Chloride Salts Doping

Various ion chloride salts were doped with POC to be compared and to find different applications for each reaction with POC. Figure 4 shows the mechanical tensile results of the different ions measuring stress (Figure 4A), strain (Figure 4B), and initial modulus (Figure 4C).

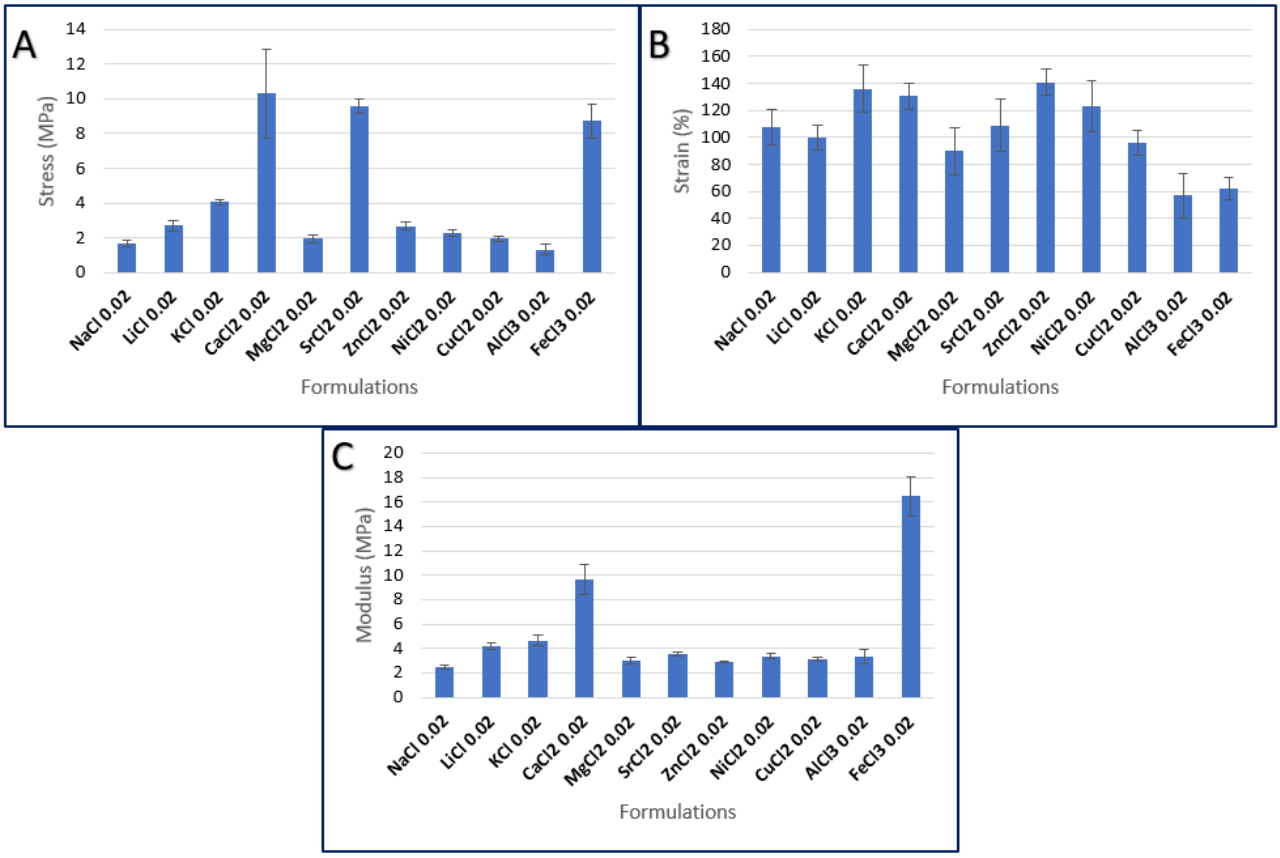

Figure 4: Various POC films doped with ion chloride salts showing A) Stress, B) Strain, & C) Initial Modulus

Calcium Species Doping

A more in depth look into calcium was taken and Figure 5 shows the mechanical tensile results of the POC doping with various calcium sources. Results measured include stress (Figure 5A), strain (Figure 5B), and initial modulus (Figure 5C).

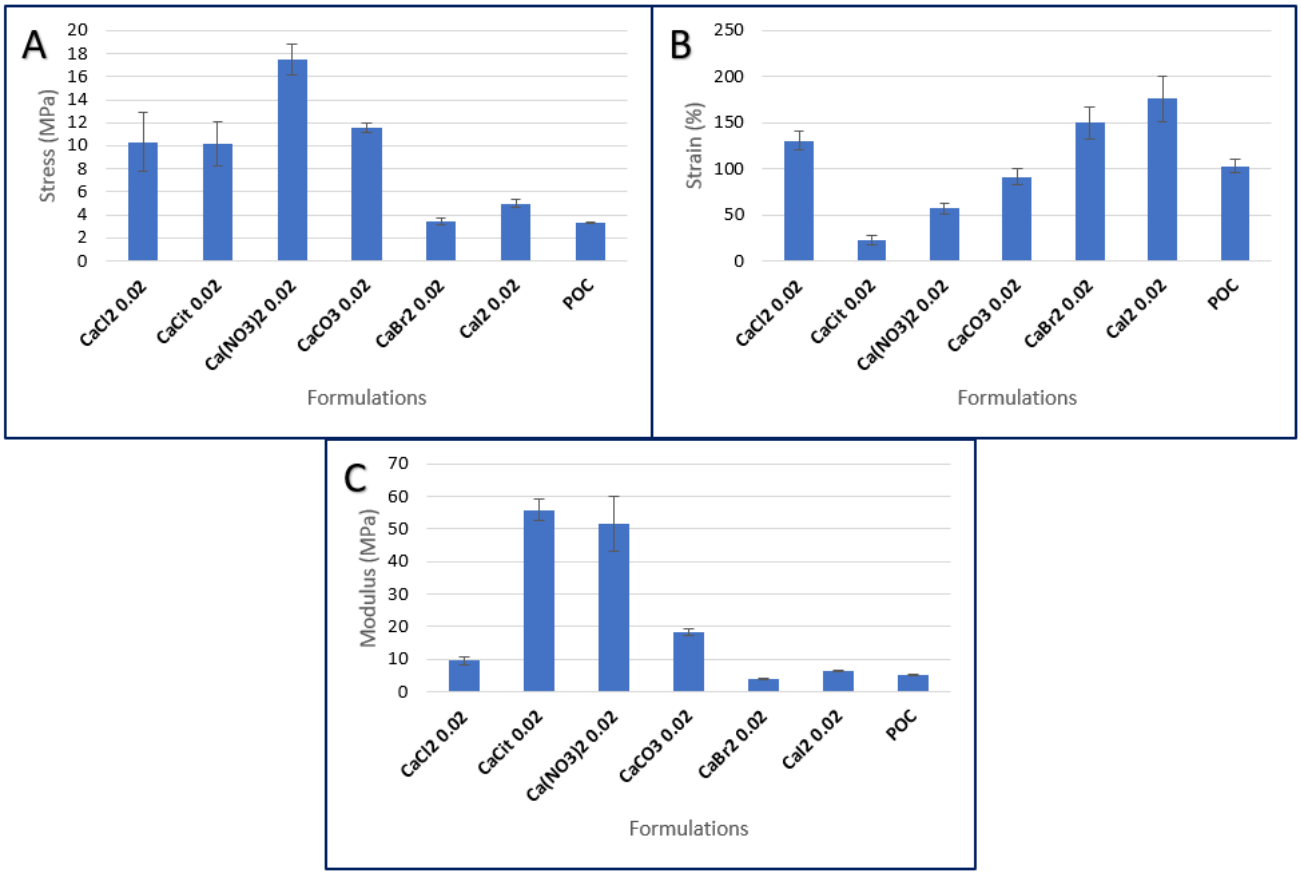

Figure 5: POC doped with various calcium species showing A) Stress, B) Strain, & C) Initial Modulus

Tensile mechanical results were also obtained on the various calcium sources doped with POC; samples were tested in a hydrated condition to match the body's environment. Results measured include stress (Figure 6A), strain (Figure 6B), and initial modulus (Figure 6C). This hydrated condition is a fully hydrated environment, meaning after two weeks of soaking the samples in PBS.

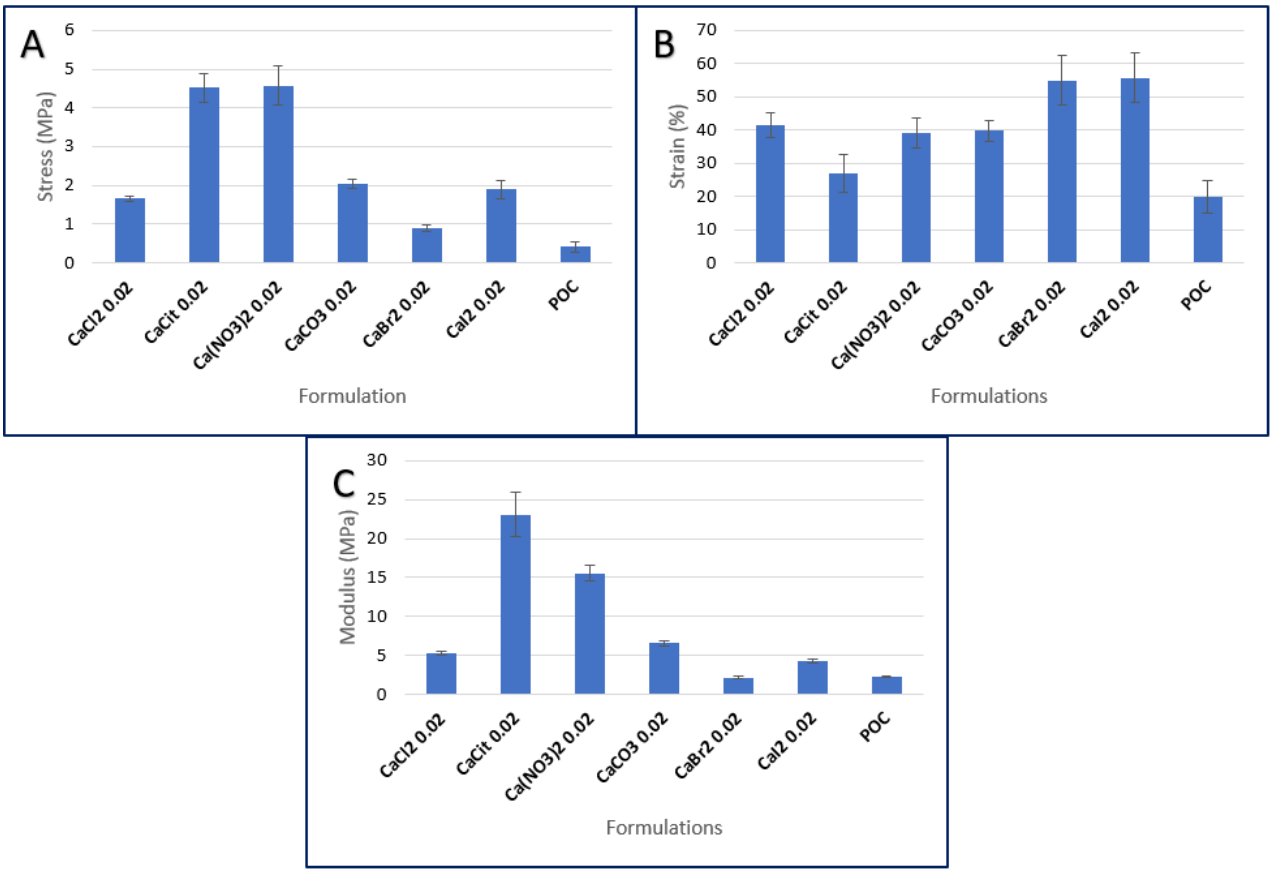

Figure 6: Hydrated POC doped with various calcium sources showing A) Stress, B) Strain, & C) Initial Modulus

Calcium Doping Reaction Modifications

Multiple variables were created within the calcium doping synthesis of POC. For the water-soluble calcium source, specifically $CaCl_2$ doping, the addition time was varied between the portions of the reaction where the synthesis stir speed was dropped. This is shown in Figure 7, where the $CaCl_2$ was added at either the original 300 rpm, 200 rpm, or 100 rpm.

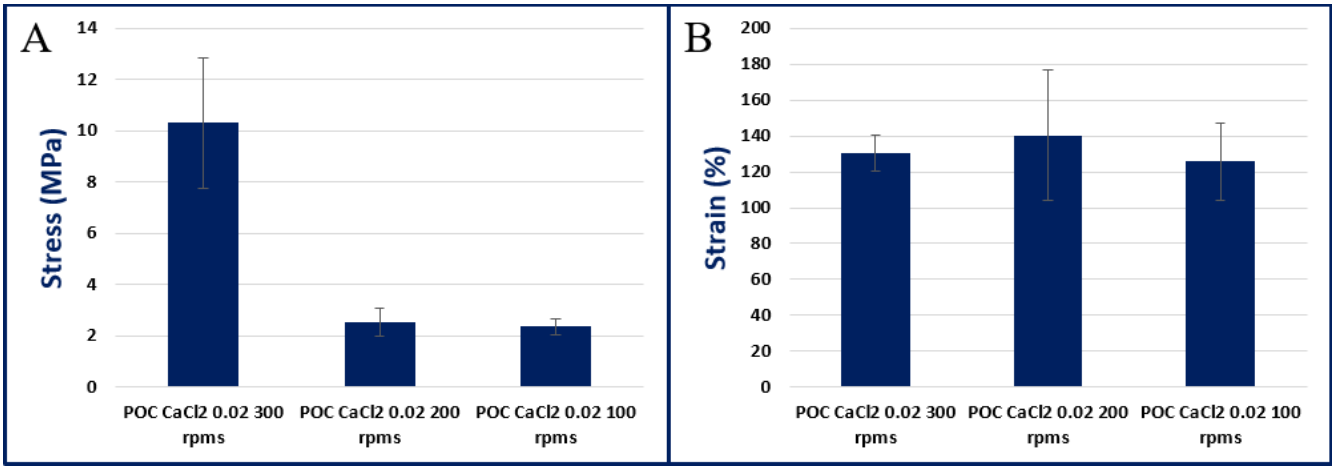

Figure 7: Different $CaCl_2$ addition times during reaction of $CaCl_2$ doped POC doping A) Stress & B) Strain

Continuing with the modifications to the calcium doping reactions, various crosslinking conditions were created and implemented to see the effect of crosslinking the pre-polymer on the mechanical tensile effect. The standard crosslinking conditions is three days at 80°C, followed by three days at 120°C. Figure 8 shows these results with plots showing the different crosslinking conditions and its effect on stress and strain. The legend reports the different crosslinking conditions in the format: temperature (°C) - days.

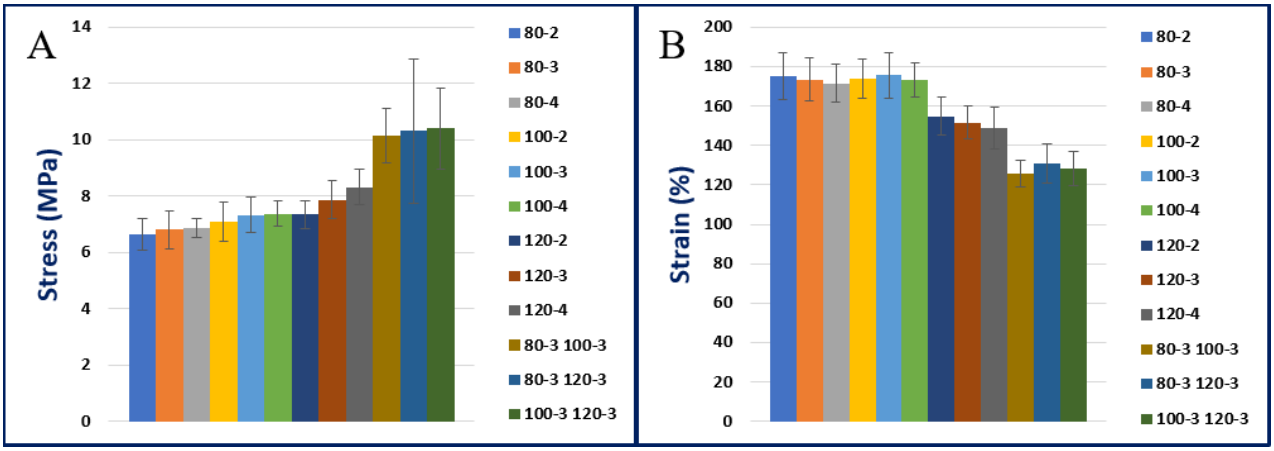

Figure 8: Various crosslinking conditions in synthesis of POC CaCl2 0.02 measuring A) Stress & B) Strain

The last of the synthesis modifications was altering the basic monomer ratios of POC, both citric acid and octanediol, with the doping of calcium. POC is usually made at a one-to-one ratio using 0.1 moles of each monomer, and that stays constant with the standard ionic doping synthesis. This test ranged the citric acid to octanediol ratio from 1:1.5 to 1.5:1 and Figure 9 shows the mechanical tensile stress and strain results of both the standard POC polymer and the POC calcium doped, $CaCl_2$, polymer.

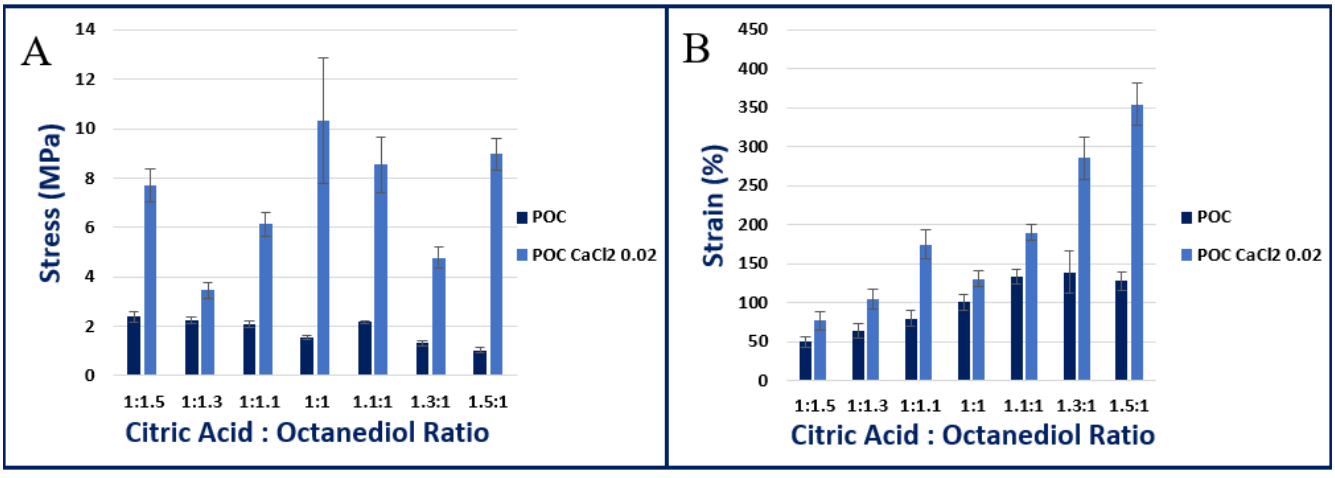

Figure 9: Varying the monomer ratio of POC in synthesis of POC and POC $CaCl_2$ showing A) Stress & B) Strain

POC Soaking in Calcium Solutions

Figure 10 shows tensile mechanical results obtained following soaking of pure POC films in calcium chloride solutions of various concentrations as well as in 0.1M phosphate buffered saline.

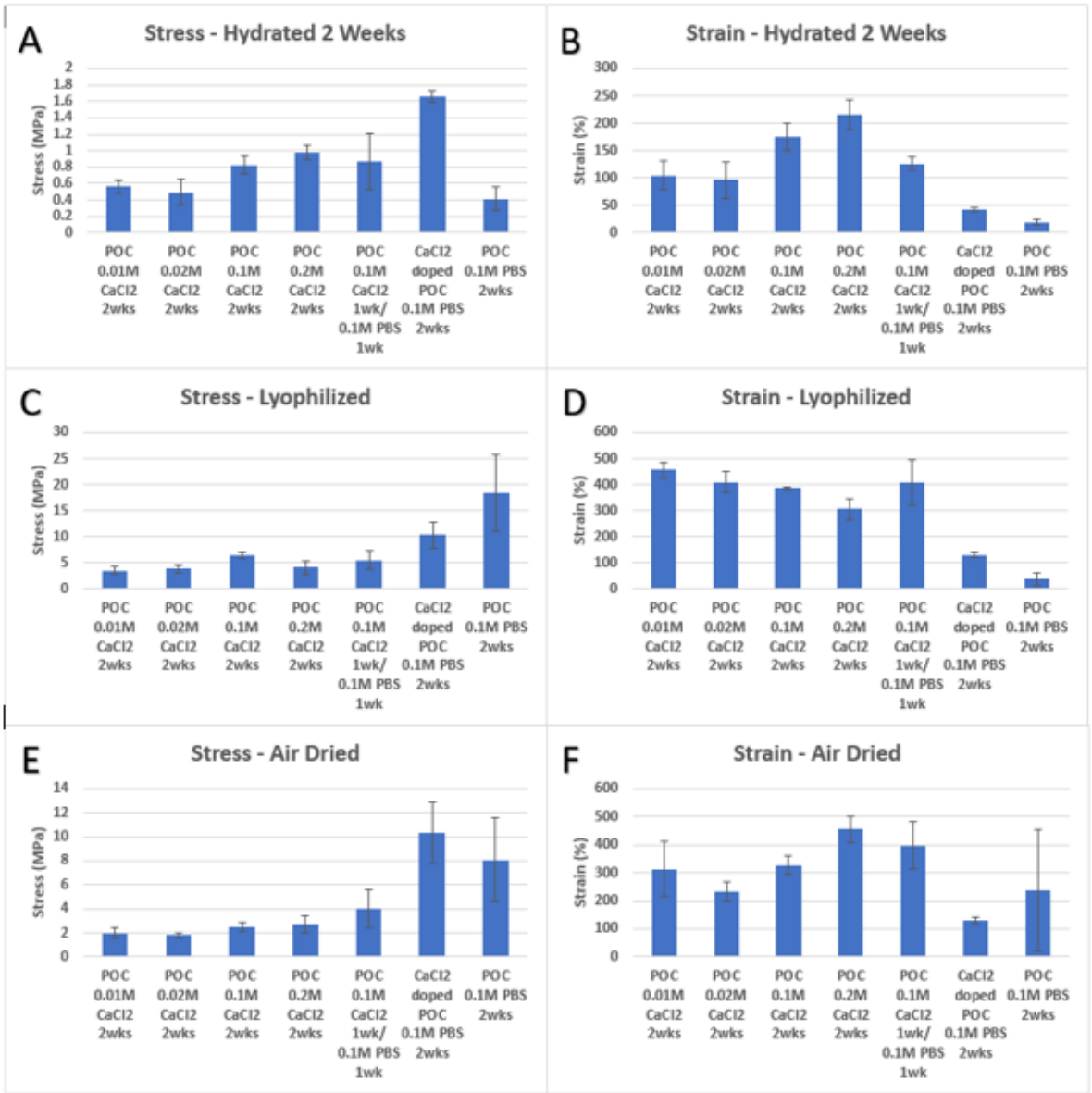

Figure 10: Tensile mechanics of calcium doped POC and POC soaked in calcium chloride or PBS solutions

Calcium Doped POC Fabricated as a Film

Morphology and Structure as Films

The morphology and structure of the calcium doped POC are shown in Figures 11 through 13. Figure 11 shows the outcome of the fabrication of films made through the calcium doping of POC with both calcium citrate and $CaCl_2$ as well as SEM (scanning electron microscopy) images of these films.

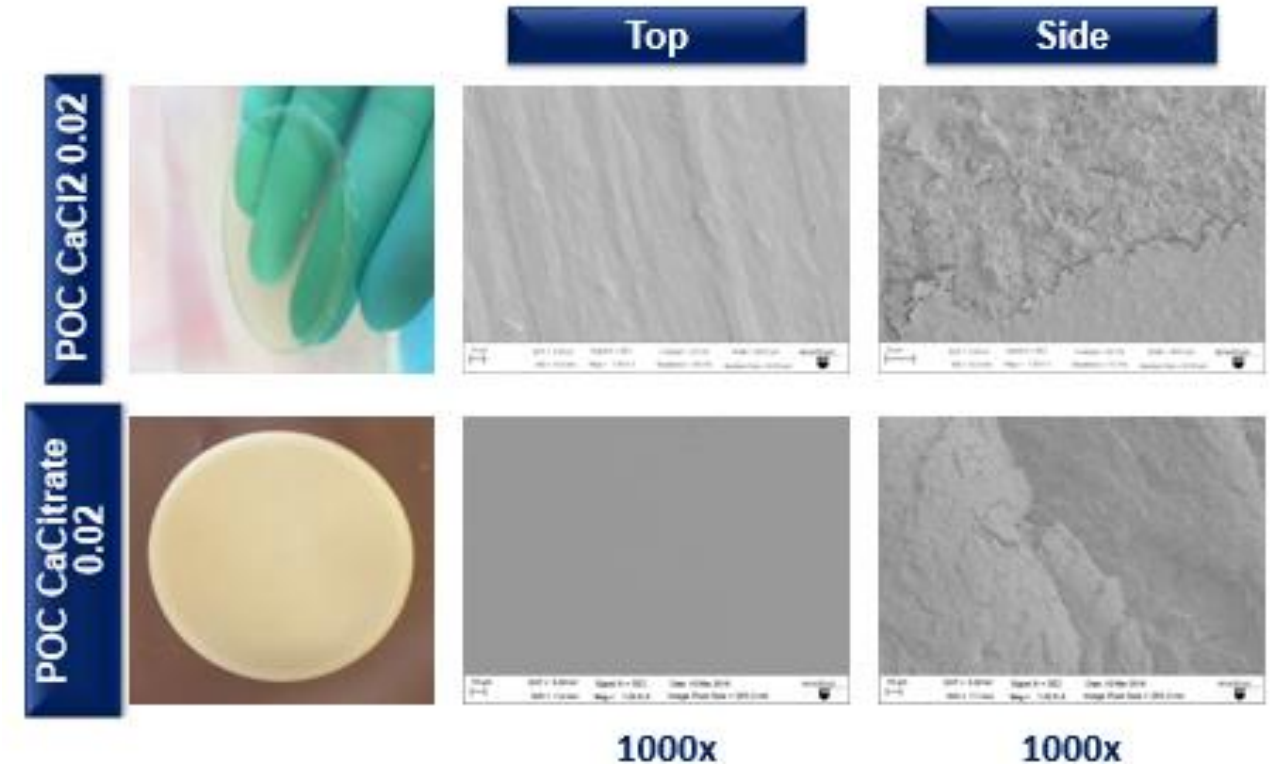

Figure 11: POC $CaCl_2$ 0.02 and POC CaCitrate 0.02 SEM images and film pictures

Figure 12 shows EDS (energy dispersive X-ray spectroscopy) images taken on film samples of calcium chloride and calcium citrate doped POC each. Figure 13 shows the results of SEM images of calcium citrate particles and calcium citrate doped POC in its pre-poylmer form.

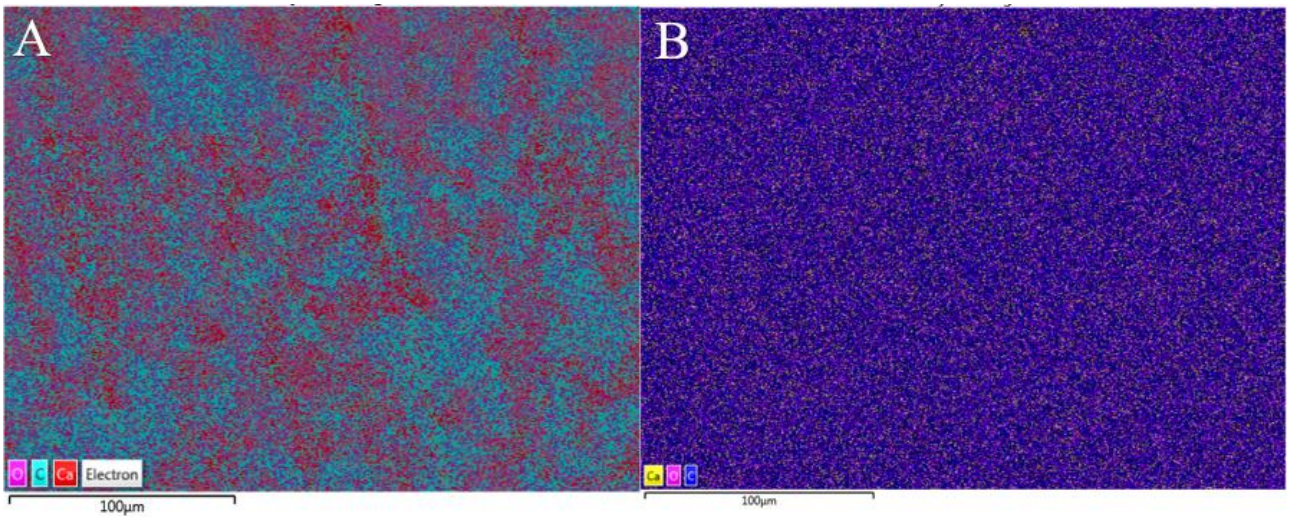

Figure 12: EDS imaging on polymer films of A) POC CaCitrate 0.02 and B) POC $CaCl_2$ 0.02

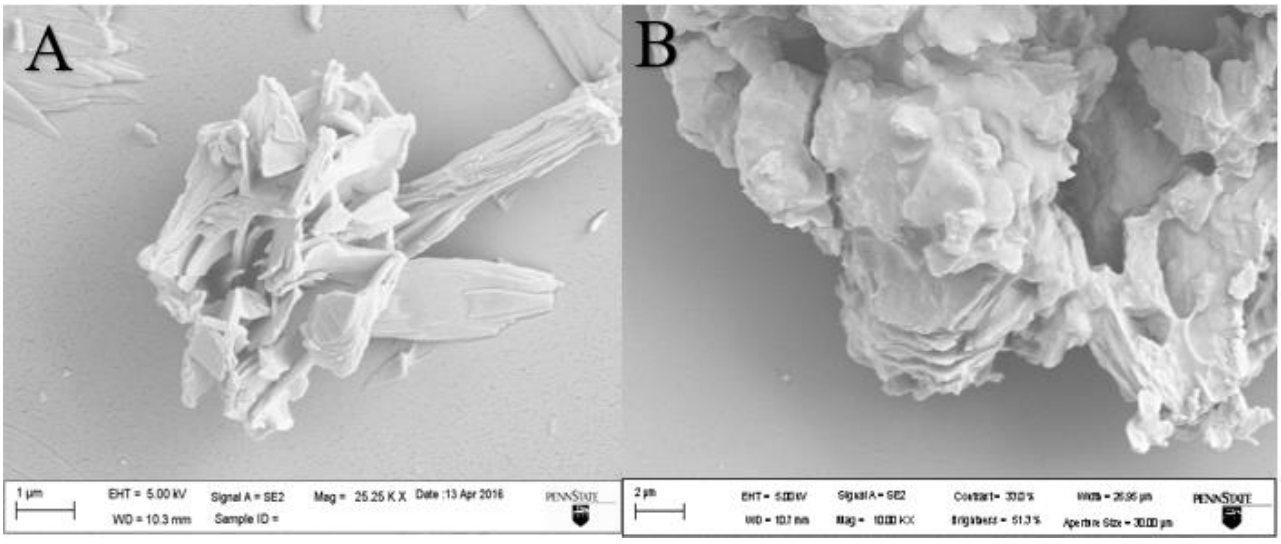

Figure 13: SEM Images of A) Calcium Citrate & B) Calcium Citrate Doped POC Prepolymer Morphology

Physical Properties of Calcium Doped POC as Films

Figures 14 and 15 show the results obtained from the mechanical tensile study. Figure 14 shows both calcium citrate and calcium chloride doped POC plots measuring stress and strain and their comparison to the standard POC polymer. Figure 15 pulls these results and depicts the stress vs. strain relationship for both calcium chloride and calcium citrate doping of POC, as well as the control, POC. Along with this plot, the elastic nature of the films is shown with pictures showing the bending of the fabricated calcium doped films.

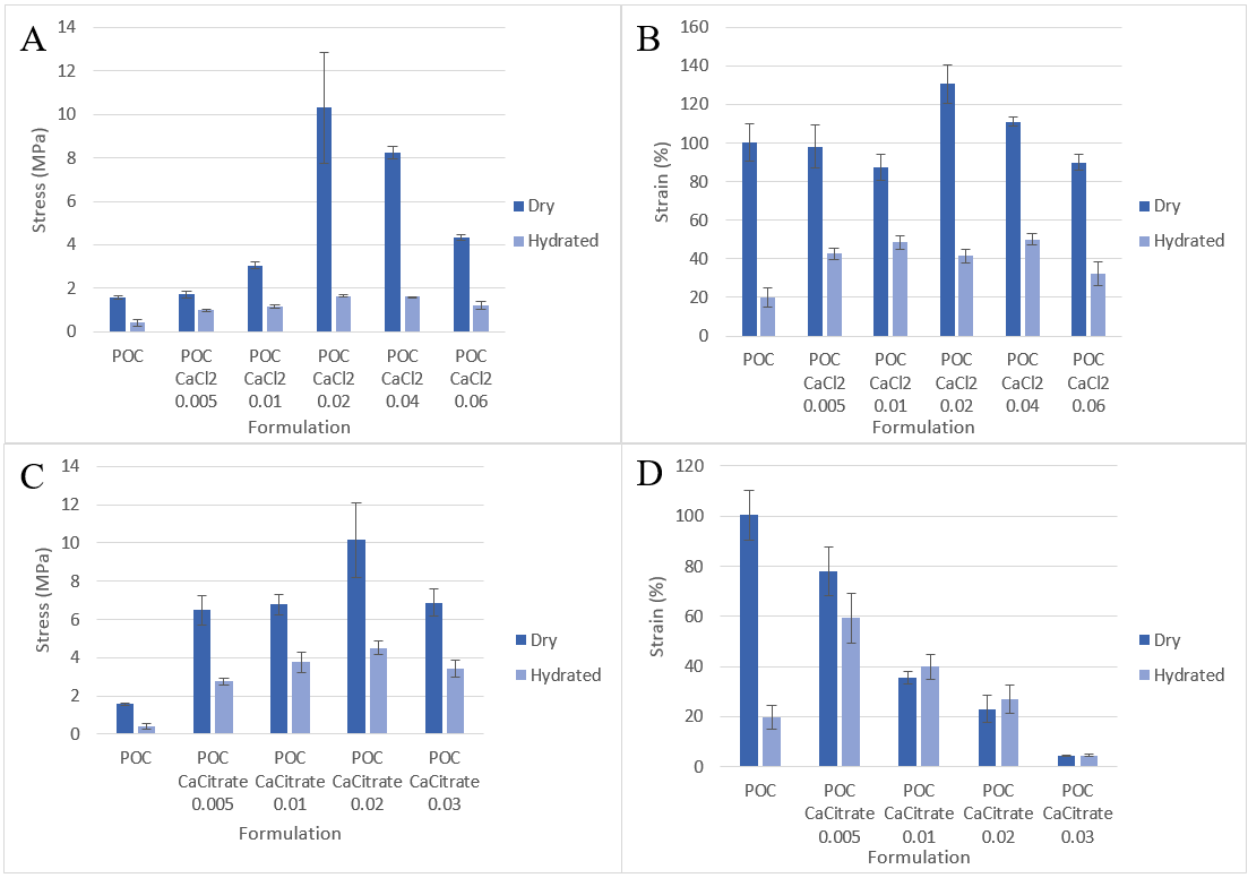

Figure 14: Varying the molar concentration of calcium doping for POC $CaCl_2$ showing A) Stress and B) Strain, and POC CaCitrate showing C) Stress and D) Strain

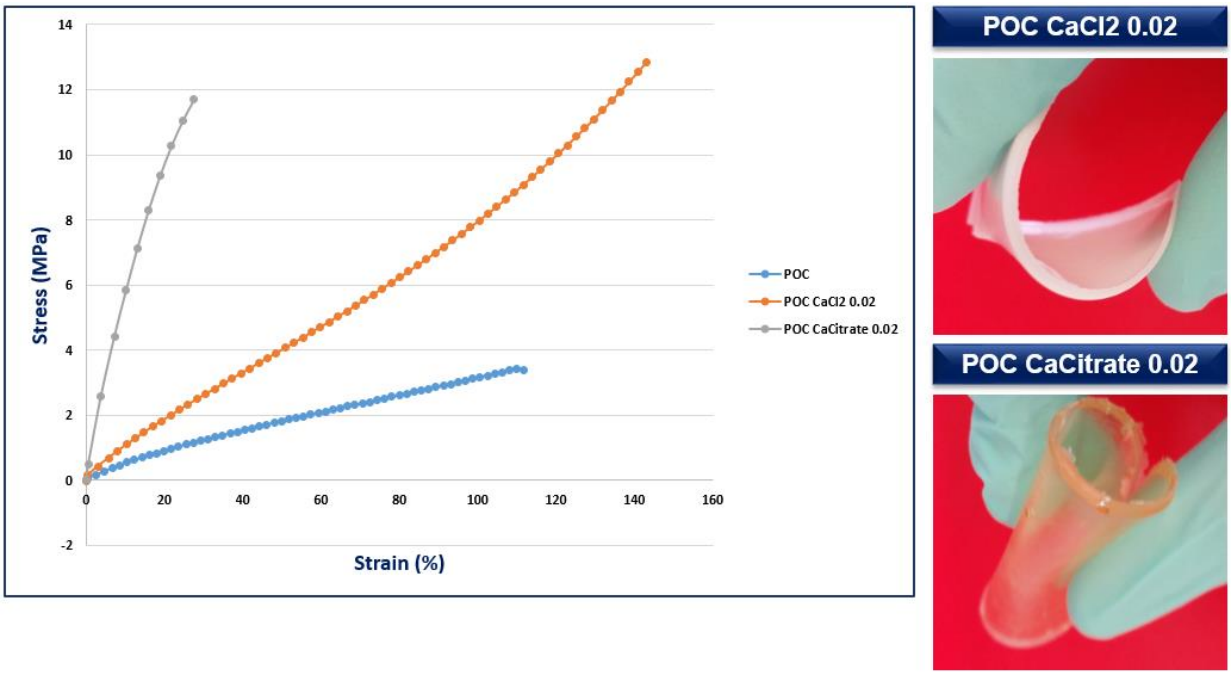

Figure 15: Stress vs. Strain plot of control POC and calcium doped POC with $CaCl_2$ or calcium citrate

Using EDS, calcium content in the film samples of $CaCl_2$ and calcium citrate doped POC were obtained. Figure 16 shows these results overlapped with the tensile mechanical strength relationship, from Figure 14, of the calcium doped POC formulations. The line graph represents the tensile stress, and the bar graph represents the relative calcium content signal.

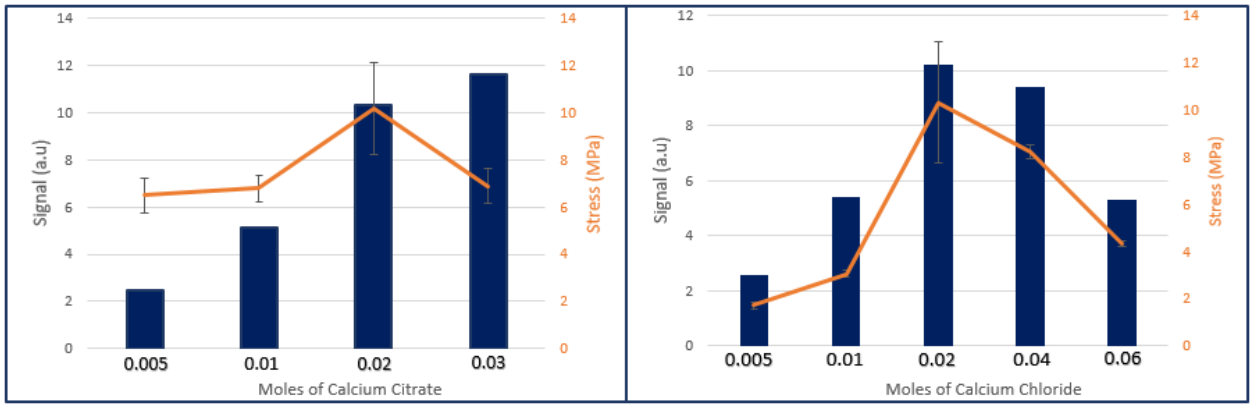

Figure 16: Calcium content obtained using EDS in film samples of POC doped with A) Calcium citrate & B) Calcium chloride

Soluble content was also tested on all different molar calcium formulations of both calcium chloride and calcium citrate doped POC after one week of soaking in either 1,4-dioxane or DI water. Figure 17 shows four plots for each solvent and each calcium source doping. Each of these obtained results are compared to the control POC polymer. A soluble content study was also conducted on the various calcium sources as seen in Figure 18.

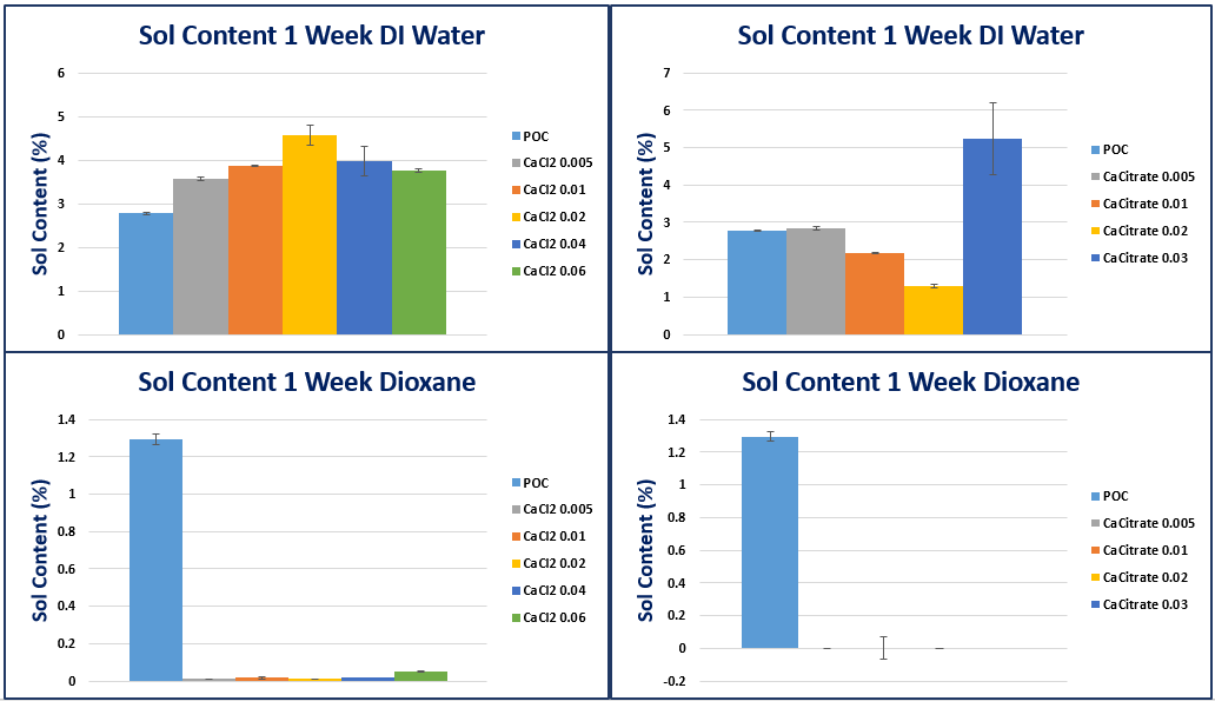

Figure 17: Soluble content of different calcium doping molar concentrations of POC soaked in water and dioxane

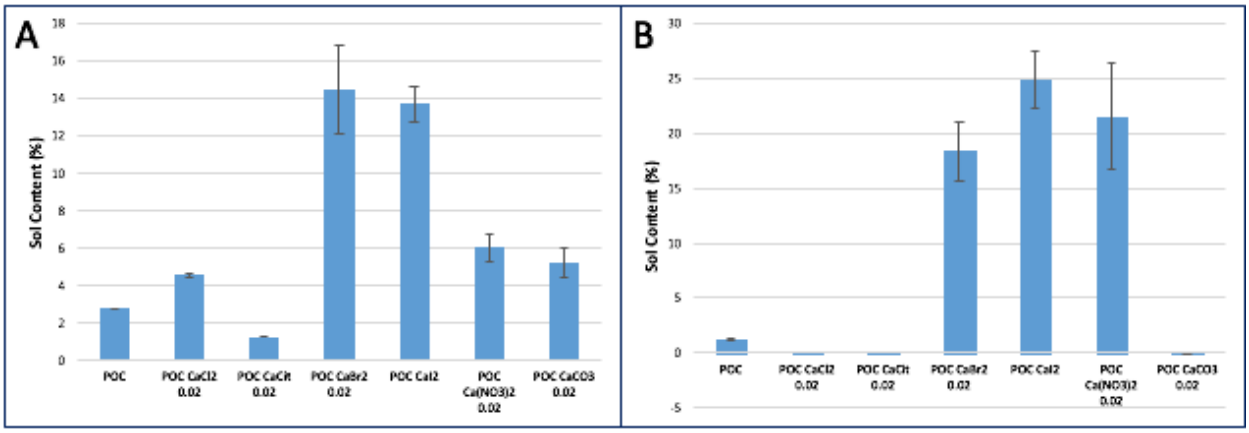

Figure 18: Soluble contents of various calcium sources doping of POC soaked in A) 1 week DI water & B) 1 week 1,4-Dioxane

A swelling study was conducted to see the behavior of the polymers in the body if used as a future implant material. To do so, different calcium doping concentrations of POC were soaked in PBS and incubated at 37°C to replicate the body's ion and osmolarity solution concentrations. These results are shown in Figure 19 and were obtained from the soaking of the samples after two weeks. Once again, POC is used as the control to compare to the new doped film samples. Figure 20 shows the swelling study conducted on various calcium sources compared to POC.

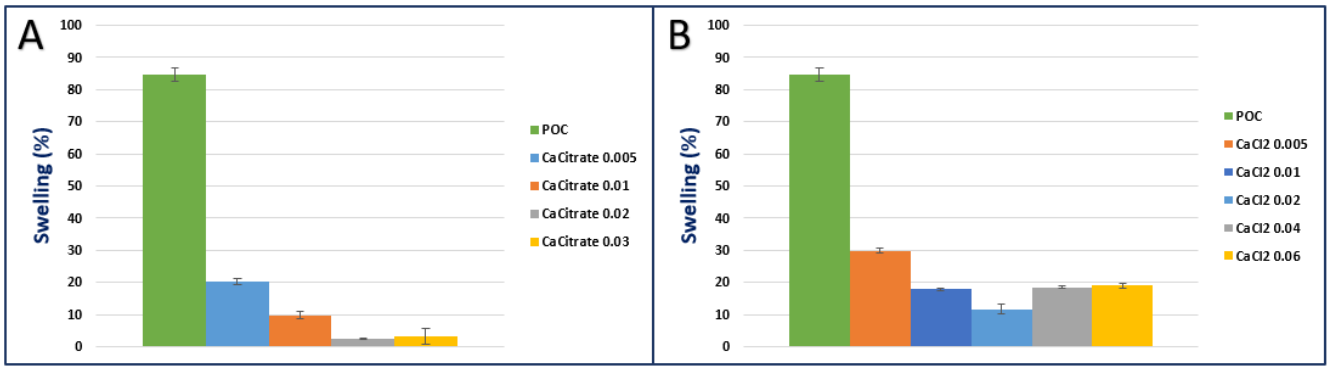

Figure 19: Swelling percentage of POC doped with A) Calcium Citrate & B) Calcium Chloride

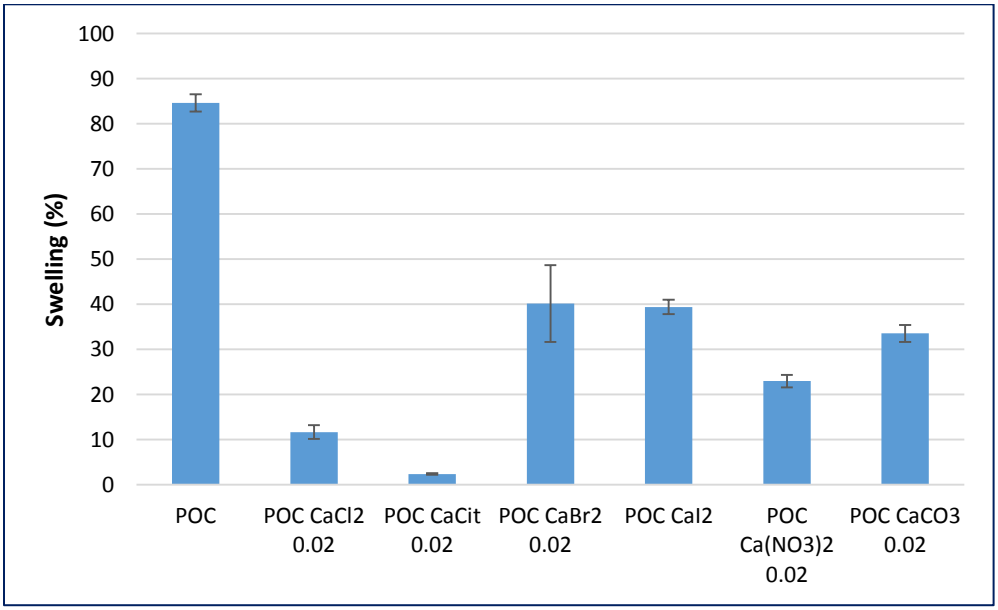

Figure 20: Swelling percentage of POC doped with various calcium species

A degradation study was conducted and the results are shown in Figure 21. The degradation study included different molar concentrations of calcium chloride POC doping and these results were compared to the control, POC. The graphic shows the percent mass loss the samples experienced over 16 weeks in PBS.

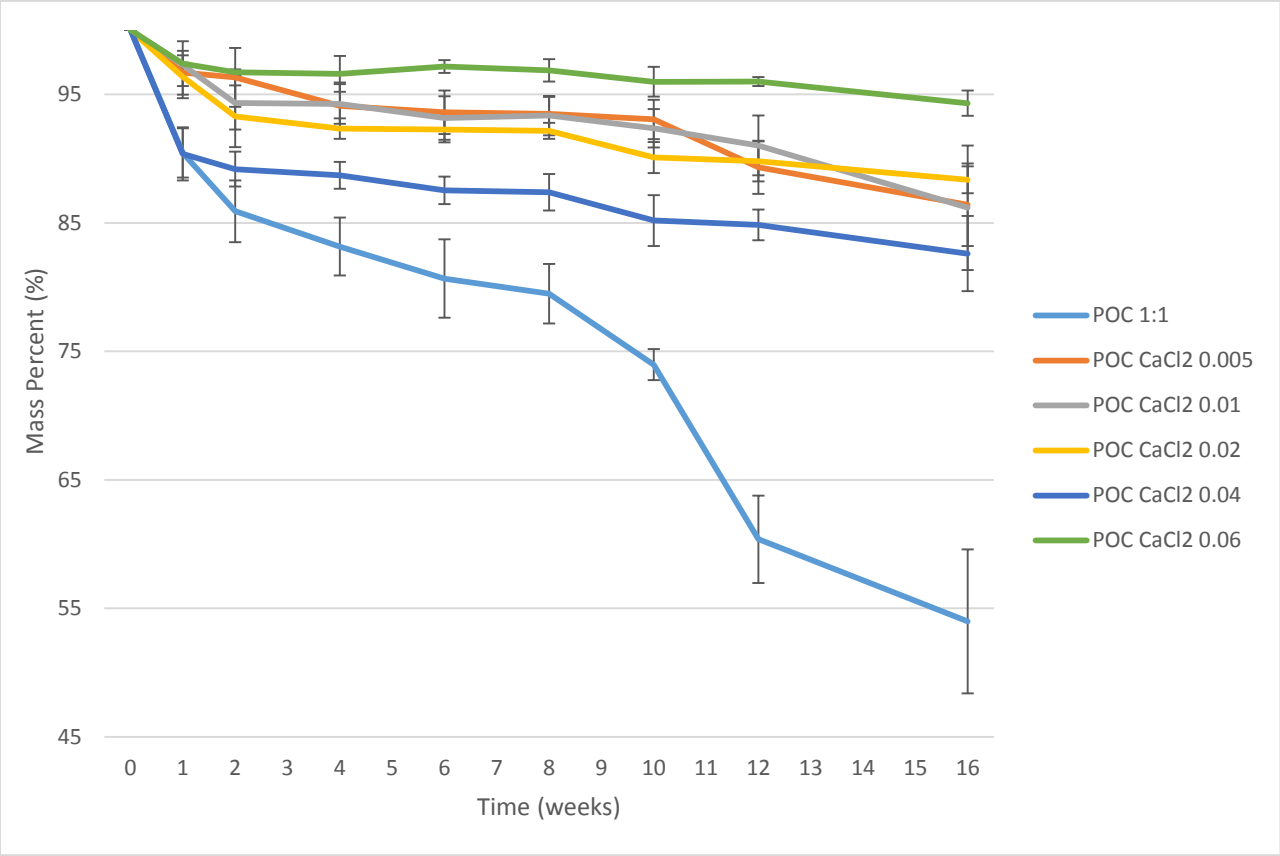

Figure 21: Degradation study of the various molar concentrations of calcium chloride POC doping over 16 weeks

Along with a degradation study over 16 weeks, a pH study was conducted over 16 weeks. The samples were also soaked in PBS and pH was measured to see the materials' effects on pH in a matching osmolarity environment to that of the body. Figure 22 shows these results.

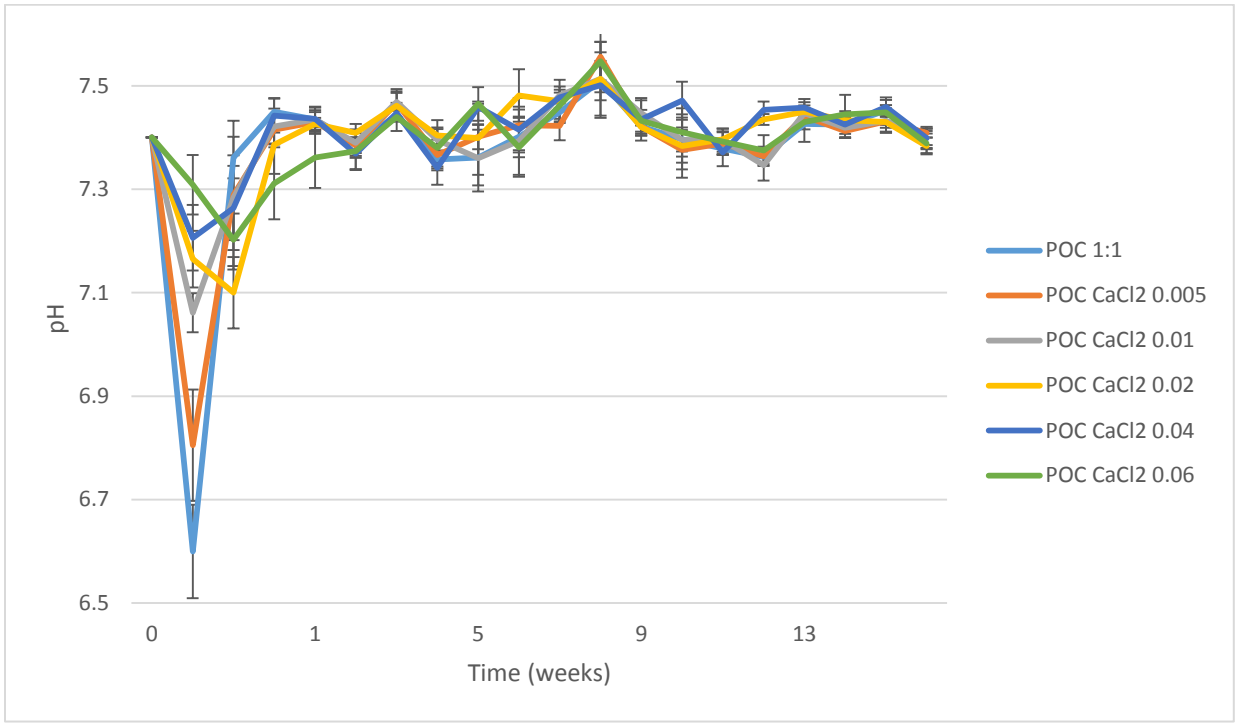

Figure 22: pH study showing POC and calcium chloride doped POC's effect on pH over 16 weeks

Figure 23 highlights the pH study's results showed in Figure 19 over the first few time points.

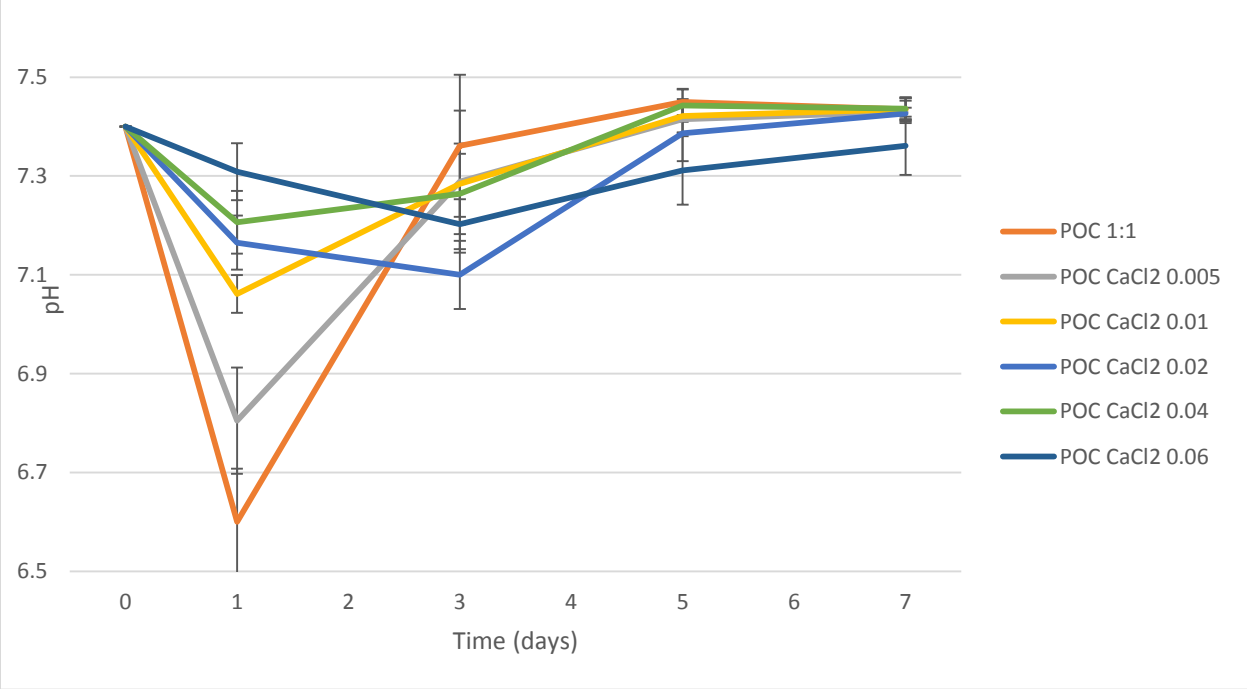

Figure 23: pH study showing POC and calcium chloride doped POC's initial effect on pH over first week

Figure 24 shows the results of contact angle studies for the doping of different calcium sources of POC, different POC calcium soakings, and the various molar concentrations of calcium chloride and calcium citrate doping of POC. The smaller the angle, the more hydrophilic the material, and the higher the angle, the more hydrophobic the material.

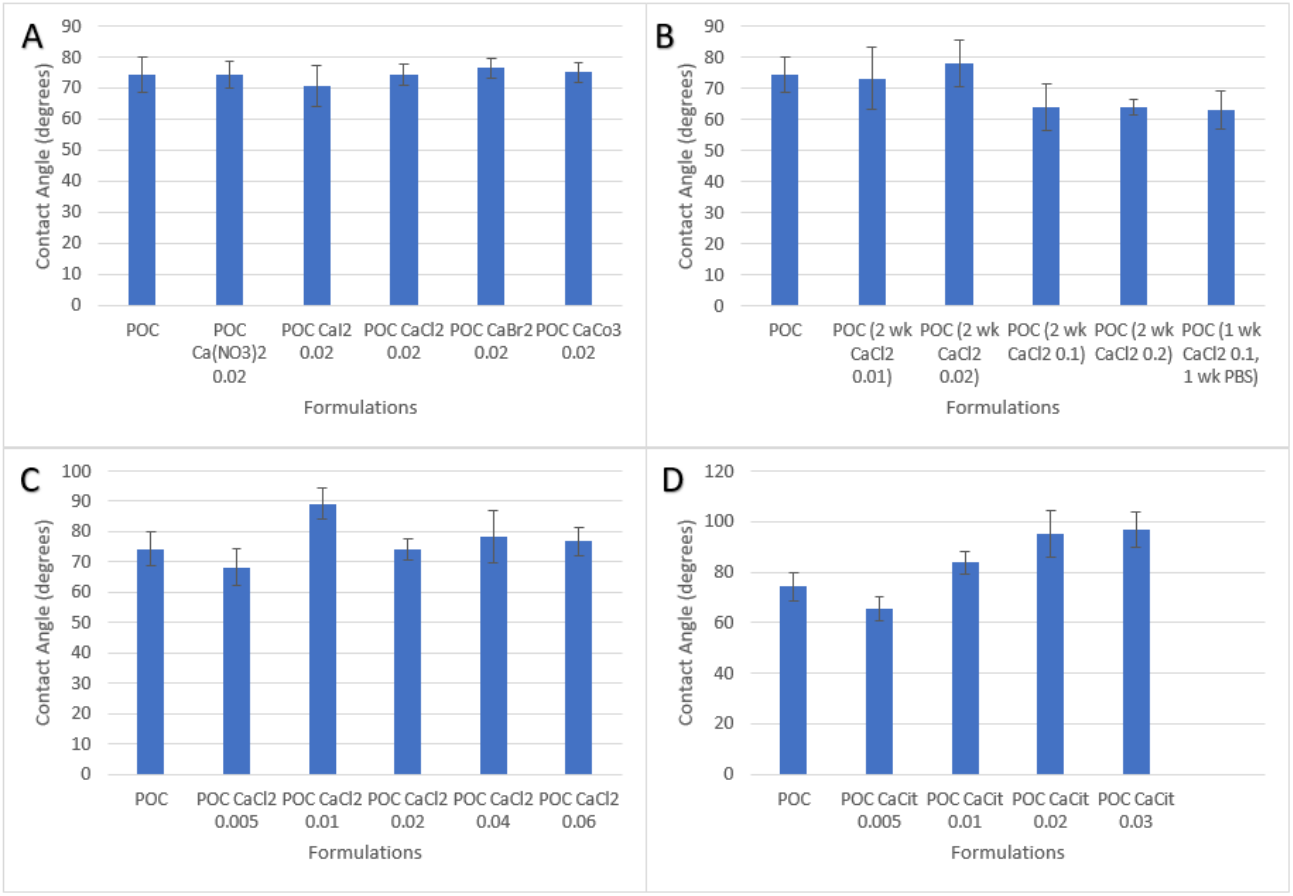

Figure 24: Contact angle results for A) Various calcium sources doped with POC, B) $CaCl_2$ soaking of POC C) various molar concentrations of $CaCl_2$ POC doping, and D) various molar concentrations of calcium citrate POC doping

Cytotoxicity on the polymer films was studied to measure the effects on cells of the leached small calcium molecules, of the fully degraded polymer, and of the film when cells were seeded on it. Figure 25 and Figure 26 show these various cytotoxicity results on a wide range of calcium doped POC materials and some controls.

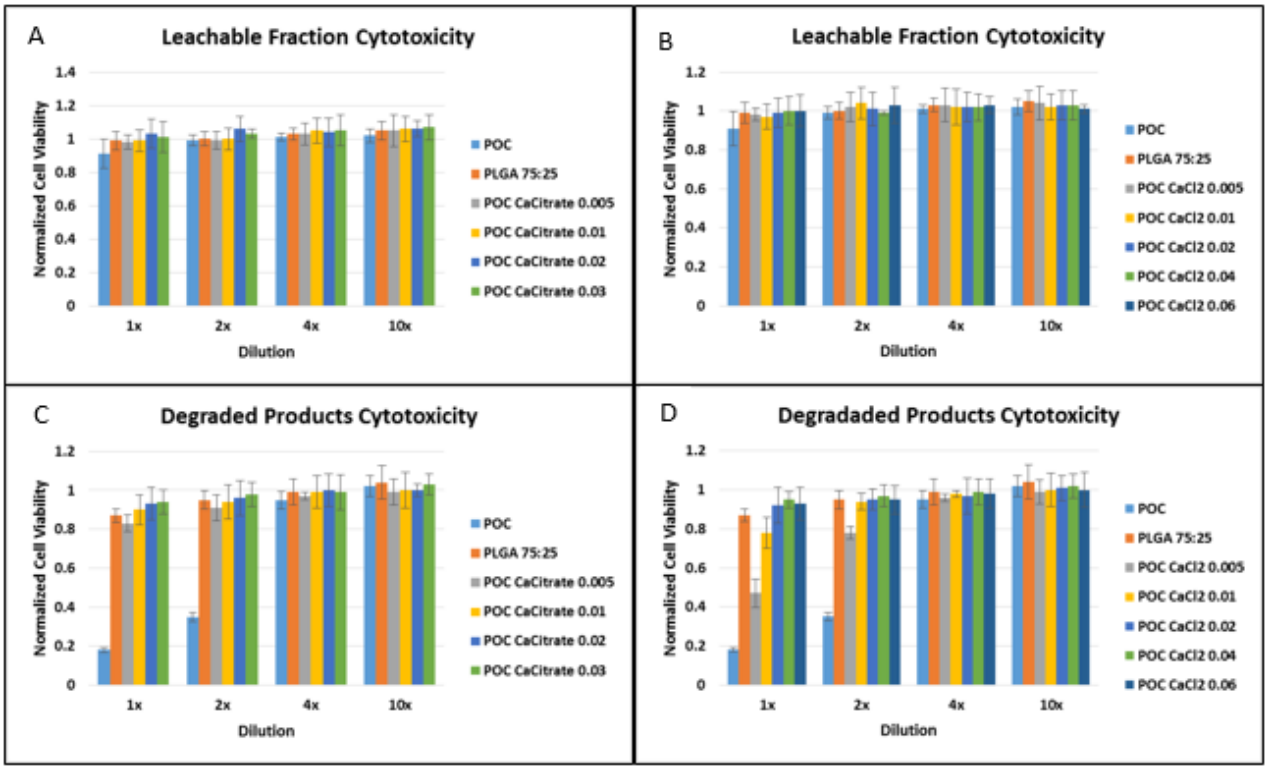

Figure 25: Cytotoxicity results on POC and calcium doped POC with both calcium chloride and calcium citrate

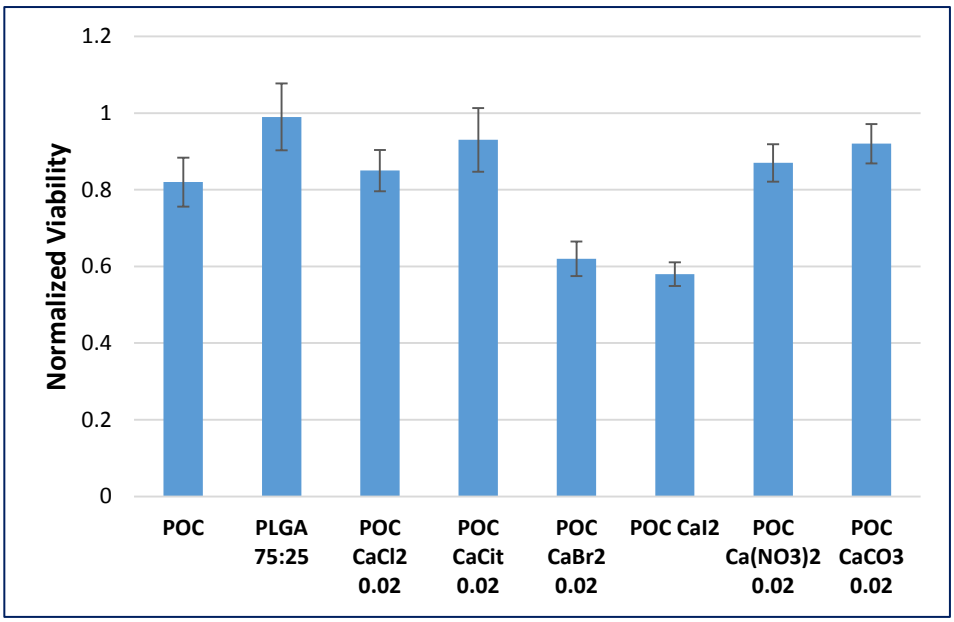

Figure 26: Cytotoxicity results on POC and various sources of calcium doped POC

Calcium Doped POC Fabricated as a Porous Scaffold

SEM images of calcium doped POC fabricated as porous samples are represented in Figure 27. Figure 28 shows the SEM images of salt leached porous samples of both calcium doped POC in $CaCl_2$ and calcium citrate at 0.02 molar quantity. At 80% porosity, the pore size is roughly between 250 and 425 micrometers.

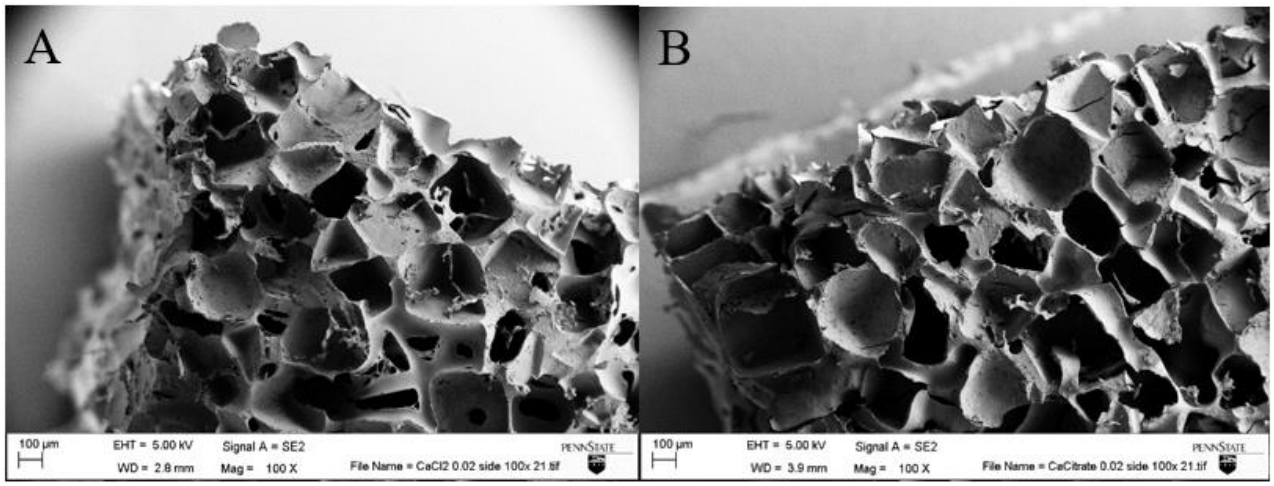

Figure 27: SEM image of porous samples of POC doped with A) $CaCl_2$ 0.02 & B) Calcium Citrate 0.02

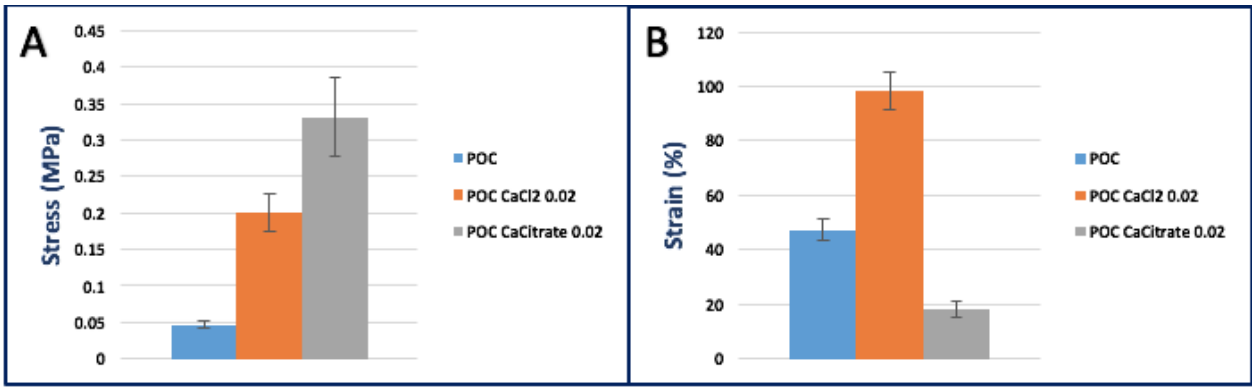

Figure 28: Mechanical results on porous scaffolds of calcium chloride and calcium citrated doped POC

Calcium Doped POC Fabricated as a Composite

An SEM image of POC doped with calcium chloride fabricated a a composite is shown in Figure 29 while mechanical results are represented of various ion doped POC composites in Figure 30. Composites contain 60% weight hydroxyapatite.

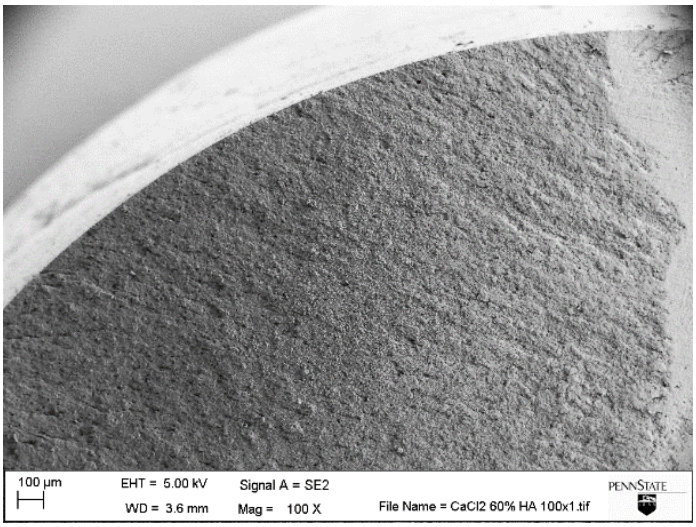

Figure 29: SEM image of composite POC doped with CaCl2 0.02 with 60% HA

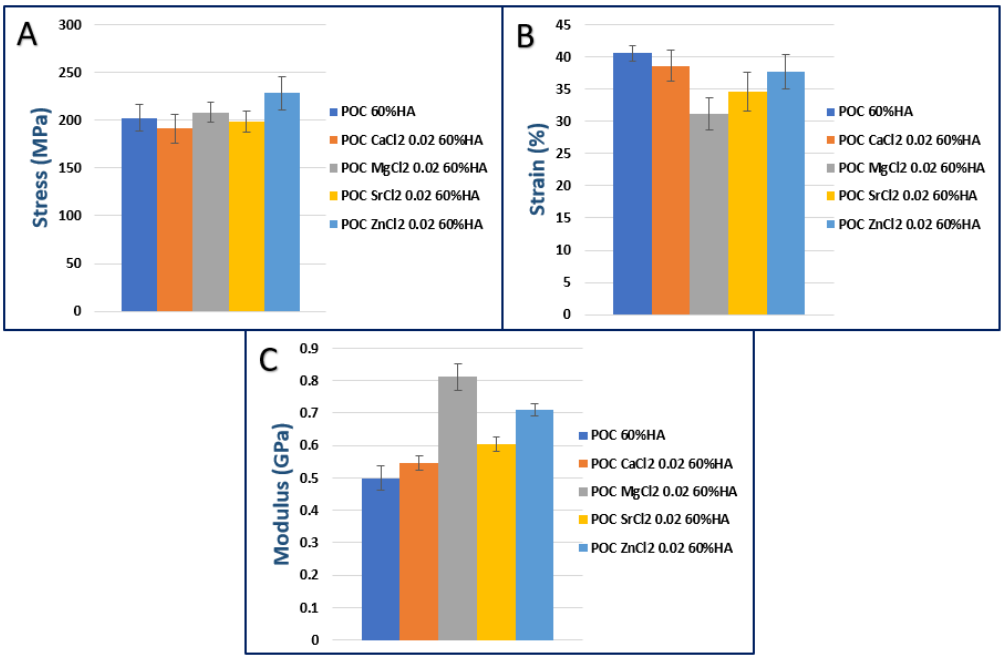

Figure 30: Mechanical results on various ion doped POC fabricated as a composite with 60% hydroxyapatite

Synthetic Periosteum Membranes Fabrication

Figure 28 shows initial results in fabricating the dual-membrane synthetic periosteum. Figure 31A shows the 3D printed PVA micro-channel and Figure 31B shows that channel imbedded into a POC film layer. This fabrication strategy is targeted for future work of the material as one of the possible orthopedic applications.

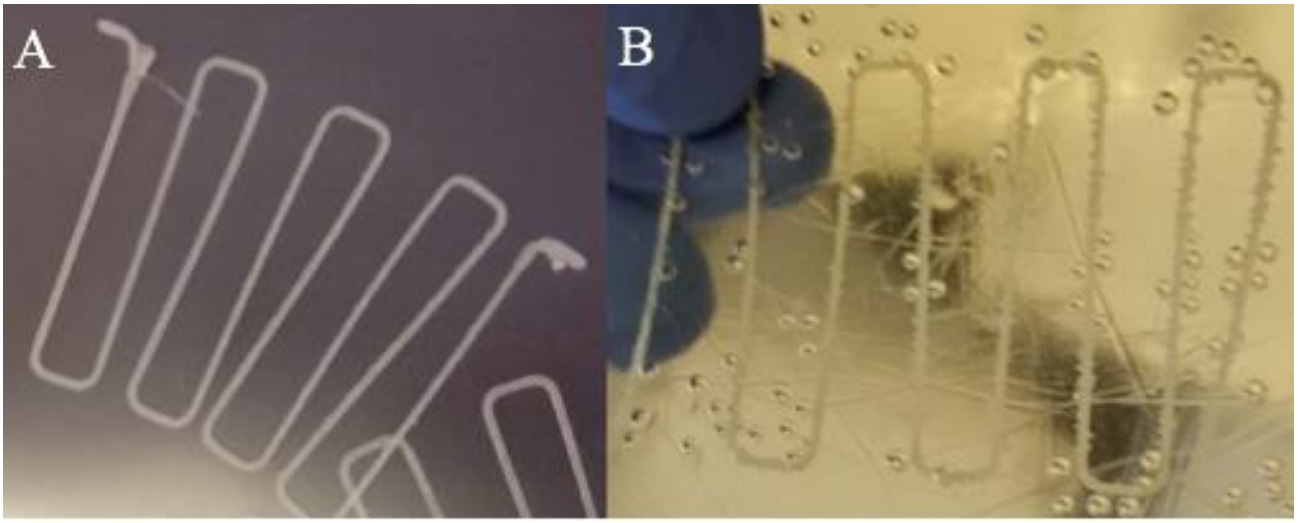

Figure 31: A) 3D printed PVA micro-channel network & B) Micro-channel cross-linked within POC film layer

Chapter 4

Discussion

Developing POC into a more functional and versatile biomaterial was sought after through the incorporation of ions to the polymer material. The conceptual proof of the increased functionality through the ionic incorporation to POC would be shown with the altered material properties to widen the range of applications, while the versatility would be proven with the flexible nature of being able to alter the synthesis and various additions to the proven reaction and fabrications.

Figure 4 shows the POC doping of various ionic species using ion chloride salts. The measurements included stress, strain, and initial modulus over all the formulations in a 0.02 molar ionic addition to the 0.1:0.1 molar ratio of the POC monomers. The ions chosen to dope POC were chosen for different versatile applications. For example, ions including calcium, strontium, and magnesium for orthopedic applications, zinc and copper for antibacterial applications, while iron is a natural occurring ion throughout the body and could hold many vital applications. Calcium doping was chosen to take on further investigation as it showed relatively favorable results in terms of tensile strength and elasticity in comparison to the other ion chloride salts as seen in Figure 4A and Figure 4B. Calcium also is one of the best reported ions in terms of its preferential binding capabilities due to its ability to chelate with carboxyl groups. These results also show the ability to expand the synthesis as various metal ions in different salt compositions were doped with POC and outputted different mechanical results, which can be linked to different functional applications.

Various calcium species were then chosen to dope POC. Figure 5 and Figure 6 show the mechanical results of the materials in both dry and hydrated conditions. Calcium chloride and calcium citrate showed the relatively best combination of results between both dry and hydrated conditions. POC doped with calcium chloride was chosen to be further investigated for similar reasons to the calcium ion was further investigated, for the more favorable combination of tensile strength and elasticity results. POC doped with calcium citrate was also chosen for further investigation so there were calcium species with representation of both water soluble, calcium chloride, and water insoluble, calcium citrate, calcium ions. These two calcium sources were also the most controllable under the reaction protocols. Formulations including calcium carbonate and calcium nitrate doping of POC showed interesting mechanical results, but their synthesis had a difficult reaction to control.

Investigations into the synthesis of POC doped with calcium was taken to see how modifications of the reaction would alter the material properties. Figure 7 shows the varying of addition times of $CaCl_2$ to the POC synthesis. $CaCl_2$ was added at the various times where the stirring speed was changed thus doping calcium at different viscosity and molecular weight levels of the POC synthesis. Figure 7 shows that the addition of $CaCl_2$ when the temperature is initially dropped to 140°C and the stir speed was set to be 300 rpm was more favorable for the tensile strength results with roughly five times the overall strength in comparison to both addition at 200 rpm and 100 rpm each. The elasticity results showed no significant differences between the addition time.

The next modification to the POC reaction was with altering the crosslinking conditions of the pre-polymer. Figure 8 shows the tensile mechanical results of calcium chloride doping of POC with a plethora of different crosslinking conditions ranging from 80°C for just two days to

80°C at three days, followed by 120°C for three days. The data showed that there was not much variation in stress and strain results due to the variation in crosslinking conditions for the ionic addition of calcium to POC. This is a positive result showing the flexibility of the ionic incorporation to POC as the variance in this reaction factor did not have a large effect on the outcome of the polymer. On the other hand, the small variations in tensile strength and elasticity could be served to fit different desired applications if there are more specific targeted results that the slight change to the crosslinking conditions could be used to meet those desired properties.

The basic monomer ratio of POC was altered to factor as a modification to the polymer synthesis; the standard POC synthesis calls for a 1:1 ratio of citric acid to octanediol. The ratio between these two monomers was ranged from 1.5:1 to 1:1.5 and comparisons were made between the control POC polymer to the calcium chloride doping of POC. Figure 9 shows these results and it is evident that at every monomer ratio, the calcium doped POC shows significantly higher tensile strength as compared to POC. The calcium doped POC also shows better elasticity results for each monomer formulation as compared to POC. It can also be noticed that a trend exists showing that as the ratio of citric acid to octanediol transitions from octanediol dominant to citric acid dominant, the strain percentage increases. This is likely due to the increased binding sites available for the calcium ions with the more citrate. The different mechanical results can once again be valued differently for different applications of the material, and this variation of the monomer ratio allows for additional control parameters in the properties of the polymer. ‘

The standard way of adding material into a polymer has usually been done through soaking the material in a solution. This project took efforts to truly dope the polymer material by adding the material into the polymer directly in its reaction. Figure 10 shows the mechanical results obtained following soaking of pure POC film samples in calcium chloride solutions of

various concentrations as well as in 0.1M PBS to allow for the mechanical comparisons between the various possible methods of inducing calcium into POC. Significant differences in mechanical properties compared to the calcium chloride doping of POC, as shown in Figure 14, are evident. Figure 10A and Figure 10B demonstrate wet mechanical strengths of approximately one half that of calcium doped POC for nearly all calcium soaked formulations. Calcium doped POC also displays increased strain versus pure POC, however, calcium soaked POC films demonstrate increased strain versus calcium doped POC. Figure 10C and Figure 10D show nearly double the mechanical strength in dry condition following lypholization for calcium doped POC versus soaked formulations. Figure 10E and Figure 10F show similar results after one week of air drying.

These results taken together indicate that calcium soaked POC is capable of increased wet mechanical strength, while displaying decreased elasticity. Under dry conditions, soaking offers higher elasticity, but calcium doping allowed for better homogeneity and tensile strength mechanics. It is also seen that calcium doping results in less variation between samples and thus results in a more controllable execution of synthesis. This is likely due to the calcium soaking relying more on the carboxyl group bindings, and thus, crosslinking is not as consistent and can lead to more fluctuations within its reaction.

The homogeneity of the synthesized polymers is a key factor to evaluate. As seen in Figure 11, SEM images of POC doped with calcium chloride and calcium citrate display a high degree of homogeneity. These images were taken on film samples with a 0.02 molar calcium quantity. Property variability is of important consideration due to the possibility of the polymer complexity. The more homogenous the samples, the less property variability will be of concern towards the morphology and structure of the polymer material and thus the possible effects of the

structural differences among the polymer chains [28]. Figure 12 shows EDS images of calcium doped POC with either calcium chloride or calcium citrate. The important information from these results is the cluster-like morphology that is shown of the calcium citrate doping. This is also evident from the SEM images shown in Figure 13. Calcium citrate starts out in clusters as it is an insoluble particle, as seen in Figure 13A. As calcium citrate is doped with POC, the polymer coats around the particle, and thus in Figure 13B it is seen how the pre-polymer exhibits these coated particles. Once cross-linked, the polymer fuses together into a cohesive film to a limit.

As referenced earlier, the mechanical results of calcium doped POC with both calcium citrate and calcium chloride are shown in both hydrated and dry conditions within Figure 14. In both dry and fully hydrated conditions, calcium doped POC displays improved tensile mechanical properties in comparison to POC. Under every concentration of calcium doping, via both calcium chloride and calcium, the tensile strength was shown to be stronger in both dry and hydrated conditions. Other than the goal of improving the mechanical strength, another goal was to maintain the elastic properties of pure POC. For most formulations involving calcium citrate and all formulations involving calcium chloride under wet conditions, the strain percentage was improved upon in comparison to POC. Under dry conditions, $CaCl_2$ 0.02 doped POC showed significantly increased elasticity while the other calcium chloride formulations showed very similar values to strain percentage as POC. Under dry conditions for calcium citrate doping, there was a noticeable decrease in elasticity. With these results, it is also shows the ability to adjust the calcium concentration for the polymer. The overview of the elastomeric mechanical characteristics can be seen in Figure 15, represented by the stress vs. strain relationship between the calcium doped POC and POC. As there are no measurable yield points, all three materials are

characterized as an elastomer. The tensile mechanical strength was\ improved upon while maintaining the elastomeric properties of POC.

It was of interest to find out the amount of calcium that was synthesized into the films. EDS was used to evaluate the relative calcium content between the different molar concentration doping of POC with both calcium chloride and calcium citrate. Figure 16 shows these results and shows the relationship between the calcium content to the respective calcium molar concentration's mechanical tensile strength. For the calcium chloride doping, it was discovered that the 0.02 molar doping of POC held the highest concentration of calcium within the films, followed by 0.04. These two concentrations also represented the strongest tensile results. This correlation is positive as it shows the linear relationship between calcium concentration and tensile strength. This also shows that there might be a limit to the amount of calcium that can be doped in the POC polymer, and with calcium chloride, a molar concentration of 0.02 is the most efficient doping concentration. The calcium citrate results did not show the direct correlation between calcium content and mechanical results though. The results still showed higher mechanical strength for the higher calcium concentrations, but there might be other factors in those materials limiting the calcium interactions.

The soluble content study for both calcium chloride and calcium citrate POC doping showed favorable results as well in terms of calcium doped POC polymers displaying minimal soluble content. 1,4-dioxane would favor the leaching of polymer and the pre-polymer is soluble in the dioxane solvent. For both calcium sources, there were no or minimal soluble content measured after one week in 1,4-dioxane showing that there were no unreacted particles during crosslinking whereas with pure POC, there was roughly 1.3 % soluble content remaining. In water, small particles and calcium would be released, which is good up until a point for

bioactivity as calcium increases differential potential by nucleating differentiation. It is not favorable to have a lot of soluble content as that would be detrimental to degradation and cytotoxicity. The soluble content in water for calcium chloride and calcium citrate show resembling patterns to the relative calcium concentrations as well as the mechanical tensile stress. Figure 18 shows the soluble content results for the various calcium sources doped with POC. Calcium chloride and calcium citrate showed more favorable results as formulations involving bromide, iodide, and nitrate do not show minimal soluble content.

Another important aspect of materials that could be implemented as implants or serve orthopedic applications in general include their swelling properties in matching osmolarity solutions to that of inside the body. The swelling studies showed in Figure 19 and Figure 20 on both the different molar concentrations of calcium chloride and calcium citrate, as well as with the various calcium sources, showed that calcium doped POC displays decreased swelling as compared to pure POC. Calcium chloride and calcium citrate showed the least swelling of all the calcium sources. Minimizing the swelling properties of implantable materials is important so that there is more reliable consistency in its shape. Also, less swelling correlates to less water uptake and thus the increased wet mechanical results that were noticed in the mechanical study.

The longer timed studies included the investigation into the degradation properties and materials' effects on pH over time. Figure 21 shows the mass percent loss over 16 weeks for the molar concentrations of calcium chloride POC doping. POC showed the quickest degradation rate while the calcium chloride doping definitively slowed the degradation time of the material. Calcium doped POC slows the degradation due to the formed ionic bonds not being susceptible to hydrolysis like the esters in the POC polymer. As the polymer chain's covalent bonds break, the ionic network remains and may even form new bonds with the degraded carboxylic groups.

This also helps explain why the calcium doped samples maintain their shape over the course of the degradation to a greater effect than POC. Compared to commonly used polymers, both POC and calcium doped POC are more in the middle of the pack in terms of degradation rate. They both experience quicker degradation than polycaprolactone (PCL), PLLA, and PLGA, but are slower than hydrogel and biological based materials such as collagen or chitosan.

The variations in how the materials' effect pH show some variation from each other and POC in the first few days, as seen in Figure 23, but after 2 weeks, the pH trends are very similar, as seen in Figure 22. After this second week, the pH of all the tested calcium doped concentrations and POC roam around a pH of 7.4, the physiological pH. Early effects over the first day show the higher the reported molar calcium concentration doping of POC, the less variability in pH; POC takes the largest drop in pH to roughly 6.6. The calcium chloride doped POC of 0.02 and higher molar concentrations drop to a minimum of roughly 7.2.

To obtain a relative description on the hydrophobicity and hydrophilicity of the polymer films, a contact angle study was conducted and results are shown in Figure 24. There was not a significant difference between any of the tested materials to even make a distinction between the relative hydrophobicity. Calcium doping did not seem to change the surface chemistry much, which is a positive result for cell attachment as cells do not favor highly hydrophilic or hydrophobic surfaces and POC has shown strong results for cell attachment. Some of the slight distinctions that could be made came from the calcium citrate doping having slightly higher contact angles, and thus more hydrophobic tendencies, than the calcium chloride doping. This makes sense as the calcium citrate doping represented the water insoluble doping and was expected after seeing the cluster formations in the polymer.

Cytotoxicity studies were conducted on the leachable fraction, degraded products, and on the films, as shown in Figure 25 and Figure 26 on different molar concentration of calcium chloride and calcium citrate, and different calcium sources, respectively. Leachable fraction is the cytotoxicity of the twenty-four-hour soluble content of the polymer. This measured the effect on cells of the leached small molecules, specifically calcium. Since the soluble content of all the polymers was very low after twenty-four-hours, there was not much effect on the cells, thus the high cell viability. The cytotoxicity of the fully degraded polymers looked specifically on the monomers and calcium. Calcium increased the cytotoxicity. This was possibly due to the chelation effect that moderates the effect of citric acid, and possibly due to the slightly basic nature of calcium citrate, which also moderates citric acid effects. The film cytotoxicity looked at the films when cells were seeded on it. Most calcium species had improved cytotoxicity over POC, which is likely due to the reduced leaching of acidic products. In the case of calcium carbonate and calcium citrate, the water insoluble particle doping, it is likely due to the basic nature of the calcium additive.

The versatility of the ionic doping of POC was further proven with the successful fabrication of porous and composite scaffolds. Porous scaffolds fabricated with calcium doped POC display improved mechanical properties, as shown in Figure 27. Calcium chloride doping showed a significant improvement in both tensile strength and elasticity compared to POC while calcium citrate showed an even more substantial increase in mechanical stress, but took a significant decrease in elasticity. Depending on the application, there proves to be a flexible tensile mechanical range of stress and strain for the fabrication of porous scaffolds. Composites were constructed with various ionic doping of POC and 60% weight hydroxyapatite; Figure 28 shows these mechanical results. Calcium doping showed no significant changes in either

elasticity or tensile strength. There was not much significant variation in any of the various ion chloride salt doping of POC in composite form, but the versatility of the doping is proved in that the composites with 60% hydroxyapatite could be fabricated and replicate the mechanics of POC.

The fabrication of the micro-channels for the periosteum membrane synthesis was to develop a proof of concept for the future work with fabricating a dual-membrane periosteum implant for orthopedic applications. The 3D printed PVA channel could be created within the polymer film via crosslinking. This could be used for nutrient delivery, vascularization, and other advantages that the periosteum provides for bone growth and healing. This also shows early steps in the ability to create controlled geometries within the films and this could lead to scaffolds with direct connections with the native blood vessels through surgical attachment of vessels to the micro-channels. The second membrane for this fabrication strategy includes the calcium doped POC film layer with a porosity gradient. Further research and testing on the development of a periosteum membrane is of high interest.

This research has endless directions of pursuit to fully develop and characterize the potential of the material. *In vitro* bioactivity testing includes osteogenic potential testing with human mesenchymal stem cells via an alkaline phosphatase assay on the films and composites, as well as a four-week mineralization study using 5x SBF to determine mineral morphology and calcium/phosphate ratio on the films and composites. Also, testing on the antibacterial potential against E. coli (gram negative) and S. aureus (gram positive) using bacterial inhibition kinetics on the films and composites. *In vivo* animal testing is also of interest using the cranial critical size defect model and the confined critical size defect model. Additional possible research directions include the doping of multiple ions to combine the bioactive and mechanical effects of

two or more ions, the development of a mechanism for a pH responsive ion release for environmental specificity, and the fabrication of hemostat capable materials through calcium incorporation for wound dressings or general control of bleeding during implantation.

Chapter 5

Conclusion

The findings of this study ultimately show that ions can be readily incorporated as a secondary crosslinking mechanism in POC due to the intrinsic metal chelation ability of citric acid. Incorporation of calcium ions resulted in increased mechanical strength in both dry and wet conditions, maintenance of elastomeric character, shortened degradation rate, decreased swelling, minimal soluble content, and improved cytocompatibility, all with a high degree of homogeneity. Incorporation of calcium ions in POC was also found to be successful in creating strong porous scaffolds and composites to expand its versatility in orthopedic applications. Incorporation of calcium in POC is expected to enhance osteoconductivity potential and antibacterial capability as well as impart dynamic mechanical behavior to materials. Ion doping can be expanded to other citrate-based polymers and the versatility of citrate based polymers is expected to lead to a wide variety of ion doped materials for diverse applications.

BIBLIOGRAPHY

[1] Shegarfi, H. & Reikeras, O. Review article: bone transplantation and immune response. Journal of Orthopedic Surgery, 2009; 17(2): 206-211.

[2] Hu, Y., Rawal, A, & Schmidt-Rohr, K. Strongly bound citrate stabilizes the apatite nanocrystals in bone. Proceedings of the National Academy of Sciences, 2010; 107(52): 22425-22429.

[3] Shin, H. Olsen, B.D., & Khademhosseini, A. The mechanical properties and cytotoxicity of cell-laden double network hydrogels based on photocrosslinkable gelatin and gellan gum biomacromolecules. Biomaterials, 2012; 33(11): 3143-3152.

[4] Brown, A., Zaky, S., Ray, H., & Sfeir, C. Porous magnesium/PLGA composite scaffolds for enhanced bone regeneration following tooth extraction. Acta biomaterialia, 2015; 11: 1210-1216.

[5] Grayson, W.L., Frohlich, M., Yeager, K., Bhumiratana, S., Chan, M.E., Cannizzaro, C., Wan, L.Q., Liu, X.S., Guo, X.E., & Vunjak-Novakovic, G.. Engineering anatomically shaped human bone grafts. Proceedings of the National Academy of the Sciences, 2010; 107(8): 3299-3304.

[6] Hofmann, G.O. Biodegradable implants in orthopaedic surgery -- a review on the state-of-the-art. Clinical Materials, 1992; 10(1-2): 75-80.

[7] Chiarello E., Cadossi M., Tedesco, G., Capra, P, Calamelli, C., Shehu, A., & Giannini, S. Autograft, allograft and bone substitutes in reconstructive orthopedic surgery. Aging Clinical and Experimental Research, 2013; 25(1): 101-103.

[8] Ratner, Buddy D., Schoen, Frederick J., & Lemons, Jack E. An introduction to materials in medicine. Biomaterials Sciences (Third Edition): An Introduction to Materials in Medicine, 2010: Academic Press.

[9] Giannoudis, P.V, Dinopoulos, G., & Tsiridis, E. Bone substitutes: an update. Injury, 2005; 36(3): S20-S27.

[10]. Triffitt, James T. Osteogenic stem cells and orthopedic engineering: summary and update. Journal of Biomedical Materials Research, 2002; 63(4): 384-389.

[11] Pape, H.C., Evans, A, & Kobbe, P. Autologous bone graft: properties and techniques. Joint Orthopedics Trauma; 2010; 24(suppl 1): S36-S40.

[12] Zimmermann, G, & Moghaddam, A. Allograft bone matrix versus synthetic bone graft substitutes. Injury, 2011; 42(suppl 2): S16-S21.

[13] Naughton, G.K, Tolbert, W.R., & Grillot, T.M. Emerging developments in tissue engineering and cell technology. Tissue Engineering, 1995; 1(2): 211-219.

[14]. Langer, R., & Vacanti, J.P. Tissue Engineering. Science, 1993; 260: 920-926.

[15] Stark, G.B., Horch, R., & Tanczos, E. Biological matrices and tissue reconstruction. Springer, 1998: 197-206.

[16] Hutmacher, D.W. Scaffolds in tissue engineering bone and cartilage. Biomaterials, 2000; 21: 2529-2543.

[17] Rowley, J.A., Sun, Z., Goldman, D., & Mooney, D.J. Biomaterials to spatially regulate cell fate. Advanced Materials, 2002; 14(12): 886-889.

[18] Seal, B.L., Otero, T.C., Panitch, A. Polymeric biomaterials for tissue and organ regeneration. Material Science Engineering, 2001; 34: 147-230.

[19] Mano, J.F., Sousa, R.A., Boesel, L.F., Neves, N.M., & Reis, R.L. Bioinert, biodegradable and injectable polymeric matrix composites for hard tissue replacement: state of the art and recent developments. Composites Science and Technologies, 2004; 64: 789-817.

[20] Dunn, A.S., Campbell, P.G., Marra, K.G. The influence of polymer blend composition on the degradation of polymer/hydroxyapatite biomaterials. Journal of Materials Science. Materials in Medicine, 2001; 12(8): 673-677

[21] Rezwan, K., Chen, Q.Z., Blaker, J.J., & Boccaccini, A.R. Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials, 2006; 27: 3413-3431.

[22] Ulery, B.D., Nair, L.S., & Laurencin, C.T. Biomedical applications of biodegradable polymers. Journal of Polymer Science Part B: Polymer Physics, 2012; 49(12): 832-864.

[23] Tran, R.T., Yang, J., & Ameer, G.A. Citrate-based biomaterials and their applications in regenerative engineering. Annual Reviews of Materials Research, 2015; 45: 277-310.

[24] Tran, R.T., Wang, L., Zhang, C., Huang, M., Tang, W., Zhang, C., Zhang, Z., Jin, D., Banik, B., Brown, J.L., Xie, Z., Bai, X., & Yang, J. Synthesis and characterization of biomimetic citrate-based biodegradable composites. Journal of Biomedical Materials Research: Part A, 2014; 102: 2521-2532.

[25] Gyawali, D., Nair, P, Kim, H.K.W., & Yang, J. Citrate-based biodegradable injectable hydrogel composites for orthopedic applications. Biomaterials Science, 2013; 1(1): 52-64.

[26] Du, Y., Yu, M., Chen, X., Ma, P.X., & Lei, B. Development of biodegradable poly(citrate)-polyhedral oligomeric silsesquioxanes hybrid elastomers with high mechanical properties and osteogenic differentiation activity. ACS Applied Materials & Interfaces, 2016; 8(5): 3079-3091.

[27] Yang, C.H., Wang, M.X., Haider, H., Yang, J.H., Sun, J.Y., Chen, Y.M., Zhou, J., & Suo, Z. Strengthening alginate/polyacrylamide hydrogels using various multivalent cations. ACS Applied Materials & Interfaces, 2013; 5(21): 10418-10422.

[28] Ellis, B. & Smith, R. Polymers: A property database, second edition. CRC Press Taylor & Francis Group, 2009; 2nd edition: xii.

Academic Vita

John Fadel

Fadel5646@gmail.com

EDUCATION

The Pennsylvania State University *Graduation, May 2017*
Schreyer Honors College
Bachelor of Science in Biomedical Engineering (Biochemical opt)

EXPERIENCE

Undergraduate Research Assistant *Dr. Jian Yang* November 2013 - Present**,** University Park, PA
Project: Ion doped citrate bound materials for functional orthopedic engineering applications

- Design and synthesize biomaterials for orthopedic surgical implants using polymers and polymer composites
- Focus on understanding the interactions between citrate-based polymers and mesenchymal stem cells
- Synthesized novel, soon to be patented/published, polymer, Ca-POC, for orthopedic tissue engineering
- Orchestrated undergraduate group meetings to motivate, assist, and facilitate project discussions

Calculus Tutor *LionTutors* September 2014 – Present, State College, PA

- Lead private tutoring, weekly review (30 students), and exam review sessions (60-105 students)
- Assisted in construction of new math review course (Ordinary and Partial Differential Equations)

Summer Intern *GlaxoSmithKline (GSK)* Summers 2014 & 2015, King of Prussia, PA
Summer 1: Mentor: Dr. Omar Dabbous

- Designed study within GSK on physician-physician social network effects on healthcare of asthma

Summer 2: Mentor: Dr. Yevgeniy Samyshkin

- Constructed Myasthenia Gravis (MG) literature analysis for health economics prep for FDA approval

LEADERSHIP EXPERIENCE

President *Engineering Undergraduate Council* June 2015 - Present, Penn State

- Manage and lead the College of Engineering student government, serving as the representative voice
- Lead engineering town hall meetings with organizational student leaders and administration
- Collaborate with deans and faculty as the student delegate for the Academic Integrity Committee
- Established college council Chair positions to instill more leadership and student representation

Teaching Assistant *Biochemistry & Molecular Biology*, January – June 2016, Penn State

- Constructed lesson plans, led problem solving sessions, and held office hours for class of 260 students
- Facilitated learning and group work for students through active learning practices

Mission Captain *Relay for Life of Penn State* August 2014 – May 2015, Penn State

- Collaborated with American Cancer Society to execute fundraising of $111,790.13
- Led and promoted events in union building in coordination with monthly cancer awareness themes

COMMUNITY SERVICE

Hospital Volunteering:
Mount Nittany Medical (110 hrs)
Abington Jefferson (40 hrs)

Art's Fest Kid's Day
(State College, PA | 2016)
THON Hospitality Committee
(Penn State | 2013 - Present)

Field of Dreams
(Ambler, PA | 2012)
Running for Hope 5K
(Ambler, PA | 2013)

SKILLS

Technical Skills

- SolidWorks, MATLAB, Statistical Analysis, COMSOL, Mimics, 3-Matics, Characterization, Microscopy, PCR, Mechanical Testing

Character Skills

- Leadership, delegation, public speaking, teamwork, community-oriented, fast-paced learner, proficient in English and Arabic, basic in Spanish