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Sol-Gel Derived Bioceramic Poly(Diethyl Fumarate – Co – Triethoxyvinylsilane) Composite

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Abstract

Synthetic bone graft materials have become an increasingly popular choice for bone augmentation. Ceramic-based and polymer-based bone graft materials constitute the two main classes of synthetic bone graft materials. This study investigated the synthesis of novel bioactive composites for their potential use as bone graft biomaterials. Poly(diethyl fumarate-co-triethoxyvinylsilane)/bioceramic class II organic/inorganic hybrid biomaterials were synthesized via a sol gel process. These biomaterials were then reacted with an ammonium phosphate solution to prepare their respective composites. For the first time, we successfully synthesized sol-gel derived bioceramic poly(diethyl fumarate-co-triethoxyvinylsilane) composites. *In vitro* bioactivity evaluation of poly(diethyl fumarate-co-triethoxyvinylsilane)/bioceramic composites in simulated body fluid exhibited hydroxyapatite surface formation. Mechanical testing revealed that these composites exhibit elastic moduli comparable to trabecular bone. Degradation of poly(diethyl fumarate-co-triethoxyvinylsilane)/bioceramic composites in phosphate buffer solution was controlled. It is necessary to conduct further research investigating cytotoxicity, cell attachment, proliferation and differentiation characteristics of these composites.

Keywords

Composites, Bioactive Glass, Bioceramics, Class II Organic/Inorganic Hybrid Biomaterials, Free Radical Polymerization, Diethyl Fumarate, Triethoxyvinylsilane, Bone Augmentation, Bone Remodeling, Periodontal Disease.

Summary for Lay Audience

The aging population calls for the development of novel bone graft materials. Although aging results in bone loss, other causes of bone loss include periodontal disease, fractures/bony defects, cancer tumor removal and congenital disease. There are many factors that play a role in bone healing and determine whether an intervention is required or not. Some of these factors include age, medical history, size of bony defect and location. Defects that require intervention call for use of bone substitutes. The current clinical standard involves harvesting bone from the patient themselves (i.e., autograft) or from another human donors (i.e., allograft). Some disadvantages with these methods include the risk of host rejection and the limited availability of healthy bone.

Synthetic bone graft materials have become an increasingly popular choice for bone augmentation. Ceramic-based and polymer-based bone graft materials constitute the two main classes of synthetic bone graft materials. The most frequently used bone cements are bioactive glass variants and poly(methyl methacrylate) (PMMA). These materials have disadvantages that limit their usefulness as ideal bone graft materials. Some of these disadvantages include their brittle nature, low degradation rate, lack of integration/bonding, polymerization shrinkage, heat generation and toxicity. There is a requirement for the development of a novel osteoconductive, osteoinductive and osteogenic material suitable for bone augmentation that will facilitate healing and remodeling of the bone.

In this work, for the first time, we were able to successfully copolymerize diethyl fumarate with triethoxyvinylsilane, synthesize poly(diethyl-co-triethoxyvinylsilane)/bioceramic class II hybrid biomaterials and prepare their respective composites to be used as a potential bone graft. Composites were incubated in simulated body fluid to study bioactivity, incubated in phosphate buffer saline to study degradation behavior, and tested with an Instron machine for mechanical properties. The preliminary work presented in this thesis shows the potential of these composites to be used for next generation bone graft biomaterials to meet the increasing demand.

Co-Authorship Statement

This thesis was written by A. Sleiman. Drs. A. S. Rizkalla and D.W. Hamilton reviewed and edited this thesis. They also designed the experiments present in this work. A. Sleiman conducted all studies.

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List of Abbreviations

Extracellular Matrix – ECM

Rough Endoplasmic Reticulum – RER

Collagen I – Col I

Osteocalcin – OCN

Osteopontin – OPN

Human Mesenchymal Stem Cells (hMSCs)

Alkaline Phosphatase – ALP

Bone Morphogenetic Protein-2 – BMP-2

Bone Sialoprotein (BSP)

Runt-Related Transcription Factor-2 – Runx2

Simulated Body Fluid – SBF

Hydroxyapatite – HA

Bioactive Glass – BG

Tetramethyl Orthosilicate – TMOS

Tetraethyl Orthosilicate – TEOS

Triethyl Phosphate – TEP

Polycaprolactone – PCL

Poly(propylene fumarate) – PPF

Polyglycolic Acid – PGA

Poly(lactic-coglycolide) – PLGA

Poly(L-lactic acid) – PLA

Polyvinylpyrrolidone – PVP

Polyhydroxyalkanoates – PHA

Diethyl Fumarate – DEF

Chemical Entities of Biological Interest – ChEBI

Azobisisobutyronitrile – AIBN

Triethoxyvinylsilane – TEVS

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide – EDC

N-hydroxysuccinimide – NHS

(3-aminopropyl)triethoxysilane) – APTES

Poly-d,l-lactic acid – PDLLA

Poly(diethyl fumarate-co-triethoxyvinylsilane) – Poly(DEF-co-TEVS)

Bone Morphogenetic Protein – BMP

Organic/Inorganic – O/I

Polydimethoxysilane – PDMS

Room Temperature – RT

Gel Permeation Chromatography – GPC

Nuclear Magnetic Resonance – NMR

Proton Nuclear Magnetic Resonance – ^{1}H NMR

Carbon Nuclear Magnetic Resonance – ^{13}C NMR

Polydispersity Index – PDI

Solid-State Cross-Polarization Magic-Angle Spinning Silicon Nuclear Magnetic Resonance – ssCPMAS ^{29}Si NMR

X-Ray Diffraction – XRD

Molecular Weight – MW

Poly(e-caprolactone) – PCL

Phosphate-Buffered Saline – PBS

Deionized – DI

Powder-to-Liquid – P/L

Scanning Electron Microscopy - SEM

Pascal – Pa

Energy Dispersive x-ray – EDX

Thermogravimetric Analysis – TGA

Chapter 1

Introduction

1 Clinical Need for Bone Augmentation

1.1 Bone Fracture Repair and Critical Size Defect

In 2015 the total estimated number of bone fractures in Canada, both hospitalized and non-hospitalized, was 211,968 cases across the nation. In particular, the cost associated with hospital-based care of fragility fracture patients in Canada was around 1.2 billion dollars [1]. In most of these fracture cases, the natural physiologic bone remodeling process is not enough to stabilize and result in healing of the wound. Indirect and direct fracture healing are the two ways bone is repaired. Depending on the type of break it is, this affects which process of fracture repair takes place. Indirect fracture healing is the process used for most breaks due to its nature – close alignment of the break is not required [2], [3].

Upon fracture, a blood clot, also known as a hematoma, is formed and coagulates around and within the site of injury (i.e., fracture) during the first week. The hematoma comes from the injured bone along with its surrounding tissue and is made up of pooled blood, cells and marrow [2], [3]. This first step of the healing process post-injury is called the inflammatory phase. During this phase, a huge influx of cells is observed. These cells include: lymphocytes, macrophages, monocytes and mesenchymal stem cells [3]. Tissue repair is accomplished by activation of pluripotent progenitor cells which give rise to chondrocytes and osteoblasts, while inflammatory cytokines instigate the process of generating new blood vessels, angiogenesis [2], [4]. The second phase of repair is known as the reparative phase. The second phase overlaps with the end of the first phase and lasts for approximately three to six weeks. During this phase, stabilization of the fracture occurs. This process requires replacement of the hematoma with a soft callus through chondrogenesis. New blood vessels are also required to invade this newly formed tissue. Intramembranous ossification is the process that takes place during the reparative phase where woven bone is produced. This further stabilizes the injury site [2], [3]. Once

intramembranous ossification is complete, endochondral ossification takes place and calcifies the soft callus turning it into hard callus. Blood vessels continue to grow at the injury site. As the reparative phase ends, hard callus is created by endochondral ossification as woven bone if continually formed by osteoblast cells. This process is responsible for connecting the fracture together [2], [3]. Lastly, the weaker, woven bone is remodeled and converted into stronger lamellar structures during the final stages of indirect fracture healing. However, if insufficient blood vessels are formed or if the fracture cannot be stabilized, a critical size defect or non-union is results which requires surgical intervention [2].

In contrast, direct fracture healing, the second type of healing is less common than indirect fracture healing because it solely takes place if the fracture is well-aligned and the injury results in a small gap or little movement between the broken bones [2], [3]. For fractures resulting in gaps of less than 0.01 mm, contact healing takes place. This process causes osteoclasts to travel across the gap and osteons bridge the ends. For fractures resulting in gaps less than 1mm, gap healing takes place. This process causes osteoblasts to produce lamellar bone to bridge the gap. However, the deposited layers are orthogonal to the existing osteons. As a result, a remodeling step is necessary to restore the osteons' orientation [2], [3].

Surgical intervention may be required in cases of severe fractures where the bone's self-repair process is insufficient to heal the bone by itself. Surgical intervention allows for fracture alignment and stabilization, but typically requires hospitalization. In severe cases, it is often necessary to perform surgeries using biomaterials as the bone needs to be replaced and regenerated. One of the major goals in bone regenerative therapies as well as a long-term goal of this thesis is to be able to synthesize materials that can degrade and be replaced by a patient's own bone. By achieving this, the normal function can be restored to the injured site [2], [3].

1.2 Periodontal Disease and Bone Regeneration

Gum disease, also known as periodontal disease, is common in Canada, with seven out of every ten Canadians developing gum disease within their lifetime. Indeed, periodontal

disease is one of the most prevalent dental pathologies across the world. Periodontal disease typically is painless at initiation, but can develop into a serious problem ultimately resulting in bone and gingival recession and ultimately, tooth loss [5]. Although cost depends on each individual case, insurance plan availability and the severity of disease, periodontal treatment for a month average about $3,600 followed by $115 maintenance treatments. Although genetic factors are involved, in general, periodontal disease is a preventable and can be minimized with regular cleaning by health care professionals as well as maintaining proper dental hygiene care as recommended by your dentist [6].

The underlying cause of periodontal disease – classified an inflammatory disease – is bacteria [7]. Overtime, if no proper dental hygiene care – as simple as brushing and flossing regularly – is taken, bacteria normally found in oral microflora will form a biofilm around the gingiva (i.e., gums) and on the tooth root surface which activates the body's inflammatory response. Bleeding of the gums, pocket formation, destruction of alveolar bone attachment often occurs which can result in tooth loss unless the disease progression is terminated. Periodontal disease is very common, and worldwide, has a prevalence rate of 11% for its severe forms. Indeed, for individuals aged 40 and above, periodontal disease is the major cause of tooth loss [7].

Risk factors associated with developing periodontal disease include smoking, hormonal changes in women, diabetes, diseases like cancer or AIDS, medications and genetic susceptibility. Periodontal disease is usually seen in adults aging 30 and above, although teenagers can develop it. Men are more likely than women to develop periodontal disease. Some symptoms of periodontal disease include halitosis (i.e., bad breath that won't go away), red or swollen gums that are tender and easily bleed, painful chewing, loose or sensitive teeth and gum recession. Once confirmed, initial treatment for periodontal disease includes controlling the infection. One way an individual can control the disease is by visiting the dentist's office for scaling and root planning to remove tartar and plaque. Other ways this can be accomplished includes medications such as prescription antimicrobial mouth rinse, antiseptic chip, antibiotic gel, antibiotic microspheres, enzyme suppressant and oral antibiotics.

In many cases, periodontal disease is severe and the only way to treat it is through surgical intervention. Flap surgery as well as bone and tissue grafts are two surgical treatments available for periodontal disease. Flap surgery, as the name suggests, refers to lifting back the gums to remove any tartar and plaque that exists. This is usually done when inflammation persists after deep cleaning and scaling has been performed. Bone and tissue grafts may be necessary when bone loss is experienced from periodontal disease. The aim of bone and tissue grafts is to help repair and regenerate the lost bone in order to keep the tooth intact [8]. This thesis will address this challenge through development of a novel biomaterial for enhancing bone formation.

1.3 Bone Anatomy and Remodeling

Bone is an organ in the body that is composed of organic and inorganic components. For this reason, it is known as a nano composite. The organic component is primarily made up of collagen while the inorganic component is primarily made up of hydroxyapatite [9]. Bone makes up the skeleton and provides support to various organs. It protects them due to its rigid nature and allows the body to move and transport [10]–[12]. Bone also provides many functions in the body including the regulation of blood pH, production of bone marrow cells as well as storing of minerals and progenitor cells. Some of these progenitor cells include mesenchymal and hematopoietic cells [13], [14]. As mentioned above, bone loss due to bone related disorders takes a huge toll economically and health wise. In Canada, as stated by the Canadian Institutes of Health Research (CIHR), musculoskeletal tissue injuries and diseases cost the economy billions of dollars annually in spending. Moreover, other than the economic burden it causes, physical, mental and emotional stresses are suffered from those experiencing the disease as well as their family members [15]. Over time, in some circumstances, bone encounters stress and damage that normal physiological processes of bone repair and remodeling is not sufficient to restore normal function of the bone. In that case, biomaterials are used to fill the void or fix the non-union [16]. To understand the repair and remodeling mechanisms of bone, bone histology, bone microstructure and bone macrostructure must be understood.

The epiphysis and the diaphysis are the two major structural regions of long bones. The epiphysis constitutes the ends of the bone while the diaphysis represents the middle portion,

also known as the shaft of the bone [12]. Structurally, there are two types of bone found within long bones. The outer portion is known as the compact bone which is the bone visible to us. The inner portion is known as trabecular bone or spongy bone. Trabecular bone is located underneath the cortical layer and is porous [12]. Spongy bone extends to the epiphysis regions of the bone and lines the inner region of the diaphysis. In the center of the bone itself, the marrow cavity is found which is lined by endosteum. When looking at cross sections of cortical bone under a microscope, lamellae are seen. Lamellae are mineralized layers of bone that result from bone remodeling and are characterized by the presence of haversian systems or osteons, which contain central canals containing blood vessels, lymphatics and nerves [17].

Osteons have their lamellae organized in concentric circles with a central blood vessel (Figure 1.1). The diameter of these osteons range between 50 microns to a few hundred microns [17]. Haversian systems cross the entire length of the long bone creating cylinders that travel towards the periosteum. These cylinders of osteons can form multiple branches, change cross-sectional area and twist or wrap around other osteons due to constant bone remodeling. The cylinders formed by the osteons are not perfectly symmetrical and usually contain ridges and irregularities. These ridges and irregularities reside in the lamellar structures of neighboring haversian systems. Volkmann's canals (also known as orthogonal channels), which have blood vessels as well, connect these neighboring osteons with each other and terminate in the periosteum [12], [17]. Another form of lamellae, interstitial lamellae, are residues of partially remodeled osteons. Interstitial lamellae do not possess a central canal and they present as parallel lines that fill voids between non-bordering haversian systems [17]. Finally, numerous concentric lamellae line the outer and inner surfaces of cortical bone. Volkmann's canals, as aforementioned, allow the passage of blood vessels which branch off and diffuse through the entire canal network [17].

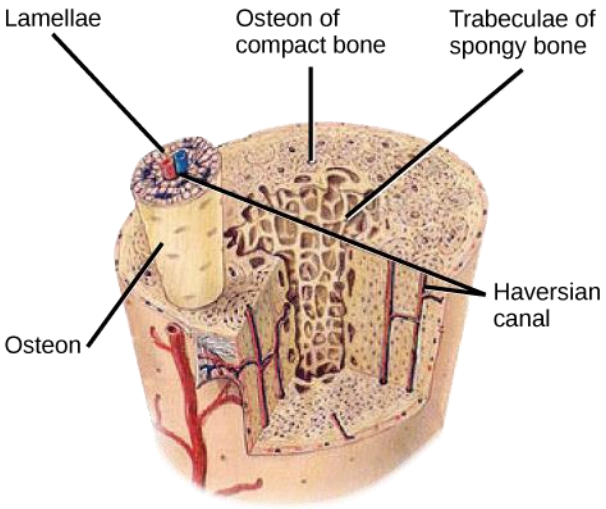

Figure 1-1: Osteons are structures found within compact bone that are organized in a parallel manner along the long axis of the bone. The Haversian canal is found within these structures and contain blood vessels and nerve fibers. This osteon image was adapted from Veiko *et al* and is licensed under CC BY 3.0 [18].

Long bones along with their trabecular structures are formed through endochondral ossification during development. The condensation of mesenchymal cells instigates this process. Chondrification, the differentiation of mesenchymal cells to cartilage cells, takes place when mesenchymal cells reach a critical mass. Once these cartilage cells have synthesized collagen extracellular matrix (ECM), the perichondrium will form a mineralized bone collar. In contrast, the chondrocytes located in the middle of the diaphysis will hypertrophy [12]. Wall structures are formed in the center of the bone. The wall structures are a result of small cavities created due to the degradation of chondrocytes. Trabeculae are formed when wall structures are mineralized by the infiltration of osteoprogenitor cells. The growth plate structure is the site where bone lengthens throughout development. This structure is formed when the ends of the growing bone meet at the diaphysis [12].

Chondrocytes play an essential role in mineralizing the collagenous ECM. As previously mentioned, mesenchymal progenitor cells are differentiated into chondrocytes once condensation occurs. At this point, ECM vesicles are produced by chondrocytes. These

vesicles can concentrate calcium and phosphate ions. This, in turn, allows mineral nucleation to be created on the interior membrane wall [12]. As this process continues and there is more deposition of calcium and phosphate ions, the vesicle ruptures which leads to the release of the mineral layer along with the concentrated ions into the collagenous ECM [12]. Due to the ion's charged nature, further ion deposition is encouraged into the surrounding extracellular fluid leading to the mineralization of the entire matrix [12].

Local ion concentration is the single most important factor for calcium phosphate nucleation. Ectopic calcification – mineralization of soft tissues – can occur in cases where a patient suffers from kidney malfunction, usually a lowered function. This results in high levels of mineral ions causing vesicle formation as cells start apoptosis [19]. In blood, normal calcium ion concentration is 2.5 mM, while phosphate ion concentration is 1mM. When concentrations of calcium in the blood raise above 2.8 mM, symptoms start to arise, however, when concentrations are above 3.0 mM, it is a critical situation [20]. In bone, calcium ion concentration can be as high as 40 mM during remodeling [21]. Parathyroid hormone and calcitonin are the two hormones in the body responsible to regulate serum calcium levels [20]. The parathyroid gland produces parathyroid hormone which is responsible for increasing serum calcium levels. This is achieved by increasing osteoclast proliferation and activity which results in bone resorption causing the release of calcium into the blood [20]. In contrast, the thyroid gland produces calcitonin which is the hormone responsible to reduce calcium concentration in the blood when they are elevated. This is achieved by storing serum calcium in bone by adjusting the activity between osteoblasts and osteoclasts [20].

The two major cells involved in bone remodeling comprise of the osteoblasts and osteoclasts. Osteoblasts are responsible for bone formation, whereas osteoclasts are responsible for bone resorption [22]. Osteoclasts are responsible for initiating the bone remodeling process. They are brought to the site of remodeling by blood vessels. Their path of action is along the diaphysis where they resorb bone longitudinally. As this process goes on, a long tunnel is formed which is known as a resorption cone [22]. Bone resorption requires lots of energy, and thus, osteoclasts have large amounts of mitochondria and are multi-nucleated [23]. The osteoclast structure is organized in a way such that the region

responsible for resorption is highly folded. This gives it its pocket appearance that is seen during cellular engulfment. This section is also lined by a clear zone that helps with bone attachment. Finally, this region does not possess any organelles [23]. As resorption takes place, a capillary makes its way through the resorption cone. This will be the center of the new osteon [22].

Compared to osteoclasts, osteoblasts contain one nucleus and have multiple large rough endoplasmic reticulums (RER) which is reflective of their main role in synthesizing protein [22]. Prior to beginning the process of bone formation, the walls of the resorption cone are coated with the cement line which is an unmineralized layer [22]. Once the unmineralized layer is placed, the remodeling process begins and the osteoblast cells deposit collagen I (col I), osteocalcin (OCN), and osteopontin (OPN). These layers of deposited matrix proteins are essential for cell adhesion and hydroxyapatite – bone mineral [22]. During the mineralization process of collagen, some of the osteoblasts become enclosed within the matrix. There are three fates for these enclosed osteoblasts. They either develop into less active osteocytes, undergo apoptosis, or line the bone. In comparison, osteoclasts have only one fate upon completion of the bone remodeling cycle and that is undergoing apoptosis [23]. Osteoclasts are not isolated although they reside in spaces known as lacunae in the mineralized matrix. Canaliculi are small channels contained within osteons which pass through the lamellae. From there, a network of osteoclasts is created [22]. The natural and physiological bone remodeling process explained above has the capability of repairing minor bone damage, however, is not able to repair fractures [22].

1.4 Markers of Osteogenesis

Mature osteoblast cells are derived from human mesenchymal stem cells (hMSCs). Differentiation is quantified through expression of certain genes including collagen type I, osteocalcin (OCN), OPN, alkaline phosphatase (ALP), bone morphogenetic protein-2 (BMP-2), bone sialoprotein (BSP) and runt-related transcription factor-2 (Runx2) [24]. When an hMSCs commits to the osteoblastic lineage these genes above are turned on in a specific temporal pattern. Runx2 transcription factor expression occurs and distinguishes the mesenchymal stem cell as an immature osteoprogenitor cells. Runx2 expression occurs first and prior to entry of the cells into the proliferative phase. Concomitantly, collagen

type I and ALP and upregulated, indicate maturation of the osteoprogenitor cells. In stages after the proliferative phase, ALP expression results in production of phosphate, which is then incorporated in the extracellular matrix by osteoblasts. Col I acts as a nucleation point for mineralization. Once full maturation has been achieved by the osteoblast, their proliferation ceases and their main function is to make bone by becoming bone-lining cells or osteocytes. One important matrix protein unique to this phase involved in helping cells attach to mineralized surfaces is OPN. One matrix protein that is produced by mature osteoblast cells is OCN, which is commonly used as a marker for mature osteoblasts and osteocytes [24].

1.5 Osteoinductive and Bioactive Materials

Osteoinductive materials can initiate the bone formation process by initiating the recruitment of progenitor cells and differentiating them into the osteoblast lineage. Out of the three bone grafting techniques discussed earlier (i.e., autograft, allograft, xenograft), autografts are the only osteoinductive grafts [25]. These has led to the development of numerous bone graft substitutes as without the addition of BMPs, allografts and xenografts have little to no osteoinductive properties [26]. There are both bioactive materials and bioinert materials that are used to replace bone [27]. Bioactive materials are designed to integrate with and bond to bone when placed into defects. Bioinert materials, in contrast, lack this ability and upon implantation are typically bordered by fibrous tissue [27].

In order to predict and quantify a material's bioactivity, simulated body fluid (SBF) may be used [27]. This is an *in vitro* study where samples are submerged in the media at 37 ºC for a specific time and hydroxyapatite (HA) mineral deposition is observed on the material's surface. The reason why SBF predicts a material's bioactivity is because it represents human blood plasma [27]. It is composed of ion concentrations and pH that mimics human blood plasma. Materials that are placed in SBF and that are bioactive will form a hydroxyapatite layer on the surface under these conditions. In the body, however, a mineral layer is formed and will form a bond with the neighboring bone. There are multiple versions of SBF and the main difference between them is the ion concentrations. Some solutions of SBF have higher ion concentrations which allows for faster mineral deposition on the material's surface [27].

Both organic and inorganic materials contribute to bioactivity. Some examples of such materials include: bioactive glasses, calcium products (i.e., calcium carbonate and calcium sulfate), collagen and demineralized bone matrix [28]. Osteoinductive properties are seen with bioactive ceramic materials such as: hydroxyapatite, biphasic calcium phosphate, tricalcium phosphate, and bioactive glasses [4]. The topography of the bioactive material's surface is altered after it has interacted with body fluid. That topography is thought to be responsible in giving the material its osteoinductive properties and can also influence cell response.

Osteoprogenitor cells along with hMSCs are activated through a pathway known as the mechanotransduction pathway. Via this pathway, they are able to attach themselves to their environment through focal adhesion proteins, express certain genes and transmit forces throughout the cytoskeleton [29]. Selimovic et al. (2012), have shown that nanofiber topographical features on a material's surface is able to promote osteogenic differentiation. This implies that the way the material behaves in body fluid and what characteristics are presented on its surface after plays a big role in promoting osteogenic differentiation [30].

Other research has shown that as the bioactive material degrades, ions such as calcium and phosphate are released into the surrounding environment and are thought to act as signaling molecules and promote cells to take the osteogenic differentiation lineage [31]. BMP is currently used to stimulate osteogenesis with materials that do not have strong osteoinductive properties as explained earlier [31]. This is important, because materials releasing calcium and phosphate ions have the potential of being used instead of BMP. To test this, a research group investigated ion-induced osteogenesis in three different media using human periosteum-derived cells [32]. In one *in vitro* study, the cells were cultured in a medium supplemented with phosphate and calcium ions. In another *in vitro* study, the cells were cultured in a typical growth medium [32]. And finally, in the last *in vitro* study, the cells were cultured in an osteogenic medium [32]. The medium containing calcium and phosphate ions demonstrated an increase in osteogenic gene expression specifically in the matrix proteins OPN, Runx2, BMP-2, and OCN [31], [32].

1.6 Bioactive Glass

Bioactive glasses (BGs) belong to a class of non-crystalline silicate glasses. In the presence of SBF or physiological fluids, BGs can stimulate hydroxyapatite formation, a bone-like mineral [33]. HA is equivalent to the inorganic component of natural bone [33]. It is believed that the hydroxyapatite integrates with the native bone in the body [33]. Bioactive glass dates as far back as 1969. The first BG synthesized consisted of SiO_2, Na_2O, CaO and P_2O_5 with ratios of 46.1 mol.%, 24.4 mol.%, 26.9 mol.% and 2.6 mol.%, respectively. *In vivo* studies have shown that the HA layer that is formed at the boundary between the bone and implant after the glass material dissolutes creates a strong bond with native bone [34]. Since 1969, silicate-based, borate-based and phosphate-based glasses have been synthesized. The only difference between the classes of BGs is the ratios of their constituents [35].

$(SiO_4)^{4-}$, in silicate-based BGs, is the main constituent responsible in making the 3D glass networks. There are other components in the glass that help with forming the glass networks. These components are known as network modifiers and they can be added in different amounts. Some examples of them include: CaO, K_2O and Na_2O [35], [36]. $(PO_4)^{3-}$, however, is the main glass forming network unit in phosphate-based BGs. In this class of glasses, the only modifiers are CaO and Na_2O. Numerous investigations have demonstrated that BGs have a great potential in bone engineering applications [37]. Bone's inorganic content contains ions such as calcium and phosphate and because of that, phosphate-based BGs can bind to bone. The P-O-P bond in the phosphate-based BGs is easily hydrated in the presence of water and thus this class of bioactive glasses possess a high dissolution rate. Components such as metal oxides (i.e., Fe_2O_3, NiO, and CuO) can be added to the glass and alter dissolution rate. Therefore, the glass composition can be adjusted depending on the type of application [38].

Another class of BGs include the borate-based glasses. They are bioactive just like the other glasses, however, in comparison to silicate-based glasses, they are superior in terms of hydroxyapatite formation because of their faster dissolution rate. The reason they have a faster dissolution rate and faster hydroxyapatite formation is because boron inhibits the formation of a SiO_2 layer, which is more stable [39], [40]. The rate of degradation of a

biomaterial is essential in hard tissue engineering. Therefore, altering composition of BG raises opportunity in controlling rate of degradation and ultimately enhancing bone regeneration. The reason why this is important is because the biomaterial should degrade at a rate similar to that of new bone formation [39]. *In vitro* studies of borate-based BGs have shown that they are able to allow cells to proliferate and differentiate. Additionally, *in vivo* studies for borate-based BGs have demonstrated that the Boron improves tissue infiltration. Boron is essential for bone health maintenance [41], [42]. In cases where bone infection is present, borate-based BGs can act as a vehicle for drug release [43], [44]. Although they have great bioactivity, research has shown that some compositions show cell cytotoxicity in a static environment. When tested under dynamic, no cytotoxicity was exhibited [44]. When initially synthesizing borate-based BGs, it is essential to optimize the concentration of the B_2O_3 component in the glass as it is the one responsible in dictating how much boron there is in the culture media [35].

Before the establishment of sol-gel synthesis techniques of BGs were introduced in the early 1990s, BG synthesis was based on a technique known as melt-quenching. Ceramic powders are melted at temperatures above 1300°C in melt-quenching techniques. Once melted, the ceramic powders are placed in cold water or graphite mold, a technique known as quenching [34], [45]. The sol-gel process of synthesizing BGs, when compared to the melt-quenching techniques, involves chemical reactions at room temperature. Bioactive glass precursors known as the sol, colloidal suspensions, become a gel after a series of hydrolysis and poly-condensation reactions take place. The glass is formed when the inorganic network of the gel dries up. The inorganic network is made up of glass constituents that are bonded covalently. Metal alkoxides which possess the generic structure of M-(OR)x make up the BG precursors. The M represents a central metallic ion that is mainly attached to alkyl (-OR) groups but may be bound to other functional groups. The SiO_2 in the BG is derived from the metal alkoxides tetramethyl orthosilicate (TMOS) or tetraethyl orthosilicate (TEOS). P_2O_5, however, is derived from triethyl phosphate (TEP). The reason why these metal alkoxides are used is because they react with water easily. Hydroxyl groups replace the alkoxy side chains once the hydrolysis reaction takes place. The acid or base catalyzed hydrolysis reactions that take place are a nucleophilic type of reaction. The oxygen atom in water attacks the silicon core atom [45].

There are several factors that play a role in dictating the result of the inorganic glass networks. These factors include: acid catalysts, base catalysts, precursor molecules, pH and solvent-reactant ratios [46]. In addition to the above differences between melt-quenching and sol-gel techniques, bioactive glasses synthesized through the sol-gel technique possess a homogenous microstructure with greater purity and a nanoporous structure. Bioactive glasses synthesized through the melt-quench process possess a dense microstructure and a heterogenous distribution [47]. Due to the nanoporous structure and increased surface area in sol-gel derived BGs, studies have shown that there is an improved cellular response as well as an improved bioresorbability [48].

Although bioactive glasses have great properties of osteoconductivity and bioactivity, there are some disadvantages associated with BGs [49]. Some of these include their stiffness properties as well as the need of high temperatures to manipulate the BG into the required shape rather than just a powder. Moreover, when sol-gel BGs are in the process of drying, cracks occur, and it is difficult to prevent this from happening due to the large shrinkage that takes place during the drying phase. When the condensation reaction liquid by-products evaporate, they make their way to the gel surface through the interconnected pore network. When the stress is too high within the pore network and the path is long, as is the case with BG scaffolds, cracking happens [49].

1.7 Biocompatible and Biodegradable Polymers

Synthetic and natural biocompatible and biodegradable polymers have been vastly studied for their use in bone tissue engineering [50], [51]. Proteins and polysaccharides are the main sources for natural biodegradable polymers. Protein based polymers are derived from gelatin, albumin and collagen while polysaccharide-based polymers are derived from chitin, cellulose, alginate and hyaluronate. Synthetic polymers have shown to be great candidates for bone tissue engineering applications [50]. Examples of synthetic polymers include polycaprolactone (PCL), poly(propylene fumarate) (PPF), polyglycolic acid (PGA), poly(lactic-coglycolide) (PLGA), poly(L-lactic acid) (PLA), polyvinylpyrrolidone (PVP), polyhydroxyalkanoates (PHA), polyphosphazene, poly (orthoesters) and polyanhydrides [50]. Polymers derived from natural sources exhibit superior cell-material interactions when compared to synthetically derived polymers. Despite this, investigators

have focused on researching synthetic polymers because naturally derived polymers are not available in large quantities and are difficult to work with and purify [52]–[54]. Compared to natural polymers, synthetic polymers can have their mechanical properties, degradation rate and porosity manipulated for specific tasks. Also, synthetic polymers can be synthesized in large quantities and possess a long shelf life. Polymers can be synthesized with mechanical and physicochemical properties (i.e., elastic modulus, tensile strength and degradation rate) comparable to that of biological tissues. These synthetic polymers can be made into different shapes and have their properties adjusted depending on the application intended [54].

In general, when polymers are synthesized with high amounts of macro-porosity, they possess limited strength and have a weakened mechanical stability. Both of these properties are essential when considering materials for bone tissue engineering [55]. The mechanical properties of the biomaterial should be comparable to that of bone, otherwise, failure may occur. Cortical bone has an elastic modulus of about 20-30 GPa. If the material implanted does not possess comparable elastic moduli, the implant or graft may loosen over time and fail. This is a phenomenon known as stress-shielding. The bone remodeling process requires a certain amount of stress and without the appropriate amount, bone resorption will occur. When this happens, the graft will loosen and fail [56]. Finally, polymers are biomaterials that are not osteoconductive and do not possess the ability to sufficiently allow bone cells to adhere, grow and proliferate [55].

Materials that are used in bone tissue engineering should possess osteoconductive and osteoinductive properties. For cells to permeate the structure and allow for tissue to grow and carry out appropriate metabolic reactions and waste removal, the biomaterial should have a proper porous 3D structure. The rate of new bone formation should be comparable to the rate of degradation of the biomaterial in order for bone to replace it. Due to the complexity of bone and the requirement of many different properties, there is no one material that meets all criteria. Therefore, composite materials with appropriate properties that possess organic and inorganic phases are promising materials in the field of bone tissue engineering.

1.8 Free Radical Polymerization

In general terms, free radical polymerization is a method of polymerization in which a polymer is formed by the consecutive addition of free-radical building blocks. These blocks can be created through a myriad of different mechanisms, such as utilizing separate initiator molecules [57]. In considering free radical polymerization, or copolymerization, it is important to consider its development. In early studies of grafting, grafting was used onto natural rubber, then to high-impact polystyrene, and grafting of multiple monomers [58]. However, the chemistry of free radicals as they are used today originates with the papers of Gomberg in 1900 [58]. For a little over a century, polymer-based materials have been more popularly used in various applications, such as biomedical applications. Graft copolymer is a category of copolymer where one or more blocks of homopolymer are grafted onto the main chain in the form of branches. Thus, it is a branched copolymer in which one or greater amount of side chains of a homopolymer is affixed to the backbone of the main chain [59].

Working alternatively to biological grafts, polymers can be used in bone repair. Self-healing biomaterial that is based on free radical polymerization is a relatively novel concept and creates a potential for safer and longer lasting restoratives [60]. In generating grafting sites within the polymer backbone, it is possible to use polymer grafting activators, like free-radical initiators [61]. However it is becoming increasingly important nowadays to utilize new materials, be more environmentally friendly and also to create better conditions for both the worker and the user [59]. Green chemistry works at a molecular level to achieve sustainability. It entails the design of chemical products and processes which either reduce or eliminate the use and generation of harmful and hazardous substances [62]. For instance, degradability of material causes less ecological harm and is a tenet of the new proposed process of this paper.

1.9 Diethyl Fumarate and its Polymerization

Diethyl fumarate (DEF) is a dieter which is obtained through the formal condensation of fumaric acid and ethanol. According to the database entitled Chemical Entities of Biological Interest (ChEBI), DEF’s role is as a metabolite and derives from fumaric acid.

Polymers that are based on fumarate derivatives are of interest in their ability to be used as biodegradable polymers for treatment of intense bone defects [63]. DEF has a history of being incorporated in the process of bone regeneration using a microstereolithography-produced customized poly(propylene fumarate)/diethyl fumarate photopolymer 3D scaffold and incorporating BMP-2 loaded PLGA microspheres [64]. Many publications regarding polymerization of fumarate describe the conventional method for polymerization or photo-polymerization with diethyl fumarate as crosslinking agent [64]. A common method is radiation polymerization of DEF, for instance DEF which is polymerized through using gamma irradiation in a dose range of 50-300 kGy, which is a dose range in which the polymerization yield increases nearly linearly [63]. DEF has also been radically polymerized under UV irradiation, which has generated considerable attention due to peculiar features of polymerization, namely to poly(substituted methylene). An important feature of diethyl fumarate's polymerization in this process is there is faster polymerization of diethyl fumarate with a bulkier ester alkyl group to higher molecular weight polymer [65]. The rate of polymerization of diethyl fumarate is affected by pressure [66]. Initiators are a source of any chemical species and react with a monomer, which is a singular molecule that is capable of forming chemical bonds [67]. The reaction forms an intermediate compound which has the ability of linking consecutively with a high number of different monomers to create a polymeric compound. The most heavily used initiators produce free radicals, which are reactive atoms or groups of atoms which possess an odd number of electrons [67]. There are a range of initiators that can be utilized in the polymerization process. Notably, azobisisobutyronitrile (AIBN), is a radical initiator that is safer to use than other initiators because the risk of explosion is much less [67].

1.10 Triethoxyvinylsilane and its Polymerization

Triethoxyvinylsilane (TEVS) is an organosilicon compound with the following formula: $(C_2H_5O)_3SiCH{=}CH_2$. It is a silane coupling agent, additionally it promotes adhesion, which may be used for cross-linking and also provides an insulating layer that has good thermal and mechanical properties [68]. Triethoxyvinylsilane can be polymerized by chemical initiation or by γ-ray irradiation [69]. Triethoxysilane, is a vinyl-functional silane that can be utilized to strengthen the bond between glass fiber or mineral fillers and resins which

are reactive towards the vinyl group. It is also used in order to functionalize resins through free radical mechanisms such as copolymerization or grafting - and in modifying surfaces [70]. TEVS has played a role in hierarchically engineered fibrous scaffolds for bone regeneration. In a recent study, hierarchically engineered fibrous scaffolds have been analyzed in their role in bone regeneration. [71]. The findings showed that surface characteristics can be tailored and mimic bone extracellular matrix. The nanofibrous scaffold is made of polylactic acid which is FDA-approved and highly utilized in regenerative medicine. This allows for a flexible structural support and a sol-gel processed organic-inorganic bioactive class (P_2O_5-CaO-SiO_2 system) provides for chemical bioactive cues. This system has been supported by various studies to have good osteointegrative properties and triggering a proper cellular response. In terms of the regeneration process, the gradual biodegradation of bioactive glass, in which ions are required in order to guide bone tissue repair, triggers regeneration [71]. Electrospun fibers mimic the fibrous contents of extracellular matrix structure of natural bone [71]. Hollow PLA fibers are made with a traditional electrospinning device through the Kirkendall effect and with 2,2,2-trifluoroethanol as a solvent. The fibers went through a succession of surface treatments such as controlled hydrolysis and applied to generate carboxyl groups at the surface of fibers. These groups activate through immersion in an 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS) solution, and (3-aminopropyl)triethoxysilane (APTES) coupling agent functionalizes it [71].

1.11 Bioactive Glass Based Biocompatible and Biodegradable Polymer Composites

The disadvantages and limitations discussed in sections 1.6 and 1.7 can be overcome by synthesizing composites of bioactive glasses and degradable polymers. Bone itself is a mixture of organic (i.e., collagen) and inorganic (i.e., HA) material. Composites derived from bioactive glasses and polymers will comprise those two components. The bioactive glass will provide the inorganic phase while the polymer will provide the organic phase. These organic/inorganic composites combine properties of both materials in one and thus have gained a lot of attention by researchers. For example, bioactivity of bioactive glass can be complemented with toughness of polymer when composite materials are being

synthesized [72]. Studies have shown that composites synthesized with BGs and polymers such as PDLLA, PLA and PGA possess superior mechanical properties when compared to pure polymers and pure bioactive glasses [73], [74].

Ceramic-based bone grafts are synthetic products which have been widely used in reducing the need for iliac crest bone grafting [75]. Ceramics differ greatly such as in differences of composition, porosity, manufacturing and structure [75]. Further, ceramic products include a variety of compounds such as for example calcium sulfate, hydroxyapatite, and tricalcium phosphate. These components have different biodegradability, binding, and mechanical properties [75]. Ceramics provide an osteoconductive matrix which is reliable however they generally lack osteoinductive potential [75]. Ceramics are biodegradable, though remodeling happens through a different process than typical bone remodeling. Some advantages of ceramics are that they are biologically inert and typically do not induce a host inflammatory response in comparison to different bone graft substitutes [75]. Ceramics may be molded and cut into a myriad of shapes which can appropriately match their environment, and the cost is usually less than other bone graft substitutes. Ceramic based bone grafts provide many advantages such as ease of sterilization, flexible shape, and inertness. Though there are limitations such as they are susceptible to fracture, and there is a need for an osteoinductive adjunct [75]. In dentistry, bone grafts have been used as a filler and a scaffold in order to generate bone formation and promote healing of wounds [76]. The grafts are bioresorbable and no antigen-antibody reaction is initiated. The bone grafts induce new bone formation as they act as a mineral reservoir. OsteoGraft for example uses calcium phosphate, calcium sulphate and bioglass in combination for their ceramic based bone graft substitutes [76]. Biologic mechanisms that rationalize bone grafting are osteoinduction, osteoconduction and osteogenesis [76]. Osteoinduction involves the stimulation of osteoprogenitor cells which differentiate into osteoblasts and this starts forming new bone. Osteoconduction happens when bone graft material begins as a scaffold for new bone growth, perpetuated native bone. Osteoblasts from the place of defect that is being grafted, use the bone graft material as a framework to generate new bone. Lastly, osteogenesis occurs when vital osteoblasts originate from bone graft material and contribute to growth of new bone with bone formation [76]

In comparison to polymers, synthetic bioceramics are considered superior for bone repairs due to improved bioactivity, strength and biocompatibility. However, the problem of brittleness of ceramic substitutes includes a use of composite materials in which organic polymers are mixed in. Polymers are degraded typically hydrolytically or enzymatically [77]. Natural and synthetic polymeric materials have been used in bone tissue engineering because of their similarity with extracellular matrices and their considerable biodegradability and biocompatibility. Many techniques have been used to modify physicochemical, structural and biological properties of polymeric materials in order to meet the requirements of bone regeneration [78]. Natural and synthetic polymers and their composites have been used as scaffolds for bone regeneration and are considered the most promising materials in comparison to metals and ceramics. Polymers have a quite flexible design capacity, and their properties can be easily fit to meet the specific requirements needed as their chemical compositions and structures can be more easily manipulated. Many natural polymers such as collagen, gelatin and synthetic polymers such as PLA and PGA have been used for bone tissue regeneration. They are usually composited with one another or other inorganic materials such as calcium phosphates in order to increase their osteogenic performance [78]. In comparison to natural polymers, synthetic polymers can be synthesized in more controlled conditions. The physicochemical and biological properties such as degradation rate, microstructure and mechanical strength are predictable and reproducible. The desired properties can be obtained rather dexterously by cautiously designing segments and functional groups of polymers [78].

1.12 Class II Hybrids

Class II hybrid scaffolds have a high potential within bone tissue engineering because of their bioactivity, tailorable microstructure, and degradation and mechanical properties [79]. Compared to Class I hybrid biomaterials, which exhibit weak interactions between the organic and inorganic phases (i.e., Van Der Waals, electrostatic interactions or hydrogen bonding), Class II hybrids are characterized by stronger interactions, for example covalent bonding, that occurs between organic and inorganic components. Class II hybrid biomaterials are synthesized in order to enhance bone formation *in vitro* [79]. Three varying strategies are typically employed in order to synthesize class II hybrid biomaterials

which includes the following: the utilization of a coupling agent which may bond with organic and inorganic phases, the utilization of an organic polymer that already contains trialkoxysilane functional groups, and *in situ* polymerization of organic and inorganic phases from precursor monomers [79]. The disadvantages of using coupling agents for synthesis of class II hybrids is that functionalized polymers allow for only a limited amount of functional groups in relation to polymer backbone [79]. Degradable polymers can be a better choice in this regard and to synthesize class II hybrids through sol gel processes, as there is a predictability of the degree of crosslinking that does not depend on molecular weight. Whereas, when using coupling agents, polymers with a high molecular weight will have poor interaction with inorganic phases which can promote phase separation over a certain amount of organic moiety [79]. The research presented in this thesis will address the development of a novel, sol-gel derived bioactive glass poly(diethyl fumarate – co – triethoxyvinylsilane) hybrid composite to promote bone regeneration and remodeling.

1.13 Rationale, Hypothesis and Objectives

1.13.1 Rationale

Bone defects can result from various sources such as infections, traumas, tumors, surgeries, congenital etiologies and diseases [4]. Bone grafting procedures have been employed as treatments for numerous decades, however, no ideal material yet exists. Bone grafting procedures are required when a bony defect results in the inability of the natural bone to self-repair. These wounds or defects are known as critical-sized bone defects [80]. World-wide, there are more than 2 million procedures that require bone grafting procedures. In the United States, there are about 500,000 cases that require bone grafting procedures annually [4]. Today's gold standard for bone grafting procedures involve autografting [4]. Autografting refers to the process of obtaining bone from one's own body [76]. Sources usually include harvesting bone from sites such as the iliac crest, mandibular symphysis or anterior mandibular ramus and moving it to the defect site [76]. However, autografting carries the risk of morbidity at the donor site (i.e., requires surgery to be harvested) and is limited in terms of size. It is the gold standard because autografting maintains osteogenecity, osteoconductivity and osteoinductivity; three main properties of bone tissue [76]. As the bone is coming from the same person, there is a reduced chance of graft

rejection [76]. Osteogenecity takes place when osteoblasts coming from the bone graft material promotes the growth of new bone. Osteoconduction takes place when the bone graft material acts as a scaffold for new bone growth. Osteoinduction is the ability of the bone graft material to stimulate osteoprogenitor cells to differentiate into osteoblasts and start bone formation [76].

Other sources of harvesting bone include allograft and xenograft procedures. These procedures are usually performed when more material is required. Allograft procedures refer to the harvesting of bone from humans, like autograft. However, the difference between the two is that allografting involves a recipient and a donor. Allograft bone is usually harvested from cadavers. From donor to recipient; disease transmission, infection and host immune response are three concerns that arise with allografts [16], [81]. Because of that, allografts are usually processed to reduce the risk of rejection and disease transmission. After processing, though, the bone's osteoinductive and mechanical properties are affected negatively. Xenografts are bone grafts derived from bovine species; species other than human [76]. Xenografts carry more risks than allografts. Some of these include disease/virus transmission, infection, toxicity associated with sterilization techniques and host rejection. For these reasons mentioned, xenografts are considered not suitable for use in transplants [82], [83]. To restore some of the osteoinductivity, bone morphogenetic proteins (BMPs) are added at the defect site when bone-graft substitutes are used [84]. Since blood supply is necessary for the growth of bone, some grafts may require an additional source of blood supply. Those types of grafts are called free flap grafts and the blood supply is usually derived from the periosteum and its associated blood vessels [76].

Bone substitutes can be derived from biological as well as synthetic products [4]. Some bone substitutes derived from biological products include demineralized bone matrix, platelet-rich plasma, bone morphogenetic proteins hydroxyapatite and coral [4]. In contrast, some synthetic bone substitutes include calcium sulfate, calcium phosphate cements, beta-tri-calcium phosphate ceramics, biphasic calcium phosphates, bioactive glasses and polymer-based bone substitutes [4].

These materials have disadvantages that limit their usefulness as bone graft materials. Some of these disadvantages include their brittle nature, low degradation rate, lack of integration/bonding, polymerization shrinkage, heat generation and toxicity [49], [85]. There is a requirement for the development of a novel osteoconductive, osteoinductive and osteogenic material suitable for bone augmentation that will facilitate healing and remodeling of the bone. This thesis focuses on the development and characterization of a composite biomaterial prepared by reacting poly(DEF-co-TEVS)/bioceramic class II hybrid with an ammonium phosphate solution.

1.13.2 Hypothesis

The novel synthesized class II hybrid composite biomaterial will have desirable chemical and mechanical properties for non-load bearing bone applications. The addition of poly(diethyl fumarate-co-triethoxyvinylsilane) will positively influence the mechanical properties of the graft.

The biomaterial will have:

- A good degradation profile compared to the bioceramic control
- An adequate compressive modulus and strength comparable to that of trabecular bone
- The ability to form hydroxyapatite (bioactive)

1.13.3 Objectives

The main objective of this project was to develop a novel, bioactive, biodegradable and mechanically competent composite for non-load bearing bone augmentation procedures through the synthesis of a sol-gel derived bioceramic poly(diethyl fumarate – co – triethoxyvinylsilane) hybrid. The specific objectives of this investigation to achieve the main objective were:

1. To synthesize and characterize poly (diethyl fumarate-co-triethoxyvinylsilane)
2. To synthesize and characterize a bioceramic – poly (diethyl fumarate-co-triethoxyvinylsilane) hybrid.
3. To develop and characterize a bioactive composite to be used as a bone graft.

Chapter 2

Synthesis of Poly(diethyl fumarate-co-triethoxyvinylsilane)/Bioceramic Hybrids

2 Summary

Functionalizing the sidechains of a copolymer backbone with triethoxyvinylsilane silane groups results covalent crosslinking between the organic phase – made up of the copolymer – and the inorganic phase – made up of bioceramic – during the sol-gel synthesis process of class II organic/inorganic (O/I) hybrid biomaterials. DEF and TEVS monomers were copolymerized at a 70/30 (DEF/TEVS) molar ratio. As the inorganic bioceramic was being synthesized via a sol-gel process with reagents including tetraethyl orthosilicate (TEOS) and triethyl phosphate (TEP), varying amounts – by weight percent; 0%, 20%, 30%, 40% – of functionalized 70/30 (DEF/TEVS) copolymer was added to obtain different bioceramic/poly(DEF-co-TEVS) compositions. Hydrolysis and polycondensation reactions during the sol-gel process took place to form Si-O-Si and Si-O-P bridging networks between the organic and inorganic phases.

2.1 Introduction

Bone augmentation procedures are currently evolving to utilize more and more synthetic bone graft materials as an alternative to invasive allograft techniques. Ceramic-based and polymer-based bone graft materials constitute the two main classes of synthetic bone graft materials. In lieu of this, we investigated the synthesis of bioceramic, the successful copolymerization of DEF and TEVS and the successful synthesis of poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials. The copolymers were co-condensed with inorganic bioceramic constituents in a sol-gel synthesis process where ethanol was used as the solvent to synthesize the class II hybrid biomaterials in a homogenous O/I fashion. We characterized the materials using various techniques explained in detail in the materials and methods section.

Interactions at the molecular level within the organic and inorganic phases in O/I hybrid

biomaterials allows for components in both phases to synergistically contribute to the materials' overall properties [86]–[88]. When hydrogen bonds exist between the organic and inorganic phases, Class I hybrid biomaterials are achieved. However, when there is covalent chemical cross linking between the organic and inorganic constituents, Class II hybrids are obtained [88], [89]. Due to their biodegradability, bioactivity, biocompatibility and osteoconductivity, bioactive glasses synthesized through a sol-gel process with a general composition of SiO2-P2O5-CaO have been previously used for bone augmentation and regeneration [49], [90], [91]. Although BGs showed great results *in vitro* and *in vivo*, they are brittle and degrade quickly making them a challenge to use in bone regeneration procedures [90], [92]–[94].

Synthesizing class II hybrid biomaterials from inorganic bioceramics and organic polymers can overcome the challenges mentioned. There are three methods for preparing class II hybrid biomaterials. One method involves linking the organic and inorganic phases via a coupling agent. (3-aminopropyl)triethoxysilane is an example of a coupling agent that has been used in functionalizing polymers like gelatin, chitosan and polyglutamic acid before synthesizing class II hybrid biomaterials. Phase separation between the organic and inorganic phases can result from using this technique because coupling agents have limited polymer functionalization potential [49], [88], [95]–[100].

The second method to synthesize class II hybrid biomaterials involves using silane containing organic polymers such as polydimethoxysilane (PDMS). PDMS has been hydrolyzed with TEOS to yield class II hybrid biomaterials. The non-degradable nature of PDMS makes it unfeasible to use in bone regeneration procedures [101].

Copolymerizing a monomer with an alkoxysilane monomer is an alternate method of achieving a silane functionalized copolymer. Polystyrene and poly(methylmethacrylate) are two examples of polymers that have been copolymerized with trialkoxysilyl monomers. The silane containing copolymers were hydrolyzed with silica moieties to synthesize class II hybrid biomaterials. Again, the non-degradable nature of the polymers makes it unfeasible to use in bone regeneration procedures. Moreover, because silica was the only inorganic moiety used, the class II hybrid biomaterial was not able to induce osteogenesis

(i.e., not bioactive) [49], [102]–[104]

In this chapter, we investigated the synthesis of poly(DEF-co-TEVS) and poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials. A ratio of 30 mol% TEVS and 70% mol% DEF was maintained in the monomer mixture during copolymerization. When the TEVS content in the copolymer exceeded 30 mol%, the resultant copolymer exhibited a higher risk of cross-linking, therefore, making it insoluble for further use. When the TEVS content in the copolymer were below 30 mol% TEVS, the resultant copolymer did not yield with sufficient functionalized groups, therefore making it less ideal to use for the synthesis of class II hybrid biomaterials later. There were a few reasons why we chose these specific monomers to use. Firstly, they are degraded into biocompatible products that are not toxic to the cell [105]. Secondly, the injectable nature of the biomaterial makes it possible to fill irregular bony defects completely with no voids [105]. Additionally, they are easy to use, can act as a carrier for drug molecules and allow for minimally invasive surgeries [105].

Poly(DEF-co-TEVS)/bioceramic class II hybrids were prepared by co-condensing poly(DEF-co-TEVS) with inorganic bioceramic precursors of TEOS and triethyl phosphate (TEP) via sol gel chemistry where ethanol was used as a solvent. Poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials were prepared with varying copolymer content by wt % (0% copolymer, 20% copolymer, 30% copolymer and 40% copolymer). We characterized poly(DEF-co-TEVS) and poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials to confirm co-polymerization and successful synthesis of the class II hybrids since DEF has never been functionalized with TEVS before.

2.2 Materials and Methods

2.2.1 Materials

Diethyl fumarate (98%, contains no inhibitor) and calcium nitrate tetrahydrate (99%) were purchased from Alfa Aesar (MA, USA). Triethoxyvinylsilane (97%), 2,2′-azobis(2-methylpropionitrile) (AIBN, 98%), tetraethyl orthosilicate (98%) and triethyl phosphate (≥99.8%) were purchased from Sigma Aldrich (MO, USA). Methanol (99.8%) was purchased from VWR (PA, USA). Hexanes and nitric acid were purchased from Caledon Labs (Brampton, ON, Canada). Anhydrous Ethanol was purchased from Commercial

Alcohol (GreenField Specialty Alcohols Inc., Canada).

2.2.2 Synthesis of Bioceramic

A bioceramic of composition in mol % (70 SiO_2-26 CaO-4 P_2O_5) was synthesized via sol-gel chemistry. To synthesize a theoretical yield of 10g of bioceramic, a solvent of 7.43 mL distilled water and 25.28 mL of ethanol was prepared in a 250 mL beaker using a magnetic stirrer bar. 5M nitric acid was used to bring the pH between 2 and 4. The beaker was covered with aluminum foil to prevent evaporation. 23.456 mL of tetraethyl orthosilicate was then added and stirred for 30 minutes. 9 grams of calcium nitrate tetrahydrate was then added and stirred for 30 minutes. Finally, 2 mL of triethyl phosphate was added and stirred for 1 hour. The magnetic stir bar was removed and 3 holes were made in the aluminum foil to allow the ethanol to evaporate. Once gelation occurred (about 3 days), beaker was placed in vacuum oven dryer to dry at room temperature for 48 hours followed by ball milling.

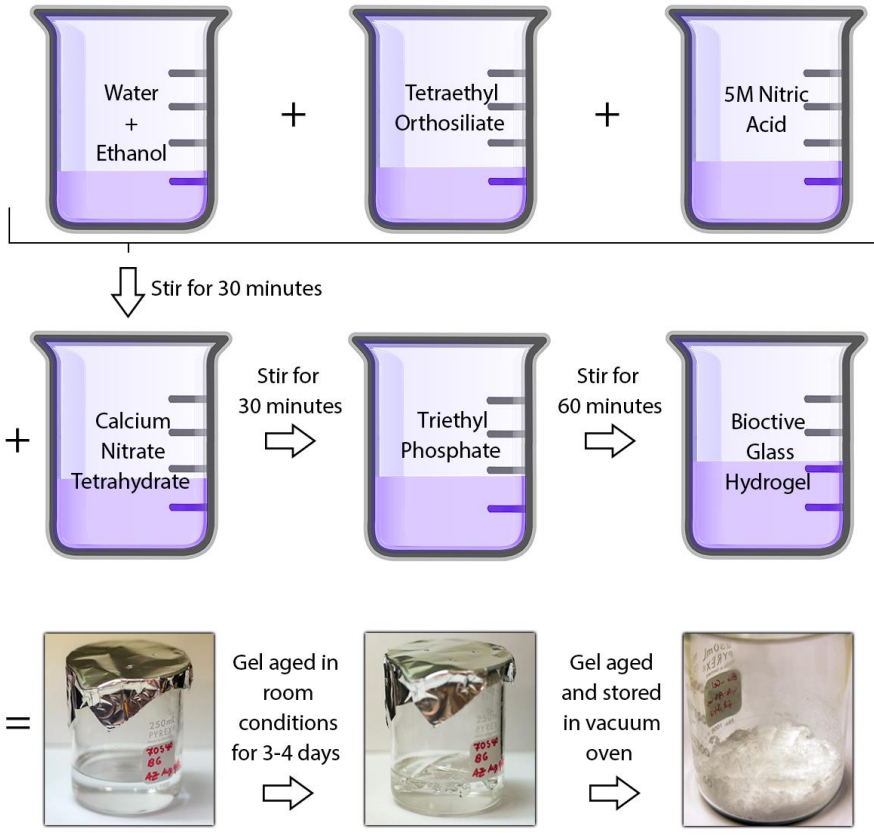

Figure 2-1: Schematic showing the synthesis process of bioceramic.

2.2.3 Synthesis of Poly(DEF-co-TEVS)

To prepare a theoretical yield of 40 grams of copolymer, 0.2304 g of AIBN (i.e., 0.02 mol/L of total DEF and TEVS) was first dissolved in 27.212 mL of DEF in a three-necked flask using a magnetic stir bar. Once dissolved, 13.7 mL of TEVS was added to the solution. The copolymer was synthesized in bulk (i.e., no solvent was used) at 80 °C and under N_2 atmosphere for 72 hours (Figure 2.1) [65]. We found 72 hours of reaction time to be optimal since less time resulted in poor copolymer yield, while longer time resulted in silane-silane crosslinking making the copolymer insoluble. The three-necked flask was connected to a condenser to reflux any potential evaporation (i.e., one neck was connected to a stopper, one neck was connected to a water condenser and one neck was connected to the N_2 line; all connections were sealed using vacuum grease).

After 72 hours, the reaction was stopped, room temperature (RT) was achieved and the following steps were used to purify the copolymer. First, 80 mL of methanol (i.e., double the copolymer amount) was added to the three-necked flask to dissolve the copolymer. The copolymer solution was transferred to a 250 mL beaker covered with aluminum foil and placed in an ice bath. Purification of the copolymer was carried out by repeated precipitation in excess (3-5 times higher than the copolymer solution) cold hexanes. After every purification, the top layer containing methanol, monomers and other impurities was decanted carefully while the bottom layer containing the copolymer was maintained. This was done for a total of 8-10 times. Finally, the precipitated copolymer was dissolved in methanol followed by transferring into a Teflon beaker and placed into a vacuum oven to dry at RT for 24 hours. Gel Permeation Chromatography (GPC) was used to determine the molecular weight and polydispersity index (PDI) of the copolymer [106]. ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy were used to confirm copolymerization [107].

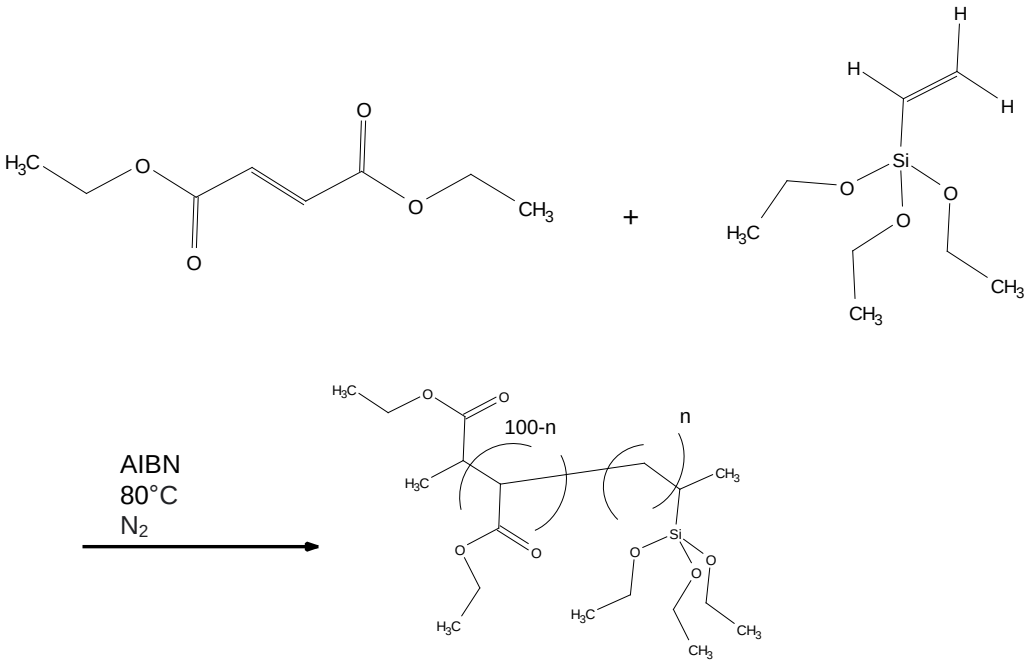

Figure 2-2: Schematic showing the copolymerization of DEF and TEVS. n=30 mol% TEVS.

2.2.4 Synthesis of Poly(DEF-co-TEVS)/Bioceramic Class II Hybrid Biomaterials

Poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials were synthesized *in situ* via a sol gel process (Figure 2.2). Pre-determined amounts of purified poly(DEF-co-TEVS) was dissolved in ethanol to obtain different compositions of poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials. The following nomenclature was be used to identify different compositions. 0% copolymer represents 0 wt% organic copolymer and 100 wt% bioceramic while 40% copolymer represents 40 wt% organic copolymer and 60 wt% bioceramic. Because the copolymer is water insoluble, we had to ensure that hydrolysis of the bioceramic inorganic precursors was completed before adding the dissolved copolymer to the sol. To ensure that there is no phase separation, the pre-determined amounts of copolymers dissolved in EtOH were added to the sol after 24 hours ensuring that the sol is mostly hydrolyzed (i.e., minimal amount of free water). They were added slowly and mixed with a magnetic stir bar ensuring a homogenous mix. The magnetic stir bar was removed and the beaker was covered with perforated aluminum foil for 72 hours at RT in the fume hood. Once gelation occurred, the beaker was kept for an additional 48 hours in the fume hood at RT. After that, the beaker was transferred to a vacuum where it was dried under a reduced pressure of 30,000 Pa for 24 hours at RT. The resultant powder was ball milled and further characterized. The bioceramic composition for all hybrid materials was maintained at 70 mol % SiO_2, 26 mol %. CaO and 4 mol % P_2O_5.

Solid-state cross-polarization magic-angle spinning (CPMAS) ^{29}Si NMR was used to confirm the synthesis of poly(DEF-co-TEVS)/bioceramic class II hybrid. The spectrum was acquired using a Varian Infinity Plus 400 NMR spectrometer (n(1H) = 399.5 MHz, n(29Si) = 79.4 MHz) equipped with a Varian triple-resonance (H-X-Y) 7.5 mm magic-angle spinning NMR probe. The samples were packed tightly into 7.5 mm outer diameter ZrO_2 rotors and rotated at 5.5 kHz. A total of 4000 scans were summed using a 6.75 μs 1H 90-degree pulse, 2 ms contact time, 10.24 ms acquisition time, 7 s recycle delay, 50 kHz spectral width and continuous-wave 1H decoupling during acquisition. For processing, two zero-fills and 30 Hz line broadening were applied to the free induction decay before Fourier

transformation. The ssNMR spectrum were referenced with respect to tetramethylsilane (δ(29Si) = 0.0 ppm) by setting the high-frequency peak of tetrakis(trimethylsilyl)silane to −9.8 ppm.

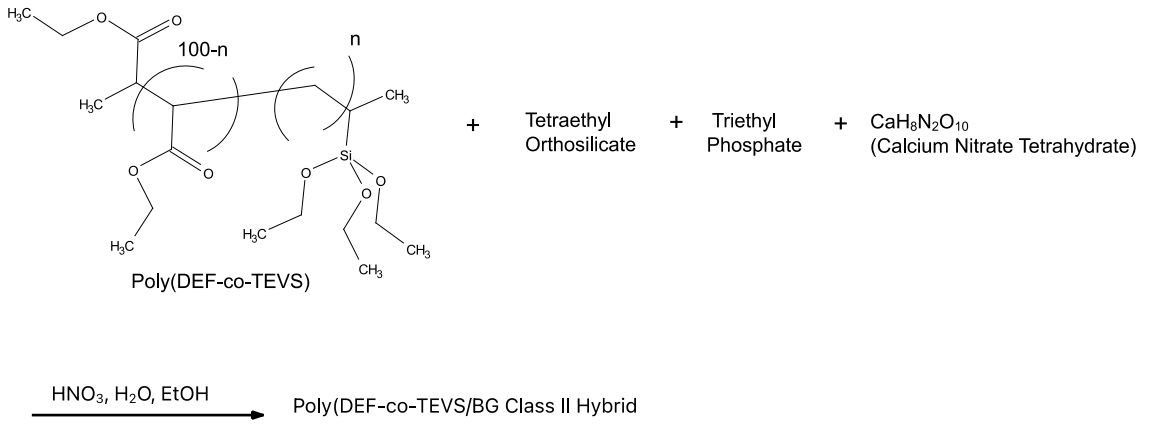

Figure 2-3: Schematic showing the synthesis process of poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials.

2.2.5 X-Ray Diffraction (XRD)

XRD was performed on the synthesized bioceramic, poly(DEF-co-TEVS) and poly(DEF-co-TEVS)/bioceramic class II hybrids using an X-ray diffractometer AXS D2 PHASER (Bruker Corporation, USA) operating on CuK_α radiation with λ=1.5418Å analysis. The diffractometer is equipped with a detector angle that measures angles ranging from 0 to 120 2θ. When testing samples, we started with the lowest incident angle possible (~ 5 degrees).

2.3 Results

2.3.1 Synthesis of Poly(DEF-co-TEVS)

Free radical polymerization of DEF and TEVS was carried out in bulk using AIBN as an initiator to prepare poly(DEF-co-TEVS). This is the first-time poly(DEF-co-TEVS) has been synthesized. GPC, % yield by wt, ^{1}H and ^{13}C NMR were used to characterize the copolymerization of DEF and TEVS. Recall that a proportion of 30 mol% TEVS and 70% mol% DEF was maintained in the monomer mixture during copolymerization. The peak

present at chemical shift of 3.17 ppm in the ^{1}H NMR spectrum labeled as 14,15 in Figure 2.5 confirms copolymerization. The absence of peaks at chemical shifts between 130 and 170 ppm referring to C=C vinyl bonds in the ^{13}C-NMR spectrum in Figure 2.8 is another successful indication of copolymerization.

From a sample of 5 copolymer batches synthesized with the conditions described above (i.e., optimized for intended application), an average weight averaged molecular weight (M_w) of 20.2 ± 1.7 kDa and an average number averaged molecular weight (M_n) of 12.2 ± 3.2 kDa was achieved according to the GPC data. Taking the ratio between M_w/M_n, a PDI of 1.649 is caluclated which is consistent with the literature of copolymers synthesized via chain reactions yielding Mw/Mn values between 1.5 and 2.0 [108]. Yield of purified copolymer was calculated as percent weight (% wt) after drying in a vaccuum oven. An average wt% yield of 32 ± 2 % was achieved.

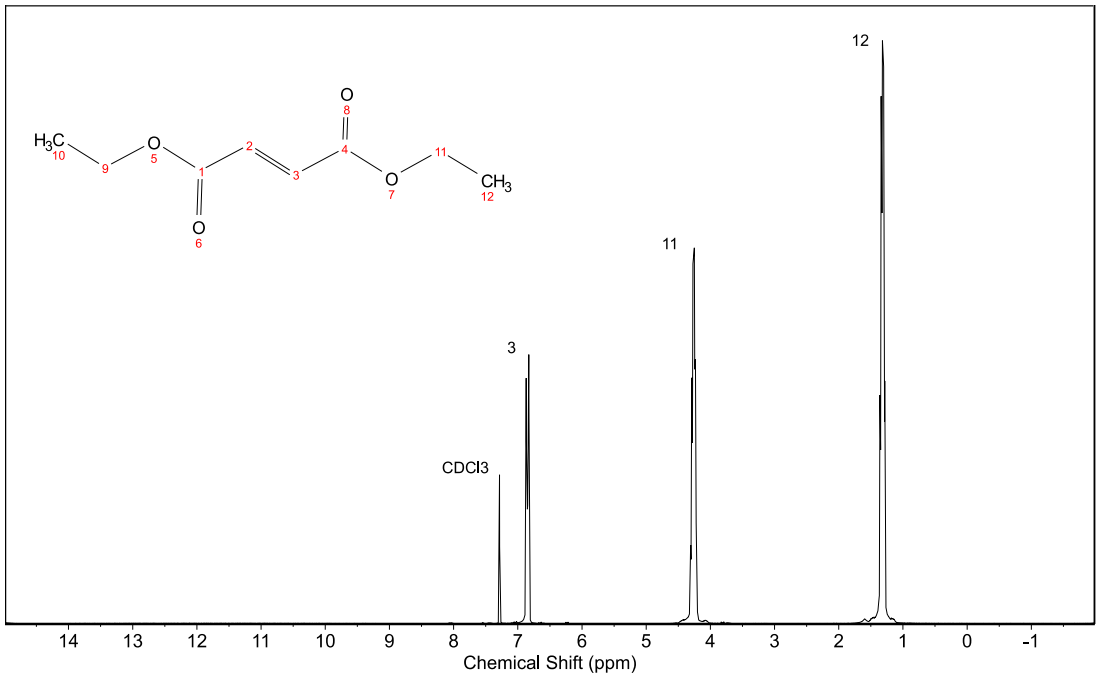

Figure 2-4: ^{1}H-NMR spectrum of DEF.

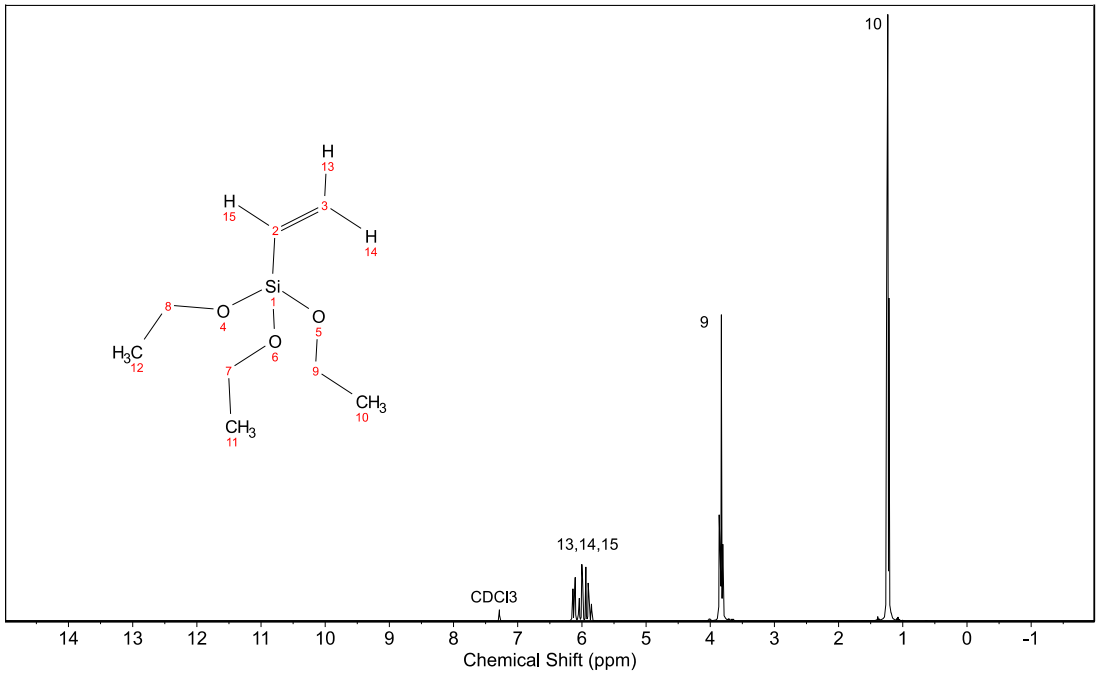

Figure 2-5: ^{1}H-NMR spectrum of TEVS.

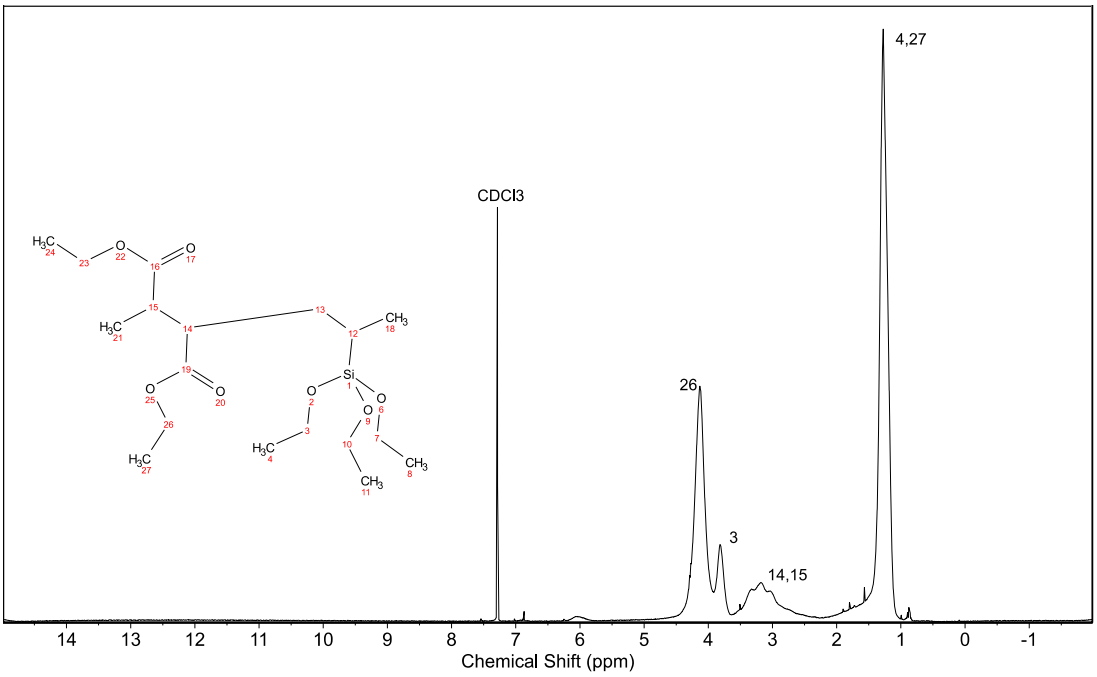

Figure 2-6: ^{1}H-NMR spectrum of poly(DEF-co-TEVS).

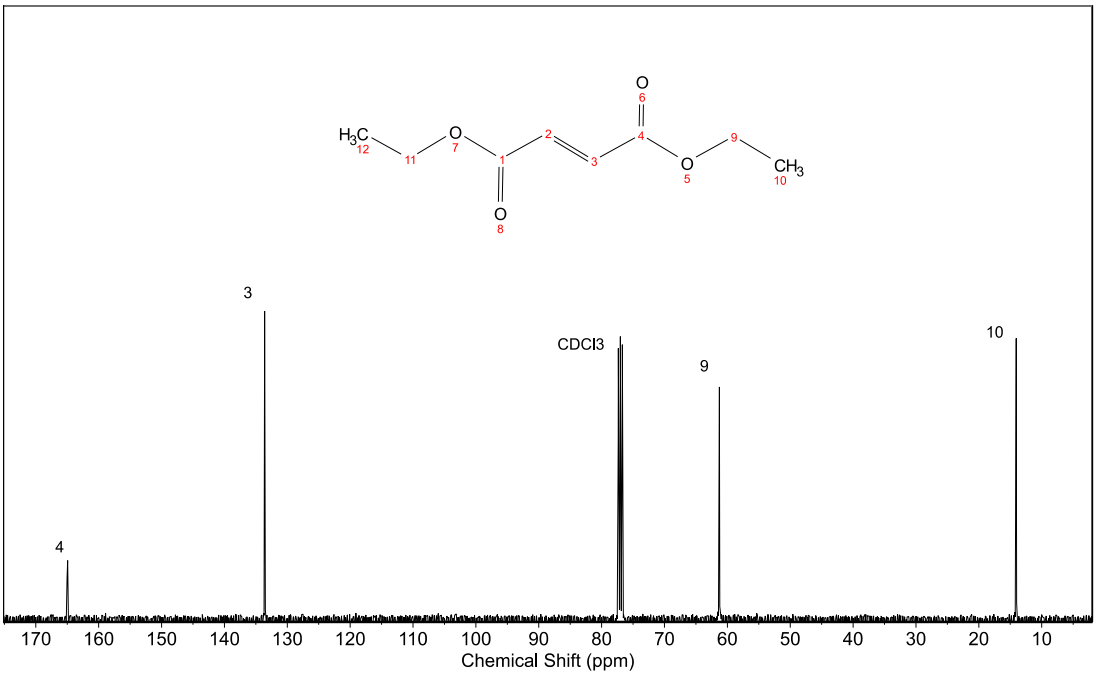

Figure 2-7: ^{13}C-NMR spectrum of DEF.

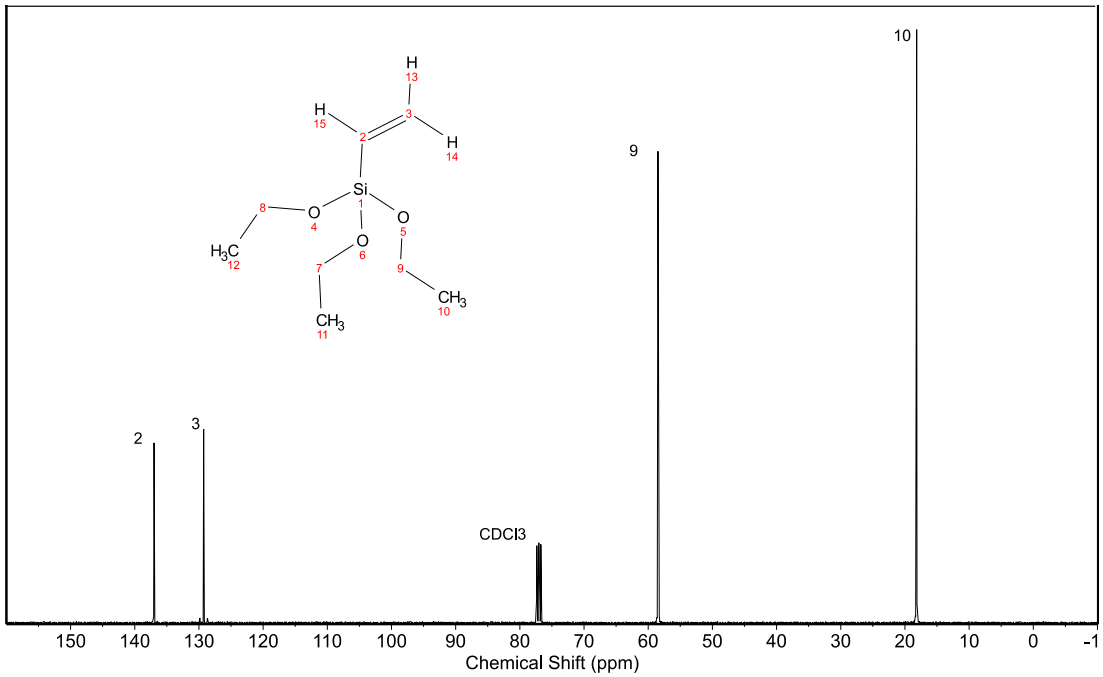

Figure 2-8: ^{13}C-NMR spectrum of TEVS.

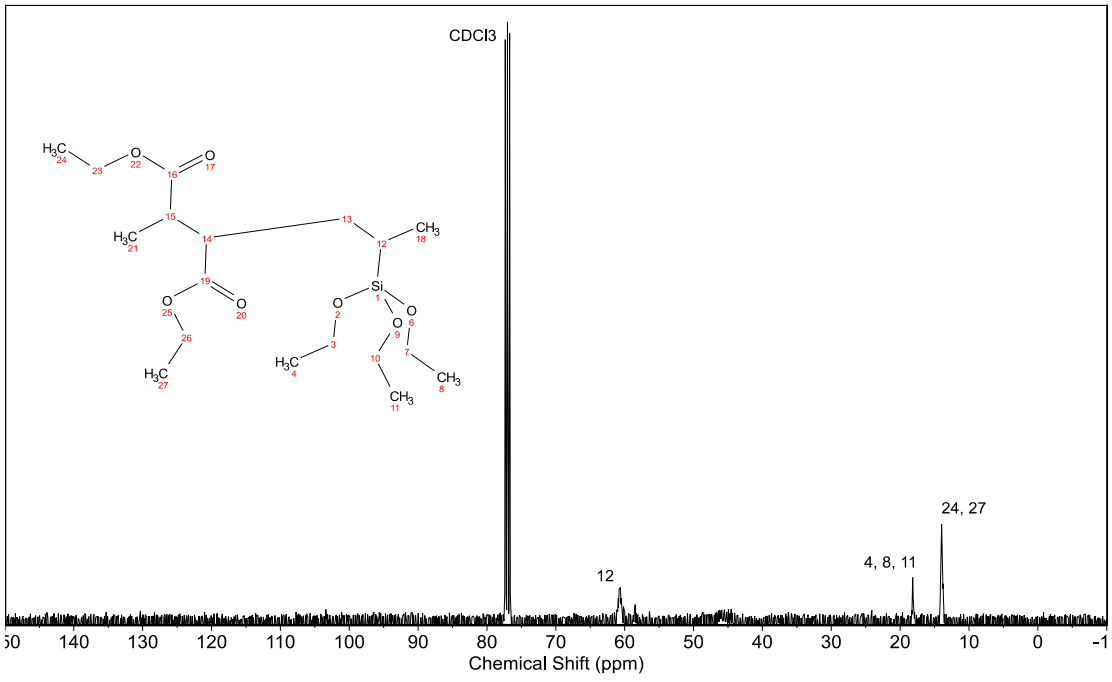

Figure 2-9: ^{13}C-NMR spectrum of poly(DEF-co-TEVS).

2.3.2 Synthesis of Poly(DEF-co-TEVS)/Bioceramic Class II Hybrid Biomaterials

Poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials were synthesized *in situ* via a sol gel process. Formation of class II organic/inorganic (O/I) hybrid networks in poly(DEF-co-TEVS)/bioceramic was characterized by solid state ^{29}Si-NMR (Figure 2.9) and XRD (Figure 2.10). T^n and Q^n peaks were present in the ^{29}Si-NMR spectra of the 40% copolymer hybrid biomaterial (Figure 2.9). T^n peaks represent chemical structures of -$CSi(OSi\text{-})_n(OH)_{3-n}$, while Q^n peaks represent chemical structures of Si-O-$Si(OSi\text{-})_n(OH)_{4-n}$. The -OH group in each structure corresponds to non-bridging oxygens. The presence of T^n peaks confirms hybrid formation since Q^n peaks are associated with inorganic networks only (i.e., bioceramic) while T^n peaks are associated with O/I covalent bonds which are present in class II hybrid biomaterials. Q^2, Q^3 and Q^4 peaks labeled in the ^{29}Si-NMR spectrum refer to chemical shifts of ~ -90, -99 and -108 ppm, respectively while the peak labeled T^2 refers to a chemical shift of ~ - 52 ppm. Q^3 and T^2 peaks correspond to terminal non-condensed Si-OH groups of Si-O- bridging networks. The XRD spectrum of copolymer revealed that it is semi-crystalline and peaks are seen in hybrid XRD spectrum further validating the synthesis of hybrid biomaterials (Figure 2.10). Crystalline peaks were present in the bioceramic and hybrid samples because bioceramic was not hydrothermally treated since high temperatures would have denatured the copolymer.

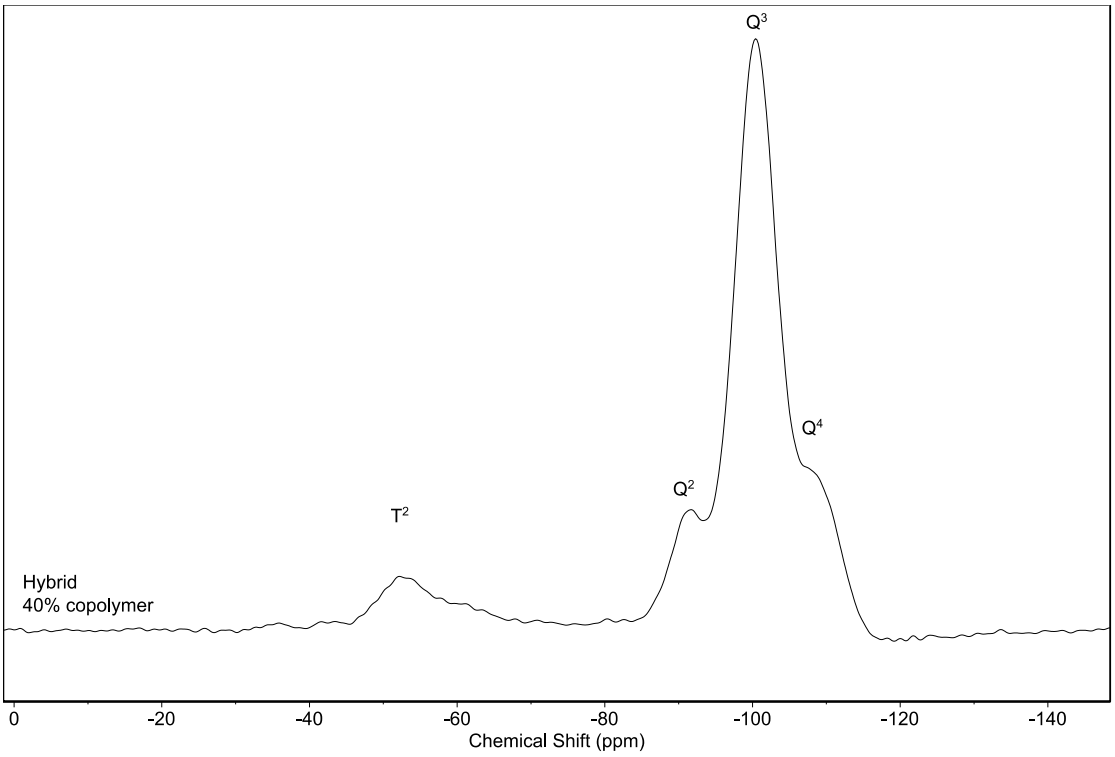

Figure 2-10: Solid State ^{29}Si-CP MAS NMR spectrum for poly(DEF-co-TEVS)/bioceramic hybrid biomaterial containing 40% copolymer by weight.

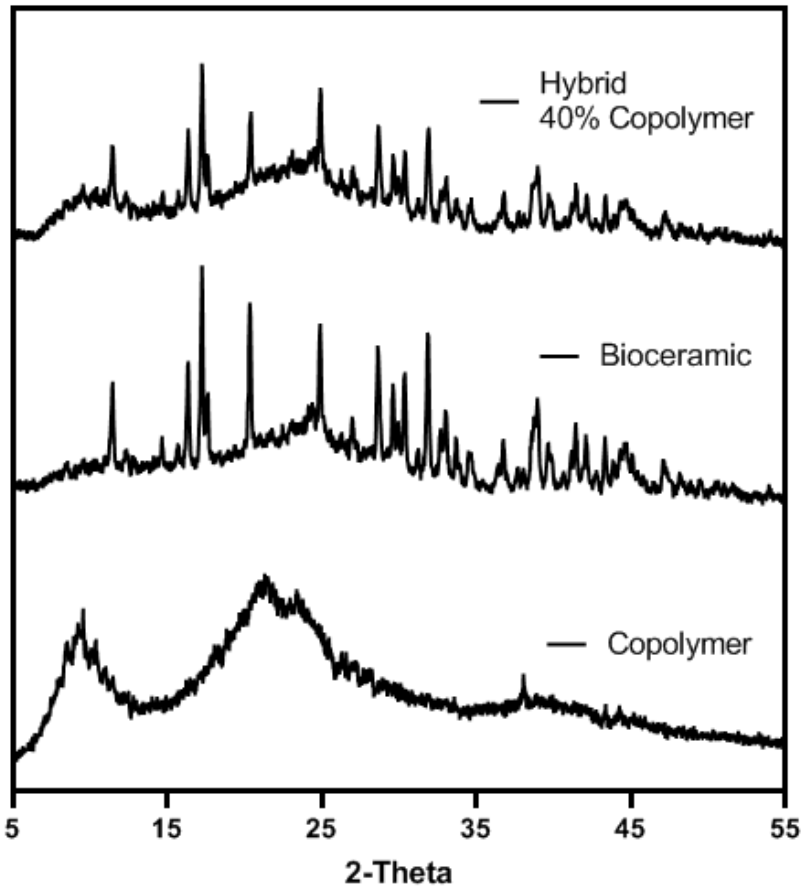

Figure 2-11: XRD of copolymer, bioceramic and 40% poly(DEF-co-TEVS)/bioceramic hybrid biomaterial. Because the material is not heated at temperatures ranging from 300-500°C, the precursors are not decomposed and that is why the resultant material is crystalline which is characteristic of ceramics, not glasses.

2.4 Discussion

O/I class II hybrid biomaterials are unique in the sense that both phases are covalently bonded. At the molecular level, they act as a single entity. This gives class II hybrid biomaterials properties coming from both precursors tailored for specific biomedical applications [88], [89], [109]. A study by Mondal et al., (2018) showed that when organic polymers were chemically bonded to inorganic BGs (i.e., class II hybrid biomaterials), they demonstrated better biological and mechanical properties along with a more predictable degradation rate than each of their constituents alone [110]. The synthesis of class II hybrid biomaterials requires a reaction between the functionalized organic polymers groups and the inorganic precursors [49], [100]. The Si-O-Si chemical covalent bonds achieved by hydrolysis and polycondensation reactions between the inorganic precursors and alkoxy-silicon functionalized organic polymer is one method class II hybrid biomaterials are synthesized. This way of preparing class II hybrid biomaterials involves end-capping which refers to the addition of alkoxy-silicon functional groups to the ends of an organic polymer. Linking functional groups only to the ends of the organic biopolymer allows for a finite extent of functionalization which may lead to O/I phase separation [98], [111]–[113]. The addition of trialkoxysilicon functional groups, however, resolves the limitation associated with end-capping. Pendant functional groups are linked to the organic biopolymer backbone resulting in a much greater number of potential reactive sites to the inorganic precursors decreasing the chance of phase separation. In the present study, we were able to successfully functionalize DEF with TEVS through a free-radical polymerization reaction. TEVS functional groups underwent polycondensation reactions with inorganic precursors of TEOS, TEP and $Ca(NO_3)_2.4H_2O$ which yielded the synthesis of highly crosslinked hybrid biomaterials. A copolymer with 30 mol% TEVS was used to synthesize poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials with 0, 20, 30 and 40 wt% organic copolymer ratios. It was hypothesized that the covalent bonds between pendant TEVS functional groups and inorganic precursors were to give rise to biomaterials possessing superior bioactivity, mechanical properties and degradation rate.

Dissolution rate and mechanical properties of class II hybrid biomaterials can be tailored to specific applications by manipulating the molecular weight (MW) of the biopolymer

organic phase. Polymers with low MW are better suited for biomedical applications involving implanted devices as compared to higher MW biopolymers. Copolymers with a MW greater than 55 kDa carry the risk of being too large to go through vasculature [114]. Although evaluating the extensive effects of biopolymer MW on class II hybrid biomaterials properties is beyond the scope of this investigation, we synthesized a copolymer maintaining a MW of about 20 kDa in this investigation.

As mentioned above, a copolymer of DEF and TEVS was synthesized with a composition of 30 mol% TEVS and 70 mol% DEF. However, poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials of varying O/I weight ratios were prepared (i.e., 0, 20, 30 and 40 wt% poly(DEF-co-TEVS)). Hybrid biomaterials with higher organic ratios would, theoretically, be associated with more T structure formation in solid state ^{29}Si-CP MAS NMR spectra. A consistent -C-Si-O network bond was maintained since a consistent monomer ratio was maintained during the synthesis of poly(DEF-co-TEVS). Sharp Q^3 and T^2 peaks were observed in the solid state ^{29}Si-CP MAS NMR spectra obtained in this study (Figure 2.9). These results were consistent with the assumptions mentioned above and suggest the formation of non-condensed -Si-OH bonds since the class II hybrid biomaterials were prepared at RT.

2.5 Conclusion

This study, for the first time, revealed that it is possible to copolymerize DEF with TEVS as well as to synthesize poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials. We were able to functionalize TEVS with DEF through a free radical polymerization procedure using AIBN as an initiator. As a result, covalent crosslinking between the organic and inorganic phases was made possible. Poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials were synthesized through a sol-gel process where hydrolysis and polycondensation reactions took place between the organic and inorganic phases.

Chapter 3

Poly(Diethyl Fumarate-co-Triethoxyvinylsilane)/Bioceramic Composites for Potential Use as Bone Graft Biomaterials

3 Summary

The composites' mechanical properties along with its' bioactivity and degradation can be altered and optimized by varying the amount of functionalized copolymer during the synthesis of the class II hybrid biomaterials. Moreover, the composites' properties can also be tailored by changing powder to liquid ratio and specimen age. This chapter discusses the reaction between the synthesized poly(DEF-co-TEVS)/bioceramic hybrids with varying copolymer composition (0% copolymer, 20% copolymer, 30% copolymer and 40% copolymer) and an ammonium phosphate solution to produce composite biomaterials. The varying copolymer compositions were used to study the effect of functionality on properties of the novel bioactive composite biomaterial for potential use as bone graft biomaterials.

3.1 Introduction

At the molecular level, bone ECM is a hybrid made up of various biopolymers and nanocrystallites [115]. These structures exhibit excellent physiochemical properties and biological activity [116]. As a result, synthesis of novel hybrid biomaterials with properties that mimic native tissues has gained a lot of interest [117]. By varying the I/O composition of these hybrid biomaterials, it is possible to tailor properties of interest [118]. Examples of naturally occurring polymers with a biodegradable nature include chitosan, hyaluronic acid, alginate and collagen. Examples of synthetic polymers include PGA, poly(e-caprolactone) (PCL) and PLA. Both types of polymers have been investigated in the past decades because their properties make them attractive for biomedical applications [119]–[122]. For example, their application can be tailored towards drug delivery (i.e., transport vehicles) and bone regeneration applications by adding osteoconductive and osteoinductive materials such as BMP-7 [123]. Despite these several advantages, on their

own, these polymers lack sufficient mechanical properties limiting their use. BGs are a class of non-crystalline ceramics that possess osteoconductive and osteoinductive properties that give them the capability of forming hydroxyapatite when in contact with SBF/physiological body fluids. Because of this unique characteristic they are able to bind to native bone and stimulate new bone regeneration/growth as the material degrades [49]. BGs, however, suffer from some disadvantages including poor mechanical strength due to their high brittle nature [124]. Therefore, BGs have been hybridized with various polymers to synthesize hybrid biomaterials in attempts to improve mechanical properties. For example, a study conducted by Sarker et al (2015) reported hybrid biomaterials synthesized from collagen and BG precursors with properties tailored towards bone regeneration applications. Collagen was chosen as the polymer of interest because it is the most abundant natural polymer in the ECM and thus can be extracted from different tissue sources. It possesses excellent healing, biocompatibility, biodegradability, low immunogenic properties [125].

In this study, we reacted the different compositions of poly(DEF-co-TEVS)/bioceramic hybrids with an ammonium phosphate solution to make a novel bioactive composite biomaterial for potential use as bone graft biomaterials.

3.2 Materials and Methods

3.2.1 Materials

Ammonium phosphate dibasic ($(NH_4)_2HPO_4$, 98%), ammonium dihydrogen phosphate ($(NH_4)H_2PO_4$, 99%), phosphate-buffered saline (PBS), sodium chloride (NaCl), sodium hydrogen carbonate ($NaHCO_3$), potassium chloride (KCl), di-potassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_2O$), magnesium chloride hexahydrate ($MgCl_2.6H_2O$), calcium chloride ($CaCl_2$), sodium sulfate (Na_2SO_4), tris-hydroxymethyl aminomethane: ($(HOCH_2)_3CNH_2$) (Tris), hydrochloric acid (HCl), and pH standard solutions (pH 4, 7 and 9) were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

3.2.2 Synthesis of Poly(DEF-co-TEVS)/Bioceramic Composites

A liquid component of an ammonium phosphate solution was prepared by dissolving 60.1g of $(NH_4)_2HPO_4$ and 5g of $NH_4H_2PO_4$ in 100 mL of deionized (DI) to achieve a neutral pH of 7.4 [126]. The powder component, poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials (0% copolymer, 20% copolymer, 30% copolymer and 40% copolymer) were mixed with the ammonium phosphate solution on dental mixing pads until a homogenous paste-like consistency was achieved. The paste-like material was then loaded into pre-cut molds, placed between two glass plates and left to harden for 24h at RT. Different powder-to-liquid (P/L) ratios of 0.35, 0.53 and 0.70 were used. Samples were aged at different time intervals of 10, 20 or 30 days post hardening. Acid-base reaction is the main mechanism responsible for composite hardening. Calcium and phosphorus ions are released from the surface of the hybrid particles and react with ammonium phosphate to form a calcium phosphate salt. The rest of the unreacted hybrid particles are embedded within the salt matrix forming a composite. Nucleation followed by crystallization then takes place and progresses as the acid-base reaction continues leading to the hardening of the composite [127], [128].

3.2.3 Mechanical Properties of Poly(DEF-co-TEVS)/Bioceramic Composites

Cylindrical composite specimens (3 mm in diameter and 5 mm in height) were prepared by loading the composite pastes into cylindrical molds and covering the two ends with microscope slides and kept at different time intervals ranging from 10 to 30 days. The specimens were then removed from the molds at each time interval and were compression tested using an Instron universal machine, model number 3345 supplied with a 5kN load cell (Norwood, MA, USA), at a crosshead speed of 0.5 mm/min. The compressive strength and modulus for each specimen were recorded. Scanning electron microscopy (SEM) images of the fractured surfaces were taken using LEO 1540XB SEM (Hitachi, Japan).

3.2.4 Degradation of Poly(DEF-co-TEVS)/Bioceramic Composites in PBS

Hardened composites disc specimens (6 mm in diameter and 2 mm in thickness) that were aged for 10 days (n=3) were placed in tightly closed polypropylene bottles containing PBS

solution for different time intervals ranging from 24h to 240h to characterize the degradation behavior of the material. PBS is a buffer solution used in biological research to study degradation behavior of various materials [129]. The solution helps maintain a neutral pH of 7.4 [129]. It is a common and well accepted medium published in literature used by many researchers because the osmolality and ion concentrations of the PBS solution matches those present in the human body [129]. Weight loss and change in morphology of poly(DEF-co-TEVS)/bioceramic composites were studied. Before incubating samples in PBS solution, each dry sample was weighed and the initial weight was recorded. The polypropylene bottles were then transferred to an orbital shaker (MaxQ4000, Barnstead Lab-line, IL) with the following settings: 120 rpm and 37 °C. Specimens were incubated for 1, 3, 6 or 10 days. Following every time point, the specimens were removed, rinsed with DI water and transferred to a vacuum oven and dried under a reduced pressure of 30,000 Pa for 24 h at RT. Specimens were then weighed and the final weight was recorded. From the initial and final weights, percentage weight loss for each composite specimen was calculated. PBS solution was replaced with fresh PBS every 24 h.

3.2.5 *In Vitro* Bioactivity Evaluation of Poly(DEF-co-TEVS)/Bioceramic Composites in Simulated Body Fluid

Disc composite specimens (6 mm in diameter and 2 mm in thickness) aged for 10 days were placed in tightly closed polypropylene bottles containing SBF solution for different time intervals ranging from 6h to 7d to study hydroxyapatite (HA) deposition. SBF was prepared according to methods described in the literature and used for the *in vitro* bioactivity tests because it resembles the composition and concentration of the inorganic component of human blood plasma [27]. The polypropylene bottles were filled with 20mL of SBF solution and 3 samples from each composition were placed inside. The tightly closed bottles were then transferred to an orbital shaker (MaxQ4000, Barnstead Lab-line, IL) with the following settings: 120 rpm and 37 °C. Specimens were incubated for 6 h, 12 h, 1 d, 3 d, or 7 d. SBF solution was replaced with fresh SBF every 24 h. Following every time point, 3 specimens of each composition were removed, rinsed with deionized water and dried in a vacuum oven at RT and under a reduced pressure of 30,000 Pa for 24 h.

3.2.6 Scanning Electron Microscopy (SEM), Energy Dispersive X-Ray Spectroscopy (EDX) and X-Ray Diffraction (XRD)

SEM and EDX were performed at the Western Nanofabrication Facility. An Osmium Plasma Coater (OPC80T, Filgen Inc. Japan) was used to coat the samples with 5 nm of Osmium before imaging. SEM images of dried specimens were taken using LEO 1540XB SEM (Hitachi, Japan). For PBS samples, this was done to examine change in morphology. However, for SBF samples, SEM images were taken to examine HA deposition. EDX was performed using the detector attached to the SEM machine to determine the Ca/P ratio.

XRD was performed using an X-ray diffractometer AXS D2 PHASER (Bruker Corporation, USA) operating on CuK_{α} radiation with λ=1.5418Å analysis to identify the diffraction peaks.

3.2.7 Statistical Analyses

GraphPad Prism 6.0 (GraphPad Software Inc., Ca, USA) was the software used to analyze data and data is shown as means ± standard deviations (SD). Statistical analysis of means was conducted using two-way analysis of variance (ANOVA) and a Tukey's multiple comparison test. Means were considered statistically significant at $p = 0.05$ (95% level of confidence).

3.3 Results

3.3.1 Mechanical Properties of Poly(DEF-co-TEVS)/Bioceramic Composites

Composites' mechanical properties play an essential role for bone applications. Compressive elastic moduli and ultimate compressive strengths of poly(DEF-co-TEVS)/bioceramic hybrid composites were determined from the compressive stress-strain curves generated using a universal INSTRON testing machine. 0% copolymer composites (i.e., bioceramic + ammonium phosphate) were made to be used as controls and compared with 20%, 30% and 40% copolymer composites. Data is presented in Figures 3.1 and 3.2. The data reveals that that the O/I ratio and specimen age influence the resultant compressive properties.

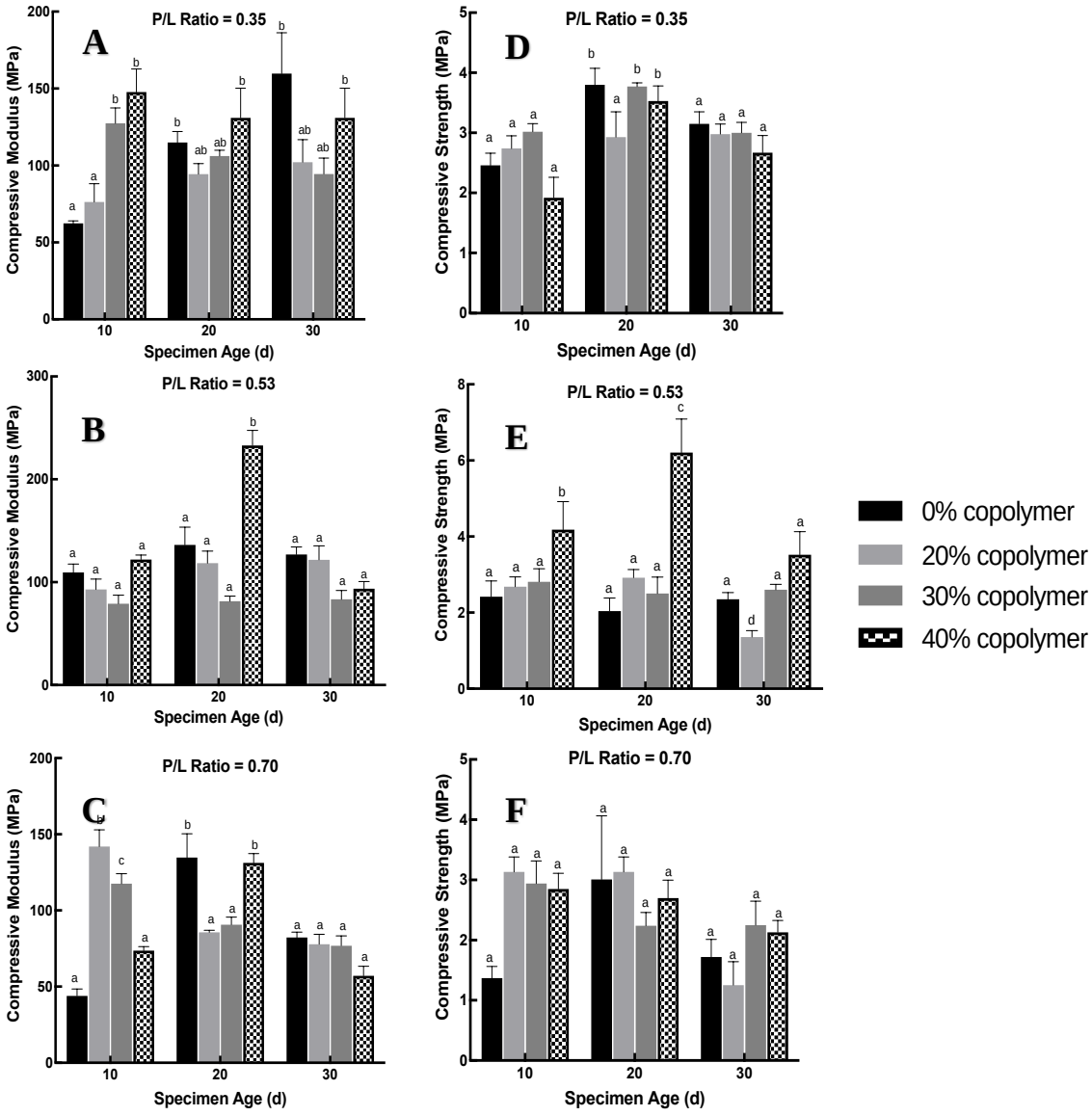

Figure 3-1: Compressive mechanical testing of poly(DEF-co-TEVS)/bioceramic composites. Left panels (A-C) represent compressive moduli of the bioactive composites with different compositions, age and P/L ratios (n=5). Right panels (D-F) represent ultimate compressive strengths of the bioactive composites with different compositions, age and P/L ratios (n=5). Different letters denote statistical significance.

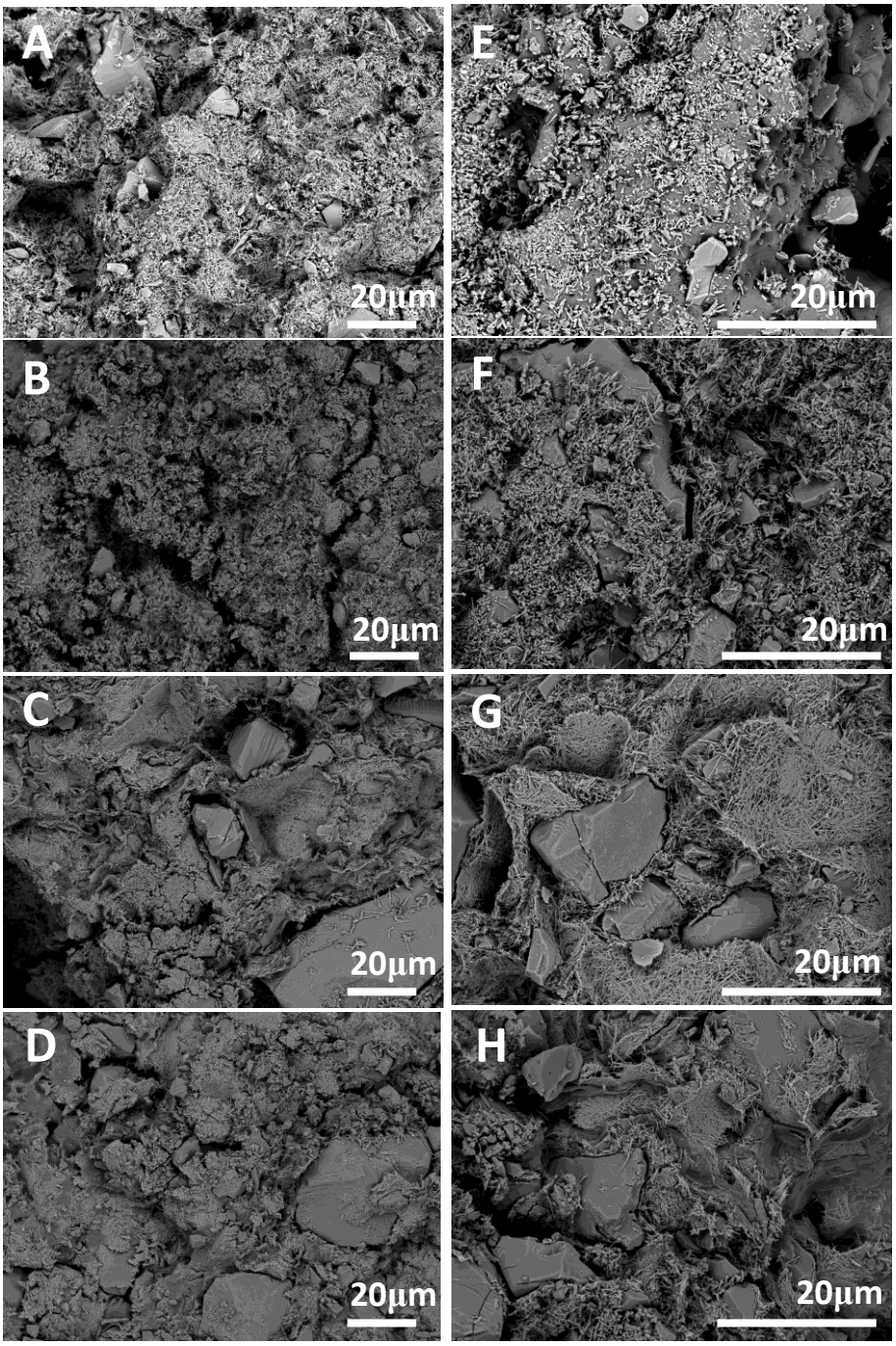

Figure 3-2: SEM images showing fracture surface of the cylindrical specimens used for mechanical testing. Left panels (A-D) represent bioactive composites with varying copolymer composition (top to bottom: 0% copolymer, 20% copolymer, 30% copolymer, 40% copolymer) at 200X. Right panels (E-H) represent the same SEM images as (A-D) but at 500X.

3.3.2 Degradation of Poly(DEF-co-TEVS)/Bioceramic Composites in PBS

Different disc specimens (n=3) of poly(DEF-co-TEVS)/bioceramic composites (0%, 20% and 40% copolymers) were made using P/L ratio of 0.53 and aged for 20 days. These specimens were incubated in PBS at 37 ºC and different time intervals over a 10-day period to study their degradation behavior (Figures 3.3 and 3.4). The percentage weight loss of the composites whose compositions were 0%, 20% and 40% copolymers were 54.05 ± 1.08, 53.98 ± 3.16 and 49.1 ± 0.52 respectively. It can also be seen that the 40% copolymer composites exhibited the lowest weight loss when compared to the 0% and 20% copolymer composites (Figure 3.3).

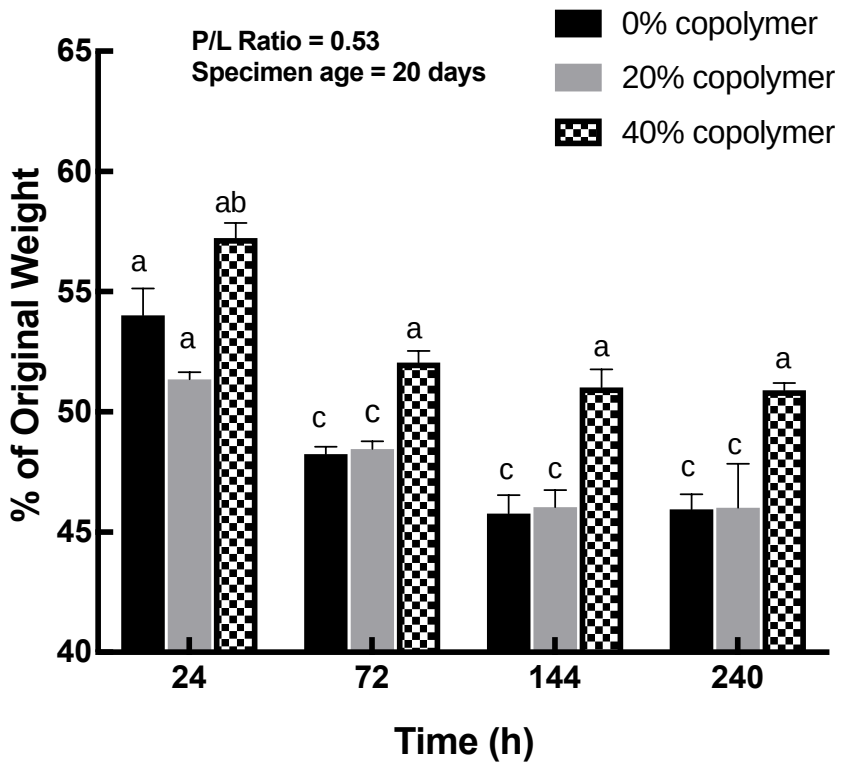

Figure 3-3: Graph showing the effect of increasing copolymer content on weight loss over time of poly(DEF-co-TEVS)/bioceramic composite samples incubated in PBS.

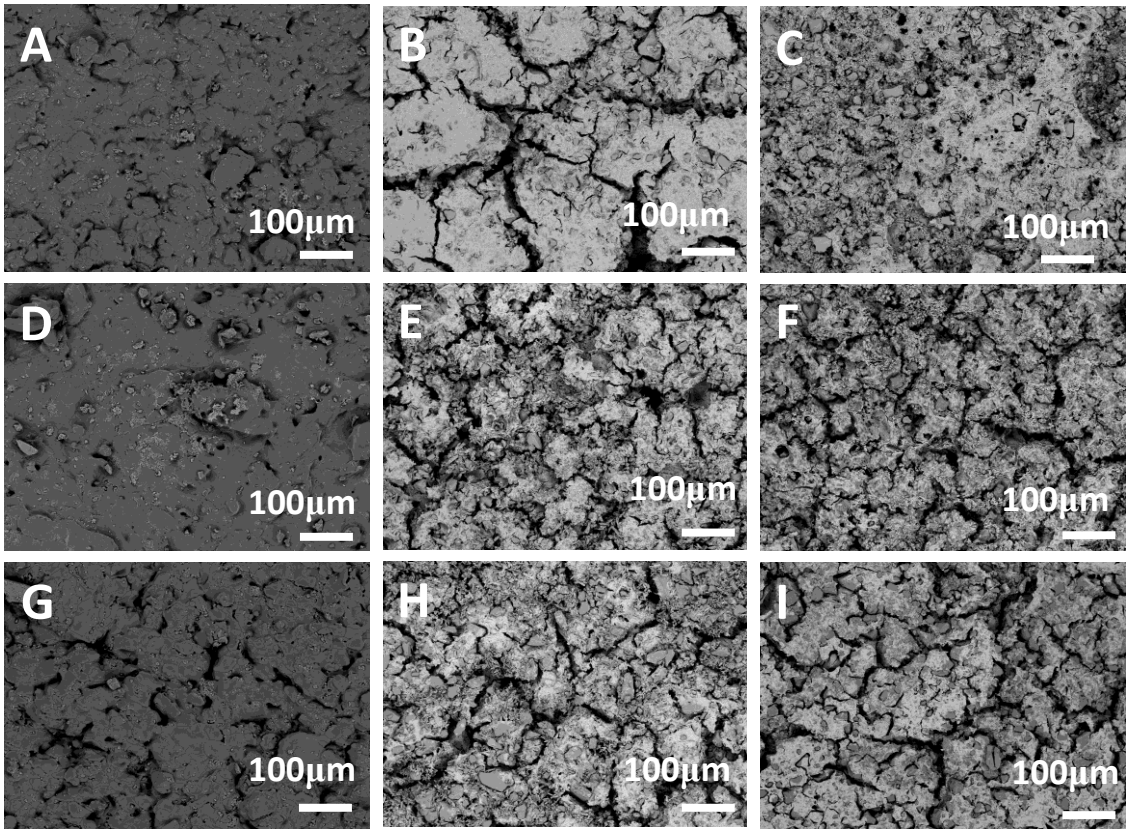

Figure 3-4: Panels (A-I) represent SEM surface images of poly(DEF-co-TEVS)/bioceramic composite disks incubated in PBS (n=3 samples per composition and data point). (A-C) represent 0% copolymer composites degraded in PBS at different times (left to right: 0 days, 1 day and 10 days). (D-F) represent 20% copolymer composites degraded in PBS at different times (left to right: 0 days, 1 day and 10 days). (G-I) represent 40% copolymer composites degraded in PBS at different times (left to right: 0 days, 1 day and 10 days).

3.3.3 *In Vitro* Bioactivity Evaluation of Poly(DEF-co-TEVS)/Bioceramic Composites in SBF

Poly(DEF-co-TEVS)/bioceramic composites were incubated in SBF for various time points to study the surface formation of biomimetic apatite on the disc specimens (Figures 3.5 and 3.6). SEM images (Figure 3.5) show composite surfaces at 6h, 3d and 7d of SBF incubation. Apatite deposition is seen at 3d and 7d. From the EDX elemental analysis, Ca/P ratios were calculated and are displayed on the SEM images (Figure 3.5). The Ca/P ratios at 3d and 7d for the different composite compositions (0%, 20% and 40% copolymer) ranged from 1.53 to 1.69 which indicated that these synthesized composites were able to induce the formation of HA once incubated in SBF. Moreover, XRD spectra (Figure 3.6) were consistent with the SEM and EDX data exhibiting HA peaks at $2\theta = 26.28^{o}$ and 31.82^{o} after the incubation of the different composites in SBF at 3d and 7d.

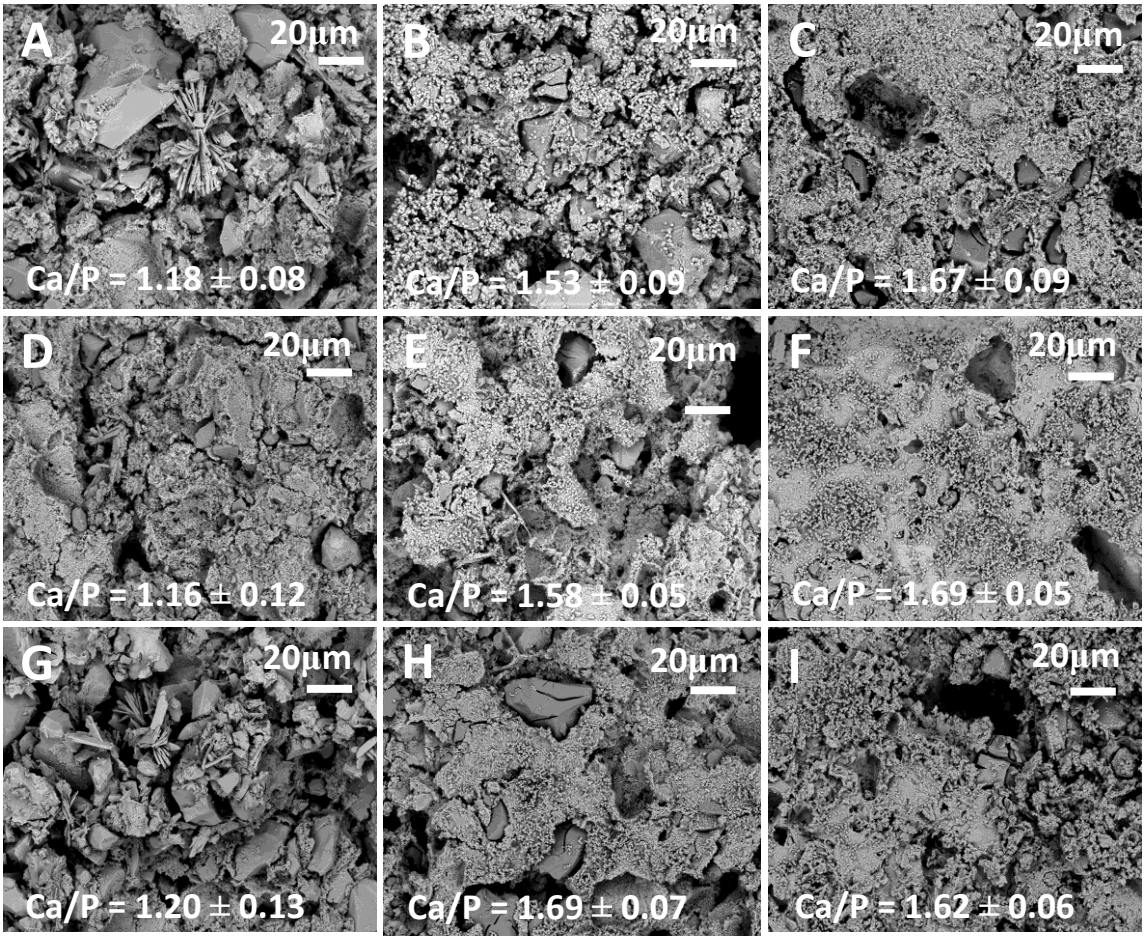

Figure 3-5: Panels (A-I) represent SEM surface images of poly(DEF-co-TEVS)/bioceramic composite disks incubated in SBF (n=3 samples per composition and data point). (A-C) represent 0% copolymer composites incubated in SBF at different times (left to right: 6 hours, 3 days and 7 days). (D-F) represent 20% copolymer composites incubated in SBF at different times (left to right: 6 hours, 3 days and 7 days). (G-I) represent 40% copolymer composites incubated in SBF at different times (left to right: 6 hours, 3 days and 7 days). Elemental analysis using EDX was performed to determine calcium to phosphorus ratios of the samples which are presented as Ca/P on panels (A-I)). Specimen age of 20 days and P/L ratio of 0.53 were held constant. SEM images were taken at 5 mm working distance along with a 3 kV electron beam voltage.

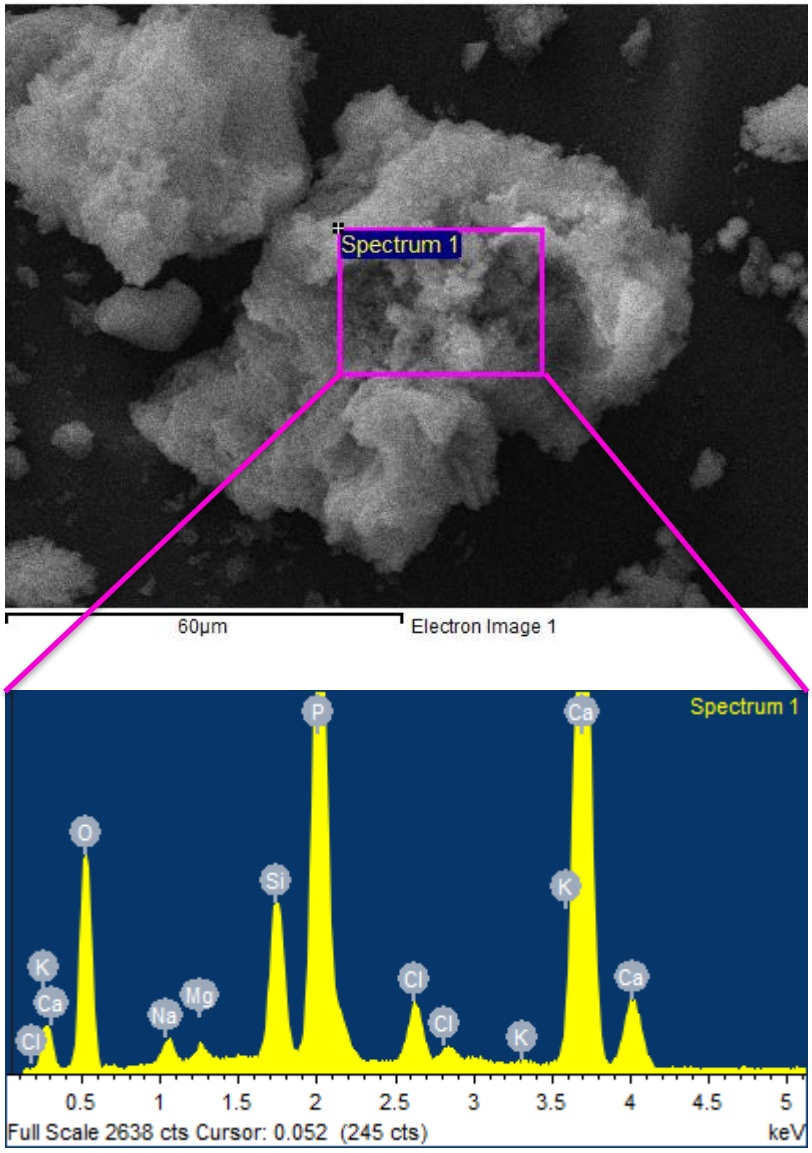

Figure 3-6: EDX spectrum of 20% copolymer composite sample after being incubated in SBF for 3 days.

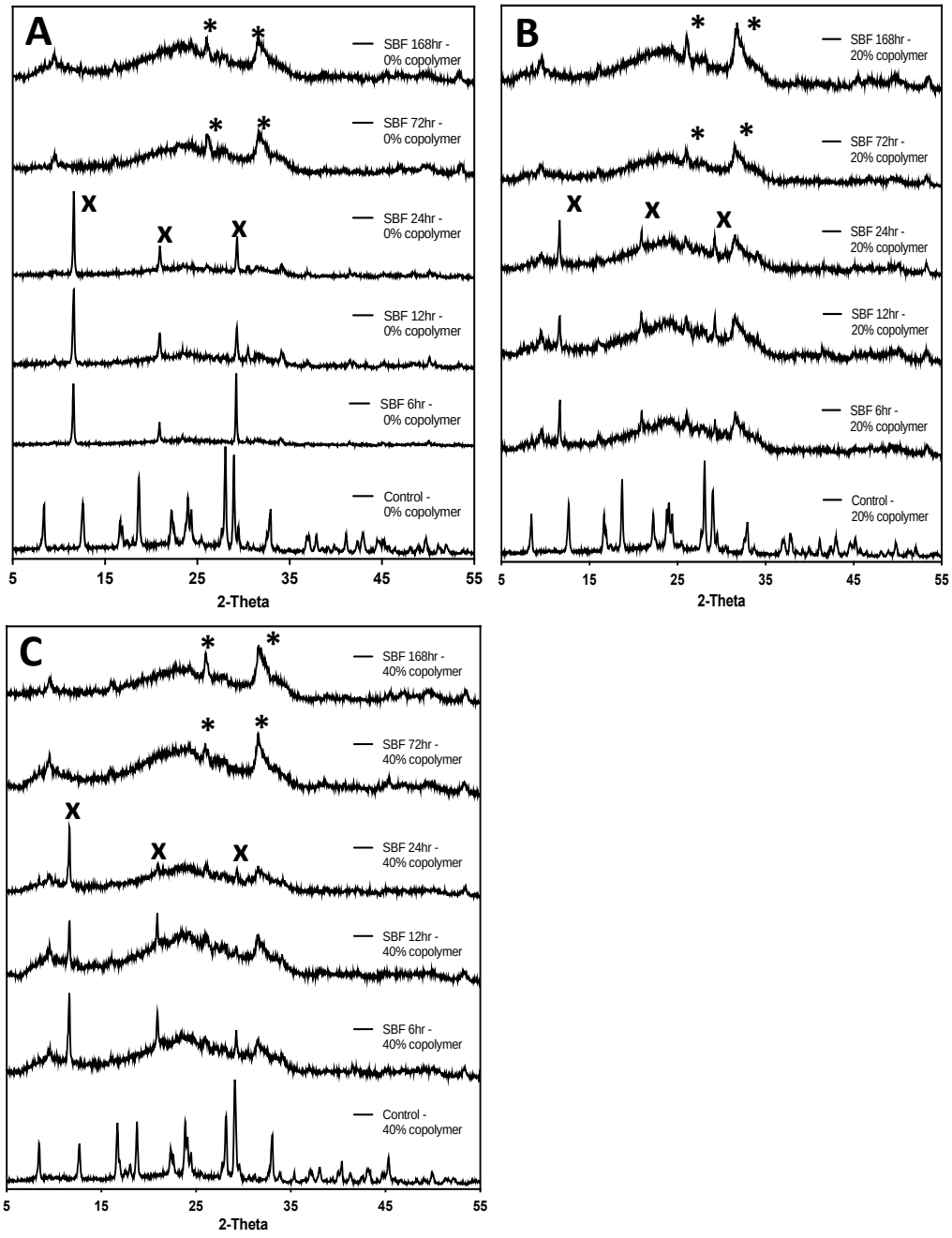

Figure 3-7: Panels (A-C) represent XRD spectra of (A) 0% copolymer, (B) 20% copolymer and (C) 40% copolymer disk bioactive composites at different SBF incubation times. (x) indicates $CaNO_3$ peaks while (*) indicates HA peaks. Specimen age of 20 days and P/L ratio of 0.53 were held constant.

3.4 Discussion

Biomaterials used as bone grafts should be bioactive and biodegradable. The synthesized poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials (i.e., powder component) in chapter 3 were mixed with an aqueous ammonium phosphate solution (i.e., liquid component) at varying P/L ratios (i.e., 0.35, 0.53 and 0.70) to make bioactive composites. Composites with 0% copolymer were used as controls. Composites were aged for 10, 20 or 30 days.

Composites which lack strong chemical interactions such as covalent crosslinking between the organic and inorganic phases are weaker when compared to those that are covalently linked [110]. The experimental composites used in this study were prepared from class II hybrid biomaterials, where the poly(DEF-co-TEVS) is covalently linked to the bioceramic. It is important to note that biomaterials used as bone grafts must possess mechanical properties matching those of trabecular bone (i.e., ~100 MPa) [130]. Because the bone remodeling process takes about 6 months, it is essential that the material replacing native bone be comparable in mechanical properties and composition [131]. The material should maintain integrity throughout the process, otherwise it may fail. We want a strong material that will integrate with bone and act as a bone substitute while new bone is being formed and replacing the material. Although results of the mechanical properties showed no clear trend due to the high scatter of the data, it was evident that the addition of 40% copolymer had a significant effect ($p < 0.01$; Figure 3.1) on compressive strength and modulus, specifically at P/L of 0.53 and specimen aged 20 days. The pendant TEVS functional groups linked as side chains to the DEF backbone during copolymerization gave rise to an increased number of reactive sites. During the sol-gel synthesis of class II hybrid biomaterials, these reactive sites interacted with bioceramic precursors and were responsible for the crosslinking between the organic and inorganic phases. Adding more copolymer to the sol-gel led to more crosslinking and thus improved composite mechanical properties.

Composites with varying copolymer ratios were incubated in PBS for a period ranging from 1 – 10 days. Degradation behavior was studied as a function of weight loss and surface morphology (Figure 3.4). In all samples, the greatest weight loss was observed after 1 day

of PBS incubation. Up to about 45% of their initial weight was lost. When mixing the powder and liquid components together to synthesize composites, unreacted bioceramic, poly(DEF-co-TEVS) and ammonium phosphate were entrapped within with composite matrix. Upon incubation in PBS, the unreacted materials leached out and thus can explain the weight loss seen at 1 day. Time points following 1 day (i.e., 3, 6, and 10 days) showed a more controlled and predictable degradation behavior. Although the weight loss between 3 days and 10 days of PBS incubation was not statistically significant within each composition, the 40% copolymer composition was statistically significant from the others and showed the least weight loss suggesting it is the most stable out of all compositions. Because these novel composites are intended to be used as bone graft biomaterials, they should show a well-controlled degradation profile. As the material degrades it should possess sufficient mechanical properties ensuring its integrity, but also, ions released from the composite matrix should either be released from the body or used by the ECM [132]. Degradation behavior of conventional O/I composites is unpredictable featuring sharp and fast weight loss in comparison to class II hybrid biomaterials composites [110]. In this study, we showed that the O/I ratios of poly(DEF-co-TEVS)/bioceramic class II hybrids influenced degradation. Hybrids with lower O/I ratios (i.e., 0% and 20% copolymer) exhibited more weight loss than the higher O/I ratio (i.e., 40% copolymer). The higher O/I ratio is associated with a greater amount of crosslinking between the organic and inorganic phases as is evident with the presence of the T^n peaks in the solid state ^{29}Si CPMAS NMR spectrum. A study conducted by Jones (2013) revealed that BGs synthesized by sol-gel resorb whether *in vivo* or *in vitro*. This implies that for a faster rate of degradation, it is better to synthesize hybrids with lower organic content. The hybrid biomaterials prepared in this study revealed that degradation behavior can be tailored by changing O/I ratios.

Studies in the literature revealed that materials containing -Si-OH networks have improved bioactivity (i.e., increased amount of apatite deposits) when incubated in SBF. This increased their reactivity due to the interaction between Ca^{2+} ions and the -Si-OH groups. Increased reactivity results in a faster degradation rate [133]–[135]. BGs incubated in SBF were shown to form a layer of HA on their surface. An animal study also showed that BG can interact and bond with bone *in vivo* [49]. Based on the Ca/P ratio derived from EDX, the experimental composites exhibited a significant increase in amount of HA after

incubation in SBF for 3 days compared to 1d incubation. (Figures 3.5 and 3.6). Theoretically, the extent of bioactivity can be altered by changing the O/I ratios during the preparation of class II hybrid biomaterials.

3.5 Conclusion

We have successfully prepared novel bioactive composites by reacting poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials with an aqueous ammonium phosphate solution. Furthermore, the composites' mechanical properties, bioactivity and degradation profiles can be tailored by optimizing sample age, P/L ratio and O/I ratios.

Chapter 4

General Discussion

4 Summary and Conclusions

This investigation aimed to develop a novel, bioactive, biodegradable and mechanically competent composite. In chapter 3, we successfully copolymerized DEF and TEVS through a free radical polymerization where TEVS was added as pendant side chains to the DEF backbone. Moreover, we were able to successfully synthesize poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials through a sol-gel reaction. The inorganic phase was composed of 70 mol% SiO_2, 26 mol% CaO and 4 mol% P_2O_5. In contrast, the organic phase consisted of poly(DEF-co-TEVS) with a composition of 70 mol% DEF and 30 mol% TEVS. In chapter 4, we reacted different compositions of poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials with an ammonium phosphate solution to make novel bioactive composites. BGs are osteoconductive and bioactive known for their ability to form hydroxyapatite *in vitro* and integrate with native bone *in vivo* [49], [136]. Despite these excellent properties, BGs have poor mechanical properties due to their brittle nature and low toughness. They also undergo fast, uncontrolled degradation *in vitro* [35]. Due to the above reasons, BGs are not ideal biomaterials for bone augmentation procedures. One way currently used to tackle these issues includes the preparation of class II O/I hybrid biomaterials. By adding an organic biopolymer to an inorganic component, mechanical properties are improved and degradation profiles are more controlled. Thus, making class II hybrid biomaterials properties more tailored towards bone augmentation and regeneration applications. Polymers that are based on fumarate derivatives are of interest in their ability to be used as bioactive, biocompatible and biodegradable polymers for treatment of bone defects [63]. Diethyl fumarate has a history of being incorporated in the process of bone regeneration [64].

The successful synthesis of class II O/I hybrid biomaterials requires that the organic biopolymer reacts with the inorganic phase (i.e., chemically bond). In some cases, as presented in this thesis, polymer functionalization is necessary. Moreover, during the sol-gel process, the organic phase (i.e., biopolymer) must be soluble in the sol. At the molecular

level, the organic and inorganic phases must act as a single homogenous entity (i.e., no phase separation). In this thesis, after numerous iterations and optimization techniques, successful synthesis of poly(DEF-co-TEVS) and poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials along with their composites addressed all requirements.

In chapter 2, ^{1}H and ^{13}C NMR spectra (Figures 2-3 – 2-8) confirmed successful copolymerization of DEF and TEVS (i.e., successful synthesis of poly(DEF-co-TEVS)). Additionally, ^{29}Si-CP MAS ssNMR (Figure 2.9) confirmed successful synthesis of poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials.

In chapter 3, poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials (i.e., powder component) was reacted with an aqueous ammonium phosphate solution (i.e., liquid component) to prepare composites with different P/L ratios, compositions, and age. Mechanical testing (Figure 3-1) revealed significant improvement in the mechanical properties of poly(DEF-co-TEVS)/bioceramic composites with 40% copolymer content, P/L ratio of 0.53 and specimen age of 20 d. Degradation behavior in PBS was more controlled and predictable for composites with copolymer added compared to conventional composites (i.e., bioceramic + ammonium phosphate). poly(DEF-co-TEVS)/bioceramic composite with 40% content lost significantly less weight compared to all other compositions (Figures 3-3 and 3-4). *In vitro* bioactivity (Figure 3-5) evaluation of poly(DEF-co-TEVS)/bioceramic composites in SBF showed HA formation/surface deposition across all conditions post 3 days of incubation in SBF with no significant differences, suggesting O/I content did not have an effect. Calcium-to-phosphate ratios were measured by EDX through elemental analysis to determine whether or not HA was being deposited on the specimen surfaces. Furthermore, XRD spectra exhibited sharp hydroxyapatite peaks post 3 days of incubation across all conditions, consistent with EDX findings (Figure 3-6).

4.1 Contribution to Current Literature

To the best of our knowledge, this is the first work to successfully synthesize a novel copolymer composed from monomers of diethyl fumarate (DEF) and triethoxyvinylsilane (TEVS); poly(DEF-co-TEVS). Poly(DEF-co-TEVS) is hydrophobic (i.e., insoluble in

water) making it unique since most organic biopolymers used for hybrid materials are hydrophilic (i.e., water soluble). Poly(DEF-co-TEVS) has the potential to resolve some of the challenges involved with water soluble polymers. Ethanol was used as a solved and where water was necessary, minimal amounts were used. We also synthesized a novel class II (i.e., covalently bonded) O/I hybrid biomaterial, poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials, through a sol-gel process. Class II O/I hybrid biomaterials are known to exhibit better overall properties than biomaterials with weaker bonding [79]. Finally, we prepared composites by reacting poly(DEF-co-TEVS)/bioceramic class II hybrid biomaterials with an aqueous ammonium phosphate solution. Three variables were manipulated:

1. Powder-to-liquid ratio (i.e., 0.35, 0.53 and 0.70)
2. Specimen age (i.e., 10, 20 or 30 days)
3. Copolymer proportion (i.e., 0 weight %, 20 weight %, 30 weight % and 40 weight %)

Mechanical properties, bioactivity and degradation behaviour were compared across all conditions to determine which is the best. A pH of 7.4 was recorded after soaking specimens in water.

4.2 Limitations

Even though the composites exhibited promising results for their potential use as bone graft biomaterials, there are some limitations in this thesis work worth mentioning. One of the precursors present in the inorganic phase was calcium nitrate tetrahydrate. Peaks of nitrate salts were observed in the XRD spectra (Figure 3.6). Because nitrates can be toxic to cells, the biomaterial may be cytotoxic. Not performing thermogravimetric analysis (TGA) is another limitation present in this work. TGA is important because it gives us data pertaining to what temperature the copolymer decomposes/denatures at. With TGA data, we can determine whether the material could be heated or not. Because we did not have this knowledge, samples were prepared at RT. Heating the hybrid biomaterials will decompose cytotoxic salts such as nitrate salts and may improve mechanical properties due to morphology changes [137]. Specimens were prepared by hand which may have introduced

sample to sample variability. The powder and liquid components were mixed on dental mixing pads. Once a homogenous paste consistency is achieved, the plastic molds were quickly filled with a spatula and covered between two microscope glass slides. We initially attempted preparing specimens by filling syringes and placing them under pressure at RT to harden, however, the paste mixture would not compress sufficiently. When pressure was released, voids were left behind and specimens could not be used. Moreover, the core of the biomaterial was still wet suggesting the acid-base reaction was not complete. This limitation could explain the inconsistent trend seen in the mechanical testing results

4.3 Future Directions

Studies conducted in this thesis can be used as preliminary findings and further investigated to develop novel biomaterials that can be used as potential bone grafts. For instance, replacing nitrates with calcium chloride or alkoxy calcium can limit toxicity. Finding a new technique/method to prepare composite specimens in a more reproducible manner can yield in more consistent results/trend. Although it is unique that the copolymer used in this work is not water soluble, modifying it to a water-soluble copolymer can facilitate hybrid preparation. Injectability and rheology studies should be conducted because they can reveal important composite properties such as working and setting time. Because these novel biomaterials will ultimately be used for clinical applications, it is essential to conduct MC3T3-E1 preosteoblast culture studies to determine cytocompatibility/cytotoxicity and evaluate cell adhesion, proliferation and differentiation. Depending on results, dynamic cell culture studies can be performed. To determine the elemental distribution and chemical composition of the hybrid biomaterial (i.e., to determine what is being degraded), it is necessary to perform EDX on the PBS solution after every time point. Determining the glass transition temperature of the copolymer is important because it can tell us more about the properties of the copolymer. Therefore, another future direction involves performing differential scanning calorimetry. Lastly, the organic component (i.e., copolymer) can be used as a drug delivery vehicle potentially enhancing material properties including bone formation. New properties can also emerge depending on the specific drug used.

5 References

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