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Sol-Gel Derived Biodegradable and Bioactive Organic-Inorganic Hybrid Biomaterials for Bone Tissue Engineering

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Graduate Program in Chemical and Biochemical Engineering

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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SOL-GEL DERIVED BIODEGRADABLE AND BIOACTIVE ORGANIC-INORGANIC HYBRID BIOMATERIALS FOR BONE TISSUE ENGINEERING

(Thesis format: Integrated-Article)

by

Bedilu Abaineh Allo

Graduate Program in Chemical and Biochemical Engineering

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
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London, Ontario, Canada

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ABSTRACT

Treatments of bone injuries and defects have been largely centered on replacing the lost bone with tissues of allogeneic or xenogeneic sources as well as synthetic bone substitutes, which in all lead to limited degree of structural and functional recovery. As a result, tissue engineering has emerged as a viable technology to regenerate the structures and therefore recover the functions of bone tissue rather than replacement alone. Hence, the current strategies of bone tissue engineering and regeneration rely on bioactive scaffolds to mimic the natural extracellular matrix (ECM) as templates onto which cells attach, multiply, migrate and function.

In this thesis work, a new class of biodegradable and bioactive organic-inorganic hybrid biomaterials were synthesized via a sol-gel process. Poly (ε-caprolactone) (PCL) and tertiary bioactive glass (BG) were used as the polymer and inorganic phases, respectively. The polymer chains were successfully introduced into the inorganic sol while the networks were formed. The PCL/BG hybrid biomaterials exhibited an amorphous structure with spatially distributed calcium atom. Hydrogen bonding was formed to link organic and inorganic phases at molecular scale via the carbonyl group of the PCL and the silanol hydroxyl group of the silica network. The presence of surface silanol groups (Si-OH) as well as the homogenously incorporated Ca\textsuperscript{2+} and PO_4\textsuperscript{3-} ions contributed to modulate the rate and total amount of bone-like HA deposition on the PCL/BG hybrid surfaces. The compressive modulus and strength of the PCL/BG hybrids increased with the decrease in PCL content. The highest values were achieved at the lowest PCL content (10 wt. %) and were around, 90 MPa and 1.4 GPa, respectively. The cell viability study revealed that the PCL/BG hybrid biomaterials (100 – 500 µg/ml) have no toxicity due to the hybridization process. The ability to tailor the bioactivity and mechanical properties of these novel PCL/BG hybrid
materials could be used as screening tool to fabricate multifunctional PCL/BG hybrid biomaterials for specific biomedical applications.

The addition of PCL provided the way to control the rheological properties of the sol by reducing the rate of gelation, which in turn facilitated the successful fabrication of 3D PCL/BG hybrid scaffolds by electrospinning process. The 3D PCL/BG fibrous scaffolds exhibited high porosity, greatly improved wettability, significantly enhanced mechanical properties, and in vitro bone-like apatite formation ability. These scaffolds demonstrated excellent biocompatibility, with an evidence of supporting cell attachment and proliferation. Furthermore, early and enhanced expressions of collagen type I (Col I), alkaline phosphatase (ALP), osteopontin (OPN), bone sialoprotein (BSP) and osteocalcin (OCN) compared with PCL scaffolds demonstrated the potential of PCL/BG hybrid scaffolds for promoting bone regeneration. The work described herein provides strong evidence that O/I hybrid scaffolds have a potential in bone regeneration, and paves a way for future studies.

**KEYWORDS:** Organic-inorganic hybrid, bioactive glass, poly(ε-caprolactone), bioactivity, tissue engineering, bone regeneration, electrospinning, biomaterial, nanofiber, 3D scaffold, mechanical properties, osteoblast.
CO-AUTHORSHIP STATEMENT

Chapters 1 and 2 entitled “Introduction” and “Literature Survey”, respectively, were written by B.A. Allo, with suggestions from A. S. Rizkalla, and K. Mequanint. Parts of sections 1.1, 2.2, and 2.3 are adapted from Allo et al., 2012. Journal of Functional Biomaterials, 3(2), 432-463, and reproduced here with permission from MDPI Publishing (see Appendix A). The publication was written by B. A. Allo, D. O. Costa, S. J. Dixon, K. Mequanint, and A. S. Rizkalla.

Chapter 3 entitled “Synthesis and Electrospinning of Poly (ε-caprolactone)-Bioactive Glass Hybrid Biomaterials via a Sol-Gel Process” is adapted from Allo et al., 2010. Langmuir, 26(23), 18340-18348, and reproduced here with a permission from The American Chemical Society (see Appendix A). The publication was written by B.A. Allo, K. Mequanint, and A. S. Rizkalla. All experiments were performed by B.A. Allo, and were carried out in the laboratories of Drs. A. S. Rizkalla and K. Mequanint.

Chapter 4 entitled “Hydroxyapatite Formation on Sol-gel Derived Poly(ε-caprolactone)/Bioactive Glass Hybrid Biomaterials” is adapted from Allo et al., 2012. ACS Applied Materials & Interfaces, 4(3), 1490-1499, and reproduced here with a permission from The American Chemical Society (see Appendix A). The publication was written by B.A. Allo, K. Mequanint, and A. S. Rizkalla. Experiments were performed by B.A. Allo, and were carried out in the laboratories of Drs. A. S. Rizkalla and K. Mequanint with a technical assistance from Ms. Elizabeth Pruski from Dr. Jeff Dixon’s lab.

Chapter 5 entitled “The Role of Bioactive 3D Hybrid Fibrous Scaffolds on Mechanical Behavior and Spatiotemporal Osteoblast Gene Expression” was written by B.A. Allo, K. Mequanint, and A.
S. Rizkalla. Dr. Shigang Lin helped on taking confocal images and RT-PCR analysis. Experiments were carried out in the laboratories of Drs. A. S. Rizkalla, K. Mequanint, and S. J. Dixon.

Chapter 6 entitled “General Discussion and Conclusions” was written by B.A. Allo, and Drs. K. Mequanint and A.S. Rizkalla reviewed and edited the Chapter.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ε</td>
<td>Elongation at break</td>
</tr>
<tr>
<td>α-MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>β-TCP</td>
<td>β-tricalcium phosphate</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscope</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BG</td>
<td>Bioactive glass</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone sialoprotein</td>
</tr>
<tr>
<td>CaP</td>
<td>Calcium phosphates</td>
</tr>
<tr>
<td>cHA</td>
<td>Carbonated-hydroxyapatite</td>
</tr>
<tr>
<td>Col I</td>
<td>Collagen type I</td>
</tr>
<tr>
<td>COOH</td>
<td>Carboxylic acids</td>
</tr>
<tr>
<td>E</td>
<td>Young’s modulus</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy dispersive X-ray spectroscopy</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
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<td>HA</td>
<td>Hydroxyapatite</td>
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</table>
HC  PCL/BG hybrid fiber (600 nm)
HCl  Hydrochloric acid
HF  PCL/BG hybrid fiber (260 nm)
HMDS  Hexamethyldisilazane
ICPS  Inductively coupled plasma atomic emission spectroscopy
IPTS  3-isocyanatopropyltriethoxysilane
JCPDS  Joint Committee on Powder Diffraction Standards
MEK  Methyl ethyl ketone
M_n  Number-average molecular weight
MTT  3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
O/I  Organic-inorganic hybrid
OCN  Osteoclastin
OD  Optical density
OH  Hydroxyl
OPN  Osteopontin
PBS  Phosphate-buffered saline
PCL  Poly (ε-caprolactone)
PDMS  Poly (dimethoxysilane)
PGA  Poly (glycolic acid)
PLA  Poly (lactic acid)
PLGA  Poly (L-glycolic acid)
PLL A  Poly (L-lactic acid)
pNPP  p-nitrophenol phosphate
<table>
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<tr>
<td>PVA</td>
<td>Poly (vinyl alcohol)</td>
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<td>PVP</td>
<td>Poly (vinyl pyrollidone)</td>
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<td>RCO</td>
<td>Rat calvarial osteoblasts</td>
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<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
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<tr>
<td>SBF</td>
<td>Simulated body fluid</td>
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<td>TCD</td>
<td>Needle tip to collector distance</td>
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<td>TCPS</td>
<td>Tissue culture polystyrene</td>
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<td>Tetraethoxysilane</td>
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<td>XRD</td>
<td>X-ray diffraction</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

1.1 OVERVIEW

Bone injuries and defects that arise from a variety of causes, including fracture nonunion [1, 2], dental and orthopedic implant fixation [2], trauma or tumor resection [1, 3, 4], and musculoskeletal disorders such as rheumatoid arthritis [5] present a significant clinical problem. In the United States alone, roughly 6.3 million bone fractures occur every year, of which about 550,000 cases require some kind of bone replacement surgery [6]. About 90% of these procedures involved the use of bone-grafting with autologous cortical and cancellous bone harvested from iliac crest, which has been considered as the “gold-standard” [6]. However, there are limitations associated with these bone-transplant therapies. Autografts harvested from iliac crest are expensive and constrained by anatomical limitation and are often associated with donor-site morbidity. Allografts introduce potential risks of transmissible diseases and infection, and can induce immunological response from the host. In addition, limited material supply and complicated surgical reconstructions are also the major issues related to these procedures [6, 7]. To overcome these limitations, over the last decade, significant effort has been devoted to the area of biomaterials and bone tissue engineering to develop an alternative bone graft substitutes that can augment or regenerate injured bone [8]. Ideally, biomaterials for bone regeneration are required to be degradable at the same rate with new tissue formation, biocompatible, osteoconductive, bioactive and must be mechanically matched with the native bone [9-12].

In the last decade, a wide range of biomaterials, including synthetic and natural polymers [13, 14], and ceramics [15-17] were intensively studied as scaffolds for bone regeneration. Among these biomaterials, sol-gel derived bioactive glasses (BG) attracted the most interest; however, their
fragility and difficulty to process in the form of 3D scaffold architecture restricted their application for bone regeneration. On the other hand, most biocompatible and biodegradable polymers have a better processability, but they also lack strength and mechanical stability to match with the bone tissue, especially when made with large volume fractions of macro-porosity [8, 13]. To overcome these limitations there has been several attempts to combine BG with biodegradable polymers to create a scaffold material with controlled degradability, bioactivity and mechanical properties. One strategy that has been considered to improve the mechanical property of synthetic biomaterials is the synthesis of organic-inorganic (O/I) hybrids via a sol-gel process. Successful hybridization processes are expected to yield biomaterials with homogeneous structure and strong interfacial bond between the O/I components at a sub-nano or molecular level. However, due to their poor solubility, many biodegradable polymers cannot be easily introduced into the inorganic sol during the sol-gel synthesis. Recently, few studies reported the use of non-degradable and water soluble polymers to demonstrate the synthesis of O/I hybrid materials via a sol-gel route [18, 19]. Other studies also attempted to introduce a biodegradable polymer in the sol-gel process, but in most cases, the inorganic content was solely silica, lacking osteoconductivity [20, 21]. Therefore, it is imperative to optimize the sol-gel synthesis procedure to introduce biodegradable polymers into the sol while the inorganic network is being formed. Besides, the choice of adequate materials, their macro- and micro-structural properties are of utmost importance [22]. Such properties affect not only cell survival, signaling, growth, and reorganization, but also their gene expression and maintaining of cell phenotype [22]. For these reasons, 3D scaffolds that mimic the natural extracellular matrix (ECM) of bone have been utilized to better serve this need [23, 24]. One such method makes use of electrospinning, which allows for the creation of sub-micron sized fibrous scaffolds. These fibrous scaffolds are very similar in structure and size to those of the natural
collagen seen in bone ECM. Furthermore, by combining bioactive inorganic components, it is possible to more closely mimic the mechanical properties and morphology of the ECM of bone, as well as enhance the biological performance of the resultant scaffold biomaterial [23].

1.2 THESIS OUTLINE

In view of the above brief overview, the work presented in this thesis focuses on the development of a new class of biomaterials for bone regeneration. In Chapter 2, pertinent literature review is presented. In Chapter 3, a sol-gel synthesis and characterization of bioactive and biodegradable organic-inorganic hybrid biomaterials consisting of PCL and BG is presented. Potential application of the PCL/BG hybrid for bone regeneration is elucidated by fabricating 3D fibrous scaffolds using the electrospinning technique [25]. Chapter 4 describes the effect of polymer content on apatite formation ability in a simulated body fluid (SBF), mechanical properties, and cell viability [26]. In Chapter 5, the evaluation of the physical properties and biological performance of the electrospun 3D fibrous hybrid scaffold, as well as the effect of fiber diameter on the scaffold properties is presented. Finally, the significance, contributions, and limitations of the study are presented in Chapter 6.
1.3 REFERENCES


CHAPTER 2: LITERATURE REVIEW*

Overview: This Chapter provides background information on the anatomy and physiology of bone, and brief overview of scaffold-based tissue engineering strategies for bone regeneration. The current state of bioactive and biodegradable biomaterials for bone regeneration is presented with a focus on biodegradable and biocompatible polymers, bioactive ceramic and glass based nanocomposite and hybrid biomaterials. In addition, a 3D fibrous scaffold design, by electrospinning technique for bone regeneration is presented. It concludes with a statement of study rationale and an outline of specific hypotheses and objectives of this study.

2.1 BONE ANATOMY AND PHYSIOLOGY

2.1.1 Structure and Function of Bone

Bone is a highly organized hierarchical connective tissue which consists of both organic and inorganic matrix components. The organic constituents of bone are primarily (~90%) collagen nanofibrils and the rest granular proteoglycans, cells and non-collagenous proteins, and are responsible for the ~40% of the bone mass [1]. The collagen matrix is characterized by a hierarchically arranged collagen multifibers in the nanometer size-range and formed into 3D networks. The inorganic component of bone consists predominately of a calcium deficient and carbonated-hydroxyapatite (HA) mineral and comprises approximately 60% of compact bone wet weight [2]. The HA nucleates and grows along the collagen fibers and forms as nano plate-like crystals approximately 40 nm long, 10 nm wide and 1-3 nm thick, producing an inorganic-organic nanocomposite [3]. A portion of HA (20-30%) remains as an amorphous phase for the rapid release of ions to the blood when needed, while the rest crystallizes into carbonated-HA. The

* Part of section 2.2 and 2.3 of this Chapter is published as: Bedilu A. Allo, Daniel O. Costa, S. Jeffrey Dixon, Kibret Mequanint, and Amin S. Rizkalla; Bioactive and Biodegradable Nanocomposites and Hybrid Biomaterials for Bone Tissue Regeneration. Journal of Functional Biomaterials, 2012, 3(2), 432-463, and reproduced here with permission from MDPI Publishing.
organization of HA and collagen fibrils contribute to the superior mechanical properties of the bone tissue to resist large compressive and tensile stresses [2, 3].

Morphologically, bone can be subdivided into two distinct types: cortical (dense, compact) and trabecular (spongy, cancellous), which are easily distinguishable by the difference in macro-porosity (Figure 2.1) [3, 4]. Cortical bone is typically the outer layer of most bones, which is 80-90 % mineralized with very few pores and void spaces, making it very strong and capable of maintaining the mechanical and protective requirements of the skeleton. It consists of densely packed and compact layers of mineralized ECM and bone cells, known as lamellae [2]. The collagen fibers within each lamellar layer are aligned parallel with one another, with those in adjacent layers running in opposite direction and rotating at an angle. This feature, in addition to the twisting of the collagen fibers, is where bone derives its exceptional ability to withstand torsional stresses, allowing it to strain ~10 % before fracturing [5, 6]. Cortical bone is comprised of several osteons in close proximity which function as weight bearing pillars [5]. Blood vessels and nerves run through Haversian and Volkmann’s canals, which are connected to each other and supply cellular nutrition to the bone. The periosteum, outer layer of cortical bone, consist a network of blood vessels and nerves that supplies the bone with nutrition, oxygen and removes cellular excretory products.

Trabecular or cancellous bone is much less dense and has a larger degree of macro-porosity (50-90 %) than cortical bone, making its modulus and ultimate compressive strength around 20 times inferior than that of cortical bone [7]. It is highlighted by honeycomb-like network, wherein lamellae surround individual trabeculae [2, 3]. Only about 20-25 % of its content is mineralized, but it is rich in bone marrow, blood vessels, and connective tissues and is involved in
hematopoiesis, making the primary role of trabecular bone metabolic as opposed to structural support [4].

**Figure 2.1**: Microscopic organization of cortical and trabecular bones. Adapted from [4].
The functions of bone are many. At a system level it provides mechanical support of soft tissues, protective shield of internal organs and the central nervous system, support during the muscular contraction resulting in motion, a reservoir for inorganic ions (calcium and phosphate) and ion homeostasis, and housing for the bone marrow and support of hemopoiesis [8].

In an adult, depending on the functional demand, bone engages in continuous cycle of remodeling process, in which old bone is replaced by new bone. A number of cell types, intracellular and extracellular molecules, and signaling pathways are involved in a well-orchestrated series during the bone formation and resorption process [9]. Thus, a clear understanding on the role of different cell types and matrix molecules in bone formation and resorption process is of paramount importance for almost all aspects of the body's reaction to biomaterials and might help to evaluate the biological performance of different materials used as bone substitutes.

2.1.2 Bone Cells and Their Function

There are various cell types involved during bone remodeling or regeneration process through the balanced activities to maintain the integrity of skeletal system. These are the bone-forming osteoblasts, osteocytes, bone-degrading osteoclasts, and bone lining cells.

Osteoblasts

Osteoblasts are fully differentiated cells that originate from either bone marrow stromal cells or mesenchymal stem cells, proliferate and differentiate to pre-osteoblasts, and then to mature osteoblasts. The developmental stages in which the osteoblasts differentiating into mature osteoblasts and subsequent matrix synthesis and mineralization is described by three biological phases: 1) cellular proliferation, 2) matrix maturation, and 3) matrix mineralization [10, 11].
During these stages, osteoblasts synthesize and secrete various bone matrix proteins including collagen type I, alkaline phosphatase, osteopontin, osteonectin, osteocalcin, and bone sialoprotein [11]. Figure 2.2 demonstrates the typical temporal expression of the different markers in the osteoblasts cells. The deposition of those proteins leads to the formation of bone matrix. Mineralization of the bone matrix completes the bone formation [2]. They are usually found lining the area of newly formed unmineralized tissue, generally comprised of collagen type I (osteoid). After mineralization they become surrounded by the mineralized extracellular matrix. The osteoblast at this point has become fully mature and is considered an osteocyte [1].

**Figure 2.2:** Osteoblast developmental sequence. Typical temporal expression of a few phenotypic markers for the osteoblasts. Adapted from Stein *et al.* [12]
Osteocyte

Osteocytes are metabolically less active cells and involved in the maintenance of local bone. Furthermore, the unique stellate shape with the canaliculi connecting adjacent osteocyte makes each of them a hub of cellular communication and nutrient delivery. New bone is formed in concentric sheets, or lamellae that surround a common center called central (haversian) canals, through which blood vessels pass and provide nutrients. As osteoblasts form new bone along the inner edge of these concentric circles, subsequent lamellae are migrated outward from the center. The canaliculi provide the transfer of nutrients from newly formed lamellae to older layer of bone (Figure 2.1).

Osteoclast

Osteoclasts are macrophage polynucleated-giant cells, which are differentiated from hematopoietic stem cells through the monocyte-macrophage lineage [13, 14]. Osteoclasts precursors and mature osteoclasts have a significant amount of the phosphohydrolase enzyme, tartrate-resistant acid phosphatase, which is commonly analyzed during osteoclast identification. Osteoclast cells reside on the surface of bone, and are responsible for bone resorption. Furthermore, they are characterized by two distinct plasma membrane regions; a ruffled border and a clear, or sealing zone [8]. The clear or sealing zone forms a barrier (resorption lacuna) around the ruffled border which is responsible for bone resorption. The osteoclasts secrete hydrochloric acid and collagenase across the ruffled border [15]. The highly acidic secretion dissolves the hydroxyapatite crystals forming the mineralized extracellular bone matrix. Then several enzymes and collagenase destroy the collagen matrix that secured the hydroxyapatite crystals to the bone.
With the matrix degraded and dislodged, the degradation products are then removed from the resorption lacuna, or resorption pits, and released into extracellular space [15].

**Bone lining cell**

Bone lining cells are the fourth types of cells that commonly covers bone surfaces. These cells are flat and elongated, contain few cell organelles. These morphological characteristics indicate that bone lining cells are hardly engaged in bone formation and resorption. In fact, these cells have little known function, but speculation states that they may be osteoblast precursors [16].

### 2.1.3 Bone Related Markers

There are no single specific proteins that are exclusively produced by bone to describe osteoblast differentiation, matrix synthesis, and mineralization. But there are small subset of proteins which are expressed in combination by bone cells and only exists in bone ECM [11, 12]. Therefore, to be able to assess the nature of bone tissue it is important to understand and examine the expression of bone-related markers. A few of the important markers for the bone cells that were selected to represent the osteoblast development cycle for the purpose of this thesis are discussed as follows.

**Collagen Type I (Col I)**

Col I is the most abundant protein and the major structural component of bone matrix and composed of three polypeptide chains with ~1000 amino acid residues, each of them forming a triple helical structure. This structure is stabilized by hydrogen bonding between hydroxyproline and the other charged adjacent molecules within the structure. Each of these linear molecules aligns with one another in a staggered fashion to form a fibril, which in turn forms bundles of collagen fibers. These collagen fibers constitute the organic component of bone ECM [17, 18].
**Alkaline Phosphatase (ALP)**

ALP enzyme is a membrane bound glycosylated protein, which is present in many organs of the human body. The presence of non-specific ALP and its activity is associated with the normal development of osteoblasts. Although its exact function in relation to bone is not completely understood, its maximum expression coincides with the post-proliferative stage of matrix maturation during the developmental stage of osteoblasts differentiation [19]. It is thought to increase local concentrations of inorganic phosphate by hydrolyzing phosphate-esters and thereby facilitating the formation of bone mineral [19-21]. Extracellular ALP possibly assists in the initial crystal formation of hydroxyapatite. Another potential function of ALP is to hydrolyze and inactivate inhibitors of mineral deposition, and inorganic pyrophosphate [2]. It has also been suggested that ALP could act as a transporter for inorganic phosphate as well as binds to Ca\(^{2+}\) to help stimulate calcium phosphate precipitation. In general, ALP is an important marker of normal osteoblasts and different stages of bone development [22].

**Osteopontin (OPN)**

OPN is one of the predominant non-collagenous ECM proteins of bone, and it is a member of the sialoprotein family due to its high sialic acid content. OPN contains an L-Arginyln-Glycine-L-Aspartic acid (Arg-Gly-Asp; RGD) amino acid sequence located near its center, which promotes cellular attachment and spreading of a variety of cells, including osteoblasts, fibroblasts, and osteoclasts [23, 24]. Other related studies showed that the expression of OPN occurs prior to osteocalcin expression, which has been linked to bone mineralization [24, 25]. Compared with polyglutamic acid in bone sialoprotein, it contains stretches of polyaspartic acid which shows high affinity for Ca\(^{2+}\), although it does not appear to nucleate hydroxyapatite [22, 24]. Rather, OPN is
a strong inhibitor of HA formation and growth in a dose-dependent manner by the mechanism of binding to the HA crystals and inhibiting its further growth [24]. Thus the presence of OPN with osteoblasts and osteoclasts during mineralization and bone resorption indicates that OPN could be serving as a control for mineralization and a promoter for resorption.

**Bone sialoprotein (BSP)**

BSP is a highly phosphorylated, acidic, non-collagenous protein in bone matrix. It contains stretches of polyglutamic acid along with a RGD sequence in its carboxy terminus. BSP marks a late stage of osteoblasts differentiation and an early stage of mineralization. It is found almost exclusively at the site of mineralization, although it can also be found in small amounts in chondrocytes, hypertrophic cartilage, and in osteoclast. It has a very high affinity towards Ca$^{2+}$ and plays a role in matrix mineralization. It has been reported as a nucleator of hydroxyapatite, the major mineral component of bone [26-28]. It also mediates bone cell attachment via an RGD dependent manner via $\alpha_v\beta_3$ integrin, which is a receptor for vitronectin [29].

**Osteocalcin (OCN)**

OCN is also known as bone gamma-carboxyglutamic acid-containing protein (BGLAP) [30]. It is the most abundant among the non-collagenous proteins. It contains three glutamic acid (Gla) residues which are carboxylated in the presence of vitamin K as a post-translational modification. Although the precise function of OCN is still not fully understood, it is believed to play a key role in bone mineralization and remodeling [25]. OCN exhibits a calcium-dependent $\alpha$-helical conformation in which the three Gla residues are aligned to facilitate the absorption to HA. Furthermore, the presence of COOH terminal beta sheet domain could also serve as the locus for
interaction with cellular receptors [22]. Another function of OCN is its role in the regulation of bone resorption and the recruitment of cells of osteoclast lineage [31].

In summary, bone is a highly organized nanocomposite material with metabolically active cells attached to, embedded in, or surrounded by a mineralized matrix [1]. Most of its properties are related to its matrix constitution. Although bone biology is a complex subject, literature pertinent to the scope of this work are reviewed. From tissue engineering perspectives, understanding the anatomy and physiology of bone provides the basis to design appropriate scaffold biomaterials that integrate with the surrounding bone tissue and provide the 3D framework upon which cells may adhere, proliferate, and eventually produce ECM proteins. Therefore, mimicking the composite structure and morphology of bone ECM with enhanced osteogenic stimulus may be achieved by combining appropriate organic matrix, and bioactive and osteoconductive inorganics, with ideal macro- and micro-structural features.

2.2 PRINCIPLES OF SCAFFOLD-BASED BONE TISSUE ENGINEERING

Bone possesses an intrinsic capacity to regenerate continuously without leaving a scar as part of the repairing process in response to injury and mechanical stimulus, as well as during skeletal development or remodeling [7, 9]. In bone regeneration, a well-orchestrated series of biological events of bone induction and conduction, including actions of different bone cell types and various molecular-signaling pathways, in an effort to optimize skeletal repair and restore skeletal function are involved [9, 32, 33]. In the clinical setting, the most common form of bone regeneration is fracture healing, during which the pathway of normal fetal skeletogenesis, including intramembranous and endochondral ossification, is recapitulated [9, 34]. However, there are cases of fracture healing in which bone regeneration is impaired, for example in orthopaedic surgery and
in oral and maxillofacial surgery in which bone regeneration is required in large quantity (beyond the normal potential for self-healing). These include skeletal reconstruction of large bone defects created by trauma, infection, tumor resection and skeletal abnormalities, or cases in which the regenerative process is compromised, including avascular necrosis and osteoporosis [9]. Recently, a tissue tissue engineering approach is becoming an alternative and promising strategy in the field of bone regenerative medicine to overcome the aforementioned limitations associated with skeletal reconstruction of large bone defects.

Bone tissue engineering is the application of biological and engineering principles towards the repair, restoration or regeneration of living tissues using biomaterials, cells and factors alone or in combination [35]. From the biological perspective, bone growth requires cells, extracellular matrix, intracellular communications, cell-matrix interactions and growth factors. However, the above mentioned components are not the only issue dealing with bone tissue engineering. Bone has a 3D configuration, and cells do not grow in a 3D fashion in vitro, which necessitating a 3D scaffold. Therefore, successful bone tissue engineering and regeneration requires well-coordinated and timely combinations of osteoconductive scaffold, osteoinductive biological molecules such as proteins or growth factors, and osteogenic cells are required (Figure 2.3).
Figure 2.3: A tissue engineering concept that involves seeding cells within porous biomaterial scaffolds. Cells are isolated from the patient and expanded in vitro, then seeded in porous scaffold together with growth factors. The tissue construct is allowed to mature in physiologic condition before transplantation of engineered construct to restore the function of the injured tissue. Adapted from [36]

The development of a porous scaffold for bone tissue engineering is needed to either induce formation of bone from the surrounding tissue or to act as a template for implanted bone cells or other agents. In this sense, the scaffold serves as a temporary matrix for cell proliferation and
extracellular matrix deposition, and bone ingrowth until the new bony tissue is totally restored/regenerated. Moreover, the scaffold would also act as a template for the vascularization of this neo-tissue and could actively participate in the regenerative process through the release of growth/differentiation factors, present within its structure.

In view of the above, the development of an appropriate 3D scaffold is an essential component for bone tissue engineering strategy. It is important to put into perspectives that the 3D scaffold ideally is expected to satisfy multi-variable design requirements to be used. In addition to having proper selection of materials, which will be addressed in the following section, the macro and micro-structural properties of the scaffold are of utmost importance. Such properties affect not only cell survival, signaling, growth, and reorganization but also their gene expression.

### 2.3 DEVELOPMENT OF SCAFFOLDS FOR BONE REGENERATION

#### 2.3.1 Desired Features of Scaffold Materials

Porous scaffolds are used for tissue regeneration to provide a supportive and conductive construct for the formation of new tissue [37]. Brekke et al. [38] compiled a comprehensive list of the critical considerations for 3D scaffold design determined from an extensive literature review. As such, scaffold constructs are to be fabricated as 3D porous structures with appropriate pore size, porosity, and interconnectivity between pores, to allow for cell and tissue ingrowths [39, 40]. Large surface area to volume ratio is desirable to promote cell ingrowths and appropriate cell density and distribution to induce vascularization of the construct from the surrounding tissue. Meanwhile, high porosity and interconnectivity are fundamental for sufficient diffusion of nutrients and oxygen and removal of metabolic waste [7, 40].
For bone tissue engineering, scaffold architecture should mimic that of cancellous bone, which is characterized by a random pore structure [38]. *In vitro* studies showed that osteogenesis is enhanced by lower porosity, which suppresses cell proliferation and promotes cell aggregation, however, *in vivo* work revealed that higher porosity and pore-size resulted in greater bone ingrowth [41, 42]. Relatively larger pores favor direct osteogenesis, since they allow vascularization and high oxygenation, while smaller pores result in osteochondral ossification, although the type of bone ingrowth depends on the biomaterial and the geometry of the pores [42]. Earlier study indicated that a pore-size of ~100 μm was thought to be a minimum requirement to regenerate mineralized bone due to cell-size, migration, and diffusion issues [43]. However, recent studies have identified that a pore-size of 200-400 μm as optimal for cell and bone-tissue ingrowths [38-41]. An increased porosity and pore-size can, however, adversely affect the mechanical properties of a scaffold, thus requiring more complex material design considerations during 3D scaffold fabrication. An upper limit is also set from the dimensions of the pores of specific bone-tissue required [42].

In addition to the porosity and pore-structure requirements, the scaffold for bone tissue engineering should exhibit controlled biodegradation or resorption rate concomitant with the new tissue formation. Furthermore, the mechanical properties of the scaffold should be compatible to the host tissue at the site of implantation. The degradation product should be non-toxic and easily excreted by metabolic pathways. Other highly desirable features concerning the scaffold processing are near-net-shape fabrication and scalability for cost-effective industrial production. A scaffold possessing a suitable surface chemistry also required to promote cell attachment, proliferation and differentiation [44-46].
2.3.2 Scaffold Material Selection

Since natural bone matrix is a composite of biological ceramic (hydroxyapatite) and polymer (collagen), it is not surprising that several synthetic and natural biomaterials based upon natural/synthetic polymers, bioceramics and their composites, and hybrids have been used to prepare scaffolds for bone tissue engineering application [46-50]. The following section will discuss some of the basic characteristics of these materials.

2.3.2.1 Biocompatible and Biodegradable Polymers

Various types of natural (collagen, hyaluronic acid, chitosan and alginate, etc.) [47, 49-51], and synthetic polymers (poly (glycolic acid) (PGA), poly (L-lactic acid) (PLA), PCL, etc.) [47, 52] have been investigated for bone regeneration. Although the preliminary results are promising for naturally derived polymers [49, 50], concerns about the feasibility of finding large quantities of natural materials needed for clinical applications has prompted researchers to explore the use of synthetic polymers. These materials can be easily manufactured into various shapes. In addition, their physical and degradation properties can be tailored for specific applications. The remarkable property of these polymers is their ability to support the mechanical needs for a wide variety of applications such as screws and fixation devices in orthopedics [53]. In particular investigators have concentrated on synthetic biodegradable polymers that are approved by the United States Food and Drug Administration (FDA) as suture materials. These polymers are mainly poly (α-hydroxy esters) that are degraded by hydrolysis, and can be metabolized and excreted. The most common of biodegradable polymers are PGA, PLA, PCL and their co-polymers [39, 46, 54]. However, in spite of their wide application in tissue regeneration, poly(α-hydroxy esters) have suboptimal biocompatibility due to their acidic degradation products. In addition, they also have
limited strength and mechanical stability that are inferior to those of bone when made with large volume fractions of macro-porosity, which is a critical design requirement for tissue regenerative materials. Furthermore, they are not osteoconductive and do not directly bond to bone. The most commonly used biocompatible and biodegradable synthetic polymers for bone tissue engineering applications are summarized in Table 2.1.

Table 2.1: Physical, mechanical, and degradation properties of selected biodegradable polymer scaffolds [52, 53, 55-58].

<table>
<thead>
<tr>
<th>Scaffolds</th>
<th>Melting Point (°C)</th>
<th>Glass Transition temperature (°C)</th>
<th>Tensile Modulus (GPa)</th>
<th>Degradation Time (months)</th>
<th>Degradation Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(L-lactic acid)</td>
<td>173–178</td>
<td>60–65</td>
<td>1.5–2.7</td>
<td>&gt; 24</td>
<td>L-lactic acid</td>
</tr>
<tr>
<td>Poly (D,L-lactic acid)</td>
<td>Amorphous</td>
<td>55–60</td>
<td>1.9</td>
<td>12–16</td>
<td>D,L-lactic acid</td>
</tr>
<tr>
<td>Poly (Glycolic acid)</td>
<td>225–230</td>
<td>35–40</td>
<td>5–7</td>
<td>3–4</td>
<td>Glycolic acid</td>
</tr>
<tr>
<td>Poly (ε-caprolactone)</td>
<td>58–63</td>
<td>– 60</td>
<td>0.4–0.6</td>
<td>&gt; 24</td>
<td>Caproic acid</td>
</tr>
<tr>
<td>Poly (D,L-lactic-co-glycolic acid) (50/50)</td>
<td>Amorphous</td>
<td>50–55</td>
<td>1.4–2.8</td>
<td>3–6</td>
<td>D,L-lactic acid and glycolic acid</td>
</tr>
<tr>
<td>Poly (D,L-lactic-co-glycolic acid (85/15)</td>
<td>Amorphous</td>
<td>50–55</td>
<td>1.4–2.8</td>
<td>3–6</td>
<td>D,L-lactic acid and glycolic acid</td>
</tr>
<tr>
<td>Poly(D,L-lactic-co-glycolic acid) (90/10)</td>
<td>Amorphous</td>
<td>50–55</td>
<td>–</td>
<td>&lt; 3</td>
<td>D,L-lactic acid and glycolic acid</td>
</tr>
</tbody>
</table>

Particularly, scaffolds used for tissue regeneration are required to be at the very least, capable of supporting cell attachment and provide sufficient mechanical strength to resist tractional forces produced by cells and contractile forces exerted by the natural healing process in vivo [41, 59]. For bone tissue engineering, the defect must be shielded from intrusion of competing cell types and formation of non-osseous tissue such as scar tissue, which forms as a result of a rapid repair sequence and can be a site for failure [38, 60]. The scaffold material should be biodegradable and
bioresorbable, allowing for excretion of the initial foreign material and its degradation by products. Ideally, the scaffold degradation rate is expected to be in consort with, or lower than the remodeling rate of the tissue, under physiological loading [47, 59]. Bone regeneration scaffolds are thought to maintain their physical and mechanical properties for 3–6 months with mass loss only to occur after 12–18 months [41]. The majority of degradable polymer systems undergo bulk degradation, which is highlighted by a two-stage degradation process [47]. Initially, biodegradation begins with slow reduction in viscosity and molecular weight of the polymer. The second stage is mass loss, which is characterized by diffusion of molecular chains out of the bulk polymer, resulting in an accelerated degradation and resorption kinetics. The release of acidic by-products often associated with mass loss degradation of polymer systems could be a potential cause of inflammatory reactions [47].

2.3.2.2 Bioceramics and Bioactive Glass

2.3.2.2.1 Calcium Phosphates

Calcium phosphates (CaP) are biocompatible, osteoconductive, and possess remarkable ability to bond directly to bone [61, 62]. In particular HA, has attracted a great deal of attention in dental and orthopedic applications due to its similarity to the mineral phase of bone and teeth [63, 64]. Synthetic HA powder can be produced by a variety of wet chemical methods and solid state reactions [65, 66]. Wet precipitation represents a common commercial route for HA production where the drop-wise addition of phosphoric acid to a suspension of calcium hydroxide, or reactions between calcium nitrate and ammonium phosphate, both under alkaline conditions, results in a calcium deficient apatite precipitate [66-68]. Hydrolysis methods are also used to prepare HA, using acid calcium phosphates such as dicalcium phosphate dihydrate, octacalcium phosphate or
dicalcium phosphate anhydrous [66]. Commercially available CaP, such as β-tricalcium phosphate (β-TCP), are also easily hydrolyzed to produce HA [69]. Sol-gel chemistry, involving the hydrolysis of phosphorous containing alkoxides and calcium salt and subsequent polycondensation, is also a well-known and widely studied synthesis route. Advantages of sol-gel techniques include molecular level mixing providing a high degree of control over the composition and chemical homogeneity of the final product. However, production of crystalline HA powders from sol-gel synthesis typically require calcinations at elevated temperature, which is associated with the formation of secondary phases such as β-TCP and granular particle shapes [70, 71]. Alternatively, hydrothermal processes synthesize crystalline HA at relatively low temperatures (<250 °C) by subjecting calcium and phosphorus precursors to high pressure steam in an acid digestion bomb [72, 73]. Recently, HA nanowires with tunable aspect ratio were synthesized by a combination of sol-gel chemistry and hydrothermal treatment in aqueous solvent [74].

The synthesis of compact and dense HA and TCP scaffolds for bone regeneration often requires high temperature sintering and are poorly degradable in their highly crystalline form, while their amorphous counterparts are mechanically too fragile to be used for fabrication of highly porous scaffolds. The dissolution rate for calcium phosphates is in the following order:

Amorphous HA > α-TCP > β-TCP > crystalline HA

the crystalline HA, which is sintered at high temperature, has high chemical stability in contact with tissue fluids, which leads to limited bioactivity and osteoconductive effect [75]. Alternatively, their amorphous counterpart are characterized by a high dissolution rate in vivo, which accelerates material desorption and elicit immunologic response. Consequently, the dissolution rate and subsequent bioactivity has been improved by synthesizing biphasic calcium phosphates (BCP)
consisting of varying mixtures of HA and the more soluble β-TCP. BCP in the form of granules, blocks, and specifically designed shapes are commercially available and are used in numerous orthopedic and dental applications [76]. In vitro studies using human bone marrow cells showed improved cellular attachment, proliferation and differentiation when cultured on HA as compared to other commonly used biomaterials, titanium and high molecular weight polyethylene [77]. In vitro culturing of osteoblasts-like cells on to porous PCL scaffolds showed significant increase in osteoconductivity and bone formation when embedded with HA particles or coated with biomimetic HA [78]. Osteoconductivity is clearly evident from in vivo experiments. Improved bone ingrowths into porous implant materials was obtained when coated with CaP [79-86], as well as, eliminating fibrous tissue encapsulation commonly seen at the tissue/material interface of implanted polymer scaffolds [87, 88]. Indeed clinical applications of calcium phosphate coatings for total joint arthroplasty has shown improved osseointegration at bone/implant interface resulting in superior implant stability [89]. Further, in vivo studies have shown potential osteoinductivity of biomimetic CaP coatings where ectopic bone formation occurred when coated implants were inserted in nonosseous sites [90-92].

2.3.2.2.2 Bioactive Glasses

Bioactive glasses (BG) are amorphous and biologically active silicate-based glasses. They can react with physiological fluids to form tenacious bonds to bone through the formation of bone-like HA layers when implanted into living tissue [93-95]. The bonding mechanism involves a sequence of reaction steps leading to the precipitation of a carbonated HA layer on the implant surface. Furthermore, these reactions, which lead to the release of critical concentrations of soluble ions, induce favorable intracellular and extracellular responses leading to rapid bone formation [96]. These reactions are summarized and reported elsewhere [96]. Bioglass® 45S5 (46.1 mole % SiO₂,
24.4 mole % Na₂O, 26.9 mole % CaO, and 2.6 mole % P₂O₅) was the first material seen to form an interfacial bond with host tissue, when it was implanted in rats [93]. The strength of the interfacial bond between Bioglass® and cortical bone was equal to or greater than the strength of the host bone. Bioactivity is not an exclusive property of bioactive glass; hydroxyapatite and related calcium phosphates also show an excellent bone-bonding ability [62]. The interest in bioactive glasses has been expanded since their initial discovery, other compositions and glass types have also been found to be bioactive.

Early BGs were prepared by quenching of melts comprising SiO₂ and P₂O₅ as network formers and CaO and Na₂O as network modifiers [93]. This was the route followed until the early 1990s when sol-gel processing was introduced for the synthesis of bioactive glasses [97]. The sol-gel synthesis consists of a series of hydrolysis and polycondensation reactions of metal alkoxides followed by ageing, drying and thermal stabilization. A metal alkoxide has the generic structure M-(OR)x, and is a molecule consisting of a central metallic ion (M) bound to functional organic groups (R) through an oxygen linkage (O). Metal alkoxides, such as tetraethoxysilane (TEOS) and tetramethoxysilane (TMOS) are often used as silica precursors due to their ability to readily react with water. The acid catalyzed hydrolysis reaction results in the replacement of alkoxy side chains with hydroxyl groups. Hydrolysis occurs through a nucleophilic attack on the silicon atom by the oxygen atom in the water molecule [98].

\[
\text{Hydrolysis: } M-(OR)_4 + 4(H_2O) \rightarrow HO-M(OR)_3 + R-OH
\]

Where -R represents an alkoxy functional group, e.g. C₂H₅OH.

The ratios of the reagents can be adjusted to control the degree of hydrolysis, ultimately leading to the formation of either clusters or branched polymeric chains. Subsequently, the polycondensation
reaction results an increase in viscosity as the interconnectivity of the inorganic network grows [97, 98]. These reactions are illustrated as follows:

\[
\text{Condensation: } (\text{OR})_3M-\text{OR} + \text{HO-M(OR)}_3 \rightarrow (\text{OR})_3M-\text{O-M(OR)}_3 + \text{ROH}
\]

\[
\text{HO-M(OR)}_3 + \text{HO-M(OR)}_3 \rightarrow (\text{OR})_3M-\text{O-M(OR)}_3 + \text{H}_2\text{O}
\]

The condensation reaction liberates alcohol and water as a by-product. The water remains in the pores of the gel. The ageing process holds water in the pores, enabling localized solution and reprecipitation of the solid network. This increases the thickness of interparticle necks and increases the density and strength. The aging process usually takes place for several hours/days at elevated temperatures [99]. The pore liquid and residual alcohol is removed from the monolith in the drying stage, leaving small interconnected pores with diameter in the range of 1–20 nm [99]. Stabilization at increased temperatures results further drying and removing of surface silanol groups and formation of three-membered silica rings from the network. The process also increases density, strength, and hardness and converts the glass network to resemble that of the melt-derived counterpart [97, 100]. Addition of reagents such as tri-ethylphosphate (TEP) and calcium chloride or calcium nitrate yield the oxides of phosphorous and calcium, respectively.

The sol-gel route allows glasses of higher purity and homogeneity to be obtained, and the ranges of their compositions and textural properties to be expanded. In addition, since all the steps in sol-gel synthesis are carried out at temperatures notably lower than those required to obtain glasses by the melting method (ca. 25-40 °C), no longer is required for the addition of components intended to decrease the melting temperature \(i.e., \text{Na}_2\text{O}\) [97, 101, 102]. Therefore, the sol-gel-derived BGs have more simple compositions than melt-derived BG. Furthermore, due to the mesoporous structure, the sol-gel derived BG exhibited enhanced bioactivity and resorbability. The presence
of large number of surface silanol groups in the silica network also enables the organic modification of BG based biomaterials.

Most of the current studies on BGs are not only focusing on bone bonding, but also on their osteogenic potential and applications in bone regeneration. In addition to precipitating bone mineral, BGs have also been found to support enzyme activity [103], vascularization [104] as well as promote osteoblasts adhesion, growth, and differentiation [105, 106]. BGs were also shown to induce the differentiation of mesenchymal cells into osteoblasts and to provide osteoconductivity [107]. The dissolution products of BGs have shown to exert a genetic control over osteoblast cycle and rapid expression of genes that regulate osteogenesis and the production of growth factor [108]. Silicon has been found to play a key role in bone mineralization and gene activation [109], which has led to the substitution of silicon for calcium in synthetic HA. *In vivo* investigation has shown that bone ingrowth into silicon-substituted HA granules was remarkably greater than that of pure HA [110]. Despite their advantages, BGs are much more brittle than natural bone, thus making them unsuitable for load-bearing applications. Investigation of new strategies to enhance their mechanical property has been one of the main research interests. Coating of BG on organic polymer substrates or producing a composite of BG with organic polymer has been developed to “mimic” the composite nature of natural bone [102].

2.3.2.3 Nanocomposites and Organic-Inorganic Hybrids

Despite the availability of materials with appropriate biological and structural properties they still need improvement to satisfy all the requirements for bone regeneration. A major stumbling block in the development of scaffolds for bone regeneration is that most materials are not mechanically competent, bioactive and biodegradable all at the same time. Typically, mechanically strong
materials are bioinert [111], while bioactive and biodegradable materials tend to be mechanically weak, when produced with large volume fraction of porosity [46, 111]. Therefore, combining biodegradability, bioactivity and mechanical competence, hybrid and nanocomposite materials offer an exceptional opportunity to produce scaffolds with desired biological, physical and structural properties. O/I hybrid biomaterials differ from their nanocomposite counterparts wherein the inorganic components and the polymer chains interact through chemical bonding at the molecular level. Furthermore, O/I hybrids form a single phase material, consisting of a homogenous mixture between the organic and inorganic components. As such, the intimate nature of the organic-inorganic interfaces in O/I hybrids results in superior mechanical properties.

From a biological perspective, the constituents of O/I hybrids and nanocomposites resemble the structure of bone tissue, where the inorganic component mimics the carbonated HA and the polymer component mimics the collagen rich ECM. Biodegradable polymers and bioceramics that have the ability to degrade in vivo, are ideal candidates for composite scaffolds, which gradually degrade while a tissue is formed. The release of acidic degradation by-products from polymers can cause inflammatory reactions, while the basic degradation of CaP or BG could buffer the acidic by-products. This may help avoid the formation of an unfavorable environment for cells due to the low pH.

Mechanically, bioceramics are stronger than polymers and play a critical role in providing mechanical stability to constructs prior to formation of new bone. However, most bioceramics are very fragile and prone to catastrophic failure due to their intrinsic brittleness and flaw sensitivity. The synthesis of O/I hybrids and nanocomposites capitalizes on the beneficial properties of both material types. Increasing the content of the inorganic filler is generally proportional to an increase in stiffness. On the other side, nanoparticle fillers have the tendency to aggregate and be incompatible
with the organic polymer matrix. This leads to an increase in the number of interfaces, which may give rise to more fracture surfaces resulting in crack propagation. Therefore, in order to optimize the mechanical properties of nanocomposites the surface of inorganic nanoparticles has been modified by grafting with organic molecules, which promotes polymer/inorganic-nanofiller compatibility and nanoparticle dispersion [112].

Nanocomposite materials can be prepared by adding inorganic nanoparticles or nanofibers into different polymer matrices. The size of the filler particles is an important parameter. The nano-sized fillers have a large surface area as compared to conventional (micro-sized) fillers. Nano-sized fillers can form a tighter interface with polymer matrix in composites, and hence, a high performance in mechanical properties is expected [113]. Furthermore, the intrinsic properties of nano-sized fillers contribute towards the different interactions between the filler particles and the polymer matrix. This leads to an increase in the mechanical strength and stiffness of composites in comparison to the properties of the unfilled polymer and of composites with micron-size reinforcement [114, 115]. In particular, the particle size [116, 117] and morphology [118] have measureable influences on the ability of HA nanoparticles to reinforce materials, with smaller diameters and larger aspect ratios (length/diameter) having the most profound effect on mechanical properties.

The increased specific surface area of nanoparticles showed an improved bioactivity compared to micron-sized particles. Webster et al. [119] have reported that a significant increase in protein adsorption and osteoblast adhesion has also been observed on nano-scale ceramic materials compared to micron-sized ceramic materials and composites. In related study, the bioactivity, degradation rate and mechanical properties of PLGA doped with nano-scale amorphous CaPs were strongly improved when compared to the pure polymer [120]. However, problems associated with
poor interfacial bonding and particle agglomeration may be more pronounced when using nanosized particles. As it is highlighted in the following sections, different strategies have been employed to improve the interfacial interaction between inorganic particles and polymer matrix, including silane coupling agents and polymer grafting on the surface of inorganic fillers. Recent studies [121-123] have also indicated that a sol-gel method can also be used to produce organic-inorganic hybrid materials with tailorabile mechanical properties, controlled degradation profile and improved interfacial bonding between the inorganic and organic phase. In the following section, the state of art of nanocomposites consisting of BG inorganic fillers in polymer matrices and sol-gel derived O/I hybrids for bone regeneration is reviewed.

2.3.2.3.1 *Bioactive Glass-Based Nanocomposites*

Bioactive and biodegradable nanocomposites, which combine sol-gel derived BG nanoparticles/nanofibers and biodegradable polymers, have become very promising systems for bone regeneration because of their high osteoconductivity, osteoinductivity and biodegradability. They combine the strength and bioactivity of the BG and the ductility and toughness of the biodegradable polymers. In order to yield nanocomposites with high bioactivity and strong mechanical properties, various nanocomposites containing BG nanoparticles and biodegradable polymers were developed. Hong *et al.* [124-126] prepared a 3D porous PLLA/BG nanocomposite scaffolds containing different concentrations of sol-gel derived BG nanoparticles. Addition of BG nanoparticles up to 20 wt% did not alter the morphology of the scaffold. Whereas, the *in vitro* bioactivity study demonstrated that the scaffold containing 20 wt% had the best bone-like apatite forming ability. The compressive modulus of the PLLA/BG nanocomposite scaffolds increased from 5.5 to 8.0 MPa, while the compressive strength showed a minor increase from 0.28 to 0.35 MPa as the BG content was increased from 0 to 30 wt%. The inclusion of BG nanoparticles
increased the water uptake of the nanocomposite scaffolds at lower BG content and greatly influenced the degradation rate of the PLLA matrix [124].

BG nanofibers (BGNF) prepared by electrospinning have also been used to prepare nanocomposites. The high surface area-to-volume ratio of nanofibers has been hypothesized to provide more cell attachment sites (and therefore a higher cell density per unit of area) compared with nanoparticles having lower aspect-ratio. Kim and co-workers developed well dispersed nanocomposites from PLA [127], collagen [128], PCL [129] matrices and a sol-gel-derived electrospun BGNF [130]. These nanocomposites showed good bioactivity, inducing HA precipitation on their surfaces when exposed to a SBF [128]. It was also observed that the presence of BGNF in nanocomposites improved the osteoblast-like cells attachment, spreading and proliferation.

The effect of aspect ratio of the sol-gel derived BG fillers on the biocompatibility and mechanical properties of PCL/BG composites was investigated [114]. In this cited study, PCL/BGNF nanocomposites were compared with PCL micron-sized BG particle (BGp) composites. At 20 wt% filler content, the BG nanofiber-PCL composites displayed significant improvement in both biological and mechanical properties as compared to composites with the micron-sized fillers. Tensile test results indicated that the elastic modulus of the PCL/BGNF nanocomposites was significantly higher than the PCL/BGp composites and the unfilled PCL. In addition, PCL/BGNF nanocomposites exhibited enhanced in vitro biocompatibility and osteoblast activity as compared to the PCL/BGp composites. Furthermore, in vivo animal test results revealed good biocompatibility and bone forming ability of the PCL/BGNF nanocomposite when implanted in a calvarial critical-size bone defect. In general, results from this study demonstrated the benefits of using fillers with
high aspect ratio and surface area to volume ratio (i.e., BGNF) instead on particulate filler in preparing composite scaffolds.

Surface modification of BG nanoparticles with biodegradable polymers represents a unique approach to improve the interface compatibility between the BG nanoparticles and the polymer matrix. In order to achieve this objective, a low-molecular weight PLLA was grafted to the surface of BG nanoparticles by using a diisocyanate coupling agent [131]. The enhanced interaction and adhesion between the grafted BG nanoparticles and the PLLA matrix resulted in improved mechanical properties. At lower BG content, the grafted-BG/PLLA composites exhibited greater tensile strength than ungrafted-BG/PLLA composites. However, no significant difference in tensile modulus between grafted-BG/PLLA and ungrafted-BG/PLLA nanocomposites was observed. The morphology of the tensile fractured surface of the composite also showed that surface-grafted BG nanoparticles were dispersed homogeneously in the PLLA matrix. The in vitro studies also revealed that the addition of nanoparticles improved the bioactivity of nanocomposite scaffolds [113].

2.3.2.3.2 Sol-Gel Derived Organic-Inorganic Hybrids

Organic-inorganic hybrid materials can be either homogeneous systems derived from monomers and miscible organic and inorganic components, or heterogeneous systems (nanocomposites) where at least one of the components’ domains has a dimension ranging from some angstrom (Å) to several nanometers [132]. Aside from the intrinsic physical properties of the components, hybrid materials can also display special new properties as a result of the nature and degree of interfacial interaction between the two components. Since the traditional processing conditions for inorganic materials usually involve high temperature, it precludes the incorporation of organic compounds. Thus, the
low-temperature (25-40 °C) synthesis of sol-gel process allows it to be well adapted for the preparation of organic-inorganic hybrid materials and have proven to be effective [133]. The intimate molecular mixing promotes the organic and inorganic components to form a hybrid with small grain sizes and large interfaces [133]. These interactions result in a new material, with tailorable mechanical, chemical, and physical properties depending on the desired application [132]. The chemical reactivity of organic and inorganic species is usually quite different and phase separation tends to occur during the synthesis. Therefore, it is imperative that chemical bonds are formed between the organic and inorganic components in order to produce organic-inorganic hybrids. The nature of the interfacial chemical bond has been used to categorize these materials into two distinct classes. In class I, the organic and inorganic phases exchange weak interactions such as van der Waals and hydrogen bonds. In class II materials the two phases are linked through strong covalent bonds [132-134].

Class I O/I Hybrids

Monolithic and porous O/I hybrids consisting of BG and water soluble polymers were prepared via sol-gel route. Martin et al. [123] incorporated poly(vinyl alcohol) (PVA) into the sol-gel synthesis of BG. The results of this study showed that the addition of polymer favored the synthesis of bioactive and crack-free O/I monoliths. However, an increase in PVA content resulted in disintegration of the hybrid material when exposed to a buffer solution [123]. In other studies, up to 30 wt% PVA was incorporated to prepare PVA/BG hybrid foam scaffolds with interconnected pore networks and pore size of 500 μm [113, 135]. The compression mechanical test showed that the strain at failure and compressive strength were increased for the PVA/BG hybrid as compared to pure BG foam. Conversely, lower compressive modulus was obtained for the PVA/BG hybrid foams as compared to the pure glass foam. The applicability of PVA/BG hybrid scaffolds towards
bone regeneration could be limited because of two major reasons. First, PVA is not biodegradable and second, due to the weak hydrogen bond, which links PVA and BG, the O/I hybrid is likely to fail in a physiological environment [113, 123, 135].

**Class II O/I Hybrids**

To overcome the limitations of water soluble polymer based hybrids, and improve the stability and performance under physiological conditions, linking the polymer and inorganic phase by a strong chemical bond is an alternative strategy. For this purpose, coupling agents are used to functionalize the polymer to form a covalent bond with the inorganic phase and create a class II hybrid material. One of the widely studied sol-gel derived organic-inorganic hybrid biomaterials used is Poly(dimethylsiloxane) (PDMS) precursor [136, 137]. These hybrids can be structurally described as a silica network covalently bonded to PDMS. However, the *in vitro* apatite formation ability of these hybrids was not satisfactory unless Ca\(^{2+}\) ions are incorporated in the network [138, 139]. The hybrids showed relatively large amount of an apatite-like phase deposition on their surfaces within only 12 to 24 h in simulated body fluid (SBF). From these studies, it was observed that the apatite formation ability is increased with the inorganic content, whereas PDMS provides better mechanical properties. In general, PDMS-derived hybrids show high toughness, however, their strength (< 15 MPa) and Young’s modulus (< 300 MPa) are much lower than those of natural bones. Although excellent coupling can be achieved, PDMS is not a degradable polymer. It is preferable to have a biocompatible and biodegradable polymer with a strong coupling potential.

Biocompatible and biodegradable polymers have also been incorporated in attempt to prepare O/I hybrids. PCL/Silica hybrids were successfully synthesized via sol-gel process, in which PCL is intimately mixed into the silica network [140-143]. The silica network was achieved using 3-
isocyanatopropyltriethoxysilane (IPTS) as the coupling agent. The IPTS only reacts with the
terminal hydroxyl groups; thus the amount of cross-linking in the hybrid is controlled by the
molecular weight of the polymer [144]. To increase the cross-linking in PCL hybrid, a reduction
in the molecular weight of the polymer is required. Faster and more uniform nucleation and growth
of apatite crystals was observed in the hybrid using lower molecular weight PCL. It was
hypothesized that this behavior was mainly caused by the evenly distributed and well dispersed
silica-rich domains, which acted as nucleation sites for the formation of the apatite crystals, and
partly caused by the fast degradation of the PCL phase, which induced the fast release of calcium
ion into SBF [134, 145]. The PCL content in the hybrid system affected the bioactivity and
mechanical properties of the PCL/silica hybrid material [122]. The higher PCL content in the
hybrid resulted in lower apatite-forming rate and higher toughness. On the contrary, the lower PCL
content in the hybrid exhibited higher apatite-forming rate and lower toughness. The highest values
of tensile strength, Young’s modulus, and strain at failure were achieved in the hybrids with 60
wt% PCL content and were around 21 MPa, 600 MPa, and 50%, respectively [122]. These
materials had tailorable bioactivity, degradability and mechanical properties, but the potential is
limited by the coupling sites, which are at the end of the polymer chains. The lack of Ca\(^{2+}\) ions in
the hybrid system, which is essential in providing osteogenesis and improved bioactivity of the
hybrid material, might also limit its potential application in bone tissue engineering.
Experimentally, incorporation of Ca\(^{2+}\) in the hybrid system exhibited good osteoconductivity as
hybrids are coated with bone-like apatite layer [146].

In fact, hybrid materials demonstrated some of the advantages of combining polymers with
inorganic and bioactive materials. As many of the tissues within the body are nano-scale
composites, it seems logical that this be considered when developing scaffold biomaterials for
bone regeneration and repair. The ability to use a single phase or material for such purposes may be impractical, and composites may be utilized to yield better results. Such is the case with organic-inorganic hybrids, which can exhibit a range of bioactive, resorbable, and mechanical properties. Tailoring of material chemistry and morphology can thus be employed to match these properties with the host tissue, in an effort to give better incorporation and enhanced efficacy.

2.3.3 Fibrous Scaffolds for Bone Regeneration

In view of critical scaffold design parameters and their application in bone tissue engineering, a number of techniques have been investigated to fabricate 3D scaffolds with high porosity and surface area. The conventional methods for scaffold fabrication include drop-on-demand printing [147], gas foaming [148-150], solvent casting/particulate leaching [151-160], precipitation casting [161], electrospinning [162, 163], microsphere sintering [164], particulate leaching [151, 159, 165-168], freeze-drying [48] and a combination of these techniques.

Among the various scaffold fabrication methods, the electrospinning process has recently gained significant attention as a methodology for synthesizing micro-/nano-scale fibrous structure based scaffolds for bone tissue engineering [163, 169, 170]. Electrospun fibrous scaffolds offer great advantages because they, to certain extent, mimic the nanofibrous architecture of the collagen matrix as well the possibility of the wide range of chemicals that can be chosen from to meet different application requirements. Also, the simple set-up and the low operation cost made this technique preferable for preparation of tissue engineering scaffolds [170-172].

In this non-mechanical, electrostatic technique, a high electric field is generated between a polymer solution hanging from a capillary tip or a spinneret and a grounded metallic fiber collector. When the voltage reaches a critical value, the charge overcomes the surface tension of the deformed drop
of the polymer solution formed on the tip of the spinneret, and a jet is produced. Although the jet is stable near to the tip of the spinneret, it soon enters a series of electrically induced bending instability stage with further stretching of the solution jet under the electrostatic forces in the solution as the solvent evaporates. This stretching process is accompanied by the rapid evaporation of the solvent molecules that reduces the diameter of the jet, in a cone-shaped volume called the ‘‘Taylor-cone’’. The dry fibers are accumulated on the surface of the collection plate resulting in a non-woven mesh of nano to micron diameter fibers. The process can be adjusted to control the fiber diameter by varying solution viscosity, conductivity, applied voltage, spinneret tip-to-collector distance, and humidity; whereas the duration of electrospinning controls the thickness of fiber deposition. For example, by reducing the spinneret tip-to-collector distance, mesh with interconnected fibers can be collected [173], while reducing the solution concentration will reduce the electrospun fiber diameter [130]. Although polymer chain entanglement is an important criterion for fiber formation in polymers [174, 175], the viscosity of a solution is also more general parameter. To achieve various fiber assemblies, there are generally two main methods, one is to control the flight of the electrospinning jet through the manipulation of the electric field and the other is to use a dynamic collection device. Nevertheless, by using different static collection devices, it is possible to achieve some form of fiber assemblies. To overcome various limitations of the typical electrospinning set-up and to further the performance of the electrospun fibrous mesh, researchers have come out with other modifications to the set-up [176]. Figure 2.4 illustrates a typical set-up of electrospinning process assembled with a rotating mandrel collector.
Figure 2.4: Schematic diagram of a typical electrospinning set-up.

The materials used in the development of electrospun nanofibers are considered to have properties that are specifically suitable for the calcified hard tissues, in terms of mechanical and biological aspects. In the last decade, various scaffold architectures suitable for the recruitment of osteoprogenitor cells have been designed to mimic the composition, morphological traits and mechanical properties of the native bone ECM [169]. The biomimetic architecture of nanofibrous structure provides high surface area-to-volume ratio, which allows more substrate for cell attachment (and therefore a higher cell density per unit of space) compared to other structures. In addition to the initial cell responses, further osteoblastic differentiation and mineralization have also been reported to be improved on nanofibrous surfaces compared to a dense substrate of the same material [169].
A wide range of synthetic and natural polymers was electrospun to fabricate nanofibrous scaffolds intended for bone regeneration application [17, 177]. Synthetic polymers, such as PLLA, PGA, and PCL, have been commonly used. These polymers provide great flexibility in synthesis, processing, and modification. However, their application in tissue engineering is limited due to lack of bioactivity, lower stiffness, and poor cellular affinity. On the other hand, the inherent bioactivity of the natural polymers associated with peptide sequences may influence positively the cell adhesion, proliferation, and differentiation [169, 177]. Various natural polymers, such as collagen [178], gelatin [179], silk [180], and chitosan [181] are also investigated to fabricate nanofibrous scaffolds, but their weak mechanical properties, cost, and availability for large scale production might be an issue. Since both synthetic and natural polymers have advantage and disadvantages, research has progressed to fabricate nanocomposites and O/I hybrids consisting of these polymers and bioactive inorganic fillers. However, electrospinning of composite solution is not easily implemented for the formation of a nanofibrous structure. In many cases, fibers become discontinuous due to beads and destroy continuous nanofibrous morphology, by agglomeration of the inorganic fillers. Therefore, one of the biggest challenges in electrospinning process is maintaining a homogeneous dispersion of the filler and fabricating continuous fibrous structures. 

[Ref]

In HA based nanocomposite fibers uniform dispersion and a uniform sized, bead-free fibrous morphology was obtained by using a surfactant that mediates the interface of the hydrophilic nanocrystals and the hydrophobic solution [182]. Subsequently, many publications have reported nanocomposite electrospinning using biodegradable synthetic polymers with bioactive inorganic nanoparticles, typically nano-HA and silica xerogels, and most of the nanocomposite nanofibers demonstrated some improvement in the mechanical properties and/or bone cell functions [183,
Prabhakaran et al. [185] fabricated a nanocomposite polymeric nanofibers containing nano HA by electrospinning for bone tissue engineering. They suggested that the nanocomposite PLLA/collagen/HA nanofibrous scaffold could improve the proliferation and mineralization of osteoblasts, resulting in the enhancement of bone regeneration. Recently, Li et al. [186] also reported a bioactive PCL/nano-HA fibrous scaffold for bone regeneration, which employed a coupling agent to improve the dispersion of HA in the PCL matrix. Consequently, the good dispersion of HA nanoparticles in the PCL matrix enhanced the tensile strength and modulus of the scaffold. Although the particulate bioactive inorganics were considered as a suitable fillers to fabricate composite fibrous scaffolds for bone regeneration, the size of the particles, their homogeneity/dispersability, and the maximum filler content have still been an issue to improve for the successful electrospinning.

To overcome the challenges and limitations observed during composite fiber fabrication, sol-gel chemistry based organic-inorganic hybrid scaffolds preparation has been pursued. As one example, a silicon-based inorganic precursor (glycidoxypropyl trimethoxysilane) was homogenized with a natural polymer gelatin, which was then aged to form siloxane groups and linkages with the amino acids of gelatin to generate a hybridized structure [187]. The siloxane-gelatin hybrid solution was electrospun into a continuous nanofiber, and the hybrid nanofiber exhibited an excellent ability to form bone mineral and demonstrated improved osteoblastic activity in vitro, thus proving to be a candidate substrate for bone regeneration [187]. Compared to the composite approach, only few reports discussed on hybrid fibrous scaffolds. Therefore the main objective of the current research is to develop biodegradable and bioactive O/I hybrid fibrous scaffolds via a sol-gel process, and study the effect of hybridization process on physicochemical, mechanical and biological properties of the resultant hybrid scaffolds.
2.4 SUMMARY

In the preceding sections, literature review pertinent to this thesis is presented. Due to rapid advances made in bone tissue engineering and regeneration, it was not possible to include all aspects of the field. However, every effort is made to ensure that seminal works and significant research findings are included, with minimal bias, in this Chapter. Notwithstanding the different challenges and opportunities presented by bone tissue engineering strategies, this thesis will only attempt to address limitations on development of bioactive and biodegradable fibrous scaffold materials for bone regeneration.

2.5 HYPOTHESIS AND OBJECTIVES OF THE STUDY

The overlying hypothesis of this work is that organic-inorganic hybrid biomaterials consisting of BG and biodegradable polymers will overcome the limitations associated with the current synthetic bone grafts, in turn; they will have improved bioactivity, mechanical properties and osteogenic potential as ultimately used for bone regeneration. It is also hypothesized that the sol-gel process provides a suitable way to combine the BG and biodegradable polymers on a molecular level and tailor the physical and biological property of the resultant hybrid biomaterials. It is further hypothesized that 3D organic-inorganic fibrous scaffolds prepared by combined sol-gel/electrospinning process will potentially mimic the micro-nano architecture of the bone ECM and become a suitable platform for bone regeneration. In order to test the above hypotheses, the following objectives are formulated:

I. To synthesize and characterize biodegradable and bioactive organic-inorganic hybrid biomaterials consisting of BG and PCL via a sol-gel process for bone tissue engineering.
II. To study the effect of polymer content on *in vitro* hydroxyapatite formation ability (bioactivity) and mechanical properties of hybrid biomaterials for bone tissue engineering.

III. To fabricate and characterize biomimetic 3D organic-inorganic hybrid fibrous scaffolds by the electrospinning technique.

IV. To investigate the benefits of electrospun 3D porous organic-inorganic hybrid scaffolds in terms of cell attachment, proliferation and bone related markers gene expression.
2.6 REFERENCES


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CHAPTER 3: SYNTHESIS AND ELECTROSPINNING OF POLY(ε-CAPROLACTONE)/BIOACTIVE GLASS HYBRID BIOMATERIALS VIA A SOL-GEL PROCESS*

Overview: This Chapter discusses about the synthesis and characterization of poly(ε-caprolactone)/Bioactive glass hybrid biomaterials via a sol-gel process for bone regeneration. The presence of functional groups and interfacial bond between the organic and inorganic phase by FTIR spectroscopy, structural properties by XRD, surface morphology and elemental analysis by SEM/EDX technique, thermal properties and organic-inorganic ratio determination by TGA are described. Furthermore, the 3D PCL/BG hybrid scaffold fabrication by electrospinning technique and its potential for bone regeneration is elucidated.

3.1 SUMMARY

Strategies of bone tissue engineering and regeneration rely on bioactive scaffolds to mimic the natural extracellular matrix (ECM) as templates onto which cells attach, multiply, migrate and function. For this purpose hybrid biomaterials based on selective combinations of biodegradable polymers and bioactive glasses are of particular interest, since they exhibit tailored physical, biological and mechanical properties as well as predictable degradation behavior. In this study, hybrid biomaterials with different organic-inorganic ratios were successfully synthesized via a sol-gel process. Poly(ε-caprolactone) (PCL) and tertiary bioactive glass (BG) with a glass composition of 70 mole % SiO₂, 26 mole % CaO and 4 mole % of P₂O₅ were used as the polymer and inorganic phases, respectively. The polymer chains were successfully introduced into the inorganic sol while the networks were formed. Fourier transform infrared spectroscopy (FTIR), X-ray diffraction

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XRD), thermogravimetric analyses (TGA), scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX) were used to investigate the presence of different chemical groups, structural crystallinity, thermal property, elemental composition and homogeneity of the synthesized hybrid biomaterials. Identification of chemical groups and presence of molecular interaction by hydrogen bonding between the organic and inorganic phases was confirmed by FTIR. The XRD patterns showed that all PCL/BG hybrids (up to 60% polymer content) were amorphous. The TGA study revealed that the PCL/BG hybrid biomaterials were thermally stable and good agreement was observed between the experimental and theoretical organic-inorganic ratios. The SEM/EDX results also revealed a homogeneous elemental distribution and demonstrated the successful incorporation of all the elements in the hybrid system. Finally, these synthesized hybrid biomaterials were successfully electrospun into 3D scaffolds. The resultant fibers have potential use as scaffolds for bone regeneration.

3.2 INTRODUCTION

The holistic approach in bone tissue engineering involves the combination of progenitor or primary cells and biocompatible scaffold materials with or without appropriate growth factors, to initiate tissue repair and regeneration [1-3]. Scaffold materials designed for bone tissue regeneration are desired to be biodegradable, biocompatible, osteoconductive, bioactive and must be mechanically matched with the native bone [4, 5]. Several materials including synthetic and natural polymers [6, 7], and ceramics [8-10] were developed with the objective of regenerating diseased and damaged bone tissue structures.

Bioactive glasses (BGs) and biodegradable synthetic polymers were among the most widely studied materials for orthopedic applications. BGs are known for their excellent biocompatibility,
biodegradability, osteoconductivity and ability to form a bone-like mineral phase at the interface when in contact with living tissues (i.e. bioactivity) [11, 12]. BGs are produced either by melt-derived approach at high temperature (ca. 1400 °C) or by sol-gel process at low temperature (typically 25 °C - 40 °C). The sol-gel chemistry is generally divided into two steps: hydrolysis of metal alkoxides to produce hydroxyl groups followed by polycondensation of the hydroxyl groups and residual alkoxyl groups to form a three-dimensional (3D) network (see detailed chemistry in ref.[13]). However, BGS are stiff and brittle making them difficult to be formed into complex shapes and are susceptible to fracture under mechanical loads. On the other hand, most aliphatic polyesters are also biocompatible, biodegradable and can be readily processed into 3D porous structures [7] but, they too lack strength and mechanical stability to match with bone tissue, especially when made with large volume fractions of macroporosity [2, 6]. Hence, despite the availability of materials with appropriate biological and structural properties for bone tissue engineering they still need improvement in their mechanical behavior. One strategy that has been considered and studied to enhance the toughness of synthetic biomaterials is the synthesis of organic-inorganic hybrid materials [14, 15]. These materials are based on selective combination of biodegradable polymers and bioactive glass and, exhibit tailored physical, biological and mechanical properties as well as predictable degradation behavior [16]. This type of structural organization of synthetic materials resembles the structure of bone tissue, where the inorganic component mimics the carbonate hydroxyapatite and the polymer component mimics the collagen-rich extracellular matrix.

Recently, the sol–gel synthesis route has been reported to be a viable approach to combine organic-inorganic components at the nanoscale and/or molecular level [17, 18]. The idea is to introduce polymer chains into the sol while the inorganic network is being formed. The polymer type to be
incorporated influences the structure and properties of the resulting hybrids because of the chemical interactions established between the organic and inorganic components. Unfortunately, due to their poor solubility many biodegradable polymers cannot be easily introduced into the sol. Therefore, investigators studied only a non-degradable and water soluble poly(vinyl alcohol) (PVA) to synthesize polymer/BG hybrid materials via the sol-gel route [15, 18]. Only few studies attempted to introduce a biodegradable polymer, PCL, in the sol-gel process [19, 20]. In these studies, either silica was used as a sole inorganic component or, where a binary inorganic components were used, the use of calcium nitrate meant that the polymer must be burnt off before the BG can be used due to the toxicity of the nitrate [21]. Whereas the incorporation of calcium in BGs via a sol-gel process has been reported with a limited success (due to calcium segregation) [22-24], further studies are needed to prepare a homogenously distributed calcium into organic-inorganic hybrid materials.

In view of the above, the objectives of the present study were twofold: (i) to synthesize and characterize homogeneous bioactive and biodegradable organic-inorganic hybrid materials from BG and PCL via a sol-gel process and; (ii) to demonstrate the potential application of the PCL/BG hybrid system for 3D scaffold fabrication by electrospinning. This study is the first to report the synthesis and characterization of sol-gel derived hybrid biomaterials from PCL and BG with tertiary glass composition.

3.3 EXPERIMENTAL SECTION

Poly (ε-caprolactone) (PCL) (CAPA® 6800, MW 80,000 g/mol) was kindly supplied by Solvay Chemicals Inc. (Houston, TX). Tetraethyl orthosilicate (TEOS, 98 %), triethyl phosphate (TEP, 99.8 %), and calcium chloride dihydrate (CaCl₂·2H₂O, 99 %) were purchased from Sigma-Aldrich
Hydrochloric acid (HCl) was purchased from Caledon laboratory chemicals (Georgetown, ON). Methyl ethyl ketone (MEK) and ethanol (EtOH) were obtained from a solvent purification system.

### 3.3.1 Synthesis of PCL/BG Hybrids

Schematic flowchart for the synthesis of PCL/BG hybrid biomaterials by the sol-gel process is presented in Figure 3.1. A polymer solution was first prepared by dissolving known amount of PCL pellets in MEK at 35 °C under moderate stirring for 12 h. After the PCL is completely dissolved, TEOS (8.87 mL), DI water, TEP (0.69 mL) and CaCl$_2$·2H$_2$O (1.91 g dissolved in 5 mL ethanol) were added successively under vigorous stirring in the presence of a catalytic amount of 1N HCl. The molar ratio of H$_2$O/TEOS was maintained at 8:1. Hydrolysis of TEOS was carried out while the sol/polymer mixture was stirred at 35 °C for 72 h until a homogeneous and transparent sol was obtained. Solvent loss during the sol formation was prevented by fitting a condenser. Following this, the sol was sealed with parafilm and kept at 50 °C in an oven for 72 h for polycondensation and network formation. Ageing and drying of PCL/BG hybrid gels were carried out at 60 °C under controlled humidity of 95% for one week. The compositions of PCL/BG hybrids and reagents used are summarized in Table 3.1.
Figure 3.1: Schematic flowchart for the synthesis of PCL/BG hybrid material via a sol-gel process.
Table 3.1: Compositions of PCL/BG hybrid biomaterials.

<table>
<thead>
<tr>
<th>PCL/BG (wt. %)</th>
<th>Sample code</th>
<th>PCL/MEK (wt./vol)</th>
<th>H₂O/MEK (vol/vol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/100</td>
<td>BG</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10/90</td>
<td>H1090</td>
<td>0.05</td>
<td>1.00</td>
</tr>
<tr>
<td>20/80</td>
<td>H2080</td>
<td>0.10</td>
<td>0.91</td>
</tr>
<tr>
<td>40/60</td>
<td>H4060</td>
<td>0.20</td>
<td>0.63</td>
</tr>
<tr>
<td>50/50</td>
<td>H5050</td>
<td>0.20</td>
<td>0.40</td>
</tr>
<tr>
<td>60/40</td>
<td>H6040</td>
<td>0.20</td>
<td>0.31</td>
</tr>
<tr>
<td>100/0</td>
<td>PCL</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

3.3.2 Electrospinning of PCL/BG Hybrid Biomaterials

The viscosity of the sol was closely monitored by Brookfield DV-II+ Programmable Viscometer (Brookfield Engineering Laboratories, Middleboro, MA) during the polycondensation and network formation. Once the viscosity reached 27 mPa.s, 1 mL of the viscous solution was transferred to a plastic syringe equipped with a stainless steel needle, which was connected to a high-voltage (15 kV) supply. The flow rate was 0.25 mL/h and it was controlled by a syringe pump. A piece of flat aluminum foil was placed at the grounded stationary collector 6 cm far from the needle tip to collect the hybrid fibers. For the electrospinning study, a hybrid composition of 50 wt. % PCL and 50 wt.% of BG (H5050) sample was used.
3.3.3 Characterization of PCL/BG Hybrids

3.3.3.1 Fourier Transform Infrared spectroscopy (FTIR)

Bruker IFS 55 FTIR (Bruker Optics, Billerica, MA) was used to characterize the presence of specific functional groups in the PCL/BG hybrid material and hydrogen bond formation between the organic and inorganic phases. A small amount of each sample was mixed with potassium bromide (KBr) powder, pressed as a pellet, and analyzed at a resolution of 4 cm$^{-1}$ and sample scans of 32. All spectra were analyzed utilizing the OPUS software version 4.0.

3.3.3.2 X-ray diffraction (XRD)

XRD measurements of the samples were carried out using a rotating anode X-ray diffractometer model RTP300 (Rigaku Rotaflex, Japan) operating on CoK$\alpha$ radiation with $\lambda = 1.7891$ Å and run at 45 kV and 160 mA. Samples for XRD measurements were prepared by crushing the PCL/BG hybrid monolith into powders and by chopping PCL/BG hybrid fiber mat to reduce the aspect ratio. Pure PCL cast film prepared from an MEK solution was used as a control. XRD measurements were conducted in the $2\theta$ range from 2 to 82° with steps of 0.02.

3.3.3.3 Thermogravimetric analyses (TGA)

TGA experiments were carried out under air using a Mettler TGA850 thermal analyzer (Mettler-Toledo Inc., Columbus, OH). Samples weighing approximately 20–40 mg were heated from 25 °C to 800 °C at a rate of 10 °C/min. The onset and maximum degradation temperatures for each sample are reported. The residual masses at 800 °C were recorded to estimate the organic/inorganic ratio in the hybrid material.
3.3.3.4 Scanning electron microscope (SEM) and energy dispersive X-ray spectroscopy (EDX) analyses

The surface morphology of the hybrid samples were visualized by using S-2600N SEM (Hitachi, Japan). Elemental distribution and chemical composition of the hybrid sample were further analyzed by using an EDX detector attached to the S-2600N SEM. Specimens cut from the monolithic hybrid sample and from the electrospun fiber mat were sputter coated with gold using K550 sputter coater (Emitech Ltd, UK) and scanned at a working distance of 11 mm and a constant accelerating voltage of 25 kV. High resolution SEM images were captured using model FIB/SEM LEO 1540XB microscope (Carl Zeiss, Oberkochen, Germany) operating at electron beam voltage of 1 kV. The fiber diameters and pore sizes of the electrospun mat were determined using ImageJ software (National Institutes of Health, USA).

3.3.3.5 Atomic Force Microscope (AFM) analysis

The surface roughness of the mat was measured using AFM. Specimens were first glued on glass coverslips and, in a second step, individual coverslips were attached to a steel sample holder disk. Images were acquired in contact mode with a Multimode AFM with Nanoscope IIIa controller using an NP-S cantilever of spring constant 0.12 N/m (Veeco Instruments, Santa Barbara, CA).
3.4 RESULTS

3.4.1 Synthesis of PCL/BG Hybrid Biomaterials

Organic-inorganic hybrids consisting of different ratios of PCL (10% to 60 wt. %) and tertiary BG (with glass composition of 70 mole % SiO$_2$, 26 mole % CaO and 4 mole % of P$_2$O$_5$) were synthesized via a sol-gel process. As shown in Table 3.1, PCL was used because it is a biocompatible and biodegradable aliphatic polyester with good mechanical properties and, is widely used for various biomedical applications [25-27]. Furthermore, the ester carbonyl groups of PCL are capable of forming intermolecular hydrogen-bonding with the silanol groups (Si-OH) on the surface of the inorganic network produced in the sol–gel process [28]. The glass composition used in this study was chosen based on a previous report that demonstrated its bioactivity [29].

The addition of water (H$_2$O: TEOS molar ratio of 4:1 to 8:1) to the inorganic components is required for complete hydrolysis of TEOS. The presence of water combined with the hydrophobic nature of PCL limits the solubility of PCL in the inorganic sol. In fact this was a major limitation for the synthesis of a hybrid material using biodegradable hydrophobic polymers. For this reason, previous studies used a non-biodegradable PVA polymer because of its water solubility [15]. In the current study MEK was used to dissolve PCL and, when the tertiary inorganic components and water were added to the polymer solution, mixing was accomplished without phase separation. In addition to its water miscibility, MEK has a relatively low boiling point and an ability to perform as a co-solvent to dissolve the inorganic precursors without affecting the hydrolysis of TEOS. Despite the miscibility of MEK at all proportions with water, it was kept to the optimum amount (Table 3.1) because it affects the success of the sol-gel reaction and homogeneity of the hybrid material. For example, it was observed that at higher H$_2$O: MEK ratio, the polymer phase started
to separate from the sol whereas at lower ratios the rate of hydrolysis was reduced and the gelation time was increased. Finally, temperature had a significant effect on the synthesis of the current hybrid biomaterials by sol-gel chemistry. Although an increase in temperature improved the homogeneity of the organic/inorganic components [30], TEOS hydrolysis was favored at low temperatures because the reaction is exothermic and hence, thermodynamically not favored at high temperatures [31]. In the present study, the optimum sol/polymer mixture temperature for the TEOS hydrolysis was found to be 35 °C.

3.4.2 FTIR characterization of PCL/BG Hybrid Biomaterials

FTIR was used to investigate the presence of hydrogen bonding interaction between PCL and the inorganic phase. The carbonyl functional groups of the PCL can form hydrogen bonding with the silanol (≡ Si-OH) groups on the surface of the silica network of the inorganic phase. The source of the silanol groups is from the incomplete polycondensation of the TEOS [30]. Figures 3.2 and 3.3 display the FTIR spectra of pure PCL, BG gel, and PCL/BG hybrids. Additional peak assignments are summarized in Table 3.2.

In Figure 3.2, the peak at 1640 cm\(^{-1}\) is attributed to –OH groups from residual moisture whereas the peak at 1072 cm\(^{-1}\) is due to of Si-O-Si stretching. The characteristic peaks of PCL are observed at 1730 cm\(^{-1}\) (unbonded C=O), 732 cm\(^{-1}\), 840-1470 cm\(^{-1}\), 2892 cm\(^{-1}\), 2930 cm\(^{-1}\), and 2974 cm\(^{-1}\). The FTIR peaks in the range of 3000–3700 cm\(^{-1}\) (Figure 3.3A) and centered at 3400 cm\(^{-1}\) is assigned to the hydroxyl stretching vibrations of the self-associated silanol groups and the width of the peak reflects the wide frequency distribution of the hydrogen bonded –OH groups. With an increase in PCL concentration the -OH peak broadened, indicating the formation of hydrogen bond interactions between silanol hydroxyls (Si-OH) of the silica network and carbonyls of the PCL.
Figure 3.3B shows the FTIR spectra of the pure PCL and PCL/BG hybrid materials in the wavenumber range of 1650 to 1800 cm\(^{-1}\). The peaks observed in this region are ascribed to the stretching carbonyl (C=O) of PCL. The peak at 1730 cm\(^{-1}\) is the characteristic peak for pure PCL. New shoulder peaks appeared at low wavenumbers (\(\approx 1700 \text{ cm}^{-1}\)) for all compositions of PCL/BG hybrid materials. The shoulder peaks are assigned to the stretching vibration of the carbonyls of PCL that are hydrogen bonded with the silanol hydroxyls of silica. This observation indicates that intermolecular hydrogen bonds are formed between the carbonyls of PCL and the silanol hydroxyls of the silicon oxide network.

**Figure 3.2**: FTIR spectra of pure PCL, BG gel and PCL/BG hybrid biomaterials.
Figure 3.3: (A) FTIR spectra of the hydroxyl stretching vibration in the range of 3000–3800 cm\(^{-1}\) for BG gel and PCL/BG hybrids; (B) FTIR spectra of the carbonyl stretching vibration in the range of 1650–1800 cm\(^{-1}\) for pure PCL and PCL/BG hybrids.
Table 3.2: Summary of Major FTIR peaks associated with PCL, bioactive glass and the PCL/BG hybrid systems.

<table>
<thead>
<tr>
<th>Material, PCL/BG hybrid</th>
<th>Wavenumber, cm⁻¹</th>
<th>Peak assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG</td>
<td>1076 -1232</td>
<td>Si-O-Si stretching [32, 33]</td>
</tr>
<tr>
<td></td>
<td>945</td>
<td>Si-OH stretching</td>
</tr>
<tr>
<td></td>
<td>1640</td>
<td>O-H bending (molecular water)</td>
</tr>
<tr>
<td>BG, PCL/BG hybrid</td>
<td>3000-3600</td>
<td>O-H stretching</td>
</tr>
<tr>
<td>PCL/BG hybrid</td>
<td>1700</td>
<td>-C=O (H-bonded carbonyl)</td>
</tr>
<tr>
<td>PCL</td>
<td>1730</td>
<td>-C=O (free carbonyl)</td>
</tr>
<tr>
<td></td>
<td>2892, 2930, 2974</td>
<td>asymmetric C-H stretching</td>
</tr>
<tr>
<td></td>
<td>1482</td>
<td>C-H bending</td>
</tr>
</tbody>
</table>

3.4.3 XRD Characterization of PCL/BG Hybrid Biomaterials

The XRD patterns for PCL, BG, and PCL/BG hybrid biomaterials are shown in Figure 3.4. The pure bioactive glass showed no peaks indicating that the sample was completely amorphous. In contrast, several peaks were observed from the XRD patterns of pure PCL because PCL had a semi-crystalline structure [34]. In Figure 3.4, two distinctive diffraction peaks are observed at 2θ = 25.03° and 2θ = 27.7°, which were indexed to be (110), and (200) planes, respectively, of an orthorhombic crystalline structure of PCL [35]. On the other hand, all compositions of PCL/BG hybrids showed the absence of diffraction peaks indicating that the current hybrid materials were amorphous despite the semi-crystalline PCL used in their preparation. The observed amorphous character of the PCL/BG hybrids was attributed to the confinement of PCL crystallization within the inorganic network [36].
3.4.4 Thermogravimetric Analyses of PCL/BG Hybrid Biomaterials

TGA was used to evaluate the thermal stability and to estimate both the organic and inorganic contents (calculated based on the residual weight at 800 °C) of the PCL/BG hybrid biomaterials.
Primary TGA and derivative weight loss curves for the pure PCL, BG gel, and the PCL/BG hybrids are presented in Figure 3.5.

The pure PCL sample underwent a single stage degradation whereby complete decomposition of the polymer occurred between 390 °C and 435 °C with an inflection temperature of 414 °C. In contrast, the pure bioactive glass had two distinct stages of degradation and showed approximately 19 % weight loss below 250 °C, which could be caused by the elimination of water, solvents and unreacted components (specifically TEOS) of the precursor compounds. From 250 °C to 800 °C the weight loss for the pure BG was insignificant and the residual weight measured at 800 °C was determined to be 70% of the initial weight. The TGA thermograms of the PCL/BG hybrid materials showed multiple stages of thermal decomposition. Similar to the pure BG gel, the first weight loss (below 250 °C) occurred in two stages and is associated with the elimination of water, residual solvents, and cleavage of unreacted and thermally unstable components. The second weight loss that is observed between 250 °C and 490 °C also took place in two stages with the exception of H1090 which has the lowest (ca. 10 %) PCL. The weight loss in this temperature range is primarily due to the decomposition of PCL components. Interestingly as the PCL content increased from 10 % to 60 %, the decomposition temperature range of the hybrid materials is broadened. With increased PCL content the residual weight at 800 °C is decreased. The inflection temperature, weight loss and residual inorganic contents for all the samples determined from TGA are summarized in Table 3.3.
Figure 3.5: (A) TGA curves of the pure PCL, BG and PCL/BG hybrid biomaterials synthesized by sol-gel process. (B) Derivative of weight percent versus temperature curves for pure PCL, BG gel and PCL/BG hybrids biomaterials. Where, (a) BG gel, (b) H1090 hybrid, (c) H2080 hybrid, (d) H4060 hybrid, (e) H5050 hybrid, (f) H6040 hybrid, and (g) pure PCL.
Table 3.3: Summary of thermogravimetric analyses results of pure PCL, BG gel and PCL/BG hybrid biomaterials.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Residual Mass at 800°C, Wt. %</th>
<th>Organic/Inorganic ratio based on residual mass at 800°C*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>On/End Set T, °C</td>
<td>Mass loss, Wt. %</td>
<td>On/End Set T, °C</td>
<td>Inflection T, °C</td>
</tr>
<tr>
<td>PCL</td>
<td>-</td>
<td>-</td>
<td>393 – 433 (N/A)</td>
<td>414</td>
</tr>
<tr>
<td>H6040</td>
<td>70 – 208</td>
<td>16</td>
<td>290 – 498 (498-800)</td>
<td>378</td>
</tr>
<tr>
<td>H5050</td>
<td>89 – 202</td>
<td>14</td>
<td>307 – 483 (483-800)</td>
<td>376</td>
</tr>
<tr>
<td>H4060</td>
<td>102 – 198</td>
<td>12</td>
<td>312 – 491 (491-800)</td>
<td>376</td>
</tr>
<tr>
<td>H2080</td>
<td>93 – 250</td>
<td>18</td>
<td>378 – 475 (475-800)</td>
<td>400</td>
</tr>
<tr>
<td>H1090</td>
<td>89 – 250</td>
<td>22</td>
<td>336 – 437 (437-800)</td>
<td>334</td>
</tr>
<tr>
<td>BG</td>
<td>92 – 205</td>
<td>19</td>
<td>N/A</td>
<td>-</td>
</tr>
</tbody>
</table>

* Organic/Inorganic ratios were determined as follows:

\[
\left( \frac{\% \text{ mass loss from stage II}}{\% \text{ mass loss from stage II} + \% \text{ residual mass at 800°C}} \right) \times 100
\]

The organic/inorganic ratio values obtained from the TGA (column 8, Table 3.3) results are in good agreement with the theoretical (see column 1, Table 3.3) estimation of the final organic/inorganic ratio.
3.4.5 Morphology and Homogeneity of the PCL/BG Hybrid Biomaterials

The morphology and elemental distribution for all compositions of the hybrid samples were investigated by using SEM and EDX. As an example, the SEM and EDX results for H2080 and H505 hybrid samples are presented in Figures 3.6 and 3.7. The SEM analyses of the hybrid samples revealed a smooth and homogeneous surface (Figures 3.6A and 3.7A). The homogeneity of the hybrids was further confirmed by EDX elemental analyses. As shown in Figures 3.6 and 3.7, the elemental distribution of carbon, calcium, phosphorous, and silicon atoms within the hybrid matrix elucidates the homogeneity of the synthesized hybrid materials. Since silicon and phosphorous atoms are the network formers and the calcium atom is a network modifier of the inorganic network, investigating their elemental distribution and concentration in the hybrid system is important to understand the extent of structural homogeneity of the hybrid system. The elemental analyses was conducted on three different samples (n=3) which were synthesized in separate experiments, and the experimental weight percentage of silicon, calcium and phosphorous atoms in the bulk hybrid matrix were determined. The result obtained from the elemental analyses was compared with the theoretical compositions of each element in the starting material. The inorganic elemental compositions were determined for the elements in the inorganic phase, and values are shown in Table 3.4. The silicon and calcium contents obtained from the elemental analyses closely matched with the initial composition. However, the phosphorous content was significantly higher in the hybrid materials compared with the initial composition. This is presumably caused by the small deviation of the silicon and calcium contents that resulted in a significant increase in the amount of phosphorous.
Figure 3.6: SEM image, EDX spectra and elemental mapping of H2080 hybrid biomaterial; A) SEM image, B) EDX spectra; C, D, E & F are elemental mapping of carbon, calcium, phosphorous, and silicon atoms, respectively.
**Figure 3.7:** SEM image, EDX spectra and elemental mapping of H5050 hybrid biomaterial; A) SEM image, B) EDX spectra; C, D, E & F are elemental mapping of carbon, calcium, phosphorous, and silicon atoms, respectively.
Table 3.4: Theoretical and experimental weight percent of elemental compositions of silicon, calcium and phosphorous. The experimental values were determined by EDX and data are expressed as mean ± SD for n=3.

<table>
<thead>
<tr>
<th>Elements</th>
<th>Theoretical (wt. %)</th>
<th>Experimental (wt. %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicon</td>
<td>60.30</td>
<td>61.3 ± 4.10</td>
</tr>
<tr>
<td>Calcium</td>
<td>32.10</td>
<td>27.7 ± 1.77</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>7.60</td>
<td>14.6 ± 4.67</td>
</tr>
</tbody>
</table>

3.4.6 Electrospinning of PCL/BG Hybrid Biomaterials

Rheological property is one important parameter that is known to affect electrospinning [37]. At higher PCL content, the polycondensation of the inorganic silica was slow because of a dilution effect by the polymer and solvents and, ultimately resulted in an easily spinnable sol. Conversely, when the PCL content is low, the polycondensation of the inorganic silica was relatively fast. Thus finding the right window of operation for spinnability is important. Under the experimental conditions, the viscosity increased continuously (albeit slowly) during electrospinning. The optimum viscosity of the gelling solution was determined to be between 25 mPa.s and 35 mPa.s giving a 24 h window for electrospinning. Whereas above 35 mPa.s, the solution could not spin. Based on 1mL solution, a fibrous product with mesh thickness ranging from 0.5mm to 1mm was obtained. Besides the viscosity range, flow rate (0.05–0.25 mL/h), distance to collector (5–8 cm), voltage (10 – 22 kV) were acceptable processing windows.

In Figure 3.8, the SEM image showing the morphology of PCL/BG (H5050) electrospun hybrid mat and fiber diameter distribution plot are presented. Although the other ratios could equally be electrospun, this ratio was chosen as an initial study because it represented an equal amount of PCL and tertiary bioactive glass. The mean and standard deviation were calculated based on
randomly picked 100 fibers (n=100) from the SEM image of the electrospun mat. The SEM image and the accompanying fiber diameter distribution plot of the H5050 PCL/BG electrospun hybrid mat demonstrated the successful fabrication of a continuous and uniform PCL/BG (H5050) hybrid fiber with a diameter of 0.32 ± 0.10 μm.

**Figure 3.8**: SEM image and fiber diameter distribution plot of electrospun fiber mat for H5050 PCL/BG hybrid biomaterial.

The roughness of the fibers was examined using high resolution SEM and AFM. Figures 3.9A and 3.9B show that the hybrid fibers had smooth surfaces on a single fiber level. An attempt to further zoom-in the fibers resulted in the partial melting of the PCL component (Figure 3.9B) even at a lower accelerating voltage of 1kV. The 3D roughness profile from AFM analysis of the fiber mat
presented in Figure 3.9C shows a root mean square height variation of 1.8 µm. When the individual fibers were zoomed-in, they had height variations of no more than 2 nm. The AFM data is consistent with the images obtained from high resolution SEM. Furthermore, the pore sizes of the electrospun mat revealed that the current hybrid fibers had an average value of 5 µm, which is similar to reported values for electrospun composite bioceramic fibers [38].

**Figure 3.9:** High resolution SEM images of H5050-fibers (A, B), AFM 3D roughness profile of the electrospun-mat (C) and pore-size distribution within the mat (D).
The XRD pattern (Figure 3.10) of the electrospun hybrid mat showed the absence of the diffraction peak indicating that the electrospun hybrids mat were amorphous despite the semi-crystalline PCL used in their preparation. This result is consistent with the results observed for the monolithic hybrid samples (Figure 3.4). The non-crystalline nature of the hybrid fibers is important because no confounding results will be expected when these fibers are studied for crystalline bone-like matrix formation in a simulated body fluid or under cell culture conditions.

**Figure 3.10**: XRD pattern of pure PCL and H5050 PCL/BG hybrid fiber.

The EDX elemental map of the hybrid electrospun fiber mat, shown in Figure 3.11, also revealed a homogeneous distribution of carbon, calcium, phosphorous, and silicon atoms. Bright areas in the images (as indicated by the arrows in Figure 3.11) indicate relative high concentration of the
elements, while dark areas indicate relative low concentrations of the elements. This, however, does not suggest heterogeneity but merely the effect of surface texture of the electrospun mat used in the EDX analyses. This in turn means that some of the fibers to be much closer to the electron detector resulting in an apparent intensity variation on the EDX elemental map (Figure 3.11).

**Figure 3.11:** SEM image, EDX spectra and elemental mapping of H5050 hybrid fiber; A) SEM image, B) EDX spectra; C, D, E & F are elemental mapping of carbon, calcium, phosphorous, and silicon atoms, respectively. Arrows indicate intensity variations due to surface texture.
3.5 DISCUSSION

The sol-gel process that involves the acid-catalyzed hydrolysis of alkoxide precursors followed by polycondensation to form a glass gel network is versatile for the preparation of micro and nanoporous bioactive glasses for biomedical applications. Despite their bioactivity, sol-gel derived glasses are inherently brittle and, polymers are often incorporated to impart toughness thus forming nanocomposites. In an attempt to create bioactive nanofiber/biodegradable polymer composites, Kim and coworkers adopted a three step process [39-42]. In the first step, a water or alcohol-soluble non-degradable polymer was added into the sol and electrospun to form nanofibers. In the second step, the nanofibers were heated to over 600 °C to burn off the polymer and remove the nitrate that is often used as a calcium precursor. In the final stage, the nanofibers were milled and used as fillers with biodegradable polymers to form nanocomposite scaffolds. Because the biodegradable polymer components were not added to the sol while the network is being formed during the polycondensation, these nanocomposites are not considered to be hybrid materials whereby the bioactive glass components and the polymer chains are interacting via chemical bonding on a molecular scale. In a more recent study [38], composite fibers were also reported by electrospinning of PCL mixed with hydroxyapatite and β-tricalcium phosphate. Despite the inclusion of PCL in the electrospinning process, these composite fibers were, however, two phase materials where the inorganic matrix is suspended in a continuous PCL matrix. Unlike these cited studies, the PCL-bioactive glass compositions in this study are hybrid materials with a single phase as evidenced by the complete disappearance of the PCL crystalline peak (Figures 3.4 and 3.10).
One clear advantage afforded by sol-gel process is the formation of a single phase hybrid materials from two seemingly incompatible systems. As stated in the introduction, a limited number of studies attempted to introduce a biodegradable polymer, PCL, in the sol-gel process [19, 20]. Despite these efforts, there is little practical interest for these materials to be used for bone regeneration since; either silica or binary glasses were used as inorganic component, the use of calcium nitrate meant that the polymer must be burnt off before the BG can be used due to the toxicity of the nitrate [21]. The present study is the first to successfully incorporate biodegradable PCL into the sol-gel process in the presence of tertiary glass components (i.e. SiO$_2$, CaO and P$_2$O$_5$). Through a number of pre-screening experiments, the amount of MEK required to dissolve PCL and the hydrolysis temperature for TEOS were determined while the sol is always a homogenous mixture without phase separation. The current work is also the first to report the electrospinning of hybrid materials to fabricate fiber-based 3D scaffolds using biodegradable PCL and tertiary BG. As shown in Figures 3.8 and 3.9, scaffolds with an average fiber diameter of 320 nm were obtained.

In addition to the successful incorporation of PCL into the sol which was subsequently electrospun, the calcium homogeneity throughout the monolith as well as the electrospun mat was a significant contribution of the present work. Calcium is an important component of BG-based biomaterials as it is required to stimulate osteoprogenitor cells [43, 44], but the incorporation of a uniformly distributed calcium in the network proved to be a formidable task [23]. In the preparation of bioactive glasses, calcium nitrate has been conventionally used as a precursor but a temperature of at least 600 °C is needed to remove the nitrate by-products which otherwise are toxic to cells [21, 45]. The PCL/BG hybrids in the present study cannot be heated to this high temperature because the PCL will be completely degraded. Therefore the use of CaCl$_2$.2H$_2$O as a calcium precursor due
to its solubility in water and non-toxicity to cells was used [18]. Calcium ions are known to be loosely bonded with the SiO₂ and P₂O₅ and could be easily washed out by the solvent during drying or segregate in the hybrid matrix, resulting in a heterogeneous calcium distribution [23]. To obtain a homogeneous hybrid system with uniform calcium distribution, the drying of the hybrid gel was carried out in a controlled humidity environment. Relative humidity of the drying chamber was kept at equilibrium and, the rate of evaporation of solvents was also controlled by sealing the sample container with parafilm. Because the PCL incorporated in the hybrid material has a low melting point, the temperature of the drying chamber was set at 60 °C. The final drying of the samples was conducted under vacuum in a Teflon mold covered with a parafilm.

Tissue engineering and regeneration of bone require that scaffold materials should mimic the natural tissue to be replaced. Because of their ability to mimic the ECM components of bone, nanofibrous structures prepared from hybrid biomaterials are suitable for such applications. Although not strictly similar to the current work, there are literature data to suggest that micro and nano-sized composites made from PCL and bioactive glass fibers to be biocompatible with osteoblast cells [40]. The tertiary bioactive glass used in the present study mimics the calcified tissue in bone whereas the biodegradable polymer mimics the fibrous collagen. The electrospun fibers mimic the architecture of bone ECM components and could potentially provide a better environment for tissue formation in tissue engineering systems. It has been reported that osteoblast cell attachment, proliferation, and differentiation to be facilitated by the use of nano-fibrous scaffolds with mesh sizes smaller than that of the cell [46]. By combining inorganic and biodegradable polymer phases via the sol-gel process, the current work demonstrates the feasibility of electrospun hybrid materials. A further benefit of the present work is that high-temperature treatment of the electrospun fibers followed by resuspending them with another polymer to
fabricate the scaffold is not needed. This, in turn, reduces the laborious steps and makes this approach practical and facile as biological molecules such as growth factors could potentially be incorporated.

### 3.6 CONCLUSIONS

PCL/BG hybrid materials for potential bone tissue regeneration were successfully synthesized via a sol-gel process. PCL and tertiary BG were used as the polymer phase and the inorganic phase, respectively. The polymer chains were successfully introduced into the inorganic sol while the inorganic networks were formed. The SEM/EDX result revealed homogeneity of the hybrid system and the elemental analyses result indicated all the elements were incorporated successfully. Identification of chemical groups and type of the intermolecular interaction was investigated by means of FTIR. It was shown that the organic and inorganic phases interacted at the molecular level via a hydrogen bond formation between the carbonyl of the PCL and the silanol hydroxyl. The XRD pattern showed that for all compositions (up to 60% polymer content), PCL/BG hybrid materials were amorphous. The TGA study also revealed the PCL/BG hybrid materials are thermally stable and the organic-inorganic ratios of the hybrid material were close to the theoretical ones. 3D scaffolds fabricated by electrospinning have potential applications for bone tissue regeneration.
3.7 REFERENCES


CHAPTER 4: HYDROXYAPATITE FORMATION ON SOL-GEL DERIVED POLY(ε-CAPROLACTONE)/BIOACTIVE GLASS HYBRID BIOMATERIALS*

Overview: In this Chapter, the essential biomaterial properties for bone regeneration, including bioactivity, mechanical properties and cell viability are discussed. Different characterization techniques, such as SEM, EDX, XRD, FTIR, and ICPS are used to investigate the rate of deposition, morphology, elemental composition, and crystalline structure of HA precipitated on PCL/BG hybrid surfaces. Effect of O/I ratio on the mechanical properties (compression) of the hybrid material is also presented. Furthermore, for different PCL/BG ratios, the effect of PCL/BG hybrid particulate concentrations on cell viability is investigated.

4.1 SUMMARY

Investigation of novel biomaterials for bone regeneration is based on the development of scaffolds that exhibit bone-bonding ability, biocompatibility, and sufficient mechanical strength. In this study, using novel PCL/BG hybrids with different organic/inorganic ratios, the effects of BG contents on the in vitro bone-like HA formation, mechanical properties, and biocompatibility were investigated. Rapid precipitation of HA on the PCL/BG surfaces were observed after incubating in SBF for only 6 h, as confirmed by SEM, EDX, FTIR, and inductively coupled plasma atomic emission spectroscopy (ICPS). The ICPS elemental analysis results were further analyzed in terms of the Ca$^{2+}$ and PO$_4^{3-}$ which were consumed to form the apatite layer. The results revealed that the rate and total amount of HA deposition decreased with an increase in PCL content. The compressive modulus and strength of the PCL/BG hybrids increased with the decrease in PCL.

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content. The highest values were achieved at the lowest PCL content (10 wt. %) and were around, 90 MPa and 1.4 GPa, respectively. To evaluate the cytotoxicity of PCL/BG bioactive hybrids, MC3T3-E1 osteoblast-like cells were cultured for up to 72 h. The data indicated that whereas initial cell attachment was marginally lower than the control tissue culture poly styrene (TCPS) surface, the hybrid materials promoted cell growth in a time-dependent manner. Cell viability within the different PCL/BG bioactive hybrids samples appeared to be influenced by compositional differences whereby higher PCL contents correlated with slight reduction in cell viability. Taken together, this study adds important new information to the growing knowledge on hydroxyapatite formation, mechanical properties, and cytotoxic effects of PCL/BG surfaces prepared by sol-gel process using a tertiary glass composition and may have considerable potential for bone tissue regeneration applications.

4.2 INTRODUCTION

Development of novel biomaterials represents an essential area of interest in bone tissue engineering strategies [1]. A variety of biomaterials, including natural and synthetic polymers and ceramics [2] are being used to fabricate synthetic scaffolds which act as guides and stimuli for tissue regeneration [3]. In general, scaffolds for bone tissue engineering are required to be, at the very least, capable of supporting cell attachment, provide sufficient mechanical strength, bioactivity, and biodegradability [4]. For this reason, BG and biodegradable synthetic polymers were among the most widely studied materials for bone tissue engineering [5, 6]. BG-based biomaterials are known for their biocompatibility, osteoinductivity, osteoconductivity and ability to form a bone-like mineral phase at the interface when in contact with living tissues [7, 8]. However, BGs are stiff and brittle making them difficult to be formed into complex shapes and, are susceptible to fracture under mechanical loads [9]. Although aliphatic polyesters such as PGA,
PLA, PCL, and their co-polymers have been extensively investigated as a potential scaffold materials for bone tissue engineering [10], they have shown limited strength and mechanical stability to match with bone tissue, especially when fabricated with large volume fractions of macroporosity [11]. In addition, they are not osteoconductive and do not directly bond to bone. Despite the availability of different materials, it has been a challenge to find a single material that is both mechanically competent and biodegradable. Recently, a sol-gel derived organic-inorganic hybrid materials from biodegradable polymers and bioactive inorganic materials have been developed with the aim of increasing mechanical stability and to improving tissue interaction during bone regeneration [12-14]. Organic-inorganic hybrid biomaterials are based on selective combination of either biostable [15, 16] or biodegradable [12, 17] polymers and BGs, which are expected to exhibit tailored physical, biological and mechanical properties. In addition, this type of structural organization of synthetic materials resembles the structure of bone tissue, where the inorganic component mimics the hydroxyapatite and the polymer component mimics the collagen rich extracellular matrix [18].

The requirement for the O/I hybrids to bond to living bone is the formation of biologically active HA layer on their surface when they are exposed to physiological fluid. The in vitro bone-like HA layer formation ability of bone-forming biomaterials, evaluated in SBF [19], is generally thought to facilitate recruitment of proteins such as collagen, fibronectin, and vitronectin, in which osteoblasts bind and proliferate [20], thereby allowing for the formation of an intimate bond to bone. The modification of the inorganic network by adding organic polymers in the O/I hybrid materials were suggested to influence the bioactivity and the mechanical properties of the resultant hybrid materials [15, 21]. For example, in PCL/Silica hybrid system, higher PCL content showed lower apatite-forming rate and polymer-like ductile–tough fracture behavior than lower PCL
content which showed higher rate of apatite formation and ceramic-like hard–brittle fracture behavior [15, 22]. Results from these studies imply that by varying the polymer to inorganic (i.e. Silica) ratio, the bioactivity and mechanical properties of the hybrid material could be tailored. Other studies also showed that incorporation of Ca\(^{2+}\) in the hybrid system provided an improved osteoconductivity and \textit{in vitro} bone-like apatite formation ability of the hybrid system [23].

Another aspect of vital importance in preparing a scaffold material for bone regeneration is biocompatibility of the scaffold material to the host tissue. Sol-gel derived bioactive and biodegradable O/I hybrids are presumed to be cytocompatible only after calcination (ca. 600 °C) and removal the polymer template used for the sol-gel process [24-28]. Obviously, once the polymer is degraded, the materials are no longer O/I hybrids. Sol-gel derived O/I hybrid materials are prepared at low temperature to facilitate silicate hydrolysis and avoid phase separation between the organic and inorganic components. In the absence of high temperature treatment that removes unreacted species and/or traces of trapped impurities, the sol-gel approach could be a major source of concern to the cytocompatibility of this class of biomaterials. Although data is scanty, cellular responses to sol-gel derived O/I hybrids without high-temperature treatment generally showed poor results [23] even with attempts to neutralize the residual HCl that may be present in the samples [29].

Notwithstanding the above reports, systematic data on O/I hybrid materials with biodegradable polymer and tertiary glass components is notably lacking. In Chapter 3 the synthesis and characterization of bioactive and biodegradable PCL/BG hybrid biomaterials with different organic to inorganic ratios via a sol-gel process was reported [12]. In that study, PCL/BG hybrid scaffold fabrication by the electrospinning process was demonstrated [12]. However, the suitability of these novel hybrid biomaterials for potential bone tissue engineering applications was not
studied. Therefore, the objective of the present study was to investigate the effect of composition on the \textit{in vitro} bone-like apatite formation ability, mechanical property, and cytotoxicity of the PCL/BG hybrid biomaterials synthesized via a sol-gel process. The study documented in this Chapter adds important new information to the growing knowledge of rapid hydroxyapatite formation on PCL/BG surfaces prepared by sol-gel process using a biodegradable polymer and a tertiary glass composition.

### 4.3 EXPERIMENTAL SECTION

#### 4.3.1 Preparation of PCL/BG Hybrid Biomaterials

PCL/BG hybrids were synthesized via a sol-gel process as described in Chapter 3. The sample designation for the compositions of PCL/BG hybrids used in this study are summarized in Table 4.1 [12].

<table>
<thead>
<tr>
<th>PCL/BG (Wt. %)</th>
<th>Sample code</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/100</td>
<td>BG</td>
</tr>
<tr>
<td>10/90</td>
<td>1090</td>
</tr>
<tr>
<td>40/60</td>
<td>4060</td>
</tr>
<tr>
<td>60/40</td>
<td>6040</td>
</tr>
<tr>
<td>100/0</td>
<td>PCL</td>
</tr>
</tbody>
</table>

Table 4.1: Compositions of PCL/BG hybrid biomaterials
4.3.2 *In vitro* Bioactivity of PCL/BG Hybrid Biomaterials

The *in vitro* bioactivity of the PCL/BG hybrid and control disk samples (6 mm x 2 mm) were carried out by incubating in SBF [19]. The SBF solution has a composition and concentration similar to those of the inorganic part of human plasma (Table 4.2). One liter of SBF solution was prepared by dissolving NaCl (7.996 g), NaHCO₃ (0.350 g), KCl (0.224 g), K₂HPO₄·3H₂O (0.228 g), MgCl₂·6H₂O (0.305 g), CaCl₂ (0.278 g), and Na₂SO₄ (0.071 g) into distilled water and buffering at pH 7.4 with tris(hydroxymethyl)aminomethane (HOCH₂)₃CNH₂ (6.057 g) and appropriate amount of 1N HCl. The as-prepared PCL/BG hybrid monoliths were pulverized by a ball mill for 5 min, then 0.05 g of powder was weighed and heat pressed by using a custom made stainless steel mold to prepare a PCL/BG hybrid disks with an approximate dimension of 6 mm in diameter and 2 mm in thickness. Each specimen was immersed in 20 ml of SBF (0.05 g/20 ml) contained in polypropylene bottles covered with a tight lid. The bottles were placed in an orbital shaker at a constant speed of 120 rpm and temperature of 37 °C from 6 h to 168 h without SBF refreshing. After each soaking period the disks were collected from the SBF and rinsed thoroughly with phosphate-buffer saline (PBS) and ethanol and dried overnight at 37 °C.

**Table 4.2:** Ionic compositions (mM) of SBF and human blood plasma.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Mg²⁺</th>
<th>Ca²⁺</th>
<th>Cl⁻</th>
<th>HCO₃⁻</th>
<th>HPO₄²⁻</th>
<th>SO₄²⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBF</td>
<td>142.0</td>
<td>5.0</td>
<td>1.5</td>
<td>2.5</td>
<td>147.8</td>
<td>4.2</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Plasma</td>
<td>142.0</td>
<td>5.0</td>
<td>1.5</td>
<td>2.5</td>
<td>103.0</td>
<td>27.0</td>
<td>1.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>
4.3.3 Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy

The morphology of HA-like crystals precipitated on the disks surface during the incubation period was observed by using a high resolution scanning electron microscopy (Leo 1540 FIB/SEM with Cross-Beam, 25kV, Zeiss Nano Technology Systems Division, Germany). The growth and elemental composition of the HA-layer formation were also evaluated by using an EDX detector attached to the S-2600N SEM. All disks were coated with 3 nm of Osmium metal using Filgen OPC-80T osmium plasma coater prior to SEM imaging.

4.3.4 Inductively Coupled Plasma Spectroscopy

The concentrations of calcium, phosphate and silicon ions in the SBF solution during the incubation period were determined by using the inductively coupled plasma optical-emission spectroscopy (ICP-OES; Vista-Pro Axial, Varian Inc., USA). Furthermore, Ca\(^{2+}\) and PO\(_4^{3-}\) ion consumption in SBF to form the HA-layer were calculated as follows:

\[
X_{\text{consumption}} = X_{\text{max in SBF}} - X_{\text{final in SBF}}
\]

Where \(X\) indicates either Ca\(^{2+}\) or PO\(_4^{3-}\) ion concentrations and, \(X_{\text{max in SBF}}\) and \(X_{\text{final in SBF}}\) were determined from the time-course ICPS measurements.

4.3.5 Fourier Transform Infrared Spectroscopy

The FTIR absorption spectra were collected by using the Bruker IFS 55 FTIR (Bruker Optics, Billerica, MA). After soaking in SBF the specimens were powdered and, 2 mg of each sample was mixed with 200 mg potassium bromide (KBr) powder, pressed as a pellet, and scanned at a resolution of 4 cm\(^{-1}\) and sample scans of 32. All spectra were analyzed utilizing the OPUS software
version 4.0. Identification of the absorption bands was based on previous study on synthetic and biological apatite [30].

4.3.6 X-ray Diffraction

XRD measurements of the samples were carried out by using a rotating anode X-ray diffractometer model RTP300 (Rigaku Rotaflex, Japan) operating on CoKα radiation and run at 45 kV and 160 mA. Samples for XRD measurements were prepared by crushing the PCL/BG hybrid disks before and after incubating in SBF. XRD measurements were conducted in the 2θ range from 2 to 82° with step size of 0.02. The 2θ values for equivalent CuKα radiation was obtained using Bragg’s law, \( \lambda = 2d \sin \theta \) and \( \theta_{\text{Cu}} = \sin^{-1}\left(\frac{\lambda_{\text{Cu}} \sin \theta_{\text{Cu}}}{\lambda_{\text{Cu}}}\right) \), where, \( \lambda_{\text{Cu}} = 1.54056 \) Å and \( \lambda_{\text{Co}} = 1.79026 \) Å.

4.3.7 Compressive Testing

PCL/BG hybrid and the PCL control cylindrical specimens (n=5) with 3:2 aspect ratio (9 mm in height and 6 mm in diameter) were used for compressive mechanical testing. A uniaxial compression test was conducted using an Instron Universal Mechanical testing machine equipped with 5 kN load cell (Instron model 3345, Canton, MA) with crosshead speed of 1 mm/min at ambient temperature and humidity. The compressive modulus was determined from the slope of the initial linear elastic portion of the stress-strain curve. The maximum strength of the PCL/BG hybrid specimens were also determined by using the software associated with the Instron machine.
4.3.8 Cytotoxicity Assay

For colorimetric assay of the metabolic activity of viable cells, non-radioactive Cell Proliferation Kit I (MTT; Roche, Toronto, ON, Canada) was used. In this technique, the yellow tetrazolium salt (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) is reduced to a purple formazan compound by the dehydrogenase activity of intact mitochondria. Consequently, this conversion only occurs in living cells. For the purpose of this assay, the PCL/BG hybrid powders were first sterilized by using ultraviolet (UV) light for 1 h. Following this, a known amount of PCL/BG hybrid powders were dispersed in serum-free culture media (α-MEM) by using ultrasonic irradiation for 20 min to make up 2 mg/l suspension. MC3T3-E1 osteoblast-like cells (generously provided by Dr. Jeff Dixon at the University of Western Ontario) were seeded in 96-well tissue culture plates at an initial density of 1×10^4 cells per well. A final volume of 100 µl cultured medium per well containing α-MEM supplemented with 10% FBS and 100 U/ml of penicillin and 100 µg/ml of streptomycin were used. Cultures were incubated for 24 h in a humidified atmosphere of 95% air and 5% CO₂, at 37 °C. The PCL/BG hybrid suspension at concentrations of 100, 200, 300 and 500 µg/ml was then added in the confluent layer of MC3T3-E1 osteoblast-like cells. After 24 and 72 h of incubation period, 10 µl of MTT solution (5 mg/ml in phosphate buffered saline (PBS)) was added to each well followed by incubation in humidified atmosphere at 37 °C for 4 h. For dissolution of the purple formazan crystals formed after the incubation period, 100 µl of the solubilization solution (10% SDS in 0.01 M HCl) was added and incubated overnight in a humidified atmosphere at 37 °C. The optical density (OD) of each well at the absorbance wavelength of 595 nm was determined by a microplate reader (ELX 800, Bio-Tek, USA) with a reference wavelength of 650 nm. Tissue culture polystyrene (TCPS) was used as a control. The cell-free culture medium with respective PCL/BG hybrid suspension was used as a blank.
Triplicate of each sample were tested for each incubation time. The data obtained were then normalized with respect to the OD value of the TCPS at the 6 h culture.

4.3.9 Statistical Analysis

Statistical data analyses were conducted using a one-way analysis of variance (ANOVA) and Tukey HSD Rank Order Test. A probability value of 95 % ($p < 0.05$) was used to determine the level of significance. Error is reported in figures as the standard deviation (SD).

4.4 RESULTS

4.4.1 In vitro Bioactivity of PCL/BG Hybrid Biomaterials

4.4.1.1 Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy

The SEM micrographs of PCL/BG hybrid and PCL disks surface before and after different incubation time in SBF solution are shown in Figure 4.1. Before incubation in SBF solution (0 h), all the PCL/BG hybrid samples with different compositions showed a smooth surface. After 6 h the PCL/BG hybrid surfaces were covered with heterogeneous and sparsely dispersed spherically-shaped particles. After 24 h of soaking the surfaces were covered with densely packed particles.

In Figures 4.2 and 4.3, a high magnification of the micrographs taken after longer incubation time (>24 h) for a representative 4060 and 6040 PCL/BG hybrid samples are presented. Similar to typical HA morphology, these micrographs showed evidence of needle-like crystallites covering the PCL/BG hybrid disks. The observed morphology of the HA apatite layer on the surface of PCL/BG hybrid disks was similar for all compositions. To the contrary, no apatite layer deposition was observed on the PCL control disks for all time points up to 168 h incubation.
Figure 4.1: SEM micrographs showing the evolution of HA layer on different PCL/BG hybrids and PCL control disk surfaces after incubating in SBF at different time points. 0 h represents the samples before incubation starts. (Scale bar = 5 μm)
Figure 4.2: High magnification SEM micrographs and the corresponding EDX patterns for the evolution of the HA layer on 4060 PCL/BG hybrid disks before and after incubation in SBF for different time points. (SEM scale bar = 1 μm)
Figure 4.3: High magnification SEM micrographs and the corresponding EDX patterns for the evolution of the HA layer on 6040 PCL/BG hybrid disks before and after incubation in SBF for different time points. (SEM scale bar = 1 µm)
To demonstrate the evolution of apatite layer deposition on the specimen surfaces with respect to
the incubation time, the EDX elemental intensities from Figures 4.2 and 4.3 were converted into
wt. % and are presented in Figures 4.4A and 4.4B, respectively. Before incubation (0 h), the EDX
spectrum showed that the surface of the PCL/BG hybrid had large amounts of silicon (65 wt. %)
compared with the other inorganic constituents (29 wt. % Ca and 6 wt. % P) for the PCL/BG
hybrid system. After incubation in SBF from 6 h to 168 h, the EDX analysis detected an increase
in Ca and P concentrations compared with the Si content. Both the increase of Ca and P and the
decrease of Si were sharp at the earlier incubation times before it leveled off after 96 h of
incubation. The Si content on the surface became extremely low, likely due to the consequence of
the thickness of the newly formed apatite layer and the limitation on the X-ray beam penetration
depth. The Ca/P ratio after 168 h was calculated to be 1.70 ± 0.04 which is slightly higher than the
stoichiometric \((\text{Ca}_{10}\text{(PO}_4)_6\text{(OH)}_2)\) Ca/P ratio (1.67), possibly due to the B-type \(\text{CO}_3^{2-}\) substitution
in apatite lattice [31]. The observation from SEM and EDX studies clearly confirmed that the new
surface was enriched by Ca and P, which are the main constituents of HA.

Given that at 25 keV primary electron beam used in this experiment, the penetration depth at which
the primary electrons have sufficient energy to generate characteristic X-rays is limited to about
2-5µm thick. Therefore the apatite layer thickness at different times was measured. To do this,
specimen surfaces were scratched to disrupt the deposited HA layer and cross sectional images
were taken at 45° angle. The data collectively presented in Figure 4.5 showed that the film
thickness increased significantly from 2 µm at 6 h incubation to 21 µm at 168 h of incubation with
SBF.
Figure 4.4: Composition (wt. %) profile of Si, Ca and P atoms on the surface of: (A) 4060 PCL/BG hybrid disks and (B) 6040 PCL/BG hybrid disks (as determined by EDX) before and after incubation in SBF for different time points. Note that the smooth trend lines are added for ease of visualization.
Figure 4.5: HA film thickness variation with incubation time for 4060 PCL/BG hybrid disks as determined by SEM followed by ImageJ analysis. Scale bar: A=2μm; B= 10μm
4.4.1.2 Inductively Coupled Plasma Spectroscopy

When PCL/BG hybrids are incubated in SBF, the first step is the release of Ca$^{2+}$, PO$_4^{3-}$ and Si ions from the PCL/BG hybrid disks into the SBF solution. This is followed by the re-deposition (consumption) of the released ions as well as the ions in the SBF solution to the disks to form the HA layers. Therefore, the concentration variations of these ions in the SBF with the incubation time was measured by means of ICPS. In Figure 4.6, the release profile of Ca$^{2+}$, PO$_4^{3-}$ and Si ion into the SBF solution from the PCL/BG hybrid disks and the PCL control is presented. As can be seen, a sharp increase in the Ca$^{2+}$ concentration took place in the first 6 h of the test for all samples except for the PCL control. For Ca$^{2+}$ and P, this increase was followed by a decrease and stabilized at a certain value after 24 h. The release profile of Si also showed a monotonic increase for all of the samples except for PCL control between 24 and 96 h, and remained constant until the end of the test. The SBF with PCL control showed no change in Si concentration.

The consumption of Ca$^{2+}$ and PO$_4^{3-}$ by each sample was calculated by subtracting the final concentration at 96 h from the maximum concentration at 6 h. The data presented in Figure 4.7 demonstrated that the total consumption for both Ca$^{2+}$ and PO$_4^{3-}$ ions varied with composition. Significantly lower Ca$^{2+}$ and PO$_4^{3-}$ consumption from the SBF solution were observed for 6040 and PCL disks ($p < 0.05$). In the extreme case of the control PCL, negligible amount of ions were consumed. Furthermore, the results from Figure 4.7 indicated the amount of CaP deposited on the surfaces of the PCL/BG hybrid samples, which may be used as one of the screening criteria for the evaluation of in vitro bioactivity of materials.
Figure 4.6: Ca\textsuperscript{2+}, PO\textsubscript{4}\textsuperscript{3-}, and Si ion concentration in SBF as a function of soaking time for PCL control and PCL/BG hybrids with different compositions (□ - BG, ◇ -1090, ○ - 4060, Δ-6040, ✶ - PCL). Note that the smooth trend lines are added for ease of visualization.
Figure 4.7: Total consumption of Ca\(^{2+}\) and PO\(_4^{3-}\) ions from the SBF solution during the *in vitro* bioactivity test conducted for PCL/BG hybrids with different compositions. Different letters indicate that the groups are significantly different at \(p < 0.05\).

4.4.1.3 Fourier Transform Infrared Spectroscopy

The growth of HA crystals on PCL/BG hybrid sample was also investigated by FTIR spectroscopy before and after incubation for up to 168 h (7 days) in SBF. The IR absorption spectra for PCL/BG hybrid samples and pure BG after various incubation times in SBF are displayed in Figure 4.8.
Figure 4.8: FTIR spectra of the *in vitro* HA formation on the surface of the PCL/BG hybrid and pure BG disks after incubating in SBF for different time points.
The spectra of the PCL/BG hybrid samples before incubation in SBF showed the absorption peak of the bending and stretching vibrations of Si–O–Si bonds at the wave length of 800 cm\(^{-1}\) and 1080 cm\(^{-1}\). The peak at 1630 cm\(^{-1}\) is attributed to –OH groups from residual moisture. The characteristic peaks of PCL are observed at 1730 cm\(^{-1}\) (C=O). However, after incubation in SBF for 6 h, a weak P–O vibrational band near 602 cm\(^{-1}\) was detected, indicating the initial presence of CaP-rich layer. After immersion in SBF for 24 h two crystalline P–O vibrational peaks at 564 cm\(^{-1}\) and 602 cm\(^{-1}\) was clearly observed. With increased incubation time, the other vibrational peaks of HA were also gradually detected, indicating the evolution of HA as a function of time. The IR spectra of the PCL/BG hybrid samples incubated for more than 96 h showed well-resolved vibrational peaks at 1080 and 800 cm\(^{-1}\), which were assigned to the Si–O–Si bands; the peaks at 1492 cm\(^{-1}\) were assigned to the C–O vibration bands; the peaks at 964, 602, 564 cm\(^{-1}\) were assigned to the P–O bands [30].

### 4.4.1.4 X-ray Diffraction

The XRD patterns for PCL/BG hybrid and pure BG samples before and after incubation in SBF up to 168 h are shown in Figure 4.9. The un-incubated BG and PCL/BG hybrid samples (0 h) showed no diffraction peaks indicating that the samples were completely amorphous. In contrast, after 6 h of incubation the apatite diffraction peaks appeared at \(2\theta = 21.8^\circ, 25.9^\circ, 31.77^\circ, 45.4^\circ, \text{ and } 53.45^\circ\), which were indexed to be (200), (002), (211), (222) and (004) diffraction planes, respectively, of HA (JCPDS #9-432). The apatite (002) diffraction peak became more evident and appeared sharp after 24 h of incubation. The (211) diffraction peaks also became narrower with a longer time. In addition, other diffraction apatite peaks (200, 211, 222 and 004) also became more evident.
Figure 4.9: XRD pattern of the in vitro HA formation on the PCL/BG hybrid and pure BG materials surface after soaking in SBF at different time points.
4.4.2 Mechanical Properties

The compression test results of the PCL/BG hybrids and control PCL are illustrated in Figure 4.10. Each of the curves is a representative for each composition.

![Image of stress-strain curves](image)

**Figure 4.10:** Representative stress-strain curves for pure PCL and different compositions of PCL/BG hybrid samples as obtained from uniaxial compressive testing.

The mean values and standard deviations of compressive modulus, compressive strength, and strain at failure of the PCL/BG hybrids and control PCL obtained from the current study are summarized in Table 4.3. It can be seen that the incorporation of the PCL within the inorganic network significantly increased the strain at failure from $7.67 \pm 0.52\%$ for 1090 to $10.81 \pm 1.53\%$ for 6040 ($p < 0.05$). On the other hand, the presence of the inorganic component in the hybrid
system also contributed to an increase in the compressive modulus and compressive strength. The four compositions shown in Table 4.3 were separated into three significant groups (p < 0.05).

Table 4.3: Summary of the mechanical properties of the PCL/BG hybrids and PCL samples (n=5) as obtained from the uniaxial compressive testing. Different letters indicate that the groups are significantly different at p < 0.05.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Strain at Failure [%]</th>
<th>Compressive Strength [MPa]</th>
<th>Compressive Modulus [MPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1090</td>
<td>7.67±0.52(^a)</td>
<td>88.58±9.57(^a)</td>
<td>1388.6±75.87(^a)</td>
</tr>
<tr>
<td>4060</td>
<td>9.76±0.98(^b)</td>
<td>30.89±4.08(^b)</td>
<td>633.3±45.38(^b)</td>
</tr>
<tr>
<td>6040</td>
<td>10.81±1.53(^b)</td>
<td>28.95±2.80(^b)</td>
<td>552.62±30.37(^b)</td>
</tr>
<tr>
<td>PCL</td>
<td>-</td>
<td>19.79±1.05(^c)</td>
<td>335±11.92(^c)</td>
</tr>
</tbody>
</table>

4.4.3 Cytotoxicity Assay

The cytotoxicity of the PCL/BG hybrid samples after adding different concentrations (100-500 µg/ml) of powdered hybrid materials to the cell culture were assessed by the MTT assay and the results are presented in Figure 4.11. As can be seen, the cell viability data showed that the addition of the as-prepared PCL/BG hybrid powder suspension into the cell culture demonstrated no significant toxicity (p>0.05) to the cell viability compared to the cell density after 6 h culture on the TCPS. However, slight reductions in the optical density (OD) values were observed for the higher polymer content (6040). In addition for all compositions an increase in OD value was observed after 24 and 72 h incubation periods, suggesting that while the growth rate may be lower
than the TCPS control, cells were actively metabolizing on all hybrid materials at all PCL/BG hybrid particulate concentrations over the 72 h culture period.

Figure 4.11: Normalized MC3T3-E1 cells viability after culturing in direct contact with BG, 1090, 4060 and 6040 PCL/BG hybrid particulates with concentrations of (A) 100 µg/ ml, (B) 200 µg/ ml, (C) 300 µg/ ml, and (D) 500 µg/ ml, for periods of 6, 24 and 72 h.

4.5 DISCUSSION

Organic-inorganic hybrid biomaterials synthesized by a sol-gel process are becoming one of the most sought-after classes of materials in scaffold fabrication for bone tissue regeneration [13, 32]. More specifically, the use of biodegradable polymers such as PCL in the sol-gel process would be an interesting option, due to its biocompatibility and biodegradability. Although there are literature
reports of PCL/BG glasses based on sol-gel process [33, 34], PCL is not often added to the sol while the network is being formed during the polycondensation. Instead, PCL is added after sintering sol-gel derived BG glasses. Unlike the present work, however, these nanocomposites are not considered to be hybrid materials whereby the bioactive glass components and the polymer chains are interacting via chemical bonding on a molecular scale forming a with a single phase. The most relevant work to the current study was conducted by Rhee and coworkers [22, 35, 36] who reported sol-gel derived PCL-Silica materials where the PCL was added during the Silicate hydrolysis stage. Sol-gel chemistry is inherently carried out with water as the main solvent for hydrolysis of the Silicate component. This, in turn, means that the organic polymer must be high molecular weight and water soluble. Unfortunately, these criteria are met only by non-biodegradable polymers such as polyvinyl alcohol (PVA) or polyvinyl pyrrolidone (PVP). If the polymer is degradable and water soluble but has low molecular weight, it will be leached out or segregated easily. Because high molecular weight PCL tends to precipitate in the presence of water during the sol-gel process (owing to its water insolubility), Rhee et al. [35] used a low molecular weight (2,000 g/mol) water-soluble and degradable α,ω-hydroxyl PCL. In order to overcome leaching and segregation of this low molecular weight α,ω-hydroxyl PCL, it was chemically linked to 3-isocyanatopropyl triethoxysilane which was then co-condensed with tetraethyl orthosilicate (TEOS). Contrary to these cited works, the present study did not require the use of a coupling agent since a high molecular weight PCL (80,000 g/mol) was used because it does not leach out from the hybrid. Since high molecular weight PCL was not water soluble, this challenge by using methyl ethyl ketone (MEK) as a co-solvent to ensure that a homogenous system was obtained at all times during the polycondensation was addressed [12].
In Chapter 3, the synthesis and characterization of a novel O/I hybrid materials based on PCL and tertiary BG with uniformly distributed calcium for potential applications in bone tissue engineering was discussed thoroughly [12]. However, the effect of composition on properties such as the bioactivity, mechanical properties and biocompatibility of these materials were not investigated.

In the present study, the hypothesis was that the presence of surface silanol groups (Si-OH) and the incorporation of both calcium and phosphate ions into the hybrid system accelerates the in vitro bioactivity behavior. To test this hypothesis, three different PCL/BG hybrids with different composition were prepared with anticipated bioactivity and enhanced mechanical properties as compared to studies conducted by different groups, which used either non-degradable polymers [15, 16, 37] or Silica as the only inorganic phase in the hybrid system [15, 22, 38]. The reason for restricting the maximum PCL content in the hybrid system to 60 wt. % (i.e. 6040) was due to the fact that the hydrophobic nature of PCL limited its solubility in the inorganic sol even with MEK co-solvent. The data shown in Figure 4.1 demonstrated that all the PCL/BG hybrids, except for the pure PCL, revealed a bone-like apatite forming ability in SBF. The FTIR spectra (Figure 4.8) revealed that HA formation was higher with increased inorganic content (1090 and 4060) than for the lower organic content for 6040. This result, while consistent with literature report [22], is significant due to the use of the tertiary BG as the inorganic phase and the biodegradable polymer (PCL) as an organic phase, combinations that are not reported to date. Perhaps, the most striking finding of the current study is the observation of well-structured HA layer formation at a very low incubation time of 6 h regardless of the PCL/BG composition. In PCL-Silica sol-gel materials incorporating calcium, previous studies [22, 39] have demonstrated the need for 7 days of incubation in SBF to obtain meaningful HA layers. Furthermore, an attempt to induce rapid HA deposition by annealing the samples at elevated temperature (200 °C) had detrimental effect [40].
Careful examination of these cited studies indicate that phosphate groups were notably absent in the sol-gel hybrid materials. Given that HA deposition study in the present work was done on hybrid samples containing tertiary glass components, it is believed that the phosphate was responsible for rapid HA deposition rather than the PCL/BG composition. For all compositions and incubation times, the deposited HA morphology was not different but the deposited film thickness increased significantly over time (Figure 4.5).

Since the growth of the apatite layer is known to be affected by the presence of \( \text{PO}_4^{3-} \) and \( \text{Ca}^{2+} \) ions \([41, 42]\), the amounts of the \( \text{Ca}^{2+} \) and \( \text{PO}_4^{3-} \) ions present in the inorganic phase of the present hybrid system were initially optimized based on the condition where a greater bioactivity is observed as reported in other studies \([43]\). The bioactivity data on different PCL/BG ratios used in the current study indicated that the amount of Ca and P ions in the hybrid materials influenced the HA deposition. The total amount of \( \text{Ca}^{2+} \) and \( \text{PO}_4^{3-} \) ion released from the specimens into SBF and subsequently consumed (Figures 4.6 and 4.7) to form the apatite layer on the PCL/BG hybrid surfaces were directly proportional to the inorganic content incorporated in the hybrid system. In addition to the phosphate groups present in the bioactive glass, the rapid apatite nucleation observed in this system (6 h) could also be related to the presence of high concentration silanol groups on the PCL/BG surfaces and the increased overall ionic activity of the solution \([44]\). Once the apatite nuclei are formed, the layer can grow spontaneously by consuming the \( \text{Ca}^{2+} \) and \( \text{PO}_4^{3-} \) ions from SBF; this is because the SBF is supersaturated with respect to the apatite.

The choice of PCL in this study was to provide biodegradability and to optimize the mechanical properties of the hybrid systems. It has been reported \([22, 35]\) that low molecular weight PCL accelerates the biodegradability of PCL-Silica hybrids and affects the mechanical properties whereby higher PCL contents resulted in polymer-like ductile–tough fracture behavior \([22]\).
Conversely, the low PCL content in the hybrid led to a ceramic-like hard–brittle fracture behavior. With respect to the fracture failure, the data presented herein are consistent with the above notion. In the present study, the compressive stress and modulus values increased with the increase in BG content and vice versa (Figure 4.10, Table 4.3). Maximum compressive strength and modulus values of 90 MPa and 1.4 GPa, respectively were achieved for 90% BG content. When the data in this Chapter is compared with the closest study in the literature [22], there was a notable difference since, contrary to the cited work, both the compressive strength and modulus of our PCL/BG hybrid system significantly increased with the increase in the BG content. Given the PCL molecular weight difference between these studies and the experimental conditions involved, direct comparison is somewhat difficult. However, it underscores the predictability of the mechanical properties of the current PCL/BG hybrids - collectively suggesting that by combining a tertiary BG and a biodegradable PCL polymer, the mechanical properties and HA formation could be modulated.

In addition to the bioactivity and mechanical properties, biomaterials used for bone tissue engineering also need to be cytocompatible, without eliciting adverse response from the application site or surrounding tissue. Various studies [24-28, 33, 34] demonstrated that PCL/BG hybrid materials synthesized by a sol-gel process and have undergone a thermal stabilization at high temperature (ca. 600 °C) to be non-toxic since the high temperature burns off residual and leachable toxic components including the polymer. However, the PCL/BG hybrids derived from the sol-gel process in this study could not be thermally treated at high temperature, since it would lead to degradation of the PCL. In view of this, the effect of the possible unreacted precursors on cell viability was evaluated. As shown in Figure 4.11, the result from the cytotoxicity assay of PCL/BG hybrids indicated that the present materials were not significantly toxic when compared
with the TCPS control for each time point. Cell viability studies on sol-gel derived BG hybrid biomaterials were reported primarily for PVA systems [8, 29, 45]. It seems that the cytocompatibility in the PVA hybrid systems is dependent on cell type. For example, when primary cells are cultured on these materials, cell viability was generally poor [29] whereas stem cells (both bone marrow and adipose-derived) showed excellent viability [8, 45]. Although stem cells did not utilized in this study, the osteoblast-like cells viability on PCL/BG hybrid system is better than PVA/BG hybrids on primary cell viability whereas it is comparable to the reported stem cell data.

Taken together, the PCL/BG hybrids synthesized via a sol-gel process demonstrated some of the advantages of combining biodegradable polymers with tertiary BGs. The ability to use a single material, polymer or glasses, for such purposes may be impractical, and hybrids may be utilized to yield better results. Such is the case with organic-inorganic hybrids, which can exhibit a range of bioactive, resorbable, and mechanical properties. Further tailoring of the material chemistry and morphology can thus be employed to match these properties with the host tissue, in an effort to give better incorporation and enhanced efficacy.

4.6 CONCLUSIONS

In this study, *in vitro* bone-like HA formation ability, mechanical properties, and biocompatibility of sol-gel derived PCL/BG hybrids based on tertiary glasses were investigated. It was shown that the bioactivity of sol-gel derived BG is mainly governed by the presence of surface silanol group (Si-OH) and Ca and P content. After incubating in SBF, all hybrid materials showed HA formation ability. However, the rate and total amount of HA formation decreased with an increase in PCL content (Figure 4.7). The strain at fracture increased with an increase in PCL content whereas the
compressive modulus and strength of the PCL/BG hybrids increased with the decrease in PCL content. The cytotoxicity test indicated that no significant (p> 0.05) toxicity was observed for lower polymer contents, however a slight reduction in cell viability was observed with the higher polymer content as compared to the control. Therefore, the ability to tailor the bioactivity and mechanical property of these novel PCL/BG hybrid materials could represent a potential application for bone tissue engineering.
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CHAPTER 5: THE ROLE OF BIOACTIVE 3D HYBRID FIBROUS SCAFFOLDS ON MECHANICAL BEHAVIOR AND IN-VITRO SPATIOTEMPORAL OSTEOBLAST GENE EXPRESSION*

**Overview:** This Chapter describes about the fabrication of 3D poly(ε-caprolactone)/Bioactive glass hybrid scaffold via a sol-gel combined electrospinning process for bone regeneration application. The scaffold properties, including pore-size distribution, porosity, wettability, mechanical properties and in vitro bioactivity are evaluated and compared with PCL control. In addition, the role of PCL/BG hybrid fibrous scaffolds on in vitro osteoblast cell proliferation and osteoblast phenotype markers gene expression is presented. Furthermore, the effect of fiber diameter on the physical and biological properties of the PCL/BG hybrid fibrous scaffolds is discussed.

5.1 **SUMMARY**

Three-dimensional (3D) bioactive organic-inorganic hybrid fibrous scaffolds are attractive extracellular matrix (ECM) surrogates for bone tissue engineering. In this study, fibrous scaffolds with two different fiber diameters were fabricated from PCL and tertiary BG in the system (SiO$_2$-CaO-P$_2$O$_5$) by a combined sol-gel and electrospinning processes. The physical, chemical, mechanical and biological properties including bone-associated gene expression profiles by cells seeded on these hybrid scaffolds were evaluated and compared with fibrous PCL scaffolds used as control. The PCL/BG hybrid fibrous scaffolds exhibited excellent wetting properties, enhanced pore-sizes and superior mechanical properties compared with the control. Incubating PCL/BG hybrid fibrous scaffolds in simulated body fluid (SBF) revealed bone-like apatite formation on the surface of the fibrous scaffolds. Osteoblast cells cultured on PCL/BG hybrid fibrous scaffolds attached and spread with multiple attachments on the fibers while actively proliferating suggesting

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that the sol-gel process did not have a detrimental effect. Targeted studies on early and late marker bone-associated gene expressions by rat calvarial osteoblasts cultured on the hybrid fibrous scaffolds indicated differential gene activation. mRNA expressions for alkaline phosphatase (ALP), osteopontin (OPN), bone sialoprotein (BSP), and osteocalcin (OCN) were significantly higher on the hybrid fibrous scaffolds \((p < 0.001)\) compared with the PCL control. Taken together, our results suggest that PCL/BG fibrous scaffolds may provide a favorable microenvironment and accelerate bone regeneration.

5.2 INTRODUCTION

Scaffold-guided bone regeneration holds a promise in providing an improved clinical therapy to repair critical bone defects resulting from disease or trauma [1]. The ultimate goal of scaffold design is to mimic the complex structural composition, spatial distribution, and functionality of native tissues, and to provide a 3D template for cell growth and neo-tissue formation [2]. Therefore, scaffolds for bone regeneration should be mechanically stable, biocompatible, bioactive, osteoconductive, degradable at a rate comparable to neogenesis, and have porous architectures to support bone ingrowth [2, 3]. Although fulfilling the above requirements remains a challenge, the nanocomposite structure of the bone extracellular matrix (ECM) consisting of collagen fibrils and carbonated hydroxyapatite (HA)-like nanocrystals [4, 5] provides a framework for the design of synthetic scaffolds. In an attempt to fabricate scaffolds that mimic the bone ECM using processing techniques such as electrospinning, phase separation, and self-assembly [6, 7], nanoparticles, nanofibers, and nanocomposites have received considerable attention. Previous studies have demonstrated that the high specific surface area and porosity associated with electrospun nanofibrous structures enhanced the osteogenic potential of synthetic scaffolds in
terms of protein adsorption [8], cell adhesion [9], proliferation, differentiation and mineralized tissue biosynthesis [8]. It has also been suggested that varying the fiber diameter, the pore-size, porosity and surface area of the scaffold could influence their biological performances including cell attachment, proliferation, and differentiation [10-12].

In order to make a significant progress towards tissue engineered bone, O/I hybrid scaffolds play an integral part. Notwithstanding this, however, synthetic biodegradable aliphatic polyesters (e.g. PCL) have been among the preferred and most widely studied scaffold materials for bone regeneration because of its good biocompatibility, slow degradation, and good processability [13, 14]. Because of the use of PCL scaffolds for bone regeneration led to poor bioactivity and low stiffness [15, 16], various studies attempted to incorporate bioactive inorganic nanoparticles into biodegradable polymers as fillers to form composite nanofibers [17-19]. Although some encouraging results are reported, most of these electrospun composite fibers have two major drawbacks: 1) agglomeration of nanoparticle fillers within the fibrous matrix and, 2) lack of specific interactions between the organic and inorganic phases leading to poor dispersion, compromised mechanical properties, and unfavorable cellular responses [20, 21]. One of the strategies to overcome this notable problem is the preparation of scaffolds from sol-gel derived hybrids by the electrospinning process. In Chapter 3, it was reported that tertiary BGs can be homogenously incorporated into PCL using a sol-gel process and demonstrated the feasibility of electrospinning the gelling composition [22]. In that study, the electrospinning parameters were not optimized and the biological evaluations were not carried out [22]. In view of this, one of the objectives of this study was to fabricate and evaluate property-relationships of sol-gel derived O/I hybrid fibrous scaffolds.
Scaffolds should not only serve as structural templates for cell attachment and viability, but also should guide tissue formation by regulating spatial and temporal bone-associated gene (collagen type I, alkaline phosphatase, osteopontin, bone sialoprotein, and osteocalcin) expressions leading to protein translation and matrix remodeling. Not surprisingly, osteoblast differentiation is a multistep process modulated by an integrated cascade of gene expression that initially supports proliferation and the sequential expression of genes associated with the biosynthesis, organization, and mineralization of the bone ECM [23]. Bone-associated gene and protein expressions of osteoblast and osteoblast-like cells seeded on 3D collagen and biodegradable synthetic scaffolds have been studied [8, 24-26]. These studies demonstrated that the 3D topography of the polymeric scaffolds played a role in enhanced bone-associated gene expressions. The role of 3D O/I hybrid fibrous scaffolds on bone-associated gene expression is, however, unknown. It is hypothesized that, in addition to the 3D fibrous structures, the homogenously incorporated inorganic component plays a significant role in osteogenic gene expression. Furthermore, the hypothesis that in vitro mineralization and cell viability is enhanced on 3D PCL/BG hybrid fibrous scaffolds compared with a previously widely studied PCL was tested.

5.3 EXPERIMENTAL SECTIONS

5.3.1 Fabrication of PCL/BG hybrid Scaffolds

The electrospinning of sol-gel derived PCL/BG hybrids was carried out as described previously [22]; except that instead of a stationary collector, a custom designed rotating mandrel collector was used to produce larger mat size and to control mat morphology (see Figure 2.4). Accordingly, 1 mL of the viscous 1:1 PCL/BG hybrid solution was transferred to a plastic syringe equipped with a 22 gauge stainless steel needle, which was connected to a high voltage supply. The
electrospinning parameters were optimized to produce two distinct fiber diameters. For PCL/BG hybrid scaffolds with 260 nm mean fiber diameter (hereinafter called HF): 18 kV (voltage), 0.1 mL/h (flow rate), and 10 cm (needle tip to collector distance (TCD)); for PCL/BG hybrid scaffolds with 600 nm mean fiber diameter (hereinafter called HC): 15 kV (voltage), 0.15 mL/h (flow rate), and 6 cm (TCD) were identified. For fabricating PCL control nanofiber scaffolds, 10 wt. % of the polymer solution in 4:1 chloroform/ dimethyl sulfoxide ratio was spun at 18 kV (voltage), 0.1 mL/h (flow rate), and 10 cm (TCD).

5.3.2 Scaffold Morphology by Scanning Electron Microscopy (SEM)

The morphologies of PCL/BG hybrids and PCL control were visualized by HITACHI S-3400N Variable Pressure Scanning Electron Microscope (VP-SEM) (Hitachi, Japan). Specimens were sputter coated with gold using K550 sputter coater (Emitech Ltd, UK) prior to imaging at a working distance of 10 mm and a constant accelerating voltage of 10 keV.

5.3.3 Scaffold Porosity Measurement

Mercury porosimetry measurements were made using an Autopore IV porosimeter (Micromeritics, Norcross, GA). Samples of electrospun PCL/BG hybrid mats were cut into rectangular sections about 1.5 cm in width and 3 cm in length, before being placed in the penetrometer. Care was taken to achieve measurable intrusion volumes while maintaining unimpeded access by the mercury to the entire surface of the samples. Prior to mercury intrusion, the penetrometer was degassed to approximately 4 kPa to remove air from the system. Mercury filling of the penetrometer was performed at 3.5 kPa. Logarithmically spaced data points were taken at pressures ranging from 0.35 to 410 kPa. An equilibrium intrusion rate threshold was set at 0.003 mL/gs. The pore diameter
distribution was determined according to the Washburn equation, relating the applied pressure, \( P \), to the pore diameter, \( d \):

\[
d = \frac{-4\gamma \cos \theta}{P}
\]

Where \( \theta \) is the contact angle between the intruding mercury and the pore wall with \( (\theta = 140^\circ) \), and \( \gamma \) is the surface tension of the mercury (480 mN/m).

### 5.3.4 Water Contact Angle (WCA) Measurements

Surface wettability of the electrospun PCL/BG hybrid and PCL fibrous scaffolds was studied by static WCA method at 21 °C. The WCA of different fibrous scaffolds were measured with deionized water using a Kruss DSA 100 goniometer (Hamburg, Germany) followed up by drop shape analysis software. Values were determined by averaging measured data for 5 µL droplets at three different spots on each fibrous scaffold. The Laplace–Young fitting method was used to calculate all the static contact angles.

### 5.3.5 Mechanical Testing of PCL/BG Hybrid Scaffolds

Mechanical properties of the fibrous scaffolds were determined in tension using an Instron 3345 universal testing machine (Instron, Canton, MA) equipped with a 50 N load cell. Specimens (5 mm width \( \times \) 30 mm length \( \times \) 350 µm thick) were cut from the the electrospun PCL/BG hybrids and PCL mats using a sharp blade. For wet condition measurements, prepared specimens were soaked in simulated body fluid (SBF) for 24 h prior to testing. For each group, 5 independent specimens (\( n = 5 \)) were tested at a crosshead speed of 1 mm/min. Stress–strain relationships were obtained from the load and displacement data. The Young’s modulus (\( E \)) was determined by
calculating the slope of the linear portion of the stress–strain curves. The ultimate tensile strength (UTS), defined as the maximum stress achieved prior to rupture, was obtained from the stress–strain curves of each specimen.

5.3.6 *In vitro* Bioactivity of PCL/BG Hybrid Fibrous Scaffolds

The *in vitro* bioactivity of the electrospun PCL/BG hybrid mats was carried out by incubating in SBF [27]. The SBF solution has a composition and concentration close to those of the inorganic constituents of human plasma, and was prepared according to our previous publication [28]. Electrospun PCL/BG hybrid fibrous scaffolds (1.5 cm in diameter and ~ 350 μm in thickness) were placed in polypropylene vials containing 5 mL SBF and incubated at 37°C inside an orbital shaker at a constant speed of 120 rpm for 1 and 4 days without SBF refreshing. After each incubation period, the samples were removed from the SBF and rinsed thoroughly with phosphate-buffer saline (PBS), 70 % ethanol, and dried overnight at 37 °C. Specimens were evaluated as reported in our previous study [28].

5.3.7 Sample Preparation for MC3T3-E1 Cell culture

Electrospun PCL/BG hybrid and PCL control fibrous scaffolds were rinsed with ethanol and dried before sterilizing in low-temperature radio-frequency glow discharge argon plasma using a PDC-32G plasma cleaner (Harrick Plasma, Ithaca, NY) for 4 min. Prior to the seeding of cells, the scaffolds were equilibrated in 1.5 ml of serum-free culture medium in 24 well culture plates. After 24 h, the serum-free medium was removed and newborn mouse calvaria-derived MC3T3-E1 subclone 4 pre-osteoblast cells (ATCC, USA) were seeded at a density of 2.5×10^4 cells/scaffold on PCL/BG hybrids and PCL control fibrous scaffolds. Cells were cultured for up to 7 days in alpha-MEM medium supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA),
100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Carlsbad, CA) in a humidified atmosphere of 95% air/5% CO$_2$ at 37 ºC. The medium was refreshed every 3 days.

5.3.8 Confocal Microscopy and SEM Analysis of MC3T3-E1 Cell Morphology

MC3T3-E1 cells were fixed at room temperature for 1 h with 4 % (w/v) paraformaldehyde (EMD chemicals Inc., Gibbston, NJ) and permeablized for 10 min in cation-free PBS containing 0.1 vol % Triton X-100. Cells were incubated for 1 h at room temperature in 2 % bovine serum albumin in PBS containing Alexa™ Fluor 488-conjugated phalloidin (1:50 dilution) followed by three washes with PBS. DAPI (300 nM in PBS, Invitrogen, Burlington, Canada) was used to label nuclei. Samples were mounted on slides in SHUR/Mount™ (Triangle Biomedical Systems, Durham, NC) and analyzed with a Zeiss LSM 410 confocal microscope (Carl Zeiss Canada, Toronto, Canada) equipped with an argon/neon and a UV laser. For SEM imaging, cells were fixed with 4 % glutaraldehyde solution (Sigma-Aldrich) for 15 min, rinsed three times with PBS and dehydrated in a graded ethanol (25 %, 50 %, 75 %, 90 %, 95% and 100 %) for 5 min at each concentration. Samples were then subsequently incubated in 25/75, 50/50, 75/25, 0/100 % hexamethyldisilazane (HMDS; Sigma-Aldrich) for 10 min to preserve osteoblast morphology and dried in air. Surface morphology of samples were examined using Hitachi 3400-N VP-SEM.

5.3.9 MC3T3-E1 Cell Proliferation

Cell number was determined using the CyQuant cell proliferation assay kit, with a dye that binds to cellular nucleic acids, according to the manufacturer’s protocol (Molecular Probes, OR). Briefly, after 1, 4 and 7 days culture, the samples were rinsed twice with PBS and transferred to 24 well plates, and kept at -80 ºC until the performance of the assay, at which point cells were thawed at room temperature. Whole cell lysate was obtained by the addition of 650 mL of GR
CyQUANT GR dye/cell lysis buffer to each sample followed by 5 min incubation at room temperature. After the incubation, plates were read on a FLX 800 microplate fluorescence reader (Bio-Tek Instruments Inc., USA) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The observed fluorescence intensity was then converted to cell number using a standard curve.

5.3.10 Osteoblast Differentiation and Bone-associated Gene Expression Studies

5.3.10.1 Osteoblast Isolation and Culture

Rat calvarial osteoblasts (RCO) were isolated from 0- to 5-day-old neonatal Sprague Dawley rats. All animal procedures were approved by the Council on Animal Care of the University of Western Ontario and were in accordance with the guidelines of the Canadian Council on Animal Care. Briefly, the parietal and occipital bones were dissected and washed with phosphate-buffered saline (PBS). The harvested bones were minced and subsequently digested by incubation in 700 U/ml of type I collagenase (Sigma-Aldrich, St. Louis, MO) at 37 °C. The supernatant of the first digestion was discarded and the calvarial fragments were treated five more times with collagenase (20 min at 37 °C), and the subsequent supernatants were collected, combined together, and sedimented. The resulting cell pellet was re-suspended and cultured in α minimum essential media (α-MEM) supplemented with 10 % (v/v) fetal bovine serum (FBS; Life Technologies Inc., Carlsbad, CA), antibiotics and antmycotic (AA; 200 U/mL penicillin, 200 μg/mL streptomycin, 0.5μg/mL amphotericin B) (Gibco, Carlsbad, CA). Primary RCO cultures were maintained in a humidified 5% CO₂ atmosphere at 37°C and expanded until confluent and released from 75-cm² tissue culture plastic flasks using a 0.05% trypsin and 0.2 g/L EDTA solution, and cell number was determined by using a hemocytometer. For Alkaline Phosphatase enzyme (ALP) activity assay and gene
expression experiments, RCOs (primary or passage 1) were seeded on sterilized and plasma cleaned PCL/BG hybrid and PCL control scaffolds in 24-well plates at a density of $1 \times 10^5$ cell/scaffold and cultured in α-MEM (supplemented with 10% FBS and AA) for 3 days. Subsequently, RCO’s were cultured in osteogenic medium (regular medium described above supplemented with 2mM β-glycerophosphate and 50 µg/mL ascorbic acid) and incubated for an additional 7 or 14 days (with medium changed every 2-3 days).

**5.3.10.2 ALP Activity Measurement**

ALP activity was measured using a commercially available SensoLyte p-Nitrophenol phosphate (pNPP) Alkaline Phosphatase Assay kit (AnaSpec, USA). After 0, 7 and 14 days of culture time, the control, and experimental culture scaffolds were washed gently with PBS, followed by washing twice with cold 1x lysis buffer, before lysing with 1x lysis buffer with 0.2% of Triton X-100. For extracting cell lysates, cells on scaffolds were sonicated twice for 1 min and centrifuged at $10^4$ rpm for 15 min at 4 °C. Then, the cell lysates were analyzed by measuring the ALP activity when ALP enzyme catalyzes the cleavage of a phosphate group and releases p-nitrophenol from p-nitrophenyl phosphate, which is colorless in alkaline buffer solution. Upon dephosphorylation of p-nitrophenyl phosphate for 1 h at 37 °C, the solution turns yellow, after which the reaction was terminated with stop solution, and the colorimetric determination of the product (p-nitrophenol) was detected at an absorbance of 405 nm using a microplate reader. The total intracellular protein content was quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA) in accordance with supplier’s instructions. The results of ALP activity were reported as ng of p-nitrophenol produced per hour, normalized to total protein content (ng/h/mg protein). Four electrospun PCL/BG and PCL control fibrous scaffolds were used in each experiment and was repeated twice (n=8).
5.3.10.3 Quantitative Assessment of Osteoblast Marker Gene Expression

Total RNA was extracted using TRIzol (Invitrogen) prior to the addition of osteogenic medium (day 0), and days 7 and 14. RNA was purified using an RNeasy Micro Kit (Qiagen - Canada, Toronto, ON) and genomic DNA removed using DNase I (Qiagen), according to the manufacturer’s instructions. The total RNA concentration and purity was determined from the absorbance at 260 and 280 nm using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized using 1 μg of total RNA primed with oligo(dT)$_{12-18}$ as described in SuperScript™ (Table 5.1). Quantitative real-time PCR was conducted in 10 μl reaction volumes, using a Chromo4 Real-time Thermal Cycler (Bio-Rad, Mississauga, Canada) and gene expressions of collagen type I (Col I), tissue non-specific alkaline phosphatase (ALP), Osteopontin (OPN), Bone sialoprotein (BSP), Osteocalcin (OCN) and GAPDH were then determined with iQ™ SYBR® Green Supermix (Bio-Rad) according to the recommended protocol of the manufacturer. Cycling parameters were optimized as follows: denaturation 95 °C (15s), gradient annealing 53°C/58°C (60s), extension 72 °C (30 s), and running for 40 cycles. mRNA expression in RCOs was normalized to GAPDH with at least three repeats per experimental group and expressed as a relative ratio using the Gene Expression Macro analysis software (Bio-Rad, Mississauga, Canada). Although clonal murine calvarial MC3T3-E1 cells are the predominant in vitro model for studying bone cell interactions with biomaterials, osteoblast differentiation, and ECM signaling primary RCO cells were specifically chose for bone-associated gene expression studies since our long-term objective is to implant the tissue-engineered bone construct into animal models. It is believed that this is a logical approach as the use of cell lines to engineer a tissue for eventual implantation is obviously less desirable.
Table 5.1. Primers for Rat-Specific Osteoblast mRNA Amplification

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase (ALP)</td>
<td>Forward: 5′-AGGCAGGATTGACCACCGG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-TGTAGTTCTGCTCATGGA-3’</td>
</tr>
<tr>
<td>Collagen, α 1, type 1 (Col I)</td>
<td>Forward: 5′-CAACAAATCCCCCACACAC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-CACACAAAGACAAAGAAGAG-3’</td>
</tr>
<tr>
<td>Osteocalcin (OCN)</td>
<td>Forward: 5′-CTGCATTCTGCCTCTCTGAC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′CTATTCCACCTTTACTGCCC-3’</td>
</tr>
<tr>
<td>Osteopontin (OPN)</td>
<td>Forward: 5′-GTTTGCTTTTGCTTTGTT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-ATCGTCGTCGTCATCATC-3’</td>
</tr>
<tr>
<td>Bone sialoprotein (BSP)</td>
<td>Forward: 5′-CTGCTTTAATCTTGCTCTG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-CCATCTCCATTTTCTTCC-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5′-GGTGGTCTCCTCTGACTTCACA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GTTGCTGTAGCCAAATTCGTTG-3’</td>
</tr>
</tbody>
</table>

5.3.11 Statistical Analysis

All statistical analyses were performed using Instat 3.0 (GraphPAD Software, Inc., San Diego, CA). One- and two way analyses of variance (ANOVA) and a Tukey-Kramer multiple comparisons test were used to assess the statistical significance of the data at $p < 0.05$.

5.4 RESULTS

5.4.1 Morphology, Fiber-size Distribution and Porosity of PCL/BG Hybrid Fibrous Scaffolds

In this study, biomimetic and homogeneous 3D O/I hybrid fibrous scaffolds consisting of 50 wt. % PCL and 50 wt. % tertiary BG were successfully fabricated by a combined sol-gel and electrospinning processes to exploit the synergetic effect of PCL and BG when combined at a molecular level. The choice of this ratio was based on a previous finding (Chapters 3 and 4) that
this composition led to the best spatially distributed inorganic components in the O/I hybrid system from sol-gel reactions [22]. It can be seen from Figure 5.1 A-C that the sol-gel derived PCL/BG hybrid and PCL control fibrous scaffolds had randomly oriented fibers forming a porous 3D structure and are reasonably uniform in sizes with no beading, fusion or bundling effects. The SEM micrographs were further analyzed to determine the fiber diameter distribution whereby individual fiber diameters (n=100) at six random locations in the mats were measured from representative SEM images by using ImageJ software. The corresponding histograms displayed in Figure 5.1 showed that all compositions yielded reasonably unimodal distributions and good correlation with a Gaussian nonlinear fit. Accordingly, two different PCL/BG hybrid scaffolds with fiber diameter distributions of 260 ± 60 nm (HF) and 600 ± 166 nm (HC) were fabricated. The control PCL scaffold had 300±80 nm fiber diameter which was not statistically different from the HF hybrid scaffolds (p> 0.05). Scaffolds for tissue engineering are required to have high porosity and interconnected pore structure for cell infiltration, matrix remodeling, and nutrient transport to take place. Therefore, it was sought to examine the effect of fiber diameter on the porosity of PCL/BG hybrid scaffolds. Pore-size distribution and pore parameters of electrospun PCL/BG hybrid scaffolds determined by a mercury porosimetry are presented in Figure 5.2 and Table 5.2.
**Figure 5.1** Representative SEM micrographs and fiber diameter plots for PCL/BG hybrid and PCL fibrous scaffolds. The fiber diameter distribution plots were generated from six random SEM micrographs (n=100) acquired at random locations, and ImageJ software was used to measure individual fiber diameters. (A) PCL/BG hybrid scaffold with 263 ± 60 nm fiber diameter (HF), (B) PCL/BG hybrid scaffold with 600 ± 166 nm fiber diameter (HC), (C) PCL scaffold with 300 ± 80 nm fiber diameter. Scale bar represents 10 μm.
From Figure 5.2, the mean pore diameter for HF hybrid scaffolds was 33.5 μm; however, the HC scaffold exhibited a bimodal pore diameter distribution, with one major peak at 52 μm and a minor secondary peak at 3 μm. The intrusion and subsequent extrusion cycles during the mercury intrusion experiments resulted in negligible hysteresis, indicating that scaffold compression at the operating pressures was minimal [29]. Porosity analyses of HF and HC electrospun hybrid scaffolds revealed 77.26 % and 83.47 % respectively (Table 5.2) suggesting the utility of these scaffolds for bone tissue engineering applications.

Figure 5.2. PCL/BG fibrous scaffolds pore-size distribution plot obtained by mercury intrusion porosimetry as a function of differential and cumulative intrusion volumes.
Table 5.2: Mercury Intrusion Porosimetry (MIP) analyses data for the electrospun PCL/BG hybrid fibrous scaffolds.

<table>
<thead>
<tr>
<th>Attributes</th>
<th>HF</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Intrusion Volume</td>
<td>3.89 mL/g</td>
<td>3.94 mL/g</td>
</tr>
<tr>
<td>Penetrometer Weight (m_p)</td>
<td>62.24 g</td>
<td>62.24 g</td>
</tr>
<tr>
<td>Assembly Weight (m_a)</td>
<td>a143.45 g</td>
<td>a143.45 g</td>
</tr>
<tr>
<td></td>
<td>b138.43 g</td>
<td>b133.65 g</td>
</tr>
<tr>
<td>Sample Weight (m_s)</td>
<td>0.08 g</td>
<td>0.16 g</td>
</tr>
<tr>
<td>Sample Bulk Volume (V_b)</td>
<td>0.41 mL</td>
<td>0.76 mL</td>
</tr>
<tr>
<td>Porosity</td>
<td>76.54 %</td>
<td>83.23 %</td>
</tr>
<tr>
<td>Average Pore Diameter</td>
<td>33.06 μm</td>
<td>51.52 μm</td>
</tr>
<tr>
<td></td>
<td>d2.98 μm</td>
<td></td>
</tr>
</tbody>
</table>

*Assembly weight without sample: m_a = m_p + m_{Hg}
*bAssembly weight with sample: m_a = m_p + m_{Hg} + m_s
*cFirst Peak (large pore size region) of the bimodal distribution
*dSecond Peak (small pore size region) of the bimodal distribution

5.4.2 Surface Wettability of PCL/BG Hybrid Fibrous Scaffolds

Given that the current hybrid scaffolds were fabricated with two distinctive fiber diameters, it is expected that the wetting properties to be affected by both composition and fiber diameter. The water sessile drop deformation and contact angles with respect to time are presented in Figure 5.3 from which it can be inferred that the water droplet maintained its round shape on the control electrospun PCL scaffolds (Figure 5.3C) and its WCA was found to be 126.3° ± 1.6° during a 45 seconds observation. On contrary, the water sessile droplets deformed quickly and spread on the surface of the electrospun PCL/BG hybrid scaffolds, and penetrated into the scaffold meshwork at the end of a 45 s observation. Furthermore, the WCA measurements with respect to exposure time demonstrated that the spreading velocity of water droplets on HC fibrous scaffolds was higher than
HF fibrous scaffolds, Figures 5.3A and B. The time for the spreading of 5μL water sessile droplet on the scaffolds was 40 s on HF and 22 s on HC.

**Figure 5.3.** Water contact angle (WCA) measurements of electrospun PCL/BG hybrids and PCL control fibrous scaffolds. Representative time-frame images of water sessile drop on PCL/BG hybrid and PCL fibrous scaffolds and WCAs as a function of time for electrospun HC scaffolds (A), HF scaffolds (B), and PCL scaffolds (C). Error bars represent means ± SD for n=3.
5.4.3 Mechanical Properties and *in vitro* Mineralization of PCL/BG Hybrid Fibrous Scaffolds

In Chapter 4, the composition-dependent mechanical properties of PCL/BG hybrid monoliths (solid cylindrical discs) was reported [28]. These solid discs, however, cannot be used as tissue engineering scaffolds because of their 2D surfaces. Since mechanical properties of the PCL/BG hybrid nanofibrous scaffolds were important design considerations in this study, the tensile properties of the 3D fibrous scaffolds were investigated and the data are collectively presented in Figure 5.4. As can be seen from representative stress-strain curves (Figure 5.4B) both the fiber diameter of the PCL/BG hybrid fibrous scaffolds and the test condition (dry or wet) influenced their mechanical properties. HF fibrous scaffolds had significantly higher UTS in both dry and wet testing conditions than both HC fibrous scaffolds and the PCL controls (Figure 5.4C; p < 0.001). Within the three different scaffolds, the testing condition did not have a significant effect on the UTS (p > 0.05). Both HC and HF fibrous scaffolds showed significantly higher E (a measure of stiffness) compared with PCL controls (Figure 3D; p < 0.001). Whereas the testing condition had no significant effect on the E values for the control PCL fibrous scaffolds, it had a significant effect on the hybrid fibrous scaffolds whereby the wet condition increased the modulus. This finding is attributed to the plasticizing effect of the soaking solution and is consistent with the WCA data (Figure 5.3) that showed enhanced wetting for the hybrid fibrous scaffolds. Finally, the HC fibrous scaffold had significantly lower elongation at break than both HF and PCL control scaffolds (Figure 5.4E; p < 0.01). Furthermore, it is evident that the wetted HC scaffolds had significantly diminished elongation at break (p < 0.05).
Figure 5.4. Tensile mechanical properties for PCL/BG hybrid and PCL control fibrous scaffolds. (A) Tensile test set-up; (B) Representative stress-strain curves; (C) Ultimate tensile strength (UTS); (D) Young’s moduli (E), and (E) Elongation at break (ε) of PCL/BG hybrids and PCL control fibrous scaffolds measured in dry and wet testing condition. The wet condition measurement was taken after soaking in SBF for 24 h. Data are mean ± SD (n=5). Statistical analysis of the data were conducted using two-way ANOVA and Tukey Kramer multiple comparative test, p < 0.05.
Collectively, these data suggest that the overall mechanical properties of the PCL/BG hybrid fibrous scaffolds are superior as compared to PCL, which is the dominantly used fibrous scaffold for bone tissue engineering [30, 31]. It is also evident that the HF scaffolds had higher tensile strength than HC scaffolds due to its smaller average pore diameter, Table 5.2. Given its attractive mechanical properties, the bioactivity of HF scaffolds and compared it to PCL controls were tested. As shown in Figure 5.5, HF scaffolds were covered with heterogeneous and sparsely dispersed spherically-shaped particles after 24 h of incubation in SBF (Figure 5.5B) which became denser at longer incubation times. Conversely, no apatite layer deposition was observed on the PCL control scaffolds.
Figure 5.5: Representative SEM micrographs showing the evolution of HA deposit on electrospun PCL/BG hybrids and PCL control fibrous scaffold surfaces after incubating in SBF at different time points. (A) Scaffolds before incubation starts; (B, C) Scaffolds after incubating in SBF for 24 h and 96 h respectively.
5.4.4 MC3T3-E1 Cell Spreading and Proliferation on PCL/BG Hybrid Fibrous Scaffolds

Favorable cell attachment and spreading on scaffolds is an important requirement for subsequent cellular activities. MC3T3-E1 cell interactions data with the electrospun PCL/BG hybrid fibrous scaffolds at 1, 4, and 7 days are collectively shown in Figure 5.6. At day 1, on PCL/BG hybrid and PCL control scaffolds, cells spread well and were in intimate contact with the scaffold surfaces. At day 4 and 7 cells were observed with enhanced cytoplasmic extensions, suggesting favorable cell-scaffold interactions. The proliferation of MC3T3-E1 cells on the PCL/BG hybrid fibrous scaffolds and the PCL control at different time points demonstrating MC3T3-E1 cell growth for up to 7 days of culture is shown in Figure 5.7. There was no significant difference in cell number between all scaffolds at day 1 ($p > 0.05$). Similarly, there was no significant difference in cell number between HC and PCL scaffolds at days 4 and 7 ($p > 0.05$). However, HF fibrous scaffolds promoted cell growth significantly at days 4 and 7 ($p < 0.05$).
Figure 5.6. MC3T3-E1 cell morphology on electrospun PCL/BG fibrous scaffolds. MC3T3-E1 cells at 2.5×10^4 cells/scaffold were seeded on electrospun PCL/BG hybrids and PCL control. Osteoblast morphology on the scaffold surface was obtained by confocal microscopy and SEM after 1, 4 and 7 days of culture. (A, B) Representative SEM micrographs of MC3T3-E1 cells on HC and HF scaffolds respectively; (C) Confocal micrographs of MC3T3-E1 cells on HF scaffolds (green= F-actin and blue = nuclei). (Scale bar=40 μm).
Figure 5.7. MC3T3-E1 cell proliferation on electrospun PCL/BG and PCL control fibrous scaffolds. MC3T3-E1 cells at $2.5 \times 10^4$ cells/scaffold were seeded on electrospun PCL/BG hybrids and PCL control. Cell proliferation per scaffold as determined by CyQUANT fluorescent assay protocol after day 1, 4 and 7 of cultures. Data are the mean ± SEM (n=5). Statistical analysis of the data at each time point were conducted separately using one-way ANOVA and Tukey Kramer multiple comparative test ($p < 0.05$).

5.4.5 ALP Activity and Bone-associated Gene Expression

ALP is an enzyme routinely used as an early marker for osteoblasts differentiation and plays a critical role in regulating mineralization of the extracellular matrix [32]. Given its importance, the ALP activity of RCO cells on PCL/BG hybrid scaffolds was assessed by a colorimetric method. To do this, RCOs were seeded on HF, HC and PCL fibrous scaffolds and cultured for 3 days. Medium was then supplemented with β-glycerophosphate and ascorbic acid to induce osteoblastic differentiation (day 0) and cells were cultured for an additional 7 and 14 days. As shown in Figure 5.8A, ALP activity of RCO cultured on HF and HC fibrous scaffolds was significantly higher than
the PCL control for 0 day (p < 0.01). No significant differences on ALP activities were observed in all scaffolds on day 7 (p > 0.05). Between days 7 and 14, ALP activity of RCO cells cultured on HF and HC were significantly higher than the PCL control scaffold (p < 0.01 for HC; p < 0.001 for HF). Furthermore, a significantly higher ALP activity (p < 0.05) was observed for HF scaffolds compared to HC scaffolds. These results indicate that the PCL/BG hybrid scaffolds promoted ALP activity by RCO cells which is a strong marker of osteoblasts phenotype and mineralization [33].

To further evaluate osteogenic differentiation on electrospun PCL/BG hybrid and PCL fibrous scaffolds, quantitative real time PCR measurements were performed. The expression of ALP, Col I, OPN, BSP, and OCN were determined using primers (Table 5.1) specific for each osteogenic marker gene and were normalized to GAPDH and expressed as fold changes relative to the gene expression at day 0 of the PCL control (Figures 5.8 B-F). Consistent with the enzyme activity data (Figure 5.8A), RCO cells cultured on HF and HC scaffolds expressed significantly higher ALP compared with the PCL control following days 0 and 7 cultures in osteogenic media (Figure 6B; p < 0.05). Between HF and HC, there was a significantly higher ALP gene expression on HF scaffolds at early culture time (day 0) (p < 0.05), and on HC scaffolds at day 7. After 14 days of culture, ALP gene expression increased modestly but not significantly in HF and HC hybrid scaffolds in comparison to day 7, p > 0.05. The expression of Col I on the HF and HC scaffolds increased significantly for 7 and 14 days of cultures compared with the PCL control (p < 0.05). While Col I gene expression by RCO cells seeded on HF and HC hybrid scaffolds showed an upward trend with culture time, its expression on PCL control scaffolds decreased on day 7 (0.87-fold) then slightly increased on day 14 (1.2-fold). Unlike ALP and Col I gene expressions, OPN, BSP, and OCN gene expressions were significantly delayed until 14 days of culture, Figures 6 D-F. Furthermore, while both HF and HC scaffolds up-regulated OCN gene expression significantly
at day 14 (p < 0.001), OPN and BSP were only significantly up-regulated by cells cultured on HF scaffolds (p < 0.05 and p < 0.001, respectively) compared with the PCL control suggesting a spatiotemporal regulation of gene expression.

**Figure 5.8.** Alkaline phosphatase (ALP) enzyme activity assay and gene expression of bone-associated mRNAs by primary rat osteoblastic cells (ROCs) grown on PCL/BG hybrid and pure PCL (control) fibrous scaffolds. RCOs were seeded on HF, HC and PCL fibrous scaffolds and cultured for 3 days. Medium was then supplemented with β-glycerophosphate and ascorbic acid.
to induce osteoblastic differentiation (day 0) and cells were cultured for an additional 7 and 14
days. (A) ALP activity quantification, and (B-F) Spatiotemporal expressions of ALP, collagen type
I (Col I), Osteopontin (OPN), Bone sialoprotein (BSP), and osteocalcin (OC) mRNAs by qRT-
PCR. The mRNA levels of the genes of interest were normalised against the expression of
GAPDH, and are presented as fold changes relative to PCL at day 0. The bars represent mean ±
SEM for two independent experiments and four scaffolds in each experiment for ALP activity
(n=8) and triplicate samples from three independent experiments for gene expressions (n=3).
Statistical analysis of the data at each time point were conducted separately using one-way
ANOVA and Tukey Kramer multiple comparative test (p < 0.05).

5.5 DISCUSSION

This study documented in this Chapter demonstrates the potential application of novel sol-gel
derived and electrospun PCL/BG hybrid fibrous scaffolds and the effect of fiber diameter on both
scaffold properties and cellular responses for bone regeneration. Although the combination of BG
nanoparticles or nanofibers with polymeric systems enables the production of nanocomposites as
scaffolds for bone tissue engineering (reviewed in detail in ref [34]), sol-gel hybridization of the
polymer with the inorganic glass components at a molecular scale is a new enabling strategy [22].
In this strategy the inorganic components are strongly hydrogen bonded with the polymer matrix
creating a homogenous material which can be electrospun into fibrous scaffolds before the network
crosslinks. The data documented in this Chapter (Figure 1 and Table 2) demonstrated that the
electrospun PCL/BG hybrid scaffolds with 260 nm average fiber diameter (HF) and 600 nm fiber
diameter scaffold (HC) yielded an average pore-size of 33 μm and 52 μm respectively.
Furthermore, the porosity of HF and HC scaffolds was 77 % and 84 %, respectively. It is believed
that the lower total pore volume and porosity observed in the HF scaffolds resulted from the denser
structure formed by the lower fiber diameter.
The three important parameters that play a considerable role in the functional and mechanical performance of electrospun fibrous scaffolds are fiber diameter, interconnected pore-size, and percentage porosity; as these influence cell infiltration and the transport of oxygen and nutrients to the cells and removal of waste products [35]. Smaller fiber diameters (<1µm), pore-sizes in excess of the size of the cell (>20 µm), and porosities in excess of 70% are deemed to be requirements for electrospun scaffolds [36]. Oftentimes, one parameter is optimized at the expense of the other and even the application of ultrasonic methods and sacrificial polymers did not appear to improve all of the above parameters [36, 37]. Pore-size and porosity are particularly problematic to optimize in electrospun BG scaffolds [22, 38]. By combining sol-gel chemistry and electrospinning in situ, the findings of this study indicated that all the required scaffold parameters were within the desired ranges. The wettability of scaffolds is another useful parameter that influences cell adhesion and spreading and determines subsequent processes such as cell morphology, proliferation, and differentiation [39, 40]. Osteogenic cells tend to prefer hydrophilic surfaces rather than hydrophobic surfaces [41]. Results from WCA study (Figure 5.3) of the electrospun PCL/BG hybrids and PCL fibrous scaffolds revealed that the wetting was governed by both the composition and the fiber diameter. It is known that fibrous scaffolds fabricated from a given material will have a higher WCA than the film counterpart due to the rough surface topography of electrospun fibers compared with relatively smooth topography observed in films [42]. It was not, therefore, surprising that a higher WCA was observed for the electrospun PCL fibrous scaffold (126°) than the 76° WCA value that was measured for PCL films. Consequently, the test water droplet could not easily penetrate into inter-fiber pores on the rough surface of hydrophobic fibrous scaffolds. Thus, the water droplet was supported by a semi-solid and semi-air plane surface, resulting in a significant increase in the water contact angle [42, 43]. In contrary
to this, the hybrid fibrous scaffolds fabricated for this study showed a reduced WCA, thus improving the scaffold wettability [44]. Given that the PCL average fiber diameter was within a comparable range to the HF fibrous scaffolds, the reduced WCA or the higher wettability observed on the PCL/BG fibrous hybrids is mainly contributed from the hydrophilic surface of the BG phase in the hybrid system [22].

The electrospun PCL/BG hybrid scaffolds in the present study showed significantly higher stiffness (p < 0.001) compared to the pure PCL scaffolds owing to the strong hydrogen bonding at molecular scale and homogeneously distributed inorganic network within the PCL matrix [22]. Recent studies showed that electrospun scaffolds filled with bioceramic nanoparticles in excess 25% resulted in weakened scaffolds compared to the PCL control [18, 45] suggesting poor dispersion and absence of interfacial bonding between the nanoparticles and the PCL matrix. Since the current hybrid fibrous scaffolds were prepared with 1:1 ratio of tertiary BG and PCL, it is noteworthy that the observed higher mechanical strength was the consequence of homogeneous distribution. Although data on the effect of fiber diameter on the mechanical properties of electrospun BG/polymer hybrid scaffolds has not been reported in the literature, few studies have shown conflicting results where fiber diameter both increased [46] and decreased [47] the mechanical properties of polymeric fibers. Given that tissue engineering scaffolds are to be used in contact with biological fluids, the wet condition mechanical properties performed after soaking in SBF for 24 h to simulate the effect of physiologic environment revealed that the PCL/BG hybrid scaffolds showed relatively higher moduli as compared to their dry counterparts. Despite the increase in stiffness, the wet condition did not change the UTS significantly. Conversely, no significant difference in stiffness and UTS (p > 0.05) were observed for PCL control during the dry and wet condition measurements. These findings elucidate that HA precipitation on PCL/BG hybrid
scaffold surfaces after 24 h of SBF soaking (Figure 5.5) might have enhanced the stiffness [48, 49]. Notwithstanding a number of reports about sol-gel derived BG fibers, the most relevant work to the current study dealing with combined sol–gel and electrospinning techniques to fabricate hybrid fibrous scaffolds was conducted by Gao and co-workers who prepared BG/gelatin hybrid fibrous scaffolds [50]. In this cited study, CaNO₃ which is presumed to be cytotoxic [51] was used as calcium precursor. In fact the presence of CaNO₃ is one of the reasons why sol-gel derived electrospun BG fibers in previous studies had to be heated to 600 °C to remove the nitrate which also burns off the polymer, necessitating the re-suspension of BG fibers into a different polymer than the one used in the sol-gel step [34]. Furthermore, swelling, flattening, and fusion of the gelatin matrix even after crosslinking was evident leading to weak hybrid scaffold material (~ 4.3 MPa of tensile strength). Our study utilized CaCl₂ as a calcium precursor that did not require heat treatment and a biocompatible and biodegradable PCL matrix that did not swell and flatten while significantly improving both the mechanical and wetting properties of the scaffolds.

In addition to providing the structural template, bone tissue engineering scaffolds should provide the 3D microenvironment that direct osteoblastic differentiation and tissue maturation. Consistent with previous studies on carbon nanofiber [52] and on PCL fibers [53], MC3T3-E1 cells seeded on all PCL/BG hybrid scaffolds attached, well-spread, and proliferated in a fiber diameter dependent manner (Figures 5.6 and 5.7). Some possible reasons for high cell proliferation on the smaller fiber diameter hybrid scaffolds are large surface area-to-volume ratio for growth factors binding and the pliability of the smaller diameter fibers. Osteoblast differentiation and subsequent bone formation is a gradual and well-orchestrated process associated with characteristic temporal modifications in gene expression and comprised of three developmental stages: i) proliferation, ii) extracellular matrix production and maturation, and iii) matrix mineralization [54, 55]. In this 3-
stage paradigm, Col I is expressed during the initial period of proliferation and ECM biosynthesis, whereas ALP which is regarded as characteristic feature of osteoblasts during bone mineralization [56] is expressed during the post proliferative period of ECM maturation, and the expression of OPN, OCN, and BSP occurs later during the third period of matrix mineralization [57, 58]. In the present investigation, RCOs on PCL/BG fibrous scaffolds had significantly higher ALP activity on early stage and continued until day 14 (Figure 5.8) due to the presence of the tertiary BG component in the hybrid system (Figure 5.8A). Notwithstanding the significant difference that exists in ALP gene expression between the two hybrid fibrous scaffolds for the first 7 days of culture, the corresponding ALP activity was not significant suggesting temporal effects in protein translation. In the ordered hierarchical sequence of events occurring during osteoblast differentiation and matrix mineralization, Col I biosynthesis and ALP activity are followed by the deposition of osteoblasts-specific non-collagenous matrix proteins, such as OPN, BSP and OCN [23, 54, 55]. In particular, expression of OPN indicates the initial growth of hydroxyapatite crystals and their ability to bind and potentially catalyze mineralization processes [59, 60]. The high BSP gene expression suggests copious formation of non-collagenous proteins, promoting efficient nucleation of mineral phases [61]. Finally, the high expression of OCN, the most specific marker for osteoblast maturation, confirms the formation of a bone-associated protein. In the present study, bone-associated gene expressions were up-regulated throughout the 14 days of culture for PCL/BG hybrid scaffolds as compared to the PCL control. Particularly, the PCL/BG hybrid scaffolds with smaller fiber diameter favored significantly higher gene expression of OPN, BSP and OCN on day 14. Taken together, the current study demonstrated that PCL/BG hybrid scaffolds significantly improved tensile properties, in vitro bioactivity and increased bone-associated gene
expression when compared to the commonly used PCL scaffolds and that these gene expressions were up-regulated by the topography of smaller diameter fibers.

5.6 CONCLUSIONS

A highly porous sol-gel derived O/I hybrid fibrous scaffolds with two distinct fiber diameters were fabricated for bone tissue engineering application. Subsequently, scaffold morphological and mechanical properties relationships, and bone-associated gene expressions by osteoblasts were studied. When compared with the PCL fibrous scaffolds, the PCL/BG hybrids exhibited a hydrophilic surface and increased stiffness. A significantly higher tensile strength (p < 0.001) was found for the lower fiber diameter PCL/BG hybrid scaffolds as compared to the other scaffolds. Incubation of hybrid scaffolds in SBF resulted in the formation of bone-like apatite on the surface of the PCL/BG fibrous scaffold within 24 h. The PCL/BG hybrid scaffolds supported an earlier and enhanced osteoblast phenotype when compared with PCL scaffolds, including proliferation, alkaline phosphatase activity, and changes in genes associated with the osteoblast phenotype. The findings of this study demonstrated that the PCL/BG fibrous scaffolds with a controlled fiber diameter may serve as superior scaffolds versus PCL fibrous scaffolds for promoting bone regeneration.
5.7 REFERENCES


CHAPTER 6: GENERAL DISCUSSION

Overview: This Chapter provides a general summary and conclusions of the overall work presented in this thesis. Significant contributions of the research are also briefly summarized. Finally, the limitations and few future directions are presented.

6.1 SUMMARY AND CONCLUSIONS

The main goal of this study was to develop biodegradable and bioactive organic-inorganic hybrid biomaterials with tailorable physical and biological properties for bone tissue engineering. The materials selected for this study were a sol-gel derived tertiary bioactive glass (with a composition of 70 mole % of SiO₂, 26 mole % of CaO, and 4 mole % P₂O₅) and poly (ε-caprolactone). BGs are known for their ability to elicit hydroxyapatite formation, osteoconductivity, and ability to up-regulate gene expression in osteoblasts [1-3]. However, because of their lower fracture toughness and difficulty to fabricate into 3D macro-porous structure, their application has been significantly limited. Therefore, in an effort to improve their mechanical properties and processability, PCL were incorporated during the sol-gel synthesis to form O/I hybrid with BG. PCL was selected for this study, because it is biocompatible, resilient and biodegradable, which has been used in numerous biomedical applications [4-6]. Kangming et al. also demonstrated that the carbonyls in the macromolecular backbone of the PCL have shown the capability to form intermolecular hydrogen-bonding with silanol groups (Si-OH) of the silica inorganic network [7]. Despite demonstrating the aforementioned potential, there are various complex issues associated with the synthesis of O/I hybrids consisting of biodegradable aliphatic polyesters and BG using a sol-gel process that needed to be addressed such as the improvement of PCL solubility in the sol, choice of proper calcium precursors, incorporation of calcium atoms, identification of the type and mechanism of intermolecular interaction between the inorganic and organic phases, and
production of homogenous O/I hybrid biomaterials. These issues were addressed in this work during the synthesis of PCL/BG hybrid biomaterials [8].

To the best of the author’s knowledge, Chapter 3 is the first study to describe the synthesis of O/I hybrid biomaterials consisting of synthetic aliphatic polyester and a tertiary bioactive glass [8]. As such PCL chains were successfully incorporated into the inorganic sol while the inorganic networks were forming. Five different compositions (10 - 60 % PCL) in PCL/BG hybrids were successfully synthesized and thoroughly investigated. In all PCL/BG hybrid materials an amorphous structure with spatially distributed calcium atom in the hybrid matrix were observed. The organic and inorganic phases interacted at molecular level via a hydrogen-bond formed between the carbonyl group of the PCL and the silanol-hydroxyl group of the inorganic network. The addition of PCL improved the control over the rheology of the sol by reducing the rate of gelation, which helped preserve the desired viscosity range for a longer period of time. On the contrary, sols, which were synthesized without the inclusion of PCL, were very difficult to process into fibers, due to the rapid change in viscosity during the gelation process. The delay in gelation rate observed during the presence of PCL could be attributed to the steric and electrostatic interactions between the PCL and the inorganic network. Accordingly, the PCL/BG sol remained as a viscous solution for longer period of time (~ 2 weeks), whereas the pure BG sol lasted only for few approximately 2 days. Therefore, the longer gelation time observed in PCL/BG system provided the means to control the viscosity, which facilitated the fabrication of larger amount of sample with a controlled fiber diameter by using the electrospinning process. Overall, the findings of this study demonstrated the potential applications of the PCL/BG hybrid biomaterial for bone regenerations.
In order to evaluate the synergic effect due to the hybridization on PCL/BG hybrid biomaterials properties, such as \textit{in vitro} hydroxyapatite formation ability, mechanical properties, and biocompatibility; three representative PCL/BG hybrid compositions were selected [9]. In this study (Chapter 4), it was hypothesized that the presence of surface silanol groups (Si-OH) and the effective incorporation of both calcium and phosphate ions into the hybrid system enhances the \textit{in vitro} bioactivity behavior and mechanical properties. Accordingly, the results of the \textit{in vitro} bioactivity test indicated that bone-like HA was precipitated in all PCL/BG hybrid compositions after 6 hours of soaking in SBF, however, the rate and amount of HA deposition increased with an increase in BG content. The compressive tests of cylindrical specimens indicated that the BG content contributed for the higher strength and modulus observed in the PCL/BG hybrid system. The addition of the PCL also increased the percentage strain at a maximum stress. The observations from the preliminary cell study by adding different concentrations of PCL/BG particulates (100 – 500 µg/ml) in MC3T3-E1 cell cultures indicated that the PCL/BG hybrids have no significant toxicity. Taken together, this study documented in this thesis demonstrated the ability to tailor the bioactivity and mechanical property of these novel PCL/BG hybrid materials, which may suggest the potential to fabricate tunable PCL/BG hybrid scaffolds with different physico-chemical, mechanical and biological properties for various biomedical applications.

Despite the promising results using 2D PCL/BG hybrid surfaces (Chapter 4), the application of the PCL/BG hybrids for bone tissue engineering requires porous 3D scaffold architectures to mimic the trabecular bone structure of the native tissue. Therefore, 3D PCL/BG hybrid constructs were prepared by a sol-gel/electrospinning process [8]. In Chapter 5, a biodegradable and bioactive O/I hybrid fibrous scaffolds were designed to mimic the morphological functions of ECM and to provide favorable microenvironment for cells to adhere, migrate, proliferate, and differentiate. In
this work, 3D PCL/BG fibrous scaffolds with two different fiber diameters were successfully fabricated by a combined sol-gel and electrospinning process. Some of the critical scaffold properties for bone regeneration applications, including wettability, pore-size distribution, porosity, mechanical properties and bioactivity were evaluated. It is also required for bone tissue engineering scaffolds to support the osteoblast cell attachment, proliferation and differentiation. Hence, the role of PCL/BG hybrid fibrous scaffolds on osteoblast cell proliferation and osteoblast phenotype markers gene expression were evaluated *in vitro*. In addition, the effect of fiber diameter on the physical and biological properties of the PCL/BG hybrid fibrous scaffolds was demonstrated.

The fibrous scaffolds characterization results indicated that the PCL/BG hybrid scaffolds exhibited high porosity, greatly improved hydrophilicity, significantly increased tensile properties, and enhanced *in vitro* bone-like apatite formation ability as compared to the PCL fibrous scaffolds. The PCL/BG fibrous scaffolds supported the attachment and proliferation of osteoblast cells. In order to evaluate the osteogenic potential of the PCL/BG hybrid scaffolds, ALP activity and the gene expression level of bone-associated protein markers were assessed by a colorimetric technique and RT-PCR analysis, respectively. It was observed that ROCs cultured on the electrospun PCL/BG hybrid fibrous scaffolds maintained their phenotypic expression and exhibited an increased alkaline phosphatase activity, and enhanced expressions of bone-associated markers as compared to the pure PCL fibrous scaffold over the period of the culture time. In general, results of this study demonstrated that the PCL/BG fibrous scaffolds showed superior physical, mechanical and biological properties compared with PCL fibrous scaffolds for promoting bone regeneration.
6.2 CONTRIBUTIONS OF THE RESEARCH TO THE CURRENT STATE OF KNOWLEDGE

In this thesis work, a new class of biomimetic O/I hybrid fibrous scaffolds with an enhanced bioactivity, mechanical properties and osteogenic potential were developed for bone tissue engineering. Previous studies by other groups demonstrated that nano/composites of biodegradable polymers and bioactive inorganic fillers were viable options to design scaffolds that can potentially mimic structural and functional properties of bone ECM. Although some encouraging results are reported, most of the nano/composites lacked specific interactions between the organic and inorganic phases, and associated difficulties to achieve a uniform dispersion of fillers within the polymer matrix [10, 11]. The weak interaction between the organic and inorganic phases and poor dispersion led to compromised mechanical properties and unfavorable cellular responses [10, 12, 13]. Hence, the choice of appropriate materials and improving the structure-property relationships are important components in scaffold design as the interfacial interaction between the inorganic phase and the polymer matrix contribute significantly to the final mechanical properties and biological response. These design approaches were expected to capitalize the combined effects of both the biodegradable polymers and bioactive inorganic fillers. In lieu of this, the hypothesis in this work was that scaffold material based on O/I hybrids consisting of biodegradable polymers and BG will overcome the limitations of conventional composites. Aside from the intrinsic physical properties of the components, these hybrid materials can also display distinct properties as a result of the nature and degree of interfacial interaction between the two components. Given that, this thesis advances the current state-of-the-art in organic-inorganic hybrid biomaterials.
The traditional processing conditions for inorganic materials usually involve treatments at high temperature. In such cases thermal stability of organic compounds is impossible. Thus, in this study, the sol-gel process that in mild-condition was preferred to introduce the organic phase while the inorganic network is being formed [14]. The intimate mixing of inorganic precursors and the polymer of interest in organic solvents allowed the organic and inorganic components to be associated at the molecular level [15]. In most cases, the chemical reactivity of organic and inorganic species is usually quite different and phase separation tends to occur during the synthesis. Strong bonds have then to be formed between organic and inorganic components in order to produce molecular composites or O/I hybrids [16]. In the present study, strong hydrogen-bond was formed at a molecular level between the PCL and BG phases, which resulted in a new class of bioactive and biodegradable O/I hybrid material exhibiting multifunctional properties [14, 17].

Calcium is an essential component of BGs since it serves as a network modifier in a silica network and plays a vital role in the mineralization process. Previous studies revealed that segregation of calcium in sol-gel derived BGs was a major problem [18-20]. Thus, one of the goal of this work was to achieve a homogenous distribution of calcium atom in the O/I hybrid matrix. As described in Chapter 3, proper choice of calcium precursor combined with controlled drying procedure resulted in O/I hybrid monoliths and fibrous scaffolds with a spatial distribution of calcium atom. The successful incorporation and homogeneous distribution of calcium could potentially play a significant role to stimulate osteoblast cells at the genetic level during the mineralization process [21].

The observed in vitro bone-like HA formation ability by the PCL/BG hybrids is anticipated to solve the persistent limitations seen in single biodegradable polymer-based scaffold materials. When pure polymer scaffolds are used, it is often necessary to modify the surface of the polymer
such as NaOH hydrolysis to create COOH functional groups to induce HA precipitation on its surface [22]; however, the PCL/BG hybrids were able to precipitate HA without surface modification. In the present study, the presence of Si-OH group on the surface of the BG component as well as the inorganic constituent were the main reason to the observed bioactivity of the PCL/BG hybrids [9]. Hence, the ability to tailor the physico-chemical properties of the hybrid materials by adjusting the O/I ratio for the desired application provides platform strategies to develop scaffolds with unique characteristics.

The addition of biodegradable polymers during the sol-gel synthesis of O/I hybrids in part improved the control over the rheological properties of the sol thus allowing it to be electrospun in the gel state. Therefore, 3D PCL/BG hybrid scaffold was easily fabricated by using the electrospinning technique. The ultimate goal of the combined sol-gel and electrospinning process was to mimic the structural composition and functionality of the native tissue, subsequently to provide a 3D template for cells to grow on and synthesize a new tissue [23]. It is believed that the resemblance of the PCL/BG hybrid system to the bone ECM will improve the efficacy of cellular responses as compared to the conventional composites. Depending on factors such as materials and processing method, the mechanical properties will also be improved. This work is the first to demonstrate in situ synthesis of amorphous and homogenous electrospun O/I hybrids from the sol-gel solution, which can be used in bone tissue engineering without the requirement of high temperature treatments. The other significant contributions of this work are the extensive characterization of the PCL/BG fibrous scaffold properties, in terms of bioactivity, wettability, mechanical properties, porosity, pore-size distribution, and interconnectivity [23, 24]. Taken together, the findings from this study could be used as a groundwork to further improve the
performance of bioactive and biodegradable O/I hybrid scaffolds by tuning the synthesis and/or fabrication parameters.

6.3 LIMITATIONS OF THE RESEARCH AND FUTURE DIRECTIONS

6.3.1 Limitations

The present thesis work delivered the concepts and groundwork on the development of novel O/I hybrid scaffold materials with bioactive and osteogenic properties. Although the preliminary development of PCL/BG hybrid scaffold presented in this work has demonstrated the great potential of the sol-gel derived O/I hybrids for bone regeneration, it is worth noting to discussing the limitations of the work, which were not accomplished due to time constraints.

1. **Biodegradation study of the PCL/BG hybrid scaffolds:** One of the limitations of this work is the absence of detailed biodegradability study. In Chapter 2, the *in vitro* resorption/degradation mechanism of the inorganic phase in SBF during the HA deposition process was discussed. Although both PCL [4] and BG [25] are known for their degradability in physiologic environment, it is useful to study the biodegradation mechanism of PCL/BG hybrid scaffolds and the effects of degradation products during short- and long-term culture studies. Understanding of biodegradation process could bring new concepts, which could be beneficiary to improve further the design and performance of O/I hybrid scaffolds.

2. **Scaffolds with a relatively smaller pore-size:** Although the data in Chapter 5 showed that the pore-size of the electrospun PCL/BG fibrous scaffold that were better than those reported in the literature, it could still be one of the limitation of this thesis work. Ideally, 3D scaffold architectures are designed to have a minimum pore-size of 100 μm, which is required for effective cell migration and infiltration. However, the electrospinning process has an inherent
limitation to fabricate scaffolds with a macro-pore structure. Although the pore-size reported in the current work is sufficient for cells to grow and differentiate on the surface of the electrospun mats [26, 27], further improvement might be required to fabricate scaffolds with an ideal pore-size to support osteoblast cell infiltration. This might be one future study direction to maximize the pore-size of the electrospun PCL/BG hybrid fibrous scaffolds to get an efficient cell infiltration and migration.

### 6.3.2 Future Directions

The current thesis work provided the groundwork on the concepts, design, and potential applications of a 3D bioactive and biodegradable O/I hybrid scaffold materials for bone tissue engineering. It is essential to further investigate the following areas in order to justify the notion that bioactive and biodegradable hybrid scaffolds are among the potential next-generation scaffold materials for bone tissue engineering and regeneration.

1. **Preparation O/I hybrids based on biodegradable and hydrophilic polymers**: The hydrophobicity of aliphatic polyesters has always been a cause of concern to dissolve them in a sol-gel solution. In most cases, it tends to separate from the sol-gel solution, this limiting their application significantly. Although a progress was made to address this issue in the current study, it would also be useful to explore the possibility of incorporating hydrophilic biodegradable polymers that can easily dissolve in a sol-gel solution. Such polymers may include poly (amido amine)s and Poly (γ-glutamic acids).

2. **Surface functionalization of PCL/BG Hybrids**: The presence of silanol groups in the surface of the PCL/BG hybrids combined with the mild condition of the electrospinning process could be suitable to modify the PCL/BG fibrous scaffolds. In doing so, careful
selections of appropriate functional groups could enable the formation of strong chemical 
bonds between the material surface and different osteoinductive agents, such as certain 
peptides, proteins or growth factors. In addition, different osteoinductive agents may also be 
incorporated in the fibrous structure that will further facilitate to have a controlled release 
system to induce and support bone growth.

3. **In vivo studies:** The next logical step of the scaffold development is the extension of the *in vitro* studies using a suitable animal model, which is required before testing these scaffolds in a clinical setting. It has been reported that the *in vitro* expression of the osteogenic markers can be used as an indicative of *in vivo* bone formation [3], however, the effect of optimized biomimetic fibrous architectures of the O/I hybrid has never been tested. Therefore, it would be beneficial to use animal models to ascertain the bone formation efficacy of the O/I hybrid scaffolds.
6.4 REFERENCES


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Author: G. S. Stein, J. B. Lian, J. L. Stein, A. J. Van Wijnen, M. Montecino

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Figure 1: An example of a tissue engineering concept that involves seeding cells within porous biomaterial scaffolds.

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**PUBLICATION**

1. **Allo, B. A.;** Lin, S., Mequanint, K., Rizkalla, A. S., The Role of Bioactive 3D Hybrid Fibrous Scaffolds on Mechanical Behavior and *In-Vitro* Spatiotemporal Osteoblast Gene Expression, 2013. (Submitted for publication)


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