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On Demand 3D Printed Hybrid Scaffolds for Tissue Engineering Applications

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ON DEMAND 3D PRINTED HYBRID SCAFFOLDS FOR TISSUE ENGINEERING APPLICATIONS

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Dedication

I dedicate this research to my parents who because of their life lessons, I have become what I am today and also would like to thank them for pushing me and encouraging me to follow my dreams and teaching me to never give up when times are difficult.

I also want to dedicate this work to my brother and my sisters for always supporting me throughout my career and always believing in me.

I am always doing what I cannot do, in order to learn how to do it

-Vincent van Gogh
ON DEMAND 3D PRINTED HYBRID SCAFFOLDS FOR TISSUE ENGINEERING APPLICATIONS

by

IVAN D. HERNANDEZ, B.Sc.

MASTER’S THESIS

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Abstract

A composite 3D printed polymer scaffold with in-built porosity and filled with a hydrogel can provide an ideal support system for cell growth, proliferation and vascularization. Therefore, a hybrid system of 3D printed polycaprolactone (PCL) scaffold and a hydroxyapatite based hydrogel was developed for application in reconstruction of bone defects, which are inherently difficult to repair without any guided therapies. In the present study, a 3D printed gyroid structure of PCL allowed the loading of higher amount of hydrogel as compared to conventionally used 3D printed mesh structure of the same volume and strut thickness. The hydrogel was composed of alginate, gelatin, nano-hydroxyapatite, and human mesenchymal stem cells (hMSCs) to enhance the osteoconductivity and biocompatibility of the composite implant. The adhesion and proliferation of the hMSCs within the hydrogel matrix and the migration of the cells from the gel towards the solid scaffold surfaces confirmed the cytocompatibility of the designed scaffold/hydrogel system. Further, the bio mineralization test in simulated body fluid (SBF) showed the nucleation and growth of apatite crystals on the samples, which confirmed the bioactivity of the hybrid system. Moreover, the dissolution study, in SBF revealed a continuous sustained dissolution of the hydrogel with time. Overall, the present study provides a new approach in bone tissue engineering for repair of bone defects with a bioactive hybrid system of biodegradable scaffold and hydrogel.
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Chapter 1: Introduction

1.1 Clinical Background

Collagen and calcium phosphate compose the soft and hard framework of the bone, making the bone strong but at the same time flexible to withstand certain amount of stress. The healing potential of bone, whether in a fracture or fusion model, is influenced by a variety of biochemical, biomechanical, cellular, hormonal, and pathological mechanisms. A continuously occurring state of bone deposition, resorption, and remodeling facilitates the healing process[1]. It is known that bone is one of few tissues that can heal without forming a fibrous scar. As such, the process of fracture healing recapitulates bone development and can be considered a form of tissue regeneration. However, despite the regenerative capacity of skeletal tissue, this biological process sometimes fails and fractures may heal in unfavorable anatomical positions, show a delay in healing or even develop pseudo-arthritis or non-unions [2]. High energy trauma, tumor resection, revision surgery, developmental deformities and infection can lead to significant bone loss and large defects with poor intrinsic healing potential. These large bone defects pose a major clinical and socioeconomic problem, as they have negative impact on patients’ quality of life due to consecutive reoperations and prolonged hospitalizations [3]. According to the American Academy of Orthopedic Surgeons, there are approximately 6.3 million fractures each year in the United States, with more than 500,000 procedures of bone grafting, costing approximately $ 2.5 billion[4].

The severity of a fracture usually depends on the force that caused the bone to break. If the bone's breaking point has been exceeded only slightly, then the bone may crack rather than break all the way through. If the force is extreme, such as in an automobile crash or a gunshot, the bone may shatter. There are different forms of bone fractures that can occur depending on the situation
(Trauma, osteoporosis, overuse, and cancer surgeries such as curettage\(^1\)), some examples of types of fractures are shown in Fig. 1.1. Common types of fractures are greenstick fracture, linear fracture, transverse fracture, spiral fracture, oblique fracture, and comminuted fracture.

![Common types of bone fractures](image)

Fig. 1.1- Common types of bone fracture [5].

If the bone fracture is too severe even if it goes through the healing process it will not be able to restore its original mechanical and physical properties. The bone goes through a several stages of bone restoration or bone healing; these stages will make the best to restore the bone’s original properties. It will go through a process of systematic and local factors that will help the bone regenerate. Healing occurs in three distinct but overlapping stages: 1) the early inflammatory stage; 2) the repair stage; and 3) the late remodeling stage.

In order for bone to regenerate, specific mesenchymal stem cells (MSCs) have to be recruited, proliferate and differentiate into osteogenic cells. Exactly where these cells come from is not fully understood.

\(^1\)Curettage: to remove tissue by scraping or scooping.
understood. Although most data indicate that these MSCs are derived from surrounding soft tissues and bone marrow, recent data demonstrate that a systemic recruitment of circulating MSCs to the injured site might be of great importance for an optimal healing response [5].

1.2 Structure of bone

A natural bone matrix consists of an organic and inorganic parts, arranged in a well-organized manner and cells cover its internal and external surfaces [6]. On the basis of density, bone can be subdivided into cortical (compact) and cancellous (trabecular) bone [7-12]. Cortical bone surrounds the cancellous bone (fig. 1.2).

Fig. 1.2 - Schematic of the structure of cortical bone [6].
Although both types of bones have similar matrix composition and basic structure, cortical bone has ~10% porosity and cancellous bone has ~50-60% porosity [6]. In accordance with the porosity aspect, ultimate compressive strength of cortical bone is 10 times greater than cancellous bone of similar volume. Due to high porosity, the cancellous bone has more surface area than cortical bone. Also, as compared to cortical bone, cancellous bone shows higher rate of remodeling and metabolic activity under the external mechanical stress [6]. In the central part of a mature bone, bone marrow is surrounded by the bone tissue and periosteum. Bone marrow is the source of bone cells and hosts the blood vessels, which is a part of circularity system in bone [6].

1.3 Overview

In the field of bone tissue engineering (TE) there have been several approaches to reinforce and renovate the bone repair techniques that are currently being used by orthopedic surgeons. Bone is well known for its self-healing abilities [6]; however large-scale bone defects cannot be healed completely due to fibrous tissue growth in the defect area [7], in these cases biomedical engineering of implant or surgical modifications are needed to restore the normal function of the bone. Currently, one of the most common bone repair techniques is the transplantation of fresh autologous bone. Although effective for relatively small, non-load bearing defects, autografting (bone taken from the same person’s body) and allografting (bone tissue from a deceased donor) are limited by the small volume of available material, lack of structural integrity, and significant donor site morbidity [8]. For bone to regenerate there are different clinical problems that need to be addressed not only in the biological aspect of the challenges, but also in the mechanical and structural challenges. To overcome these challenges, TE approaches being developed for better and more reliable alternatives to promote bone tissue restoration.
In the last decade one of the most important advances in tissue engineering has been the design of temporary support structures called “scaffolds”. These scaffolds are designed in a three-dimensional structure and exhibiting high porosity, high pore interconnectivity and uniform pore distribution [9]; these scaffolds are suitable for the non-load bearing applications and are intended to provide structural support for cells and the new tissue being formed, acting as a temporary extracellular matrix inducing the natural processes of tissue regeneration and development[10].

Over the past two decades, significant advances have been made in the development of biomaterial scaffolds for the repair and regeneration of skeletal tissues via tissue engineering strategies[11]. There are a variety of scaffolds being proposed, ranging from ceramic to metallic and alloys. In this context polymeric biomaterials are ignored due to load-bearing capabilities. It is important to mention that the scaffold material for the bone defect filling should be highly bioactive and to recruit the bone cells as well to suppress the growth of the soft tissue in different areas. In addition to this, independent of type of scaffold material (metallic, ceramic, polymeric or composite), a dynamic compression plate is necessary for the implant to be stable. Therefore, to correct a bone defect the choice of the material’s biological properties are more important than the mechanical properties.

Different tissues being used scaffolds produced from a variety of biomaterials and manufactured using a many kinds of fabrication techniques have been used in the field in attempts to regenerate different tissues and organs in the body. Regardless of the tissue type, a number of key considerations are important when designing or determining the suitability of a scaffold for use in tissue engineering: Biocompatibility, biodegradability, mechanical properties, scaffold architecture, and manufacturing technology[12]. For a tissue engineered scaffold to be successful they need to fall under those characteristics, for example, if it is not biocompatible and the
architecture is not fit for cell growth, cells will not proliferate within the scaffold and will not enhance tissue regeneration.

Fig. 1.3- Patient derived stem cells are subsequently mixed with hydrogels and placed in a 3D scaffold to initiate differentiation [12].

1.4. Biomaterials

The most exciting and rewarding area of research in the biomaterial science is the development of “biomaterials” for health care. A biomaterial is a natural or synthetic material used in the close proximity to the living tissue for a specific motive. Importantly, these biomaterials should not show any cytotoxic effect during their service period and facilitate the desired function of the host tissue [13].
The choice of biomaterials for a particular application totally depends on the anatomical location and function. In connection with orthopedic applications, the success story of an implant biomaterial depends on many important parameters, for example, physical and mechanical properties, cytocompatibility, biocompatibility, osteointegration property (in case of bone application) and non-cytotoxicity [14].

The field of biomaterials is interdisciplinary; and the designing of a biomaterial depends on the interaction of materials science, biological science, chemical science and medical science.

1.4.1 Bio-material interaction

In order to evaluate the in vitro cytotoxicity, a biomaterial needs to be tested in simulated body environment. The specific cell line needs to be used for a specific purpose and the interaction of the cells to the biomaterials surface depends on many parameters e.g., surface chemistry, surface roughness, surface energy and adsorbed protein [15]. A cell interacts with a material surface through the adhesion receptors of the cell membrane, which interact with proteins adsorbed on the material surface [16, 17]. After getting attached to the sample surface, a cell starts releasing signaling molecules, then grows in size, proliferate, communicates with other cells, secretes extracellular molecules to fill the space between the cells and die through apoptosis in general [16]. For most of the biomedical applications, the contact of a biomaterial with cells/tissue is required for relatively longer period. Therefore, the designing of a biomaterial requires the basic knowledge of cell-material interaction in vitro as well as in vivo.
1.4.2 Hydroxyapatite

Hydroxyapatite (HA) is chemically similar to the inorganic component of bone matrix. The close chemical similarity of HA to natural bone has led to extensive research efforts to use synthetic HA as a bone substitute and/or replacement in biomedical applications [15]. Since HA has chemical similarity to the inorganic component of bone matrix, synthetic HA exhibits strong affinity to host hard tissues. Chemical bonding with the host tissue offers HA a greater advantage in clinical applications compared to most other bone substitutes such as allografts or metallic implants [16, 17]. The main advantages of using HA is its biocompatibility, slow biodegradability, and good osteoconductive and osteoinductive capabilities [18].

In this study, HA will be used to induce osteoconductivity, but HA that will be used will be using nano scaled crystalline HA. These nanocrystalline HA show improved porosity and also greater density due to greater surface area, which can improve better bone formation and give better mechanical properties [19]. Moreover, Nano HA, compared to coarser crystals, is expected to have better bioactivity [19]. Therefore, we expect that by using Nano HA for bone regeneration applications it will improve biocompatibility when implanted in vivo. The nanocrystalline HA will be infiltrated by using a gelatin-alginate based hydrogel that will be loaded with human mesenchymal stem cells (hMSCs).

1.4.3 Hydrogels

Hydrogels are three-dimensional networks composed of hydrophilic polymers crosslinked either through covalent bonds or held together via physical intramolecular and intermolecular attractions.
Hydrogels can absorb huge amounts of water or biological fluids, up to several thousand %, and swell readily without dissolving [20]. Hydrogels can be ultimately beneficial in cell transplantation due to their unique ability to offer immune isolation while still allowing nutrients, oxygen, and metabolic products to diffuse easily into their matrices.

Hydrogels can be used in tissue engineering either directly after their preparation (with or without cell entrapment) or after formulation as scaffolds. Hydrogel-based scaffolds are a very important class of scaffolds due to the ability to tailor their mechanical characteristics to mimic those of natural tissues [20]. However, when using hydrogel-based scaffolds the gel can disintegrate faster and it also can move where it is not necessary, meaning that they are giving support to the cells but depending on the type of application, the hydrogel would not stay in place. Therefore, in this study it was preferred to suspend the hydrogel within the scaffold to promote inclusion of appropriate peptide moieties on the surface or throughout the bulk of the hydrogel and scaffold to significantly increase the amount of cellular proliferation and attachment.

When talking about scaffold design we discussed that scaffolds need to have certain criteria to be able to enhance cellular proliferation. Hydrogels, need to follow certain criteria as well, they must be highly porous with an open interconnected geometry, to allow a large surface area relative to the scaffold’s volume. This high, interconnected porosity will encourage cell ingrowth, uniform cell distribution and assist the neovascularization of the matrix. Because we are injecting the hydrogel inside a supporting structure, 3D printed polymeric scaffold, it is not important to design the hydrogel to mimic the natural tissue. But it is important to include both classical mechanical and physicochemical parameters (such as biodegradation, porosity and proper surface chemistry), and biological performance parameters (such as biocompatibility and cell adhesion), as well as demonstrating enhanced vascularization [21].
1.5 Biomaterials as scaffolds in TE

Scaffolds play an important role in tissue engineering and have been known to enhance cellular regeneration. Scaffolds provide structural support for cells attachment and support for tissue ingrowth. Most of the cells in human body have to anchor themselves on to the extracellular matrix (ECM) to be able to proliferate. ECM is a collection of extracellular molecules secreted by cells that provide structural and biochemical support to the surrounding cells. The primary goal of making a successful scaffold is to mimic a ECM and enhance cellular regeneration wherever the scaffold is needed in the host’s body. For the scaffold to mimic a ECM it should provide the functions shown in Table 1.0 and provide the following characteristics: a) It should provide structural support and physical environment for cells residing in that tissue to attach, grow, migrate and respond to signals. b) It should give the tissue its structural and therefore mechanical properties, such as rigidity and elasticity that is associated with the tissue functions. c) It should provide bioactive cues to the residing cells for regulation of their activities. d) Should be able to store growth factors and other bio factors. e) Should provide a biodegradable characteristics to allow tissue ingrowth [22]. As table 1.0 shows, the scaffold function and features that need to be considered are: architecture, bioactivity, cytocompatibility and tissue compatibility, and mechanical property. All of these are essential for a successful TE scaffold, for example, if a badly designed scaffold is used for a TE application it could be unstable or if it does not have the porosity that is needed, cells would not be able to grow and expand inside the scaffold. The design is the most important part of working with scaffolds and as well as the material you are using to make the scaffold. Without the right material the scaffolds mechanical properties could be unstable, in other words, it could degrade before the cells even grow or it could not resist any type of stress when implanted into the host. The material also has to be biocompatible and bioactive for cells to grow and proliferate on the scaffold itself.
Table 1.0- Functions of extracellular matrix (ECM) in native tissues and of scaffolds in engineered tissues [22].

<table>
<thead>
<tr>
<th>Function of ECM in native tissues</th>
<th>Analogous functions of scaffolds in engineered tissues</th>
<th>Architectural, biological, and mechanical features of scaffolds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural support for cells</td>
<td>Structural support for exogenously applied cells</td>
<td>Biomaterials with binding sites for cells</td>
</tr>
<tr>
<td>Mechanical properties</td>
<td>Provides the shape and mechanical stability</td>
<td>Biomaterials with sufficient mechanical properties</td>
</tr>
<tr>
<td>Bioactivity</td>
<td>Interacts with cells actively</td>
<td>Biological and physical cues</td>
</tr>
<tr>
<td>Retain growth factors</td>
<td>Serves as delivery vehicle and reservoir for growth factors</td>
<td>Microstructures for bioactivity</td>
</tr>
<tr>
<td>Flexible physical environment</td>
<td>Provides a void volume for vascularization and new tissue formation</td>
<td>Porous microstructures for nutrients and diffusion and biomaterials</td>
</tr>
</tbody>
</table>

1.6 Role of additive manufacturing in scaffold fabrication for TE applications

Scaffolds have been around for a couple of decades, but the method to approach the desired TE scaffolds has evolved and has been divided into different categories throughout the years. Some of the approaches have been pre-made porous scaffolds, decellularized extracellular matrix, cell sheets with secreted extracellular matrix and cell encapsulated in self assembled hydrogel. The method that will be used in this study will be by making a pre-made porous scaffold by using fused deposition modeling (FDM) 3D printer. This rapid prototyping technology allows the development of manufacturing process to create the desired porous structure that is needed in TE applications. FDM works by using a thermo-polymer filament, which is heated to its melting point and then extruded, layer by layer, to create the desired three-dimensional structure (Fig. 1.4). The nozzle
melts the filaments and extrudes them onto a base, sometimes called a build platform or table. Both the nozzle and the base are controlled by a computer that translates the dimensions of an object into X, Y and Z coordinates for the nozzle and base to follow during printing.

Figure 1.4- Fused deposition modeling schematic [9].

This tissue engineering approach will be by using a biodegradable polymer that will be extruded by the 3D printer. By making a biodegradable scaffold, it is expected to break down due to macromolecular degradation with dispersion in vivo but no proof for the elimination from the body. The polymer should also be bioreabsorbable, meaning that it can dissolve in in body fluids without any polymer chain cleavage or molecular mass decrease. Therefore, the polymer that is chosen for the scaffold needs to degrade and resorb at a controlled rate at the same time as the specific tissue cells seeded into the 3-D construct attach, spread and increase in quantity (number of cells/per void volume) as well as in quality [9].
1.6.1 Scaffold Design

Apart from material biocompatibility the scaffold architecture is a key component for cellular proliferation. Skeletal tissue is a complex three-dimensional structure composed of cells, protein fibers, and minerals. Therefore, the design of the scaffolds needs to mimic the three-dimensional structure. For the repair and regeneration of skeletal tissue the scaffold need to have a high elastic modules in order to be supported where it is needed inside the body and also to provide the tissue with sufficient space for cellular growth [23]. To produce a scaffold with significant strength, the material that is being used to produce the scaffold must have sufficient high interatomic and intermolecular bonding, but also at the same time have a physical and chemical structure which allows for a cleavage of chemical bonds and breakdown of molecules. Another important factor that the 3D scaffold needs to consider when it comes to the design is interconnectivity and the porosity. Having a good interconnected macro pore structure will help the cells and the nutrients to diffuse and expand evenly through the entire structure. It will also create channels for the bioreactors to pass, therefore mimicking the interstitial fluid conditions present in natural bone and cartilage. Bioreactors permit in vitro culture of larger and better organized 3-D cell communities than can be achieved using standard tissue culture techniques [24]. The vascularization of a scaffold may be composed by completely depending on capillary ingrowth into the interconnecting pore network from the host tissue. From a biomechanical and clinical point of view, the tissue-engineered bone or cartilage transplant should allow for a mechanically secure and stable fixation on or to the host tissue. For bone, the currently available medical devices, such as pins, screws, and plates might be used. However, the integration of a device-like part into the 3-D scaffold design can be advantageous [22].
The design of the scaffold can be created using a computer-aided design (CAD) software, such as Solid Works, AutoCAD, Sketch Up, Blender, and other software that are capable of developing 3D designs. CAD designs can later be converted into a STereoLithography (STL) file, so it can be printed using the FDM printer. By using a CAD software, the scaffold that is being designed can be modified in size and in specific characteristics that are needed to create the TE scaffold, such as the porosity and the diameter of the porous that will more resemble a human tissue.

1.7. Background & hypothesis

Due to disease associated with the bone resorption/ trauma, the natural bone fails to perform normal function [18]. This leads to the loss in bone density and therefore failure of joints and/or fracture of bone. To maintain the function of bone and relief from pain, the replacement of damaged bone with suitable substitute is required. Autogeneic bone can be used for the replacement of damaged bone. But, due to the involvement of secondary wound and therefore excessive loss of blood, the use of autogenous bone is not preferred [19-23]. Although, the allogeneic bone can be used, the high probability of rejection and risk of life threatening disease limit their use [4, 24]. Therefore, it is recommended to use the synthetic biomaterials to make the scaffold or implant.

Furthermore, the objective of this study is to design a bioactive and biocompatible composite material for the bone defect restoration in in vitro studies. Among various biomaterials, hydroxyapatite (HA) has been widely used in orthopedic application due to its chemical similarity to the bone mineral [25-31]. In the context of the orthopedic application, hydroxyapatite is found to be a highly bioactive material, which supports the bone ingrowth and osteointegration.
1.8 Objective

In tissue engineering the primary goal is to enhance and to reduce the time it takes for any damaged tissue to repair. In this study the main focus will be to enhance the repair time frame form a bone defect such as a segmental bone defect (Injuries in which a section of bone is completely shattered and/or absent). The first TE approach will be by making a 3D printed scaffold using a FDM printer with an existing, inexpensive, biodegradable material. The goal by using this technique is to replace or to temporarily support the defect area and stabilize the wound with the 3D printed scaffold. The scaffold will provide the mechanical support and stability and will provide the exchange of nutrients and waste material during metabolic activity of the cells growing inside the pores. This approach will be by using polycaprolactone (PCL) polymer, this polymer is a biodegradable polyester with a low melting point of around 60 °C [32]. PCL is degraded by hydrolysis of its ester linkages in physiological conditions (such as in the human body) and it has been approved by the Food and Drug Administration (FDA) in specific applications used in the human body as (for example) a drug delivery device, sutures, and tissue repair methods [32]. PCL has been used in bone tissue engineering for a few years now and has had positive results when it comes to biocompatibility and mechanical stability.

The second objective, will be by making a hydrogel made by forming a compound of alginate with gelatin that will be reinforced with nanocrystalline hydroxyapatite particles. By having a hydrogel, it will work as an extracellular matrix for the cells that will be implanted into the scaffold. After having the hydrogel, it will be loaded with human mesenchymal stem cells (hMSCs) and then it will be infiltrated into the 3D printed PCL scaffold. Mesenchymal stem cells (MSCs) are adult stem cells which can be isolated from human and animal sources. Human MSCs (hMSCs) are the non-haematopoietic, multipotent stem cells with the capacity to differentiate into
mesodermal lineage such as osteocytes, adipocytes and chondrocytes as well ectodermal (neurocytes) and endodermal lineages (hepatocytes).

After injecting the hMSCs loaded hydrogel into the PCL scaffold it will then be cross-linked using calcium chloride (CaCl2) solution. The CaCl2 will further enhance the strength of the hydrogel and maintain a balanced state inside of the scaffold. To get better results, varied scaffold designs will be tested to proof that our primary design can better improve osteoconductivity and cell proliferation. In figure 1.5, it will give a brief explanation on how the process will be done.

Therefore, in this study polymeric scaffolds were use, loaded with human mesenchymal stem cells (hMSCs), encapsulated in a bioactive hydrogel. Polymeric scaffolds are easy to process and more cost effective in comparison to metallic, ceramic and other composite material parts. A combination of cells, bio-factors and scaffolds will be applied, to undergo a period of ex vivo pre-implantation differentiation culture or directly implanted in-vivo for tissue regeneration of defect or diseased tissues.
Fig. 1.5- Hybrid 3D printed scaffold approach for bone defect applications.
Chapter 2: Materials and Methods

2.1 Materials

Alginate (alginic acid sodium salt, Cat. No. 218295) and Type-A gelatin (Cat. No. 901771) was obtained from MP Biomedical, France. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Cat. No. 22980) and N-hydroxy-succinimide (NHS, Cat. No. 24500) were procured from Thermo Scientific, USA. Calcium chloride (Cat. No. C79-500) was purchased from Fisher Chemicals, USA. Nanocrystalline hydroxyapatite was synthesized using previously reported method of suspension-precipitation using calcium oxide and orthophosphoric acid as a precursor [33]. The polycaprolactone (PCL, Cat. No. B01M81DB07) filament with 1.75 mm in diameter and with an operating temperatures of 70-100 °C was obtained from the Shenzhen Esun Industrial Co., a high-tech enterprise specializing in researching, developing, producing and operating degradable polymer materials for the 3D printing.

2.2 3D printed PCL scaffold

In the present study, a cylindrical-shaped (height: 5 mm, diameter: 15 mm) PCL scaffolds of gyroid structure was designed using CAD software (65% porosity and 1.2 mm pore size) and then was printed using a Fused Deposition Modeling printer (MakerBot, Model. Replicator Mini 5th generation). Prior to use, the scaffold was cleaned in distilled water and sterilized by soaking in 70% ethanol for 20 min, followed by UV irradiation for 30 min. The percentage porosity of the scaffold was measured by comparing the weight of porous scaffolds with scaffolds without designed porosity.
2.3. Synthesis of bioactive pre-hydrogel

The composition of hydrogel and methods of synthesis was based on a study carried out by Kumar et al. and Wang et al. [34, 35]. Briefly, 50 mg nanocrystalline powder of hydroxyapatite was mixed in 10 ml distilled water. After magnetic stirring (100 rpm) at room temperature for 15 min, 200 mg gelatin and 200 mg sodium alginate were added and stirred again at room temperature for 15 min at 100 rpm. After, mixing of gelatin and alginate, 25 mg EDC was added and stirred at room temperature for 10 min, followed by addition of 15 mg NHS and stirring for another 5 min to make the hydrogel. Prior to use, hydrogel was irradiated with UV light for 60 min for the sterilization. After sterilization, human mesenchymal stem cells (hMSC, Cat. No. SV30110.01, HyClone™, USA) of a 50,000 cells/ml cell density were mixed with hydrogel.

2.4. Hybrid PCL scaffold/hydrogel system

Cell-loaded hydrogel was filled in a sterilized syringe (needle inner diameter: 0.7 mm) and injected in the porous gyroid scaffold of PCL. This hybrid system of scaffold/hydrogel was crosslinked with 1 M CaCl2 for 10 min, followed by washing with 1×PBS for 10 min. Furthermore, hydrogel was washed two more times for 5 min using complete culture medium. The crosslinked, bioactive hydrogel-loaded PCL scaffold was transferred to a 24-well plate and incubated in the presence of complete culture medium in 5% CO2 and 95% humidity at 37 ºC. After 48 h of incubation, samples were characterized for the cell adhesion and viability. The hydrogel without cells was used for the dissolution study, bioactivity test, and phase analysis.

Furthermore, to investigate the hydrogel loading efficacy of 3D printed scaffold with gyroid structure, hydrogel without cells was filled in the 3D printed gyroid structure. To compare the hydrogel loading capability, gyroid structure was compared with previously used designs such
as mesh and honeycomb structures. For this, scaffolds of same size were 3D printed and weight was measured, followed by weight measurement of scaffolds with the hydrogel. The difference in weight of scaffold with and without hydrogel was used to estimate the amount of loaded hydrogel in the scaffold.

2.5. Phase assemblage and pore architecture

For the pore morphology and phase analysis, hydrogel was stored at –80 °C for 12 h, followed by freeze drying for another 12 h. The dried samples were investigated by X-ray diffraction (XRD, D8 Discover, Bruker’s diffractometer, Germany) the phase and by scanning electron microscope (SEM) in secondary electron (SE) mode for the pore morphology. Prior to SEM, samples were gold-coated to minimize the charging during scanning. The operating condition for the XRD was 40 kV voltage and 40 mA current with CuKα wavelength (1.54056 Å) and 2θ ranges from 20º to 90º at a scanning rate of 2º/min with a step size of 0.02º.

2.6. Dissolution behavior of hydrogel and evaluation of bioactivity of the hybrid system

To determine the stability and bioactivity the PCL/hydrogel hybrid system in physiological solution, samples (hydrogel-loaded hydrogel without cells and crosslinked with 1 M CaCl2 for 10 min) were immersed in 2 ml simulated body fluid (1×SBF) in 24 well plate and incubated for 3, 6, and 12 days. During the incubation period media was not changed. Simulated body fluid was prepared according to the method described by Oyane et al. [36] and has been reported elsewhere [33]. At least three samples were used in the study to generate a statistically significant data.
2.6.1. Calculation of dissolved amount of hydrogel

After incubation, samples were carefully removed from the media and stored at -80 °C for 12 h in an air-tight container, followed by freeze drying. After removal of samples, SBF solution was transferred to a 96-well plate to record the optical density at 630 nm using ELISA plate reader (Model: ELx800, BioTek, USA). The 1×SBF without any treatment was used as a reference during optical density measurement.

To calculate the weight of dissolved hydrogel in SBF during incubation, a standard curve between the known amount of hydrogel in distilled water (0.5, 1, 1.5 mg/ml) and corresponding optical density (at 630 nm) was plotted. An equation corresponds to trend line on the curve was used to estimate the amount of dissolved amount of hydrogel in the SBF by measuring the optical density (Supplementary Figure 1). This equation was used to estimate the amount of dissolved alginate from hydrogel during dissolution.

\[
\text{Dissolved amount of hydrogel (mg/ml) = (0.001 + optical density)/0.0163} \quad (1)
\]

2.6.2. Analysis of apatite formation efficacy in the presence of SBF

As mentioned in previous section, after dissolution study, incubated samples were removed from the SBF and freeze dried after keeping for 12 hrs. at - 80 °C. Freeze dried samples were gold coated and observed under electron microscope to know the morphology of deposited apatite on samples and mechanism of the apatite formation. The apatite formation data was correlated with the dissolution data to understand the inter-relationship between dynamic process of dissolution and deposition.
2.7. **Cytocompatibility assessment**

As mentioned in section 2.3, crosslinked hydrogel-loaded PCL scaffolds with hMSCs were transferred to a 24-well plate and incubated in the presence of complete culture medium for 48 h in 5% CO2 and 95% humidity at 37 °C. Briefly, for the cell culture study, hMSCs were pre-stained with green florescent dye (PKH67, Cat. No. MINI67, Sigma Aldrich, USA) by the protocol provided by the manufacturer. After multiple washing of cells in complete culture medium, cells of 107 cells/ml density were mixed in 1 ml hydrogel and injected in scaffold. The hydrogel loaded scaffolds were crosslinked with 1 M CaCl2 for 10 min and washed with two time with 1×PBS and complete culture medium. After 48 h of incubation, samples were observed under the fluorescent microscope to investigate the cell adhesion, growth and viability.
Chapter 3: Results

3.1. Hybrid PCL/hydrogel scaffolds

A hybrid system of PCL/hydrogel-loaded with hMSCs was successfully prepared. The hydrogel was composed of alginate, gelatin and nanocrystalline hydroxyapatite. The hMSCs was added to improve the osteoinductive properties of the hydrogel [37]. The hydroxyapatite is known for its bioactive and biocompatibility properties and expected to facilitate the new bone formation, in vivo [33,38-39]. The hydroxyapatite powder was characterized as a needle-shaped particles (~80 nm length and ~30 nm diameter), and detailed method of particle size analysis has been reported elsewhere [40].

To compare the hydrogel loading capability of the gyroid structure compared to other designs that have been used for scaffolding in tissue engineering. Three different structures were 3D printed and were infiltrated with hydrogel to compare the hydrogel capabilities of each one. In figure 3.1, it shows images of a mesh scaffold, a honeycomb scaffold, and a gyroid scaffold. They were 3D printed with the same dimensions (15mm x 15mm x 10mm) and with the same infill density of 40%. The results revealed that the gyroid structure was capable of loading 65% more hydrogel than the honeycomb structure and 38% more than the mesh structure.

Figure 3.1- Hydrogel loading capability test comparing a mesh, honeycomb and gyroid structure.
3.2. Microstructure and phase assemblage

To study the microstructure of the PCL/hydrogel hybrid structure, the sample was freeze-dried and gold-coated to get a better image of the morphological analysis retrieved by using scanning electron microscope (SEM). The sample was fabricated using the FDM method, a cylindrical gyroid with high porosity and interconnectivity, this allows the hydrogel to infiltrate uniformly and allow cellular proliferation. The SEM images that were obtained of the samples used in the study can be seen in figure 3.2. The images taken were from the PCL scaffold (height: 5mm, diameter: 15 mm) with the hydrogel already infiltrated into the scaffold. In part (c), of figure 3.2, it is showing how the hydrogel and PCL interact together and shows the porosity of both the PCL and hydrogel at 500 µm magnification. Part (b), of figure 3.2, shows only the hydrogel at 500 µm and we calculated the average porosity of hydrogel. The SEM images revealed a highly porous structure with ~ 5 µm wall thickness of the pores and pore size of ~ 400 µm (Figure 3.2). The PCL scaffold was characterized by a strut diameter of ~ 325 µm with visible surface irregularity which expected to provide a suitable surface for the cell adhesion. The PCL pore size was also calculated with an inner diameter of 1.2 mm and outer diameter of 1.6 mm. As figure 3.3 shows, the dimensions will help the hydrogel infiltrate better and also have higher fluid flow velocity, meaning it could facilitate the invasion of the host vasculature after implantation or be employed for pre-vascularization in vitro.

Furthermore, the phase assemblage was done by analyzing the PCL/hydrogel hybrid structure using the X-ray powder diffraction (XRD). The analysis showed presence of signatory diffraction peaks of hydroxyapatite, alginate and gelatin, confirming the correct hydrogel components. The sharp narrow peaks (Shown in figure 3.4) of the hydroxyapatite and PCL confirmed the crystallinity of the nano-hydroxyapatite and PCL in hydrogel.
Figure 3.2- Part (a) is the SEM image of the hybrid PCL/ hydrogel structure with a magnification of 1 mm and part (c) is at 500 μm magnification. Part (b) is the SEM image of only the hydrogel at 500 μm, the arrows are indicating the porosity of the structure and the box is shows the wall thickness of the pores. Part (d) is the SEM image of only the PCL strut at 100 μm magnification.
Figure 3.3- SEM micrographs of the PCL scaffold without hydrogel showing pore channels where the hydrogel loaded with hMSCs will be infiltrated. Part (a) is magnified 1 mm (x60) and part (b) is magnified at 500 μm (x120).
Figure 3.4- X-ray diffraction of hybrid PCL/hydrogel (Alginate, gelatin and hydroxyapatite) scaffold at 40 kV voltage and 40 mA current with CuKα wavelength (1.54056 Å) and 2θ ranges from 20° to 90° at a scanning rate of 2°/min with a step size of 0.02°.
3.3. **Dissolution of PCL/hydrogel system in SBF**

Careful observation of the samples after completion of dissolution study in SBF shows a uniform dissolution of hydrogel during the 12 days of test period. However, no dissolution in PCL was noted for this test period. After the test, optical density of SBF was measured to estimate the amount of dissolved hydrogel (Figure 3.5). The optical density was converted to amount of dissolved hydrogel in unit volume of SBF. The data revealed a continuous dissolution profile for hydrogel from day 1 to day 6. After 6 days, a decrease rate of hydrogel dissolution was noted until the end of test. Results show ~13 mg of dissolved hydrogel in first three days, followed by 5 mg hydrogel dissolution in next three days. After 6 days, a decrease in rate of dissolution was noted with only 2 mg dissolved hydrogel in next 6 days.

Figure 3.5, shows how the hydrogel dissolved constantly the first 6 days and then plateaued from day 6 to day 12. The following section talks about how apatite will form in the hydrogel and scaffold making the system more bioactive and more stable, therefore less dissolution occurs after day 6 as figure 3.5 reveals. Section 3.4 will expand further on how apatite deposits start forming after dissolution of hydrogel.
Figure 3.5- The dissolution table shows how hydrogel dissolved when immersed in 2 ml of SBF solution for a period of 3, 6, and 12 days, plateauing after day 6.
3.4. Nucleation and growth of apatite on the PCL/hydrogel system in presence of SBF

Samples of dissolution study in SBF was further used to study the formation of apatite on these samples and its relationship with dissolution rate. SEM study showed a uniform deposition of apatite during with higher amount on the hydrogel as compared to PCL scaffold (Figure 3.9). After 3 days of immersion in SBF, as compared to control samples (non-treated), hydrogel features were completely disappeared due to the deposition of apatite crystals. After 6 and 12 days, a thick layer of apatite formed due to continuous deposition of apatite. A strain induced crack, resulted due to drying of sample was used to estimate the apatite thickness after 12 days of dissolution. The estimated thickness of apatite after 12 days was ~13 µm (Figure 3.7). A higher magnification SEM micrographs showed a higher amount of deposited apatite during 3 days on PCL struts as compared to 6 and 12 days (Figure 3.9 a-c). In contrast, the hydrogel, as shown in Figure 3.8 d-f, an increase amount of apatite formation with time was noted with highest amount of apatite on day 12. A higher magnification images of (Figure 3.8 d-f) showed a change in morphology of apatite with time. After 3 days, a globular like morphology of apatite was noted on the hydrogel, which changed to acicular morphology on day 6. After 12 days, a denser apatite layer was observed with rode-like fine spherical-shaped apatite crystals.

As figure 3.6-a shows, there was apatite formation in the first 3 days on both the scaffold and the hydrogel, but because the PCL is not bioactive the next 6 and 12 days the apatite dissolved. Therefore, the dissolution of apatite in the PCL transfers back into the hydrogel and into the tissue (Figure 3.6-b). By HA being deposited into the tissue and hydrogel the PCL/hydrogel system becomes a bioactive system even though the PCL scaffold is not a bioactive structure.
Fig. 3.6-  a) Diagram showing apatite growth within the hydrogel from day 3 to day 12, and apatite growth in PCL the first 3 days and degradation of apatite the following 6th and 12th day. b) Mechanism of apatite formation ability [41].
Figure 3.7- Scanning electron microscope (SEM) images of the PCL/hydrogel system. Part (a) shows an SEM image of the system without being immersed in SBF solution. Part (c) shows an SEM image of the system after being immersed in SBF solution for 3 days. Part (b) shows an SEM image of the system after being immersed in SBF solution for 6 days. Part (d) shows an SEM image of the system after being immersed in SBF solution for 12 days.
Figure 3.8- SEM analysis of hydrogel composition showing apatite growth. Part (a) and (d) shows apatite morphology in the hydrogel at 20 µm and 3.0 µm magnification after 3 days of incubation. Part (b) and (e) shows apatite morphology in the hydrogel at 20 µm and 3.0 µm magnification after 6 days of incubation. Part (a) and (d) shows apatite morphology in the hydrogel at 20 µm and 3.0 µm magnification after 12 days of incubation.
Figure 3.9- SEM analysis showing apatite formation in the PCL strut and in the hydrogel. Part (a), (b) and (c) shows constant apatite formation after 3, 6 and 12 days. Part (d), (e), and (f) apatite growth after day 3 and then shows no growth after day 6 and no sign of apatite after day 12.
3.5. Cell viability and proliferation

Before infiltrating the human mesenchymal (hMSCs) stem cells into the hydrogel they were pre-stained with green florescent dye to label the cell membrane and track the presence of living cells and cellular proliferation. Figure 3.11-a showed the presence of pre-stained hMSCs (bright green spots) in the hydrogel as well as on the scaffold struts. A higher magnification micrograph this clearly showed the presence of more number cells in the hydrogel than on scaffold strut (Figure 3.11-b). White broken line showed the boundary between scaffold and hydrogel. The cells are marked with red circles in both hydrogel as well as scaffold area. Since, PCL is hydrophobic in nature and do not support the cell adhesion, a bioactive hydrogel-loaded with hMSCs was infiltrated in the pores of scaffold. Due to bioactive property of hydrogel, we have expected a higher number of viable cells in the hydrogel. This was confirmed in Figure 3.11-c, which showed a high cell number with elliptical-shaped cell morphology (Figure 3.11-d). A careful look confirmed the lamellipodia extension (marked with white arrow in inset image of Figure 3.11-d), which confirm cellular designated by growth and proliferation. It is further important to mention that, hydrogel is a three-dimensional environment and due to limited penetrability and diffraction of visible light in aqueous medium only cells present on the top surface of hydrogel can be imaged correctly. The cells present beneath few micrometers cannot be captured, although cells are present everywhere. In this situation, it is appropriate to say that cells are higher than as we can see. Therefore, some samples of the PCL/hydrogel system were sliced and studied by taking more micrographs to confirm cell viability in different areas of the PCL/hydrogel system.
Furthermore, ImageJ software was used to analyze the fluorescence microscopy micrographs to count the cells in hydrogel and PCL scaffold regions. As shown in Figure 3.10, results revealed a higher cells density on the hydrogel (2355±1598 cell/cm²) than PCL scaffold (1488±119 cells/cm²). Statistical analysis was carried out to know the significant difference in the cell density. No significant difference in cell density between scaffold hydrogel and scaffold was noted at p<0.05.

Figure 3.10- The bar diagram representing the cell density in hydrogel and scaffold area. Results showed a higher cell density in the hydrogel area than the scaffold.
Figure 3.11- PCL/hydrogel hybrid system images were taken using fluorescent microscope. Part (a) and (c) were taken at 500 μm magnification. Part (b) and (d) were taken at 100 μm magnification.
Chapter 4: Discussion

The unique combination of 3D printed PCL scaffold and bioactive hydrogel can provide a solution to restore the bone defects, which in principle difficult to heal the defect in the absence of suitable support material. Moreover, patient-specific 3D printed implants can minimize the micromotion of implant at host site and thus, facilitate the firmly anchored bone formation at the interface of implant and bone. It is important to mention here that alone both materials are incapable of repairing the bone defects due to absence of basic properties of an implant material. 3D printed PCL scaffold with designed porosity can be effectively used to fill the bone defects; however, poor cells adhesion and bio inert surface properties of PCL can only delay the osseointegration of bone. In contrast, a hydrogel with bioactive molecules can be used to promote the osseointegration in the bone defect, but, insufficient load bearing capabilities restrict the application. Therefore, in the present work, a hybrid system of PCL scaffold and hydrogel was used to test its effectiveness as a bone defect filling material using in vitro cell culture methods. To further improve the efficacy of this system to accelerate the bone formation, hydrogel was loaded with human mesenchymal stem cells (hMSCs), multipotent stromal cells, to differentiate into bone forming cells in the presence of osteogenic environment. Moreover, hydrogel can be a suitable alternative to natural extracellular matrix (ECM), provides the niche for cell growth and proliferation.
4.1 Scaffold designs

In tissue engineering there has been a great advancement in scaffold production to substitute or to enhance tissue regeneration. There has been some improvement in scaffold designs, and some sense on how large and porous the structure needs to be to be able to obtain cellular proliferation, but most of the research being done today use the same scaffold designs (Mesh, honeycomb, wood pile) because these designs have been proven to enhance proliferation and cellular migration within the structure [42]. The scaffold design used for this study has proven to have more hydrogel loading capacity compared to above mentioned designs and has shown more open and accessible pore networks than other scaffold designs. In this context, higher hydrogel loading capacity provides a larger extracellular matrix for cellular proliferation. The number, size and location of the interconnections between pores play an important role in cell seeding, and in nutrient transport and cell migration during the cell culturing phase. The highest cell densities in the scaffolds could be related to regions with larger pores, higher fluid flow velocities, and the gyroid structure has proven to have 65% porosity and having larger pores compared to other scaffold designs [43]. The scaffold architecture presented in the gyroid is designed to improve the transport of nutrients and oxygen. Furthermore, the network of larger sized channels could facilitate the invasion of the host vasculature after implantation or be employed for pre-vascularization in vitro [44].
4.2 Synthesis of bioactive pre-hydrogel

To successfully synthesize and stabilize the alginate-gelatin based hydrogel being used in this study we followed a procedure published by Kumar et al. and Wang et al. [34, 35]. Alginate is a hydrophilic anionic polysaccharide that can chelate divalent cations such as Ca2+ and Mg2+. Therefore, alginate can be used to synthesize hydrogels. Furthermore, alginate is bioinert in nature, therefore, gelatin was added to improve the cell adhesion. In addition to this, hydroxyapatite [Ca₅(PO₄)₃(OH)] was added to improve the bioactivity of the designed hydrogel for the application of bone tissue engineering. Hydrogel of alginate and gelatin, reinforced with hydroxyapatite was prepared by utilizing two-step crosslinking method. In the first step, EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and NHS (N-hydroxysuccinimide) were used for the crosslinking of gelatin with the alginate, and in the second step, CaCl₂ was added for further crosslinking of alginate. During the first step, EDC was used to activate the carboxyl groups of alginate, which react with NHS to form activated esters. (Supplementary Fig. 1.0). The activated esters of alginate react with primary amines of gelatin (lysine side chains) to yield stable amine bonds thus producing a stable network structure (pre-hydrogel). In the second step, the pre-hydrogel was treated with CaCl₂ to achieve chelation with the carboxyl groups of α-L-guluronic acid (G-block) (Supplementary Fig. 2.0). The guluronate blocks of one polymer form junctions with the guluronate blocks of adjacent polymer via ionic bridges formed between the carboxylates of the adjacent alginate. Cross-linking with CaCl₂ increases the structural stability of the hydrogel (when infiltrated into the PCL scaffold).
4.3 Microstructure and phase assemblage

The detailed phase characterization using XRD analysis indicates predominant retention of HA and PCL with detectable presence of gelatin and alginate. The sharp narrow peaks (Shown in figure 3.4) of the hydroxyapatite and PCL confirmed the crystallinity of the nano-hydroxyapatite (Calcium phosphate mineral) and PCL in hydrogel. Bone and teeth of most animals, including humans, are composed of calcium phosphate and it shares 62-65% of the total bone composition [45]. Calcium phosphates have intrinsic properties that stimulate bone regeneration.

The SEM images revealed a highly porous structure with ~ 5 μm wall thickness of the pores and pore size of ~ 400 μm (Figure 3.2). Such a highly porous structure could allow high loading of cell in hydrogel. The PCL scaffold was characterized by a strut diameter of ~ 325 μm and the pore size of 1.2 mm. These dimension will help the hydrogel infiltrate better and also have higher fluid flow velocity, meaning it could facilitate the invasion of the host vasculature after implantation or be employed for pre-vascularization in vitro.

4.4 Dissolution of PCL/hydrogel system in SBF

It has been reported that the dissolution study in SBF can simulate the condition of biomaterial dissolution and bone mineralization on the biomaterial surface in physiological environment. The dissolution study showed how hydrogel dissolved when immersed in 2 ml of SBF solution for a period of 3, 6, and 12 days, plateauing after day 6. Therefore, it shows that the hydrogel would dissolve in the first 6 days when exposed to human physiological conditions and then after day six the hydrogel would stabilize and retain its shape. By having a stable hydrogel system inside the scaffold it would give more time for the cells to grow and migrate inside the host
and also grow within the scaffold allowing the tissue to regenerate before scaffold/hydrogel system degrades inside the host.

4.5 Nucleation and growth of apatite on the PCL/hydrogel system in presence of SBF

There was significant growth of apatite on the hydrogel itself and there was a constant growth during 12 days of incubation. Figure 3.9, shows the difference of the apatite growth between the PCL and the hydrogel. It revealed that after 12 days there was apatite growth in the hydrogel but in the PCL there was growth in the first 3 days and then apatite dissolved after 12 days. Apatite nucleation and growth is a dynamic process and depends on the concentration of calcium and phosphate ions in the SBF. The entire process of apatite growth on the biodegradable biomaterials (Calcium phosphate-based) can be divided into three steps. In the first step biomaterials (hydrogel) dissolve and supplement the SBF with calcium and phosphate ions until super saturation achieved. In the second step, after super saturation, calcium and phosphate ions start depositing on the biomaterial surface. In third step, when SBF gets depleted of calcium and phosphate ions, the deposited apatite start dissolving to further supplement the solution with calcium and phosphate ions. However, in case of HA-based biomaterials such as in the present work we have used HA to supplement the hydrogel, HA act as a seed for apatite nucleation. Therefore, we have noted very high apatite nucleation and growth on the HA as compared to PCL. In the case of PCL, which is bioinert in nature [46], the apatite deposition and growth depends on the SBF properties and apatite will only form in the super saturation state of SBF in terms of calcium and phosphate ions. During the super saturation of the SBF, apatite will start depositing on the PCL surface, which was confirmed from SEM micrograph after 3 days of immersion of hydrogel/scaffold system. After 6 days we noted lower amount of apatite on the PCL scaffold as
compared to 3 days. This is due to the dissolution of apatite from PCL scaffold surface into the SBF when it becomes low in calcium phosphate ions. This dissolution of apatite was further noted after 12 days with very low apatite on the PCL scaffold, in contrast we noted a increasing amount of apatite formation on the hydrogel.

The SBF biomineralization test in simulated body fluid (SBF) showed the nucleation and growth of apatite crystals on the samples, which confirm the bioactivity of the hybrid PCL/hydrogel system.

4.6 Cell viability and proliferation

Although individual components of the hybrid PCL/hydrogel scaffold system is proven to be biocompatible and was used very frequently in bone tissue engineering [47], it is interesting to know the biological function of the entire system because the properties of a biomaterial strongly depends on the processing method. In the present study, PCL scaffold with designed porous architecture was used as a support structure for the bioactive hydrogel. Hydrogel was further loaded with hMSCs to increase the osteogenecity of the system. It is known that hMSCs have a tendency of osteogenic differentiation in the presence of bioactive environment [48,49]. Since, the designed hydrogel was composed of bioactive phase such as hydroxyapatite, therefore, it can support the osteogeneic differentiation of hMSCs and thus, can accelerate the osseointegration. Gugala et al. [50] also reported the importance of bioactive graft material and a porous support structure to restore bone defect in a sheep animal model. They found that at least one support material is required to promote the bone formation. For example, no healing was noted in the absence of any support material. However, a partial healing was found in case of perforated PLA support material used as a scaffold. In contrast to this, a complete healing was achieved when this scaffold was filled with a bioactive material viz cancellous bone.
The cells present in the hydrogel can only attach to the pore wall and it was found that cells were extended in lamellipodia after 48 hrs. of culture. This is a clear indication of cell migration. The change of cell morphology from spherical to extended shape confirmed the cell adhesion on the material surface. Hydrogel was highly porous and these pores were filled with culture medium. It means during the first few hours of cell culture cells remained suspended in the hydrogel and remained in spherical shape.

In addition to the hydrogel (2355±1598 cell/cm²), a significant number of cells were found on the PCL scaffold (1488±119 cells/cm²). In general, PCL is not considered suitable for the cell adhesion and proliferation [51]. However, in the presence of bioactive phase such as gelatin on the scaffold surface (Coated during the hydrogel loading on the scaffold surface) it may provide the suitable surface for cell adhesion and proliferation. Therefore, in the present study hybrid system of and hydrogel was cytocompatibility and maybe used for the bone tissue engineering application. However, further study is required to test the efficacy of the design system for the new vascularization and osseointegration.
Chapter 5: Conclusion

The primary objective of this study was to develop a hybrid PCL/hydrogel scaffold that could enhance osteoconductivity and bioactivity to improve tissue regeneration in segmental bone defects. The system was developed to be bioactive and biodegradable but at the same time easy and fast to develop for on demand applications. In this study using FDM technology we were able to print the PCL scaffold within a few minutes, which means that if the hydrogel and the cell culture are already prepared before printing, the scaffold the hybrid system could be done in the same day the application is required for implantation. Apart from demonstrating how fast this system can be developed for on demand bone defect applications, this study also demonstrates the results of in vitro study to show the potential application of the design hybrid structure for bone tissue engineering.

The 3D printed PCL gyroid scaffold that was designed for this study allowed the loading of higher amount of hydrogel as compared to conventionally used 3D printed mesh structure of the same volume and strut thickness. The scaffold architecture presented in the gyroid is designed to improve the transport of nutrients and oxygen.

The hybrid PCL/hydrogel scaffold indicated good cytocompatibility, showing adhesion and proliferation of the hMSCs within the hydrogel matrix and the migration of the cells from the gel towards the solid scaffold surfaces. Further, the biomineralization test in simulated body fluid (SBF) showed the nucleation and growth of apatite crystals on the hydrogel as well as the PCL scaffold, which confirmed the bioactivity of the hybrid system.

Moreover, the dissolution study, in SBF revealed a continuous sustained dissolution of the hydrogel with time. Overall, the present study provides a new approach in bone tissue engineering
for repair of bone defects the faster way with a bioactive hybrid system of biodegradable scaffold and hydrogel.

5.1 Future work

This hybrid system provided sufficient data to determine its functionality in *in vitro* studies. Some future work that is important to consider would be to perform *in vivo* studies to acquire data such as vascularization and osteointegration. Because of the gyroid design we could expect some vascularization, because of the network of larger sized channels it could facilitate the invasion of the host vasculature after implantation or be employed for pre-vascularization *in vitro* [44]. Furthermore, other studies could be made such as combining endothelia cells with the hMSCs to induce vascularization.
References

Supplementary Figure 1.0- Step#1: Crosslinking of carboxylates and amines with EDC (a carbodiimide) and NHS.
Supplementary Figure 2.0- Step#2: schematic representation of calcium induced gelation of alginate in accordance with ‘egg-box’ model. The divalent calcium cations (Ca\(^{2+}\)) bind to guluronate blocks of the alginate chains as they have high affinity to divalent cations.
Curriculum Vitae

Ivan D. Hernandez was born on June 26, 1990, in El Paso, Texas. He was raised in Cd. Juarez, Mexico where he lived most of his life. He attended Cathedral High School in El Paso, TX and graduated June 2010. In August 2010 he started his bachelor’s degree in biological sciences at the University of Texas at El Paso (UTEP) and graduated from his bachelor’s with a minor in biomedical engineering in May 2015. After graduating from his bachelor’s Ivan was interested in continuing learning more about biomedical applications, so he joined the master’s program in biomedical engineering at UTEP.

During his master’s Ivan joined the Inspired Materials and Stem Cell-based Tissue Engineering Laboratory (IMSTEL) that is directed under Dr. Binata Joddar. Ivan did research in tissue engineering application by working with 3D printing technology and then published a paper titled “A 3D printed PCL/bioactive hydrogel system for on-demand bone tissue engineering” in “Gels” journal. Also presented a poster titled “Facilitating pluripotent stem cell growth and differentiation by using 2D substrates and 3D printable materials” in the TERMIS European Chapter Meeting of 2017 for personalized therapies for regenerative medicine. Ivan graduated from his master’s degree from UTEP in May 2017.

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