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A feasibility study on using supercritical fluid technology to develop a biomimetic 3-D porous scaffold for bone tissue engineering

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Abstract of the Thesis

A feasibility study on using supercritical fluid technology to develop a biomimetic 3-D porous scaffold for bone tissue engineering

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The key to designing a suitable material for bone replacement is to mimic the mineral component and the microstructure of natural bone. The novel artificial bone scaffold should have good mechanical properties, high porosity, bioactivity, and controllable degradation kinetics. In this work, a three-dimensional scaffold based on a polymer phase consisting of only naturally-derived components (gelatin and cornstarch) and a mineral phase (hydroxyapatite (HA)) was produced using supercritical CO_2 as the foaming agent. By setting the pressure of the supercritical CO_2 at 2500 psi and the temperature at 35°C, 3-D porous scaffolds were successfully fabricated and no organic solvent was used in the entire process. The results show the amount of the cornstarch to have a direct effect on the porosity, in that, without cornstarch the scaffold could not be foamed (the total volume has not increased in this case). Pore size of

the scaffolds was influenced by the HA concentration. We also investigated the effect of two different gelatin cross-linking agents (trisodium citrate and EDC/NHS) and different cross-linking methods (infusion and immersion) on degradation kinetics and supercritical CO_2 foaming. The results show that EDC/NHS cross-linked samples (by the immersion method) lasted longest for about 7 days at 37°C in SBF.

Key words: Bone, Scaffold, Gelatin, Cornstarch, Hydroxyapatite, Supercritical Fluids.

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List of Abbreviations

HA:	Hydroxyapatite
SCF:	Supercritical Fluids
PGA:	Poly glycolic acid
PDLLA:	Poly (D, L-lactide) acid
PLLA:	Poly (l-lactide) acid
PCL:	Poly (ε-caprolactone)
EDC:	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
NHS:	N-Hydroxysuccinimide
SBF:	Simulated Body Fluids
PDMS:	Polydimethylsiloxane
CaP:	Calcium Phosphate

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Introduction

Human tissue and organ failure caused by defects, physical damage or other type of injuries is always a big issue in human health care [1]. One of the solutions is replacement of the damaged tissue with a transplanted organ, but the shortage of donors is still a widespread problem. In addition, the lifelong immunosuppression and serious complications minimize its value. Another way is artificial substitute transplantation. Although in the past few decades, huge progress has been made in addressing these problems, including total artificial substitute like heart valve and artificial kidney, limited durability and biofunctional performance of these substitutes are still a big disadvantage compared to native organs [1]. For these reasons, tissue engineering and organogenesis are becoming a promising solution that opens new perspectives for regenerative medicine.

Tissue engineering, a typical interdisciplinary science includes cell biology, materials engineering and mechanical engineering for the purposes of repairing damaged or diseased tissue and organs, now plays a very important role in modern medical clinical use. The fundamental concept of tissue engineering is to combine a biodegradable matrix and live cells to form a construct inducing the formation of a specific defective tissue in a specific location [2].

Among all cases of human tissue failure, bone disease is one of the most common and costly problems. Approximately 6 million fractures occur in the U.S. each year, and an estimated 1.5 million people suffer a fracture caused by bone disease [3]. Also, as the population ages, bone diseases like osteoporosis are a major source of pain in the elderly population [4]. To treat these kinds of diseases by using tissue engineering, the use of bioengineered three-dimensional

scaffolds is rapidly becoming the most promising experimental approach for mimicking the native structure of living tissues [5]. The scaffold should be made of biocompatible materials and have good mechanical properties, high porosity, and controllable degradation kinetics [6].

The difference between three-dimensional scaffold and two-dimensional layer is that in 3-D scaffold cells are seeded and expected to grow into the scaffold. So far, the 2-D approach has resulted in the in vitro growth of tissues with cross-sections of less than 500 μm from the external surface which means cells are only able to survive close to the surface [7]. Since 3-D tissues are required in many cases like large defects in bone, the 3-D scaffold is very important to be studied and should be designed carefully with specific pore sizes and interconnectivity pore structures [8].

Polymeric based materials blended with ceramics or bioglass have a promising future due to their good biocompatibility. Also, multiple functional groups on the polymeric chain give rise to various properties through different chemical modifications [9].

1.Review Of The Literature

1.1 Bone disease treatment

Currently, different surgical treatments have been developed for the repair of large bone defects including Ilizarov method and bone graft transplant. Ilizarov surgery, also called osteodistraction, which was developed by professor Gavril Abramovich Ilizarov, is based on the regenerative potential of bone. Briefly, the shattered bones are removed firstly and a healthy part of the upper bone is broken into two segments with a saw. The screws are turned to increase the gap between the growing zone, and the new bone can keep filling in the gap. Ilizarov surgery is extremely painful, requiring a long recovery period and may cause unsightly scar as well [10]. Fig.1-1 shows an Ilizarov transport.

Figure 1-1. Ilizarov bone transport [10]

Over 500,000 bone-grafting procedures are performed every year in the United States alone, and about twice as many are performed in the rest of the world [11]. Autografts (from the patient) are considered the best way in bone graft transplant, but requires certain amount of bone tissue from the patient's body (usually in the hip), which can also cause defects in other place. Allografts (from a donor) are a major way to heal people with dysfunctional tissue, but also limited by the supply of bone from donors, while multiple complications are involved. Xenografts (form another species) are unsuccessful because of serious health problems, tissue aging at different rate [9].

So, scientists in bone tissue engineering are looking for an alternative in order to improve all the grafts mentioned, that is by developing synthetic bone scaffold. The traditional synthetic bone grafts are mostly made of metals, such as titanium, or calcium phosphate ceramics and also glass [12]. For all types of grafts osteoconductive properties are very important, which describe the ability of the materials to serve as a scaffold on which bone cells can attach, migrate, grow and divide, further vascular invasion, cellular infiltration, and produce bone. However, they are not osteoinductive, which is the ability to promote new bone formation through various proteins such as bone morphogenetic proteins (BMPs), cytokines, and growth factors [13][14]. In addition, they cannot change shape in response to the loads and are not degradable which may lead to a second surgery.

Over the last two decades, polymeric based materials have been rapidly developed and new knowledge about cell-materials interactions in tissue regeneration show that polymeric materials have a good, if not superior, biocompatibility over metals. Also, the flexibility of polymer makes it easy to combine with other polymers, which can result in multiple functional composites.

1.2 Scaffold designing concept

Scientists in bone tissue engineering who want to develop a bone substitute in order to address the growing needs of the population are focused on scaffold design. The novel artificial scaffold should have good biocompatibility, certain mechanical property, biodegradability, porous structure, bone activity and drug delivery system, as describe below.

1.2.1 Good biocompatibility

Biocompatibility is defined as the ability of a material to perform with an appropriate host response in a specific application, or be able to integrate with a particular cell type or tissue. The biological response of a material is basically dependent on three factors: the material properties, the host characteristics, and the functional demands on the material. Therefore, the biocompatibility of a material can only be assessed on the basis of its specific host function and has to be uniquely defined for each application.

1.2.2 Certain mechanical property

The mechanical property plays a very important role in bone replacement, which means the scaffold should be able to support body of the patient during the bone regeneration period. A good mechanical property not only means the materials has good stiffness, but also stability during a certain period after it is applied both in vitro and in vivo. For this, the initial mechanical properties of the scaffold should account for both the change in properties with degradation and the change with the expected bone in-growth [15]. A final strategy is to design scaffolds such that the mechanical properties of the composite of bone and scaffold are within some percentage of the mechanical properties of the host bone. Table 1.1 shows the mechanical properties of human bone.

Table 1.1. Summary of the mechanical properties and porosity on human bone [15]

	Compressive strength (MPa)	Tensile strength (MPa)	Modulus (GPa)	Porosity (%)
Cortical bone	130-180	50-151	12-18	5-13
Cancellous bone	4-12	1-5	0.1-0.5	30-90

1.2.3 Biodegradability.

Biodegradability is very attractive in clinical applications so that the foreign materials can eventually be removed from the body as the new tissue is formed, with the intention that no second surgery will be needed. The biodegradable materials give a promising future to achieve that. In principle, the degradation rate of the scaffold should match the rate of tissue formation [16]. Research has show that a synthetic bone scaffold should maintain its mechanicall properties for at least 1 to 3 month after impantation and then should be totally resorbed after 12 to 18 month [17].Biodegradable polymers derived from synthetic aliphatic polyesters, such as poly (glycolide) (PGA), poly (D, L-lactide) (PDLLA), poly (l-lactide) (PLLA), and their copolymers, are widely used as biomaterials in surgical practice [18]. However, the acidic outcome of polymer biodegradation also negatively affects the latter stage results of bone repair [19]. Several factors, such as polymer molecular weight, polydispersity, crystallinity and morphology, are known to affect the rate of hydrolytic degradation of polymer, like PLA. Other factors, such as pH, ionic strength, temperature and buffering capacity of the medium in which the degradation occurs, also influence the degradation kinetics.

1.2.4 Porous microstructure

A suitable scaffold for bone regeneration must be designed with hierarchical porous structures to attain desired mechanical function and mass transport (permeability and diffusion). A porous structure can improve the microenvironment by creating a suitable surface for supporting cell adhesion and proliferation, and migration. In addition, the nutrition can easily diffuse though the scaffold with a good connecting porous structure, which is of great benefit to let the bone repair itself faster.

A variety of methods incorporating bone-like microstructures into orthopedic scaffold were

studied in last decade. Parameters such as porosity, connectivity and pore size were introduced and proved very important to cell adhesion and proliferation both in vitro and in vivo.

Solvent casting and particulate leaching are able to control the pore size, which is almost the same as the porogens and particles that used in the process. However, solvent casting requires high temperature or organic solvent and particulate leaching has a limited thickness of the sample. Three-dimensional scaffold can be foamed from different polymer fibers by physically bonding adjacent fibers. It is able to control the porosity and the pore size [20]. But the polymer fibers need to be dissolved or melted in the first place, which is involved in organic solvent or high temperature [20][21]. Thermally induced phase separation is also a very popular method to manufacture a porous structure due to high porosity and high volume of interconnected pores. Peter X. Ma's group achieved a high porosity (90% and above) scaffold with nano-hydroxyapatite and PLLA using a thermally induced phase separation technique. However, the residual organic solvent, in this case, dioxane could not be removed completely for certain [22].

A newly developed process by Durance et al. (cited from Sundaram et al, 2008) [23], microwave vacuum drying, was proven to be a very efficient drying method for fabricating a porous structure. But the pore size is very small using this method, which is not suitable for mammalian cell migration.

Other methods, such as 3-D printing to fabricate the scaffold layers by layers or fused deposition modeling, both are not easy to control and require high temperature [24].

Compared with all these methods, supercritical fluid gas foaming method can avoid using organic solvents and control the porosity at the same time by controlling the pressure and venting rate [25]. Moreover, Ana Rita C. Duarte et. al successfully in corporated drugs into the scaffold

using supercritical fluids gas foaming [26].

All the advantages and disadvantages of fabricating bone scaffolds with certain porosity and pore size are summarized in Table 1.2.

Table 1.2. Summary of fabrication methods for building porous scaffolds

Fabrication method	Porosity	Pore size	Advantages	Disadvantages
Solvent casting/ Particulate leaching [16][27-29]	Over 94%	Pore size can be controlled by using different porogen, like gelatin, salt, or sugar, up to 500 μm	High porosity, control over pore size	Can only produce thin film or 3-D specimens with thickness up to 2 mm; Use of organic solvent;
Melting mold/particulate leaching [30]	About 90%	Related to particle size	High porosity, control over pore size	High temperature limited application of loading drug into the scaffold; Can only produce thin film
Fiber bonding [20] [21]	Highly interconnected porosity	NA	Improved stability over non-bonded tassels and felts	Limited application as the two polymers must be dissolved or melted; No real control over porosity or pore size; Poor mechanical properties; Use of organic solvent
Freeze drying [31-34]	High porosity from 70% to 95%	From several micrometers to hundred microns	High volume of interconnected porosity	A low degree of crystallinity
Thermally induced phase separation [22] [23][35]	From 89% to 96%	NA	Can be incorporated with drugs; High volume of interconnected porosity	Use of organic solvents; It is difficult to control the micro- and macro-structure of the scaffold

Table 1.2. Summary of fabrication methods for building porous scaffolds (continuation)

Fabrication method	Porosity	Pore size (μm)	Advantage	Disadvantage
Microwave vacuum drying [36][37]	From 70% to 85%	The pore size is around 20 microns	Elimination of organic solvent and avoidance of high temperature; Can be incorporated with drugs;	The scaffold had relatively small pores around 20 μm and limited interconnectivity that may affect cell migration
3-D printing [24]	NA	500 μm	Controlled mechanical strength; High degree of spatial control of scaffold architecture	Limited application as the two polymers must be dissolved or melted; Use of organic solvent;
Fused deposition modeling [26]	NA	NA	Controlled pore size; High degree of spatial control of scaffold architecture	Polymer heated to melt state
Gas foaming (Supercritical fluid technique) [25][38-42]	60-90%	10-600 μm	Controlled pore size; Avoidance of organic solvent; Can be incorporated with drugs	It yields a largely unconnected porous structure and a non-porous surface;

1.2.5 Bone bioactivity

An essential requirement for an artificial material to bond to living bone is the formation of bone-like apatite on its surface when implanted in the living body [43]. To examine the bone bonding ability of a bone substitute, a simulated body fluid (SBF) with ion concentrations nearly equal to those of human blood plasma is typically used [44]. It is a good prediction of material bone bioactivity performance in vivo.

1.2.6 Drug delivery

The concept of delivering bioactive agent, growth factors such as bone morphogenetic protein 2 (BMP-2), directly from polymeric materials to selectively deliver those drugs to specific sites and to release them locally in order to induce a desired response of the target tissue is an attractive approach [16]. Beside the concerns in drug delivery systems such as initial burst or constant release rate, the major challenge in developing scaffold drug delivery system is to incorporate such biologically active species, without loss or change of bioactivity, into a polymeric substrate.

1.3 Polymeric materials in bone tissue engineering

Compared with the traditional bone replacement materials such as metal, glass or ceramic, polymeric based materials have a bright future in bone tissue engineering. First, polymer is flexible, and the different functional groups on the main chain or side chain provide great possibility of combinations of different properties to fill the requirements [1]. Secondly, the main protein component of bone is collagen. Showing good biocompatibility, biodegradable polymer

such as PLLA, PGA, PLGA can mimic the organic part of the bone replacement [9]. Additionally, composite scaffolds made of polymer reinforced with HA seem to be promising substrates for bone tissue engineering due to their enhanced mechanical strength [15]. Thirdly, the polymeric materials are suitable for processing by diverse techniques and into diverse shapes. Among all of these polymers, the hydrogels are gaining a lot of attention due to their hydrophilic property, suitability for direct cells encapsulation or drug delivery and similarity to natural extracellular matrix (ECM). Last but not least, some of the polymers are biodegradable, which is a very important property in the application of the bone tissue engineering, because the aim of using scaffold is to fill the defect area until new bone is formed.

Major biodegradable natural and synthetic polymers for tissue engineering are listed in Figure. 1-2.

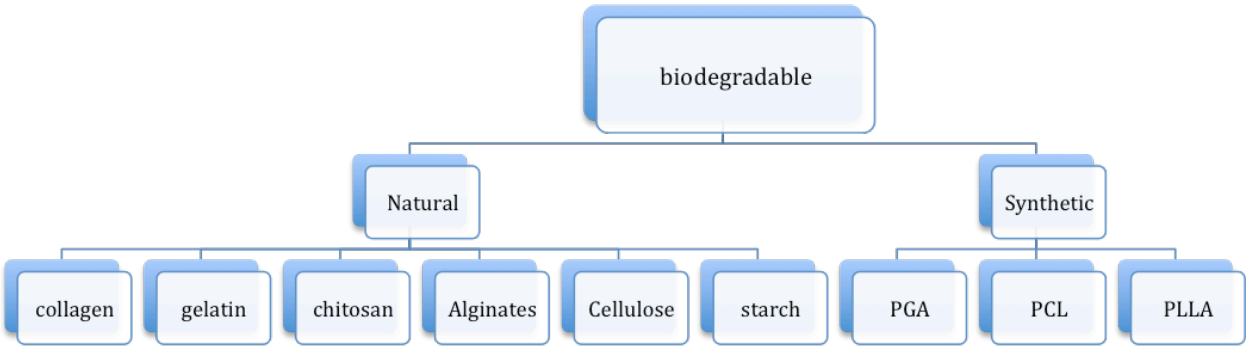

Figure 1-2. Relationship of major natural and synthetic biodegradable polymers (summarized from [9])

1.3.1 Gelatin

Gelatin is an irreversibly hydrolyzed form of collagen, which is the major organic component of bone. Overall, collagen is composed of triple helices, (three α-chains rotating counterclock-wise) and is stabilized via interchain hydrogen bonds [45]. Collagen denaturation may lead to a complete or incomplete detachment of the chains on amount of breaking of the hydrogen bonds, leading to destruction of the three-dimensional conformation of the triple helix compositions. In fact, the industrial gelatins are a mix of three compounds: α-chain (single polymer chain), β-chain (two polymer chain cross together), γ-chain (three polymer chain covalently cross-linked) [45]. It is a polyampholyte having both cationic and anionic groups along with hydrophilic group [46]. There are two types of gelatin depending on how denaturation is preformed. In general, gelatin obtained by an acid-treated hydrolysis is known as type A, and gelatin obtained by a base-treated process is known as type B [45].

Gelatin is biocompatible, biodegradable, non-immunogenic and inexpensive, which makes it suitable for food and biomedical applications, such as wound dressing [47], and in drug delivery system as soft and hard capsules, or microspheres [48].

1.3.1.1 Gelatin in bone tissue engineering

Gelatin has a high degree of biofunctional group, which means it has a great potential to react with the other biomolecules [45]. HW. Kim et al. found that gelatin–HA nanocomposites have a better efficacy in enhancing the osteoblastic phenotype expression level than pure gelatin [46]. It also has been reported that gelatin/hydroxyapatite combined with other synthetic polymers like poly (ε-caprolactone)(PCL), PLLA or poly (lactic-co-glycolic acid)(PLGA) showed good stimulants on cell proliferation [50-52]. Application of the gelatin hydrogel-

connective tissue growth factor complex, together with a collagen scaffold implanted in bone defect in a rat femur, was reported to result in remarkable induction of osteoblastic mineralization markers and distinct enhancement of bone regeneration [53].

1.3.1.2 Gelatin crosslinker

Gelatin is not suitable to be used as long term application such as bone scaffold materials mainly due to its poor mechanical property and highly water affinity. Mechanical properties, swelling behavior and thermal properties depend significantly on the cross-linking degree of gelatin. So, the studies of gelatin crosslinking agents have been widely developed in order to improve its mechanical properties. The traditional crosslinking agents are aldehyde-based cross-linkers, including formaldehyde, glyoxal and glutaraldehyde [54]. Although they have shown great efficiency in protein modification, use of these additives also causes health issues due to their toxic nature. Genipin, an iridoid compound extracted from gardenia fruits, is increasingly used due to its low cytotoxicity in order to replace glutaraldehyde and formaldehyde as a crosslinker [55]. However it imparts blue coloration after crosslinking. Caffeic acid, another naturally compound gives high thermal insistence and gel strength to gelatin [56], but is a potential carcinogen and its value in tissue engineering is limited.

EDC is a zero-length cross-linker that activates carboxylic acid groups and then forms the amide bonds with the amino groups of gelatin. Zenon Grabarek and John Gergely developed a two-step zero-length crosslinking procedure using EDC and NHS, which eliminates complications arising from the formation of crosslinks among several proteins of a multicomponent complex [57]. Chiming Yang et al. studied two different concentration of EDC/NHS, one is 17 mM EDC and 8.5 mM NHS in distilled water as the low crosslinking

solution, and the other is 34 mM EDC and 17 mM NHS in distilled water as the high crosslinking solution. The crosslinking solution was dispersed in gelatin solution and the gel solution was kept at 4°C for 24 h to produce a crosslinked hydrogel. They found that the high crosslinked scaffold could not be degraded at least 21 days after subcutaneous implantation in NOD/SCID mice while the low crosslinked one was completely degraded [14]. As we metioned, the foreign materials should be maintain mechanical properties and shape for at least 1 month, so the high crosslinking solution is better to have a more stable gelatin scaffold.

The chemical structure of EDC and NHS are shown in Figure 1-3 and Figure 1-4. The proposed crosslinking sequence between gelatin single chain and EDC/NHS is shown in Figure 1-5.

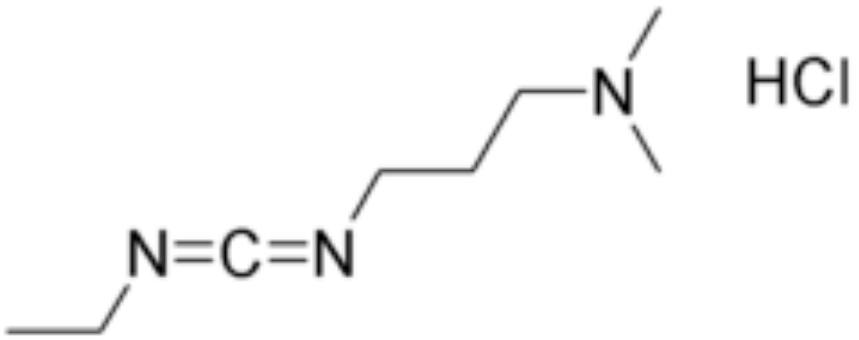

Figure 1-3. Chemical structure of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)

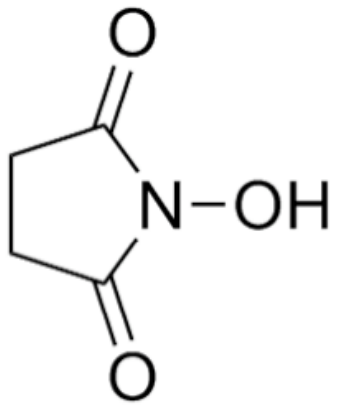

Figure 1-4. Chemical structure of N-Hydroxysuccinimide (NHS)

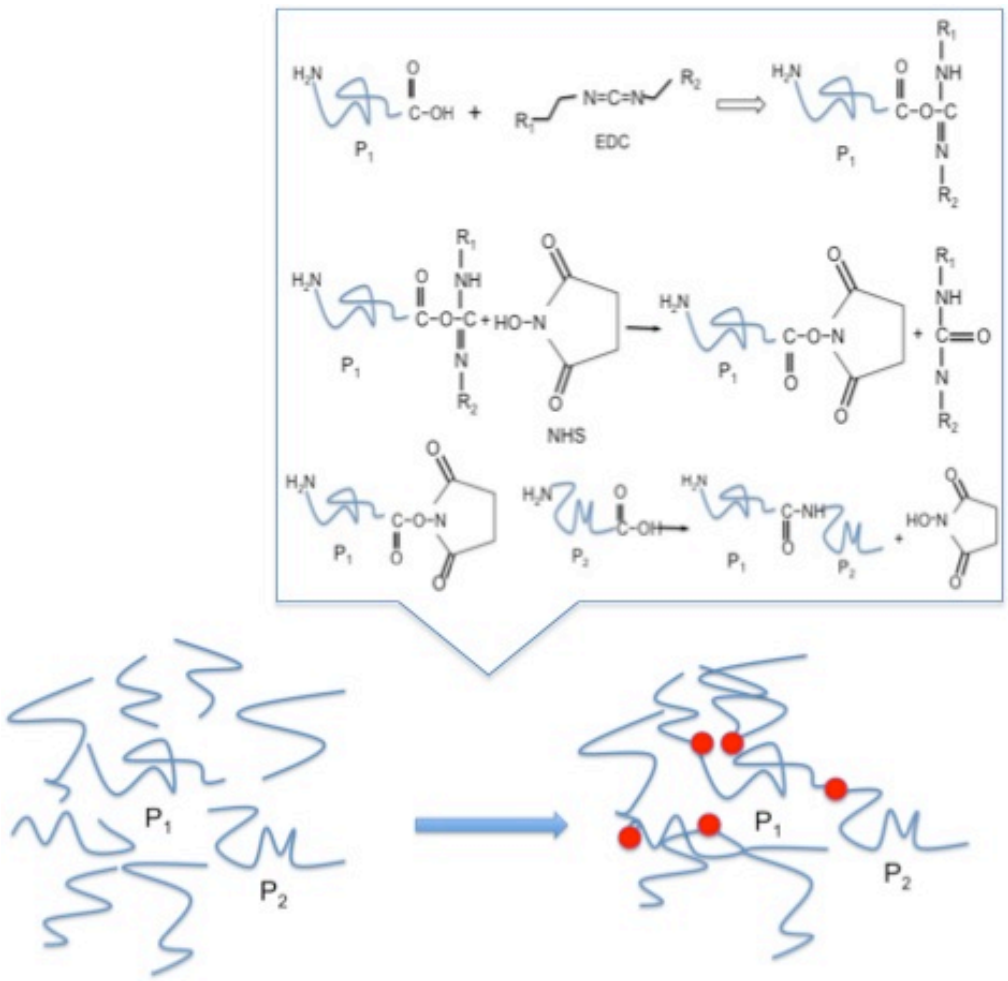

Figure 1-5. The proposed crosslinking sequence model between gelatin single chains and EDC/NHS [57]. P1, protein1; P2, Protein2.

1.3.2 Starch-based polymer

Starch is one of the most abundant and frequently employed natural materials in biomedical applications and is produced by all green plants as an energy storage compound. It consists of both linear and branched polymer [58]. Starch is naturally produced in the form of semi crystalline granules with different sizes and resource, such as potato, wheat or corn. Starch-based polymer is suitable for processing by diverse techniques and into diverse shapes, which means it has an enormous potential for 3-D porous scaffolds and bone cements. Jaya Sundaram et al. used starch blended with gelatin/HA to manufacture a porous scaffold via microwave vacuum drying, and showed the starch-based polymer to have a good mechanical property. Starch based polymer also showed great biocompatibility with human osteoblast cells in vitro and in vivo with Wistar rats [58-61].

1.4 Hydroxyapatite

In mammals calcium orthophosphates are the main inorganic component of normal and pathological solid tissues such as kidney stone, teeth, ears and bones [62].

Hydroxyapatite is one of the calcium orthophosphates with element ratio is Ca: P = 1.67 and is considered as the major component of the inorganic part of bone. The synthetic form of hydroxyapatite (HA) is osteoconductive and has a crystalline structure similar to the HA in bone but is hard to resorb and therefore may stay remain in site of implantation for many years [63][64]. As mentioned before, a characteristic of good bone bioactivity of a bone substitute is to form bone like hydroxyapatite crystals on the surface.

The properties of HA that make them excellent candidates for use in bone substitute have been given a lot of tension. However, the barrier for use of HA in loading application is their

inherent brittleness [19], although all CaP has a good stiffness and strength that is similar to those of cortical and cancellous of natural bone.

1.5 Supercritical Fluids

Supercritical fluid is a special phase when the temperature and the relative speed come to a special point that is on the basis of the molecular behavior. That special point also called critical temperature is the point at which a single substance can exist not as either fluid or gas. This was first shown experimentally 170 years ago by Baron Charles Cagniard de la Tour [64]. The special substance is called supercritical fluids (SCF).

Because the Carbon Dioxide does not leave toxic residues and is abundant, supercritical CO_2 is the most popular agent in all supercritical fluids applications, such as supercritical fluid extraction [65], dry-cleaning [66], supercritical fluid chromatography. It has been identified as prime candidates to develop alternative clean process for the preparation of drug-loaded polymeric matrix [22,67].

The non-toxic supercritical Carbon Dioxide is very suitable for tissue engineering application. Ana Rita C. group used supercritical CO_2 as a Supercritical immersion precipitation technique or supercritical assisted phase inversion to develop a bone scaffold base on starch/PLLA blend [41] and starch-poly(ε-caprolactone)[39] polymer. However, they used organic solvent such as dichloromethane and chloroform that limit the addition of biomolecules. Hongyun Tai et al used supercritical CO_2 as a foaming agent to create pore structures in PLGA and PLA matrix. They successfully controlled the pore size by changing the venting rate and the composition [25].

Figure 1-6 shows the phase diagram of supercritical CO_2.

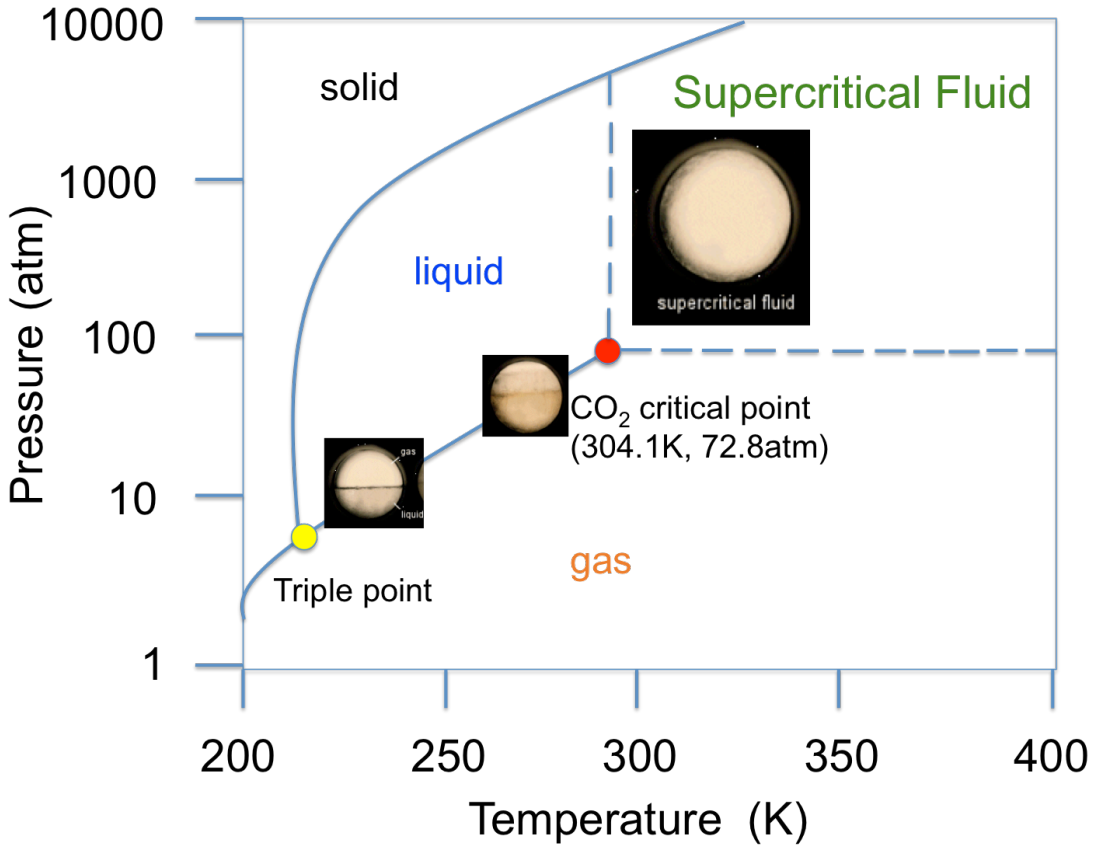

Figure 1-6. Phase diagram of Supercritical CO_2. The yellow dot and the red dot show the triple point and the critical point of CO_2 (reproduced from [51])

Objective

The aim of this thesis is to create a novel porous artificial bone replacement material in order to aid in bone repair and regeneration using only biodegradable polymer based materials.

In this work, a feasibility study on a three-dimensional scaffold based on a polymer phase consisting of only naturally-derived components (gelatin and cornstarch) and a mineral phase (hydroxyapatite (HA)) was conducted using supercritical CO_2 as the foaming agent.

First, no organic solvent will be used in this study. This alternative method of fabricating scaffold for bone implants can solve the problem of the residual organic solvent.

Secondly, we are trying to control the porosity and the pore size of the scaffold by using supercritical CO_2 foaming technique. A suitable porosity and pore size for supporting cell adhesion, proliferation and migration can be achieved.

Thirdly, gelatin and cornstarch are both biodegradable polymers. It is very attractive in clinical application that the foreign materials can eventually be removed from the body as the new tissue is formed, with the intention that no second surgery will be needed. In principle, the degradation rate of the scaffold should match the rate of tissue formation. Different crosslinking methods will be studied in this work in order to control the degradation rate.

2. Materials And Methods

2.1 HA synthesis

To synthesize HA powder, calcium chloride (anhydrous, BioReagent, suitable for insect cell culture, suitable for plant cell culture, ≥96.0%) was purchased from sigma Aldrich Chemical Co. (St. Louis, MO, USA) and sodium phosphate (Na_2HPO_4) was supplied by EMD chemicals, Inc. (US).

Briefly, 0.5 M $CaCl_2$ solution with DI water in a beaker was prepared, slowly, 0.3 M Na_2HPO_4 solution in DI water was added while stirring with a magnetic stir bar. The quantities of reactants were selected to provide a Ca/P 1.66:1 molar ratio. After adding all Na_2HPO_4 solution, NH_4OH solution was added to maintain and control pH at 10-10.5 in the solution. After that, the suspension was aged for 24 hours at room temperature. The solution was centrifuged, decanted and the suspension was washed with PBS and DI water 3 times. The obtained suspension was kept in freezer at - 80°C overnight and then freeze-dried to powder form.

2.2 Preparation of conventional simulated body fluids (c-SBF)

All chemicals that used in preparation of conventional simulated body fluids were listed in Table 2.1.

All apparatus including a magnetic stir bar and a 1000 mL polypropylene bottle were immersed in a 2000 mL glass beaker filled with 1.0 M HCl overnight and then washed with DI water. To prepare the conventional simulated body fluids, 700 mL DI water was poured into a 2000 mL glass beaker. The solution was stirred with a magnetic bar at room temperature, and all the reagents were added according to Table 2.2.

After adding all the reagents, the fluid was kept at 36°C in water bath for 15 minutes and adjusted to a final pH of 7.40 at 36°C by adding 1.0 M HCl. The solution was cooled down to room temperature and ultra-pure water was added to make the total volume 1000 mL. The fluid was transferred to a 1000 mL polypropylene bottle and kept in the refrigerator at 4°C.

Table 2.1. List of the materials for conventional body fluids (c-SBF) preparation

	Chemicals	Grade	
1	Sodium chloride	Meets ACS Specifications, Meets Reagent Specifications for testing USP/NF monographs	EMD Chemicals Inc.
2	Sodium Bicarbonate	Lab grade	Scholar Chemistry (Avon, NY 14414,USA)
3	Potassium Chloride	Meets U.S.P. & F.C.C. Requirements	Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA)
4	Potassium phosphate dibasic trihydrate	for molecular biology, ≥99%	Sigma Aldrich Chemical Co. (St. Louis, MO, USA)
5	Magnesium chloride hexahydrate	BioReagent, suitable for cell culture, suitable for insect cell culture	Sigma Aldrich Chemical Co. (St. Louis, MO, USA)
6	Hydrochloric Acid, 36.5~38.0%	Meets A.C.S. specifications	Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA)
7	Calcium chloride	anhydrous, BioReagent, suitable for insect cell culture, suitable for plant cell culture, ≥96.0%	Sigma Aldrich Chemical Co. (St. Louis, MO, USA)
8	Sodium sulfate	ACS reagent, ≥99.0%, anhydrous, powder	Sigma Aldrich Chemical Co. (St. Louis, MO, USA)
9	Tris(hydroxymethyl)aminomethane	ultrapure grade, ≥99.9%	Sigma Aldrich Chemical Co. (St. Louis, MO, USA)

Table 2.2. Reagents and Amounts for preparing 1000 mL of c-SBF

1	2	3	4	5	6	7	8	9
NaCl	$NaHCO_3$	KCl	$K_2HPO_4 \cdot 3H_2O$	$MgCl_2 \cdot 6H_2O$	1.0M HCl	$CaCl_2$	Na_2SO_4	TRIS
8.036 g	0.352 g	0.225 g	0.230 g	0.311 g	40 mL	0.239 g	0.072 g	6.063 g

2.3 Fabrication of crosslinked hydrogel (in preparation prior to SCF foaming)

2.3.1 Using Tri sodium citrate as a crosslinker

Gelatin powder, type A (porcine), was purchased from Great Lakes Gelatin Company (IL, USA). Cornstarch was supplied by ACH Food Companies, Inc. (Memphis, TN, USA).

A fixed amount of gelatin/starch polymers with three different amount of HA to get 0:100, 20: 100, 40: 100 HA: polymer ratios. The amount of composition was listed in Table 2.3. Briefly, 10 g gelatin was added to 70 mL of DI water. The solution was heated to 50°C and stirred with a magnetic stir bar. After the gelatin was dissolved, 5 g of cornstarch was dispersed into the mix with continued heating and string. The respective amount of hydroxyapatite was dispersed into the polymer solution. The mixture was then poured into a small mold made of Polydimethylsiloxane (PDMS) to obtain a disc-shaped solid gel (about 2 cm diameter and 1 cm thickness). After cooling to the room temperature, the polymer blend with different amount of HA was obtained. These solids were immersed in 0.5 M Tri sodium citrate solution for 24 hours to crosslink. All the samples were collected in a Petri dish, sealed with Parafilm and kept in refrigerator at 4°C. The process was shown in Figure 2-1.

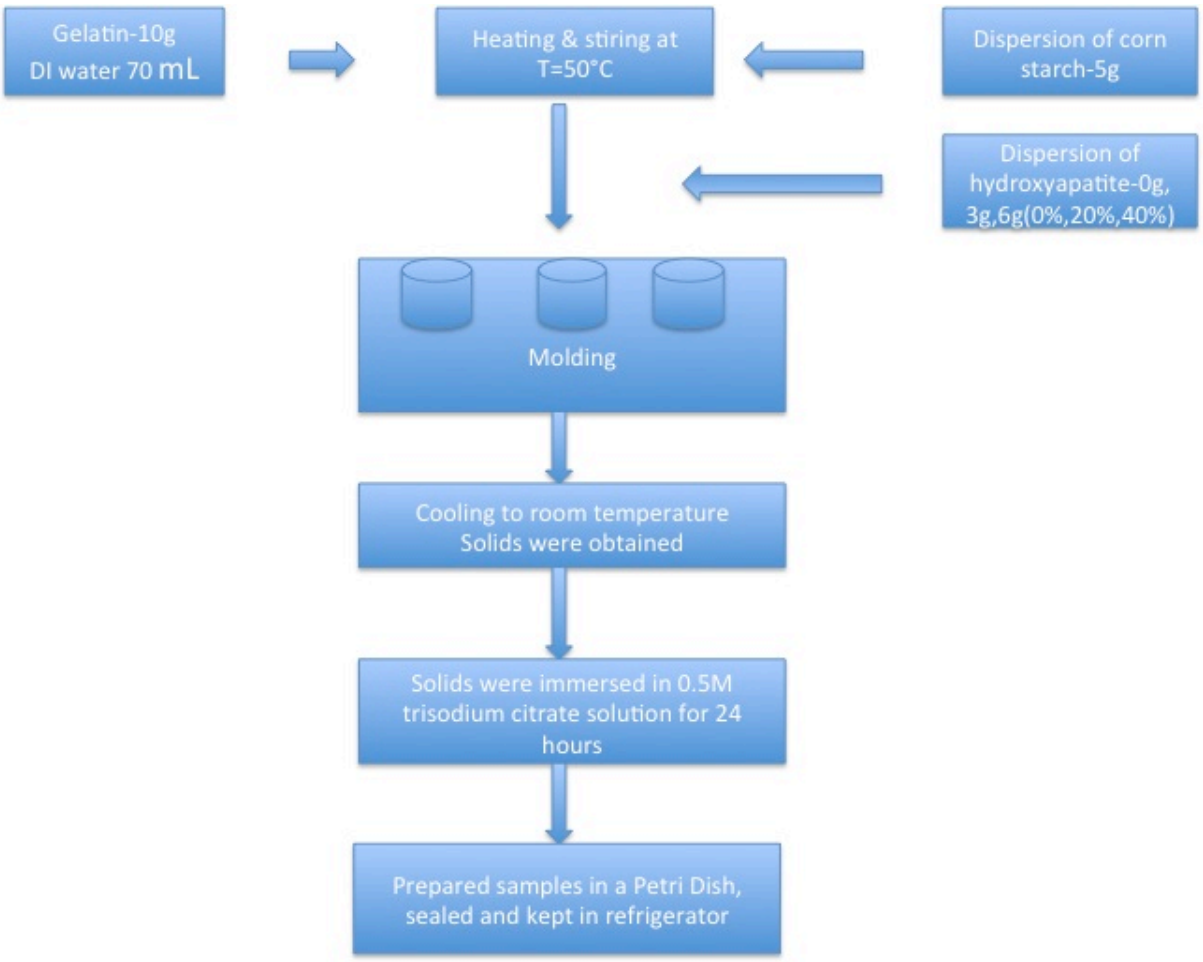

Figure 2-1. Process of hydrogel fabrication and crosslinking (Tri sodium citrate)

Table 2.3. Tri sodium citrate crosslinked gelatin/starch-HA scaffold

	Gelatin (g)	Starch (g)	HA (g)	HA: polymer
Sample 1	10	5	0	0:100
Sample 2	10	5	3	20: 100
Sample 3	10	5	6	40: 100

2.3.2 Using EDC/NHS as a crosslinker

N-Hydroxysuccinimide (NHS, 98%) was purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). N- (3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, ≥98.0%) was purchased Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

(A) Immersion method of trisodium citrate cross-linking reagent

4 g of gelatin was added to 20 mL of DI water. The solution was heated to 50°C and stirred with a magnetic stir bar.

After the gelatin was dissolved, 2 g of cornstarch dispersed into the mixture with continued heating and stirring, so that a 2: 1 mass ratio of gelatin and cornstarch was obtained. The respective amount of hydroxyapatite (0 g, 1.2 g, 2.4 g) was dispersed into the polymer. After complete dispersion of hydroxyapatite, 8 mL crosslinking solution containing 17 mM NHS and 34 mM EDC was added into the polymer mix. The mixture was then poured into a small mold made of PDMS. The solid gels were kept in refrigerator at 4°C for 24 hours. The crosslinked gel was wash three times with distilled water to remove excess cross-linking agents. All the samples were collected in a Petri dish, washed three times with DI water, then sealed with parafilm and kept in a 4°C refrigerator.

Gelatin and varied amount of cornstarch without hydroxyapatite polymer gel were also prepared in the same way.

(B) Infusion method of EDC/NHS cross-linking reagent

Instead of adding crosslinking solution, all the gels were solidified first and then immersed in DI water containing 17 mM NHS and 34 mM EDC at 4°C for 24 hours. After

crosslinking, all gels were washed three times with DI water and collected in a Petri dish, then sealed with Parafilm and kept in a 4°C refrigerator.

(C) Immersion method of EDC/NHS cross-linking reagent

We used 70% ethanol as the solvent of EDC/NHS. Briefly, all the gels were solidified and then immersed in 70% ethanol solution containing 17 mM NHS and 34 mM EDC at 4°C for 24 hours. After crosslinking, all gels were washed three times with DI water and collected in a Petri dish, then sealed with Parafilm and kept in a 4°C refrigerator.

Composition of all samples crosslinked by EDC/NHS were shown in Table 2.4 and Figure 2-2 shows the process of hydrogel fabrication and crosslinking (EDC/NHS).

Table 2.4. EDC/NHS crosslinked gelatin/starch-HA scaffold

	Gelatin (g)	Starch (g)	HA (g)	Starch: gelatin	HA: polymer
Sample 1	4	2	0	50: 100	0: 100
Sample 2	4	2	1.2	50: 100	20: 100
Sample 3	4	2	2.4	50: 100	40: 100
Sample 4	4	2	0	50: 100	0: 100
Sample 5	4	1	0	25: 100	0: 100
Sample6	4	0.5	0	12.5: 100	0: 100

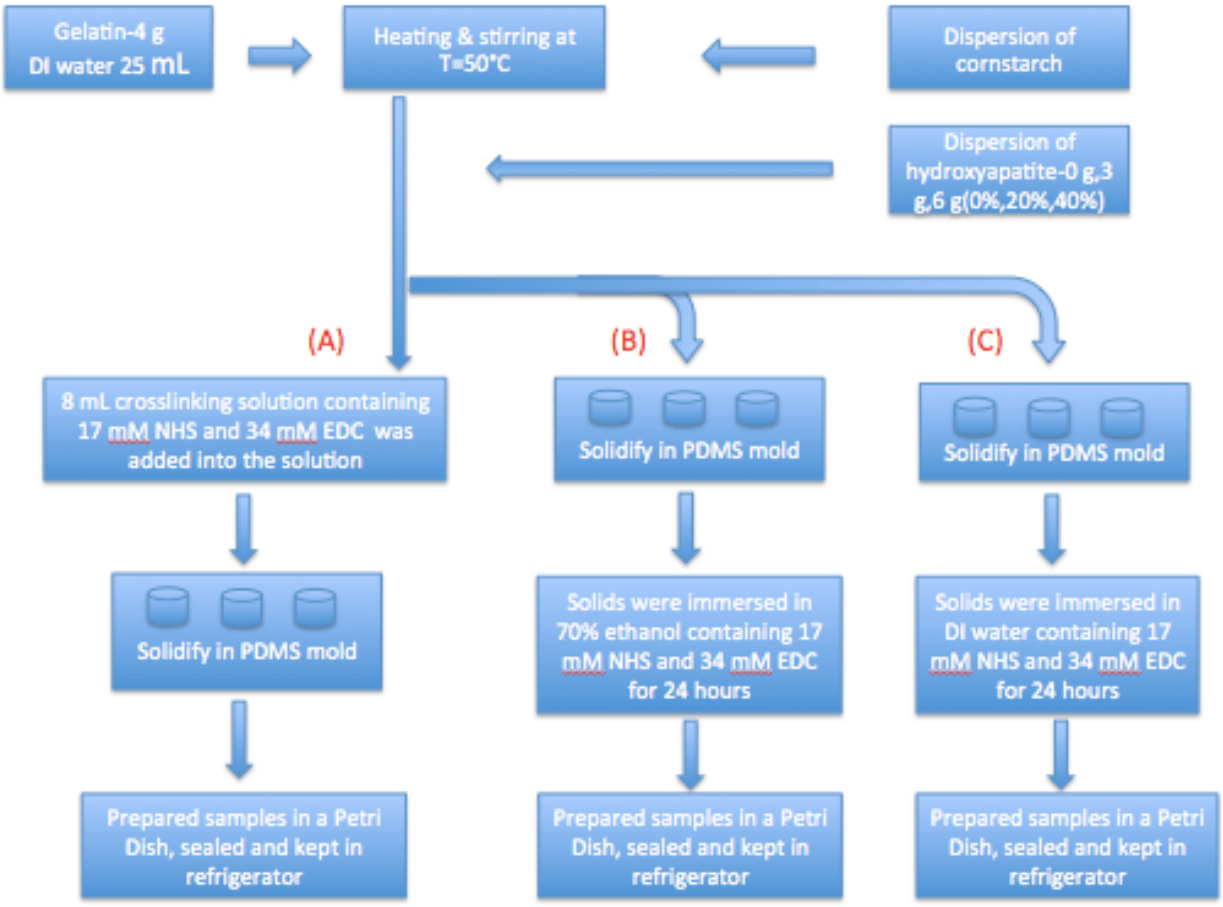

Figure 2-2. Process development of hydrogel fabrication and crosslinking (EDC/NHS)

2.4 Preparation of porous scaffold using supercritical CO_2 foaming

We set the pressure of the reaction at 2500 psi and the temperature at 35°C which is in the supercritical CO_2 region, and also 35°C is a very suitable temperature to incorporate drugs and other biomolecules while maintaining their activity. We determined that the soaking time 20 minutes is long enough for the CO_2 to diffuse into the gel in this work. We have tried keeping the sample in supercritical fluid for 40 minutes, but there was no visible difference in porosity or morphology of the foamed structure.

The crosslinked gelatin/starch/HA scaffold was placed in a metal container and put into the pressure vessel for supercritical CO_2 foaming (see Fig. 2-3). CO_2 gas was kept releasing into the vessel with constant 800 Psi pressure until the temperature of the vessel reach 4 °C. Then, the temperature of the vessel was increased to 35 °C. As the temperature increased, the pressure also began to rise. If the pressure rose over 2500 psi, the CO_2 gas was slowly vented into a chemical fume hood in order to keep 2500 psi. The condition was held for 20 minutes. Afterwards, the CO_2 was carefully vented to the hood for a 10 minutes period and the porous scaffold was gathered. Figure 2-3 and Figure 2-4 show the supercritical CO_2 system.

Figure 2-3. a) Pressure gauge used to monitor SCF process; b) Supercritical CO_2 high-pressure vessel

Figure 2-4. Supercritical CO_2 systems: a) CO_2 tank; b) steel tube; c) water bath; d) heat controller; e) pressure gauge and pressure vessel; f) safety shield; g) CO_2 venting tube; h) Fume hood

2.5 Scaffold drying methods

After SCF foaming, all the foamed gelatin/starch/HA scaffolds were carefully cut without breaking the initial structure and dried in three different ways.

1. Foamed samples were dried at room temperature for 3 days.
2. Foamed samples were dried in oven at 60°C overnight.
3. Foamed samples were kept in freeze at - 80°C overnight and then freeze-dried for 24 hours.

2.6 Degradation study

The polymer gel was cut into four pieces as shown in Fig 2-5. Using a 6-well tissue culture plate, all the samples were placed in each well separately. Then, enough SBF solution was added into each well to cover the surface of the gel. All the tissue culture plates were sealed with Parafilm and incubated at 37°C up to seven days. We checked the result every 24 hours.

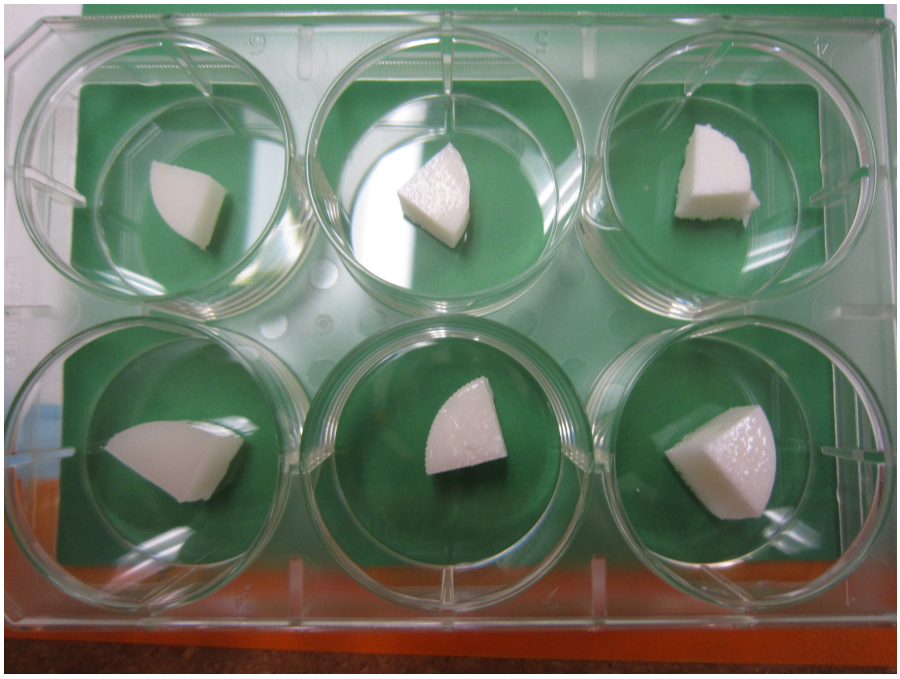

Figure 2-5. Unfoamed Gelatin/starch/HA samples placed in 35 mm tissue culture wells prepared for degradation study

2.7 Scanning electron microscopy analysis

SEM micrographs of dried samples were prepared by slowly cutting into the foamed sample without breaking the pore structure to get an interior piece, thus exposing the internal surface features. The surface was made electrically conductive by coating a thin layer of gold using a gold sputter coater under vacuum before imaging. The samples were imaged in a scanning electron microscopy (LEO 1550, 20 keV accelerating voltage).

3. Results

3.1 Morphology of the scaffold

From the SEM images we can see HA (Fig. 3-1 B) on the surface of the scaffold after supercritical CO_2 foaming. In Fig.3-2, we can see the supercritical CO_2 foaming technique is able to create connective pore structures in gelatin/cornstarch and HA (20%) scaffold. The diameter of the pore in 20% HA sample is around 100 μm.

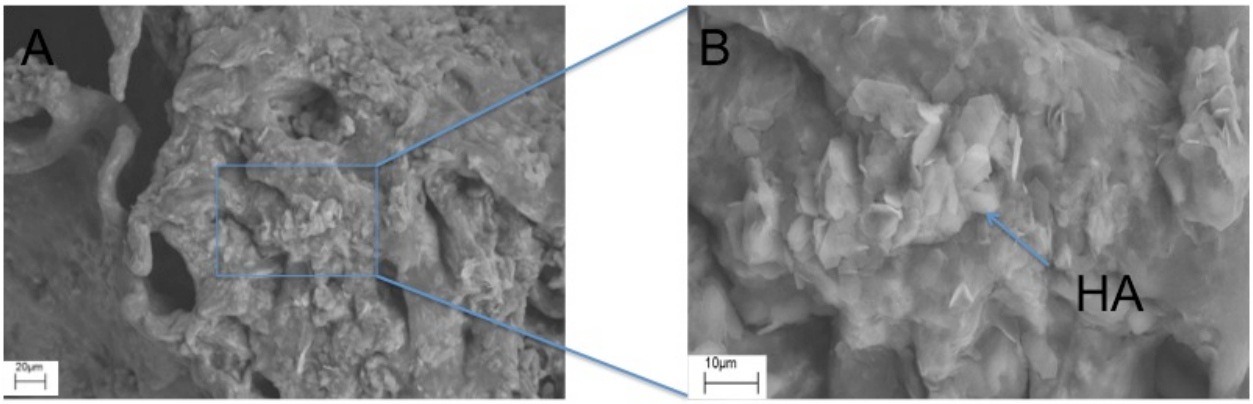

Figure 3-1. SEM images show the crystalline HA on the surface of the CO_2 foamed scaffold (gelatin: starch: HA, 10: 5: 3). A. Morphology of the scaffold; B. crystalline HA

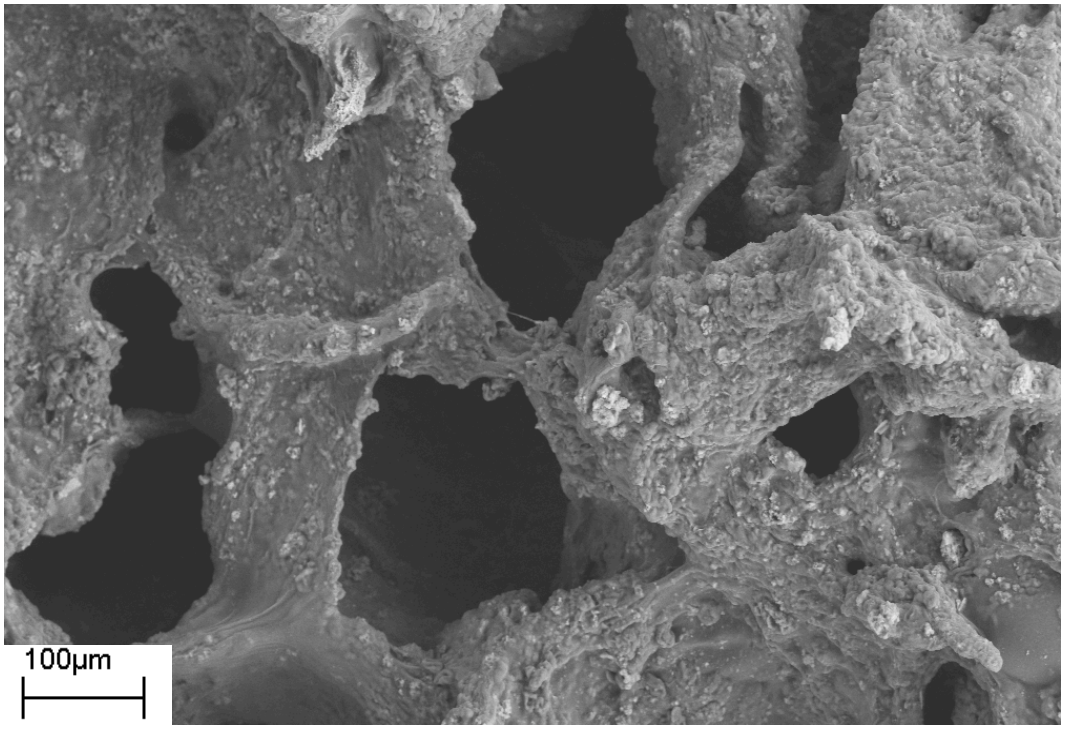

Figure 3-2. SEM image of 20% HA SCF foamed scaffold (gelatin: starch: HA, 10: 5: 3).

3.2 Effects of starch concentration on foaming process

We varied the ratio of cornstarch and gelatin from 0: 100,12.5: 100, to 50: 100. Photographs from Fig. 3-3 were taken right after foaming. Figure 3-3(A) shows the result of pure gelatin foamed scaffold. The total volume of the foamed gelatin gel was almost the same as the unfoamed gel, which suggests that the pure gelatin gel could not be foamed by our SCF gas foaming technique. Figure 3-3(B) shows the 12.5: 100 starch: gelatin sample, examination of the sample show that there is bubble-like structure in the scaffold after SCF foaming. But the bubbles were very big and weak; also, the wall of the bubbles was very thin. With more starch in the polymer, as shown in Figure 3-3(C), the number of the CO_2 bubbles increased and the walls of these bubbles were much thicker compare with the 12.5: 100 sample. The biggest volume after foaming process was achieved for 50: 100 ratio sample.

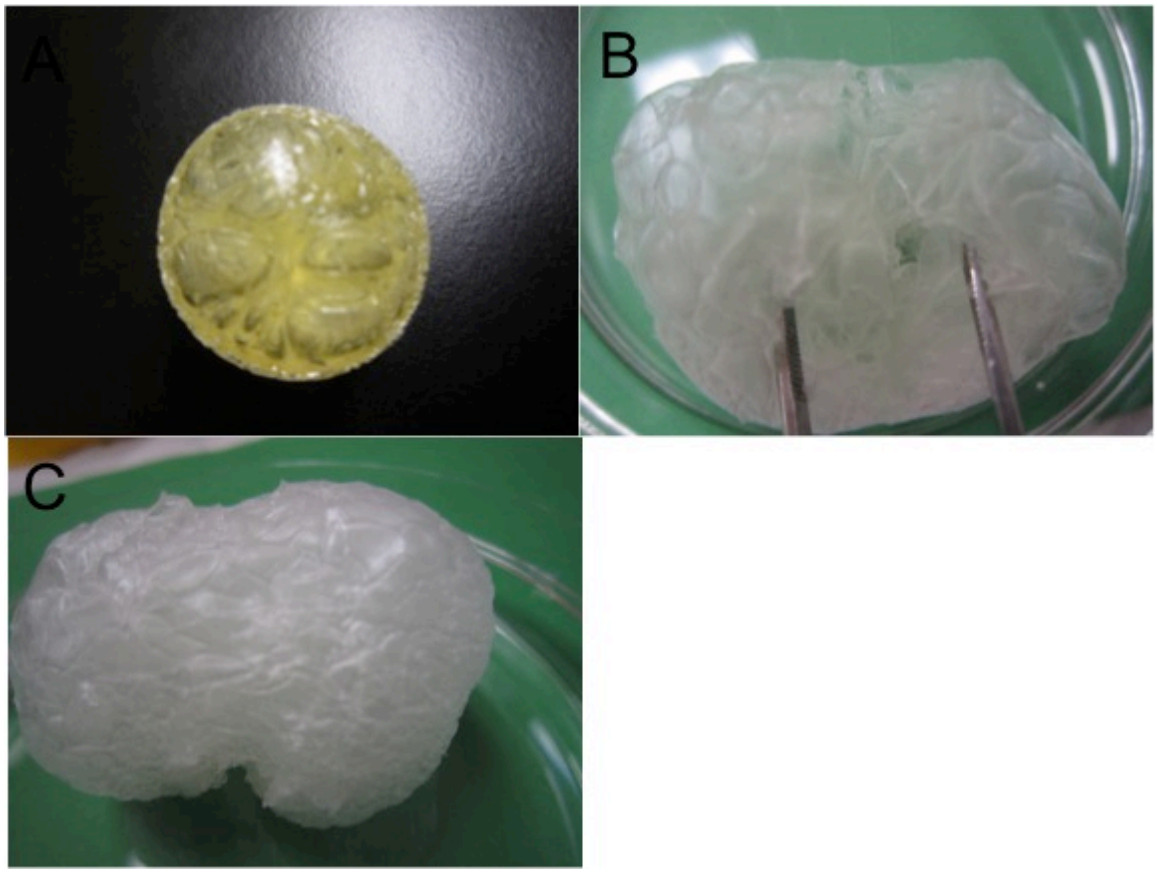

Figure 3-3. Foaming results of gelatin/starch scaffold (All samples were crosslinked by adding 8 mL crosslink solution containing 17 mM NHS and 34 mM EDC) A. Starch/gelatin (w/w)=0: 100; B. 12.5: 100; C. 50:100.

3.3 HA effects

We chose the 50: 100 ratio of cornstarch and gelatin as the polymer matrix and added different amounts of HA to vary the ratio of HA and polymer from 0: 100, 20: 100 to 40: 100. SEM images (Figure 3-4) show the morphology of the foamed sample from 0: 100 to 40: 100 under the same magnification scale. From these SEM images, we can see the sample without HA has the largest pores (around 200 μm). The 20: 100 sample had 50 μm to 100 μm pores. The sample with the most HA had many small pores in the scaffold after SCF foaming. In summary, pore size was decreased as HA: polymer ratio increased.

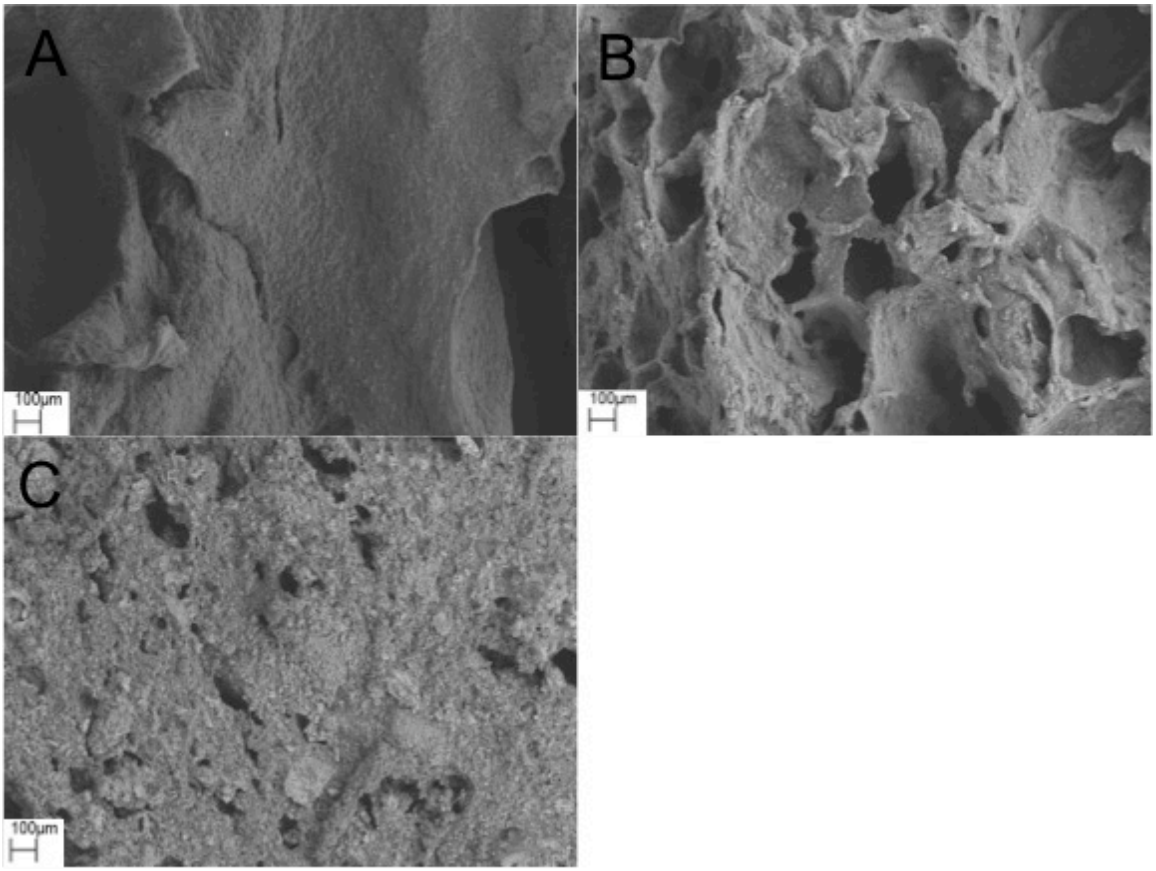

Figure 3-4. SEM images show the pore size of the CO_2 foamed scaffold with different amount of HA A. 0% (HA: polymer (gelatin: cornstarch, 2: 1), w/w); B. 20%; C.40%. (All samples were crosslinked by Tri sodium citrate)

3.4 Comparison of different crosslinking methods

Different protein crosslinking reagents and varied methods were examined using a variety of methods in this study.

Figure 3-5 shows the foaming results of the crosslinked gelatin/starch/HA gel by different crosslinkers and solvents. Figure. 3-5(A) is the sample that was soaked in 0.5 M trisodium citrate for 24 hours prior to SCF foaming. We can see the polymer could be foamed in SCF foaming technique, but the total approximate volume of the foamed scaffold was not changed too much compared with the unfoamed sample. Figure 3-5(B) is the sample that was added 34 mM EDC and 17 mM NHS crosslink solution instead of being immersed in the crosslink solution. The total volume is much greater than Figure 3-5(A), indicating higher porosity. The pores in the EDC/NHS crosslinked gelatin/starch/HA scaffold are also larger than the sample that is crosslinked by trisodium citrate.

Foaming result of sample 3 that is crosslinked by soaking in the DI water containing 34 mM EDC and 17 mM NHS is shown in Figure 3-5(C). There are some CO_2 bubbles-like structures formed from the inside of the gel and there were no CO_2 bubbles-like structures on the surface of the gel. All the walls of the CO_2 bubbles were weak and easily broken. Figure 3-5(D) shows the foaming result of sample 4 that was soaked in 70% ethanol containing 34 mM EDC and 17 mM NHS. The resulting microstructure is almost the same as sample 3, and the gel broke into two parts and a few CO_2 bubble-like structures came from inside of the gel. The surface of the gel remain the same and very hard. The foaming result of the sample 5 is shown as E, which is the non-crosslinked gelatin/starch/HA gel. The non-crosslinked gelatin was very soft after the SCF foaming process and there were no bubbles-like structure inside the scaffold. And also half

of the sample was lost during the gas foaming process, so the total volume is smallest among all the samples.

Figure 3-5. The effect of chemical crosslinking on the foaming process was examined for A. a sample immersed in 0.5 M Trisodium citrate; B. EDC/NHS crosslinked gelatin; C. sample immersed in DI water containing 34 mM EDC and 17 mM NHS; D. sample was immersed in 70% ethanol containing 34 mM EDC and 17 mM NHS; E. non-crosslinked sample. (All samples had a 100: 50 mass ratio of gelatin: starch.)

3.5 Drying effect on pore size

In this study, we have three different way of drying the foamed scaffold.

A. Foamed samples were dried at room temperature

B. Foamed samples were dried in oven at 60°C

C. Foamed samples were kept in freeze at - 80°C overnight and then freeze-dried

All samples from these drying methods are shown in figure 3-6. Fig.3-6 (B) shows the scaffold after drying at room temperature. The pore structures and pore size were kept almost the same as the foamed sample. But it took at least two days for the sample to dry completely. We then put a fresh foamed scaffold in a 60°C oven and it was completely dried over night. However, from Fig. 3-6 (A), the pore structure collapsed under the high temperature. Fig.3-6 (C) shows the freeze-dried sample, no pore structure was destroyed.

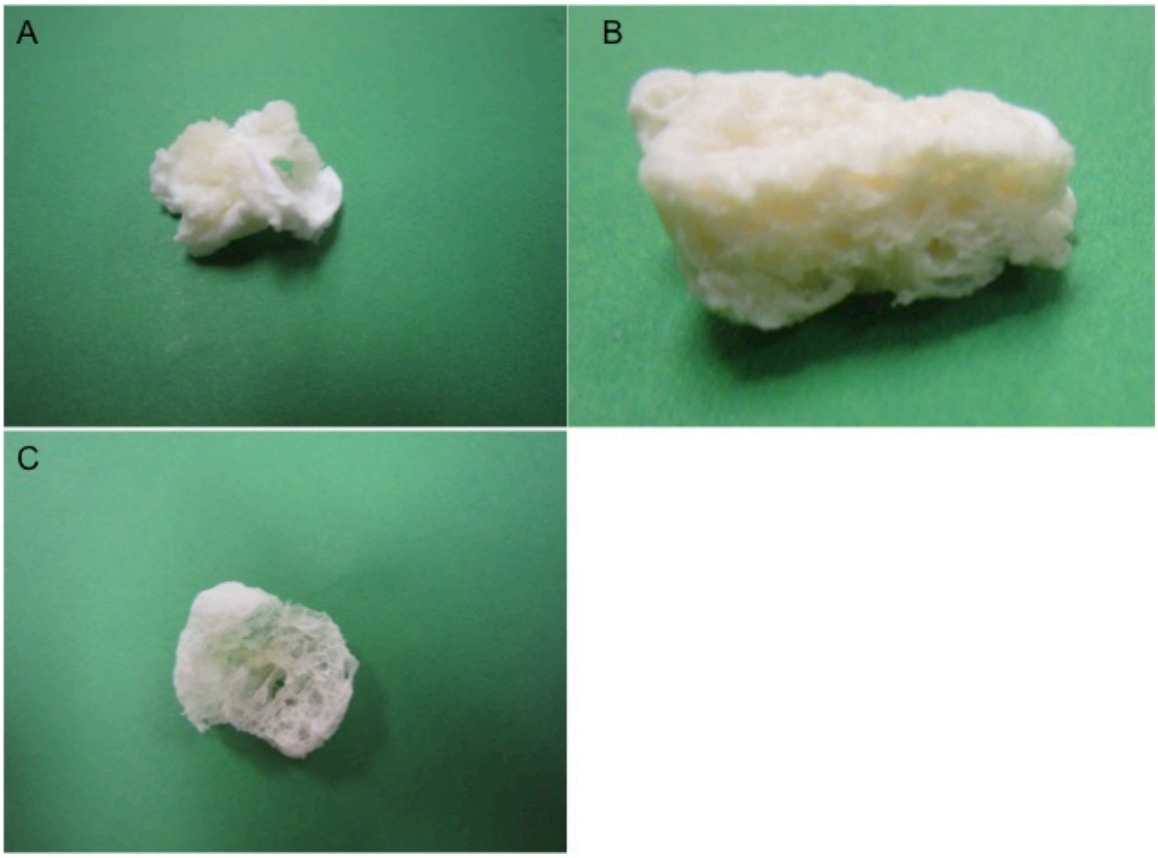

Figure 3-6. SCF foamed scaffold prepared by (A). Oven drying at 60°C (B). Air-drying at room temperature (C). Freeze drying (gelatin: starch: HA, 10: 5: 3, all samples were crosslinked by adding 8 mL crosslinking solution containing 34 mM EDC and 17 mM NHS)

3.6 Degradation study

Degradation rate is very important for bone implants, as the idea degradation rate is to match the regeneration rate of the new tissue. Bone has very strong regeneration ability. It is very important for the implants to maintain their performance at least for one month, so the bone can start curing itself. Both gelatin and starch are hydrophilic materials that may not be suitable for long-term applications in an aqueous environment. In this study, we tried different crosslinking reagents using both water and ethanol as solvents in order to achieve a more stable scaffold. To study the degradation of the crosslinked gelatin/starch/HA scaffold, we prepared a simulated body fluid (SBF) solution with ion concentrations nearly equal to those of human blood plasma and soaked the crosslinked scaffold in the SBF at 37 °C.

Fig. 3-7 shows the result of the crosslinked scaffold after soaking in trisodium citrate for 24 hours. All of the samples were degraded overnight in SBF at 37 °C. The white precipitate were the HA particles. Another way to crosslink the gelatin is adding 8 mL 34 mM EDC and 17 mM NHS crosslink solution into the hydrogel solution, but as shown in Fig. 3-8, all the samples were also completely disappeared after 3 days in SBF. The gelatin scaffold was immersed in DI water containing 34 mM EDC and 17 mM NHS for 24 hours to get a cross-linked scaffold, then a small piece of the cross-linked scaffold was cut and placed in SBF at 37 °C. As illustrated in Fig. 3-9, after soaking in SBF for 7 days, the outer layer (about 1mm thick) of the gelatin scaffold remained intact, whereas the inside of the scaffold was completely degraded. Same result of the gelatin sample that was immersed in 70% ethanol containing 34 mM EDC and 17 mM NHS for 24 hours.

Table 4.1 shows the results of the degradation and foaming.

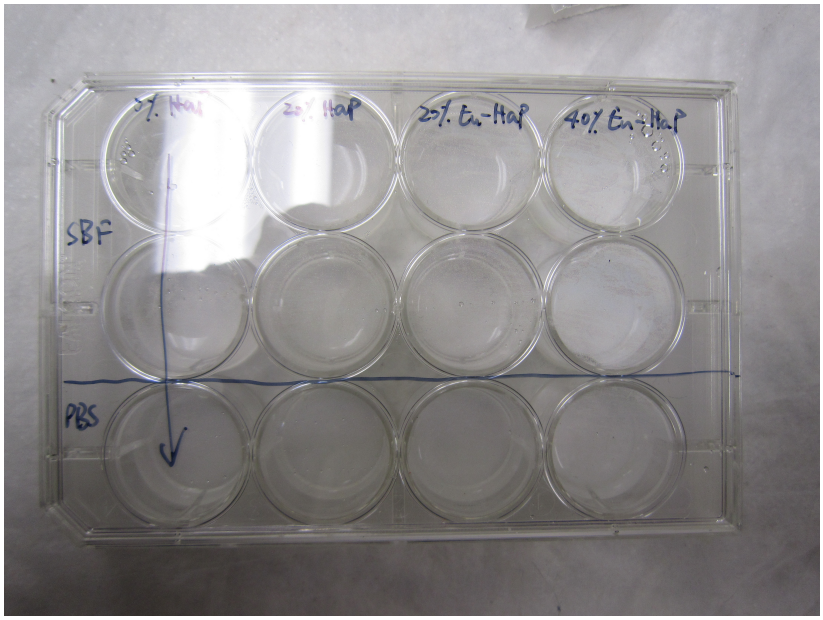

Figure 3-7. Degradation study on trisodium citrate cross-linked gelatin/starch/HA scaffold. All the samples were cross-linked by soaking in 0.5 M trisodium citrate solution for 24 hours.

Figure 3-8 EDC/NHS cross-linked gelatin/starch/HA scaffold after 7 days of immersion in SBF at 37°C. All the samples were cross-linked by soaking in DI water containing 34 mM EDC and 17 mM NHS for 24 hours.

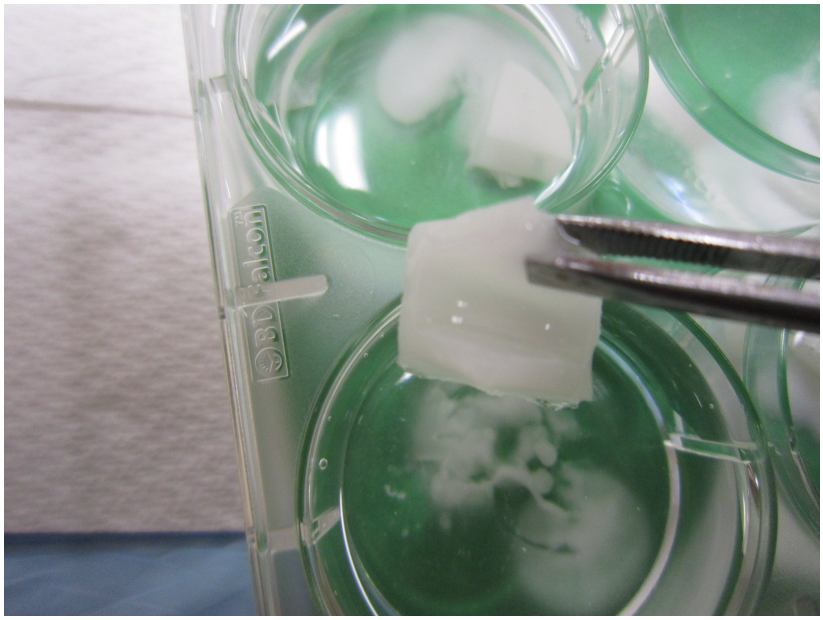

Figure 3-9. EDC/NHS cross-linked gelatin/starch/HA scaffold in SBF for 7 days at 37 °C. All the samples were cross-linked by soaking in DI water containing 34 mM EDC and 17 mM NHS for 24 hours.

Table 4.1. Degradation results

Sample (gelatin: starch: HA, mass ratio)	Crosslinking reagents	SCF foaming results	Degradation results
10: 5: 0	Immersed in 0.5 M trisodium citrate for 24 hours	✔	Completely degraded overnight
10: 5: 3	Immersed in 0.5 M trisodium citrate for 24 hours	✔	Completely degraded overnight
10: 5: 3	Infusion of 8 mL DI water containing 34 mM EDC and 17 mM NHS	✔	24 hours
10: 5: 3	Immersed in DI water containing 34 mM EDC and 17 mM NHS for 24 hours	✗	7 days
10: 5: 3	Immersed in 70% ethanol containing 34 mM EDC and 17 mM NHS for 24 hours	✗	7 days
10: 5: 3	Non-crosslinked	✗	1-2 hours

4. Discussion

We investigated a "green" process to manufacture a 3-D bone scaffold based on a polymer phase consisting of only naturally-derived components (gelatin and cornstarch) and a mineral phase (hydroxyapatite). We avoided the use of any toxic organic solvent in our process by using supercritical CO_2 as the foaming agent.

The hypothesis of manufacturing a high porosity scaffold by supercritical CO_2 gas foaming technique is that the porosity depends on the water content in the unfoamed gel. We know the supercritical fluid can act as a good solvent, but if the materials are too dense it may still be very difficult for supercritical CO_2 molecules to diffuse into the scaffold. The CO_2 molecules take the place of the water molecules when the sample is immersed in supercritical CO_2. If the sample is dry, the scaffold may contain less CO_2 after the supercritical fluid foaming process and end up with low porosity. We have tried to put dry gelatin and starch powder in the supercritical fluid but could not foam that material, as expected because there was little or no water.

4.1 Foaming results

4.1.1 Gelatin vs starch

Foaming results show the pure gelatin could not be foamed in the supercritical CO_2. By adding a small amount (0.5%, w/w) of cornstarch, bubble-like structures appeared in the scaffold after SCF treatment. We attribute this observation to the fact that gelatin is a more rigid hydrogel than cornstarch [68]. So starch may weaken the whole gel and make it easier for CO_2 diffuse into

the gel. During gas foaming by supercritical CO_2 the carbon dioxide molecules most likely diffuse into the scaffolds first and then replace the water molecule. Afterwards, when the pressure is decreased the CO_2 nucleate and develop inside the scaffold. As a result, CO_2 bubbles arose within the scaffold. The porosity of the foamed scaffold depends on the extent to which CO_2 bubbles were able to foam in the hydrogel, which is related to their thermal stability at 35°C. This may explain why starch is a better foaming agent than gelatin.

Moreover, as illustrated in Fig. 3-3, increasing the starch concentration in the scaffold, increased the porosity of the foamed product as well as the thickness of the walls of the pores (the porosity refers to the volume change in this case). That result gives us the possibility to control the porosity and pore size by modulating the starch content of the scaffold.

4.1.2 Hydroxyapatite

In figure 3-4, we can see that increasing HA concentration decreased the pore size. In this case, the crystalline hydroxyapatite particles in the gelatin/starch gel may have stopped or broken the CO_2 bubbles during the foaming process. The 40% sample has smallest pores around 20 μm or less and is much less porous than all the other samples, suggesting that supercritical CO_2 molecule did not diffuse very well in the scaffold due to the dense structure of the gel.
The small pore sizes and the lack of interconnectivity in the scaffold may be a problem for cell adhesion and growth because cell migration may be impacted. The 20: 100 ratio sample has a lot of pores around 50 μm to 100 μm, which are more suitable for cell migration.

4.1.3 Effects of crosslinking

As we mentioned before, gelatin gel needs to be crosslinked for long-term applications. We selected two different crosslinking reagents, trisodium citrate and EDC/NHS to crosslink gelatin and study the effects on the foaming results.

We observed that the non-crosslinked samples could not be foamed to a great extent. (Figure 3-5 E). We could see the volume of the gel was decreased after foaming and there was no pore structure in the scaffold. This could be because when the CO_2 bubbles started to grow, the non-crosslinked gelatin did not have enough strength to maintain the bubble structure and resulted in a low volume. The non-crosslinked gelatin is probably less resistant to temperature changes, because nearly half of the samples melted when temperature increased to 35°C during the supercritical fluids foaming process.

Comparing Fig. 3-5(A) and Fig. 3-5(B), the volume of EDC/NHS crosslinked gelatin became much greater after foaming than trisodium citrate crosslinked gelatin as we also observed greater porosity in the EDC/NHS samples. EDC/NHS is more efficient than trisodium citrate in crosslinking gelatin due to its ability to covalently bond. So, the EDC/NHS crosslinked gel is stiffer than trisodium citrate crosslinked gelatin. To achieve a better foaming scaffold, materials should have sufficient strength to withstand the CO_2 gas foaming and not collapse during the supercritical treatment.

Also, we studied the difference of infusion of EDC/NHS and immersion of EDC/NHS crosslinking reagents. The samples, which were immersed in EDC/NHS solution for 24 hours to get crosslinked in Fig. 3-5(C) and Fig. 3-5(D), were unable to foam well in the SCF technique. On the other hand, the samples that were crosslinked by adding EDC/NHS into the gel were foamed much better. Because the gel was immersed statically in the crosslinking solution, the surface of the gel was much more easily crosslinked than the interior, which means the sample

had a harder surface. This suggests that the materials should not be too stiff, otherwise, the pressure generated during supercritical fluid foaming may not be enough to form bubble structures.

4.2 Degradation results

Samples that were immersed in 34 mM EDC and 17 mM NHS crosslink solution before foaming disintegrated the most slowly in SBF. We found that it is more efficient to crosslink gelatin by immersing the gelatin in the EDC/NHS solution after the hydrogel is formed than dispersing the dry gelatin powder in crosslinking solution. But immersing the sample in crosslinking solution can only get a thin outer layer of the gel to be well crosslinked, as can be seen in resulting gel after 7 days in SBF in Fig. 3-8.

5. Conclusions

We investigated a feasible green method of fabricating a 3-D porous scaffold for bone tissue engineering using supercritical CO_2 as the foaming agent. Two natural polymers, gelatin and cornstarch were used to mimic the organic component of bone, cornstarch was used to prove foaming of SCF and synthetic HA was used to mimic the inorganic component. Trisodium citrate and EDC/NHS were used as the crosslinking reagents of gelatin in order to get controllable degradation kinetics.

In this method, we successfully manufactured a 3-D bone scaffold with high porosity and pore size that may be suitable for cell attachment. The gelatin/starch/HA was immersed in supercritical CO_2 for 20 minutes and the CO_2 was vented for 10 minutes to get a foamed gel. We set the pressure of the reaction at 2500 psi and the temperature at 35°C which is in the supercritical CO_2 region, and also 35°C is a very suitable temperature to incorporate drugs and other biomolecules while maintaining their activity. We avoided using toxic organic solvents, which is a big advantage in bioapplications.

Cornstarch can improve supercritical CO_2 foaming and HA can decrease the pore size. Changing the composition can control the porosity and the pore size. Degradation of the unfoamed samples cross-linked by immersion method using EDC/NHS crosslinking reagents lasted longest for about a week, but the samples could not be foamed. The trisodium citrate cross-linked sample can be foamed, but it can only last for 24 hours at 37°C in SBF. The samples cross-linked by infusion method using EDC/NHS cross-linking reagent can be foamed and the unfoamed sample can last for 3 days in SBF. Degradation rate of the foamed scaffolds still needs to be studied.

6. Future Work

In the future, we hope to obtain a more stable polymeric scaffold that would withstand the dynamic in vivo microenvironment. This may require crosslinking of the starch prior to foaming. More quantitative data are needed to support the conclusions. Also, other natural polymers, like cellulose or chitosan, can be an alternative component that may aid in improving the mechanical properties of the scaffold.

References:

[1] D. Puppi, F. Chiellini, A.M. Piras, E. Chiellini. Polymeric materials for bone and cartilage repair. Progress in Polymer Science 35 (2010) 403–440

[2] Bin Duan, Min Wang, Wen You Zhou, Wai Lam Cheung, Zhao Yang Li, William W. Lu Three-dimensional nanocomposite scaffolds fabricated via selective laser sintering for bone tissue engineering. Acta Biomaterialia 6 (2010) 4495–4505

[3] U.S. Markets Orthopedic Implant, Reconstruction, and Trauma Products. In LSI website (http://www.lifescienceintelligence.com/market-reports-page.php?id=A311).

[4] Peter R. McNally. Metabolic Bone Disease and the Gastroenterologist. VHJOE, VOLUME 8, ISSUE 3, YEAR 2009

[5] E. Sachlos and J.T. Czernuszka. Making tissue engineering scaffolds work. Review on the application of solid freeform fabrication technology to the production of tissue engineering scaffolds. European Cells and Materials Vol. 5. 2003 (pages 29-40)

[6] K. Rezwan, Q.Z. Chen, J.J. Blaker, Aldo Roberto Boccaccini. Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials 27 (2006) 3413–3431

[7] Ishaug-Riley SL, Crane GM, Gurlek A, Miller MJ, Yasko AW, Yaszemski MJ, Mikos AG (1997): Ectopic bone formation by marrow stromal osteoblast transplantation using poly(DL-lactic-co-glycolic acid) foams implanted into the rat mesentery. J Biomed Mater Res 36: 1- 8

[8] Langer R, Vacanti JP (1993) Tissue engineering.

[9] Antonio Gloria, Roberto De Santis, Luigi Ambrosio. Polymer-based composite scaffolds for tissue engineering. Journal of Applied Biomaterials & Biomechanics 2010; Vol. 8 no. 2, pp. 57-67

[10] Martin Chin, Distraction Osteogenesis in Maxillofacial Surgery Using Internal Devices: Review of Five Cases J Oral Maxillofac Surg 54:45-53, 1996

[11] Steven C. Ludwig Joseph M. Kowalski Scott D. Boden. Osteoinductive bone graft substitutes. Eur Spine J (2000) 9 (Suppl 1):S119–S125

[12] Joshua R. Porter, Timothy T. Ruckh, and Ketul C. Popat Bone Tissue Engineering: A Review in Bone Biomimetics and Drug Delivery Strategies. 2009

[13] Yihong Gong, Qingliang Zhou, Changyou Gao, Jiacong Shen. In vitro and in vivo degradability and cytocompatibility of poly(L-lactic acid) scaffold fabricated by a gelatin particle

leaching method. Acta Biomaterialia 3 (2007) 531–540

[14] Chiming Yang, Hanspeter Frei, Fabio M. Rossi and Helen M. Burt. The differential in vitro and in vivo responses of bone marrow stromal cells on novel porous gelatin alginate scaffolds. J Tissue Eng Regen Med 2009; 3: 601 – 614.

[15] Seheangoh. Degradation behavior of hydrophilized PLGA scaffolds prepared by melt-molding particulate-leaching method: Comparison with control hydrophobic one. J of materials science: materials in medicine 17 (2006) 131-137.

[16] Vítor. Santo, Ana Rita. Duarte, Manuela. Gomes, João. Mano, Rui. Reis. Hybrid 3-D strucuture of poly(D,L-lactic acid) loaded with chitosan/chondroitin sulfate nanoparticles to be used as carries for biomacromolecules in tissue engineering. J. of Supercritical Fluids 54 (2010) 320–327

[17] S. S. Liao, F. Z. Cui, W. Zhang, Q. L. Feng. Hierarchically Biomimetic Bone Scaffold Materials: Nano-HA/Collagen/PLA Composite.

[18] Amy J. Wagoner Johnson, Brad A. Herschler. A review of the mechanical behavior of CaP and CaP/polymer composites for applications in bone replacement and repair. Acta Biomaterialia 7 (2011) 16–30

[19] Ayako Oyane, Hyun-Min Kim, Takuo Furuya, Tadashi Kokubo, Toshiki Miyazaki, Takashi Nakamura. Preparation and assessment of revised simulated body fluids. J Biomed Mater Res 65A: 188 –195, 2003

[20] Sylvia Nuernberger, Norbert Cyran, Christian Albrecht, Heinz Redl, Vilmos Vécsei, Stefan Marlovits. The influence of scaffold architecture on chondrocyte distribution and behavior in matrix-associated chondrocyte transplantation grafts. Biomaterials 32 (2011) 10321040

[21] Qi Xing, Feng Zhao, Si Chen, James McNamara, Mark A. DeCoster, Yuri M. Lvov. Porous biocompatible three-dimensional scaffolds of cellulose microfiber/gelatin composites for cell culture. Acta Biomaterialia 6 (2010) 2132–2139

[22] Guobao Weia, Peter X. Maa,b,c, Structure and properties of nanohydroxyapatite/polymer composite scaffolds for bone tissue engineering. Biomaterials 25 (2004) 4749–4757

[23] Jaya Sundaram, Timothy D. Durance, Rizhi Wang. Porous scaffold of gelatin–starch with nanohydroxyapatite composite processed via novel microwave vacuum drying. Acta Biomaterialia 4 (2008) 932–942

[24] J.M. Taboas, R.D. Maddox, P.H. Krebsbach, S.J. Hollisterc. Indirect solid free form fabrication of local and global porous, biomimetic and composite 3-D polymer-ceramic scaffolds. Biomaterials 24 (2003) 181–194

[25] Hongyun Tai. Control of pore size and structure of tissue engineering scaffolds produced by supercritical fluid processing. European Cells and Materials Vol. 14. 2007 (pages 64-77)

[26] Ana Rita C. Duarte, Teresa Casimiro, Ana Aguiar-Ricardo. Supercritical fluid polymerisation and impregnation of molecularly imprinted polymers for drug delivery. J. of Supercritical Fluids (2006)

[27] Qingliang Zhou, Yihong Gong, Changyou Gao. Microstructure and Mechanical Properties of Poly(L-lactide) Scaffolds Fabricated by Gelatin Particle Leaching Method

[28] DongChoon Sin, Xigeng Miao, Gang Liu, Fan Wei, Gary Chadwick, Cheng Yan, Thor Friis. Polyurethane (PU) scaffolds prepared by solvent casting/particulate leaching (SCPL) combined with centrifugation. Materials Science and Engineering C 30 (2010) 78–85

[29] Xiaohua Liu a, Peter X. Ma. The nanofibrous architecture of poly(L-lactic acid)-based functional copolymers. Biomaterials 31 (2010) 259–269

[30] D. J. Mooney. Mazzoni. Breued, K. McNamara, D. Hem, J.P. Vacanti and R. Langer. Stabilized polyglycolic acid fibre- based tubes for tissue engineering. Eiomateriak 17 (1996) 115-124

[31] Ruiyun Zhang1, Peter X. Ma. Poly(α-hydroxyl acids)/hydroxyapatite porous composites for bone-tissue engineering. I. Preparation and morphology.

[32] Ahmed A. Haroun, Amira Gamal-Eldeen, David R. K. Harding. Preparation, characterization and in vitro biological study of biomimetic three-dimensional gelatin–montmorillonite/ cellulose scaffold for tissue engineering. J Mater Sci: Mater Med (2009) 20:2527–2540

[33] Alexandra Sloviková, Lucy Vo jtová, Josef Jančař. Preparation and modification of collagen-based porous scffold for tissue engineering. Chemical Papers 62 (4) 417–422 (2008)

[34] James D. Kretlow. From Material to Tissue: Biomaterial Development, Scaffold Fabrication, and Tissue Engineering. Materials interfaces and electrochemical phenomena 2007.

[35] Peter X Ma. Biomimetic Materials for Tissue Engineering. Adv Drug Deliv Rev. 2008 January 14; 60(2): 184–198

[36] Chiming Yang, Hanspeter Frei, Fabio M. Rossi and Helen M. Burt. The differential in vitro and in vivo responses of bone marrow stromal cells on novel porous gelatin – alginate scaffolds . J Tissue Eng Regen Med 2009; 3: 601 – 614.

[37] Hermann Seitz, Wolfgang Rieder, Stephan Irsen, Barbara Leukers, Carsten Tille. Three-Dimensional Printing of Porous Ceramic Scaffolds for Bone Tissue Engineering . Wiley InterScience2005.

[38] Ana Rita C. Duarte, João F. Mano, Rui L. Reisa. Preparation of starch-based scaffolds for tissue engineering by supercritical immersion precipitation. J. of Supercritical Fluids 49 (2009) 279–285

[39] Ana Rita C. Duarte, João F. Mano, Rui L. Reis. Enzymatic degradation of 3-D scaffolds of starch-poly-(3-caprolactone) prepared by supercritical fluid technology. Polymer Degradation and Stability 95 (2010)

[40] VítorE. Santo, AnaRitaC. Duarte, ManuelaE. Gomes, JoãoF. Mano, RuiL. Reisa. Hybrid 3-D structure of poly(d,l-lacticacid) loaded with chitosan/chondroitin sulfate nanoparticles to be used as carriers for biomacromolecules in tissue engineering. J. of Supercritical Fluids 54 (2010) 320–327

[41] Ana Rita C. Duarte, Sofia G. Caridade, João F. Mano, Rui L. Reis. Processing of novel bioactive polymeric matrixes for tissue engineering using supercritical fluid technology. Materials Science and Engineering C 29 (2009) 2110–2115

[42] Robin A. Quirk, Richard M. France , Kevin M. Shakesheff, Steven M. Howdle. Supercritical fluid technologies and tissue engineering scaffolds. Current Opinion in Solid State and Materials Science 8 (2004) 313–321

[43] Tadashi Kokubo, Hiroaki Takadama. How useful is SBF in predicting in vivo bone bioactivity? Biomaterials 27 (2006) 2907–2915

[44] Thomas Freier. Biopolyesters in Tissue Engineering Applications. Adv Polym Sci (2006) 203: 1-61

[45] Somayeh Hallaj Nezhadi, Peter F.M. Choong, Farzaneh Lotfipour, and Crispin R. Dass. Gelatin-based delivery systems for cancer gene therapy. Journal of Drug Targeting, 2009; 17(10): 731–738

[46] AvneshKumari, SudeshKumarYadav, SubhashC. Yadav.Biodegradable polymeric nanoparticles based drug delivery systems. Colloids and Surfaces B:Biointerfaces 75 (2010) 1–18

[47] Kezban Ulubayram, A. Nur Cakar, Petek Korkusuz, Cemile Ertan, Nesrin Hasirci. EGF containing gelatin-based wound dressings. Biomaterials 22 (2001)

[48] Roman A. Perez, Sergio Del Valle, George Altankov, Maria-Pau Ginebra. Porous hydroxyapatite and gelatin/hydroxyapatite microspheres obtained by calcium phosphate cement emulsion. J Biomed Mater Res Part B: Appl Biomater 97B: 156–166, 2011.

[49] Hae-Won Kim, Hyoun-Ee Kim, Vehid Salih. Stimulation of osteoblast responses to biomimetic nanocomposites of gelatin hydroxyapatite for tissue engineering scaffolds. Biomaterials 26 (2005) 5221–5230

[50] M. Tarik Arafat , Christopher X.F. Lam , Andrew K. Ekaputra, Siew Yee Wong , Xu Li, Ian Gibson. Biomimetic composite coating on rapid prototyped scaffolds for bone . tissue engineering . Acta Biomaterialia 7 (2011) 809–820

[51] Seung-Hwan Jegal , Jeong-Hui Park , Joong-Hyun Kim , Tae-Hyun Kim , Ueon Sang Shin , Tae-Il Kim d, Hae-Won Kim. Functional composite nanofibers of poly(lactide–co-caprolactone) containing gelatin–apatite bone mimetic precipitate for bone regeneration. Acta Biomaterialia 7 (2011) 1609–1617

[52] Jung Bok Lee, Sung Eun Kim, Dong Nyoung Heo, and Il Keun Kwon. In vitro Characterization of Nanofibrous PLGA/Gelatin/Hydroxyapatite Composite for Bone Tissue Engineering. Macromolecular Research, Vol. 18, No. 12 (2010)

[53] Takeshi Kikuchi, M.D. Promotion of Bone Regeneration by CCN2 Incorporated into Gelatin Hydrogel. Tissue engineering: Part A Volume 14, Number 6, 2008

[54] M.M Taylor. Genipin-Modified Gelatin: Preparation, Characterization, and Application as a filler for leather.

[55] Shantha Lakshmi Kosaraju, Amirtha Puvanenthiran, Peter Lillford. Naturally crosslinked gelatin gels with modified material properties . Food Research International 43 (2010) 2385–2389

[56] Ju-Ying Chang, Jia-Horng Lin, Chun-Hsu Yao, Jiunn-Horng Chen, Tung-Yuan Lai, Yueh-Sheng Chen. In Vivo Evaluation of a Biodegradable EDC/ NHS-Cross-Linked Gelatin Peripheral Nerve Guide Conduit Material Macromol. Biosci. 2007, 7, 500–507

[57] Zenon Grabarek and John Gergely. Zero-Length Crosslinking Procedure with the Use of Active Esters analytical biochemistry(1990)

[58] A.J. Salgado, M.E. Gomes, A. Chou, O.P. Coutinho, R.L. Reis, D.W. Hutmacher. Preliminary study on the adhesion and proliferation of human osteoblasts on starch-based scaffolds. Materials Science and Engineering C 20 (2002)

[59] A. J. Salgado, O. P. Coutinho, R. L. Reis, J. E. Davies. In vivo response to starch-based scaffolds designed for bone tissue engineering applications. Wiley Inter Science 2006

[60] M.E. Gomes, J.S. Godinho, D. Tchalamov, A.M. Cunha, R.L. Reis. Alternative tissue engineering scaffolds based on starch: processing methodologies, morphology, degradation and mechanical properties. Materials Science and Engineering C 20 (2002)

[61] A.P. MarquesT, R.L. Reis. Hydroxyapatite reinforcement of different starch-based polymers affects osteoblast-like cells adhesion/spreading and proliferation . Materials Science and Engineering C 25 (2005) 215 – 229

[62] S. V. Dorozhkin. Bioceramics based on calcium orthophosphates (review). Glass and

Ceramics Vol. 64, Nos. 11 – 12, 2007

[63] A S Brydone, D Meek, and S Maclaine. Bone grafting, orthopaedic biomaterials, and the clinical need for bone engineering. J. Engineering in Medicine 2010

[64] M. Bohner. Calcium orthophosphates in medicine: from ceramics to calcium phosphate cements. J. Care Injured 3 (2000) S-D3747

[65] Pradip Munshi and Sumit Bhaduri, Supercritical CO2: a twenty-first century solvent for the chemical industry. Current Science, vol. 97, No. 1, 10 July 2009

[66] Ramsey Edward, Sun Qiubai, Zhang Zhiqiang, Zhang Chongmin, Gou Wei Green sustainable processes using supercritical fluid carbon dioxide. Journal of Environmental Sciences 21(2009) 720–726

[67] S. S. Liao, F. Z. Cui, W. Zhang, Q. L. Feng. Hierarchically Biomimetic Bone Scaffold Materials: Nano-HA/Collagen/PLA Composite.

[68] P. P. Lewicki, G. C. Busk and T. P. Labuza. Measurement of gel water-binding capacity of gelatin, potato starch, and carrageenan gels by suction pressure. Journal of Colloid and Interface Science Volume 64, Issue 3, May 1978