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Hybrid Nanostructures for Bone Tissue Engineering

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Ruipeng Xue

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Ruipeng Xue

We, the thesis committee for the above candidate for the

Master of Science degree, hereby recommend

acceptance of this thesis.

Dr. Pelagia-Irene (Perena) Gouma, Thesis Advisor, Associate Professor
Department of Materials Science and Engineering

Dr. Tadanori Koga, Assistant Professor
Department of Materials Science and Engineering

Dr. Milutin Stanacevic, Assistant Professor
Department of Electrical and Computer Engineering

This thesis is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Thesis

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Natural bone is composed of natural polymers, collagen fibers and nano-crystals of minerals, mainly nano-hydroxyapatite (HA). Bone cells, which maintain the activities and metabolism of bone, are supported by and interact with this organic-inorganic hybrid matrix. Artificial bone tissue scaffolds mimicking the natural bone's extracellular matrix based on synthetic hybrid cellulose acetate (CA)-hydroxyapatite nano-composites were fabricated in this work in 3D matrix architecture for bone cell regeneration, using a single step nano-manufacturing technique. Cultured human osteoblasts were seeded on CA and CA-HA scaffolds, after which cell proliferative capacity and viability were studied using complementary assays. The interactions between the cells and the scaffolds were further characterized by scanning electron microscopy (SEM). Osteoblasts grown on these scaffolds appear to interact strongly with nano-HA clusters, resulting in cell growth and phenotype retention. The hybrids scaffolds used are shown to be ideal bone repair agents.

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CHAPTER 1: INTRODUCTION

1.1 Basics of bones and tissue engineering

1.1.1 Introduction to composites and nanotechnology

The advancement of nanotechnology has resulted in novel materials which are a combination of metals, ceramics, polymers and/or biomolecules and these hybrids exhibit some unique properties than their pure counterparts. This phenomenon is also found in nature. A well known example of a hybrid/composite material in nature is the bone, which consists of both crystalline mineral salts and collagen. For this kind of composite materials, inorganic compounds usually play the role of building blocks in the basic structure, providing strength for the whole configuration. Meanwhile, the organic parts bond with the inorganic materials to form the whole structure of the mixture. This "mixture" is called hybrid in materials science which is defined as "a material that includes two moieties blended on the molecular scale. Commonly one of these compounds is inorganic and the other one organic in nature" [1].

Composites are defined as those having a distinct phase distributed through their bulk, as opposed to modular or coated components [2]. Generally, a matrix and a dispersed phase are the two major categories of constituent materials. The continuous phase is responsible for supporting the dispersed parts by maintaining their relative positions. The dispersed phases are usually used for enhancing one or more properties for the composites than the single pure material. Most of the composites target an enhancement of mechanical properties of the matrix, transport properties, radiocapacity, density, or biocompatibility [3]. By adjusting the composition and arrangement of the

dispersed phase, the properties of the composites can be tailored for various applications.

Nanotechnology is the engineering of nanomaterials and miniaturized devices based on nanoscience, which is the study of materials systems and phenomena in the scale 1-100nm when these materials and devices exhibit size dependent properties [4]. As the medical application of nanotechnology, nanomedicine deals with the repair, construction and control of human biological systems using devices developed by nanotechnology. It is a very important research direction in nanomedicine to understand the growth, proliferation and differentiation mechanisms of living cells, and to develop advanced technologies and devices for the early diagnosis and treatment of various diseases [4].

1.1.2 Basics of natural bones and bone repair

Bone is composed of minerals, collagen, water, non-collagenous proteins, lipids, vascular elements and cells [5]. Although the inorganic part hydroxyapatite (HA) and the organic phase collagen are the major constituents of natural bones (taking up to 80% weight), the most important parts of bone are the cells maintaining the activities and metabolism of bone. Osteoprogenitor cells, osteoblasts, osteocytes, osteoclasts, and bone-lining cells are common types of cells with different functions respectively [6]. Osteoprogenitor cell, which is also called bone-precursor cell, is a mesenchymal cell and it can differentiate into an osteoblast. Osteoblasts can form new bone by generating collagen and non-collagenous proteins which have the active sites for bonding the minerals and the new bone is maintained by osteocytes which are the mature cells derived from osteoblasts and could transport agents of minerals between bone and blood [7]. Bone is constantly resorbed by the osteoclasts back to the blood stream and then replaced

by the osteoblasts in bone remodeling [8].

In nano-structure scale, bone is composed of collagen fibers and nano-crystals of minerals, mainly hydroxyapatite with dimensions about 4nm×50nm×50nm. HA crystals provide toughness and rigidity to the bone structure and on the other hand collagen, whose diameter varies from 100 to 2000 nm, acts as a structural framework giving the bone tensile strength and flexibility [9]. Through non-collagenous proteins, the minerals are bound to collagen [5]. Combination of HA, collagen and other constituents provides the bone excellent mechanic properties to support the body and makes the bone have the storage place of minerals.

It was estimated in a statistic study that about 6.3 million fractures occur every year in the United States, of which about 550,000 cases require some kind of bone grafting [10]. Autografting (tissue is transplanted from one site to another site in the same individual) and allografting (transplantation is between the patient and the donor) are common and traditional clinical treatments for these bone defects even though with many disadvantages, such as lacking of availability, complicated surgical reconstructions, possibilities of rejection and long recovery time [11]. These limitations call for a large amount of synthetic bone graft materials to replace the defect sites and help the body to recover. Table 1 lists the common grafting biomaterials which are covered by all materials categories: metals, ceramics, polymers and their composites [12, 13].

Table 1 Biomaterials for bone grafting [12, 13]

Biomaterials	**Advantages**	**Disadvantages**	**Applications**	**Examples**
Metal and alloy	Too strong, tough, ductile	Dense, may corrode	Bone plates, load-bearing bone implants, dental arch wire,	Titanium, stainless steel, Co–Cr alloys, Ti alloys
Ceramic	Bioinert, bioactive, bioresorbable, high resistance to wear	Brittle, poor tensile, low toughness, lack of resilience	Hip joints and load-bearing bone implants, bone filler, coatings on bio-implants, maxillofacial reconstruction	Alumina, zirconia, HA, bioglass,
Polymer	Flexible, resilient, surface modifiable, selection of chemical functional groups	Not strong, toxic of a few degraded products	Bone screws, pins, bone plates, bone and dental filler, bone drug delivery	Collagen, gelatin, chitosan, alginate, PLA
Composite	Strong, design flexibility, enhanced mechanical reliability than monolithic	Properties might be varied with respect to fabrication methodology	Bone graft substitutes, middle ear implants, guided bone regenerative membranes, bone drug delivery	HA/collagen, HA/gelatin, HA/chitosan, HA/alginate, HA/PLGA, HA/PLLA,
Nano-composite	Larger surface area, high surface reactivity, relatively strong interfacial-bonding, design flexibility, enhanced mechanical reliability	No optimized technique for material processing	Major areas of orthopedics,drug delivery	Nano-HA/collagen, Nano-HA/gelatin, Nano-HA/chitosan, Nano-HA/PLLA

Although these biomaterials show some good performance as artificial bones, most of these materials lack biological functions and the ability to stimulate the forming

of new bone structure. The most important aspect during this replacement procedure is that only live bone cells can ultimately generate new bone tissue and truly rebuild the bone, so the cell based method is considered promising. This revolutionary strategy, tissue engineering, focuses on promoting new tissue cell generations by bioactive materials and the cells finally lead to a new bone structure formation. One definition by Langer [14] is that tissue engineering "involves the application of the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function". The procedures of tissue engineering are that the cells are isolated from the donor, cultured on a scaffold in vitro, and transplanted with the scaffold into the defective site to guide new tissue formation into the scaffold, which would be biodegraded over time [15]. Therefore, a suitable temporary tissue scaffold and the interaction of the cells with the scaffolds are critical in tissue engineering.

1.1.3 State of the art in bone tissue engineering

The essential properties of an ideal bone tissue scaffold are porosity, biocompatibility, surface properties, osteoinductivity, mechanical properties and biodegradability [16]. Researches of new materials and fabrication methods of the scaffolds are basically focusing on improving these properties.

1.1.3.1 Materials for bone tissue engineering scaffolds

Nature derived polymers have good biodegradability and biological recognition which will be suitable for cell functions. Chitosan is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine linked in a β (1–4) manner [17]. Chitosan has been shown to promote cell growth and mineral rich matrix deposition by the culturing of osteoblasts [18]. Collagen is a fibrous protein and is a main component of extracellular

matrix of bone tissue. So it is reasonable to be used in a scaffold. It is found that *type I* collagen as the matrix of bone marrow stromal cells could make the cells differentiate into osteoblasts in *vivo* [19]. The combination of collagen and chitosan as scaffold is also reported to enhance attachment of seeded cells [20]. Also, a *collagen I* matrix with mechanical load could promote the proliferation and differentiation of osteoblastic precursor cells and enhance the quality and quantity of the generated tissue [21]. Fibrin could promote cell attachment and proliferation and may provide a more positive environment for bone regeneration [22]. Although natural polymers have been found useful as bone scaffolds, several limitations should be noted. They may exhibit immunogenicity, may contain pathogenic impurities, have limit in supply and have difficulty to control batch-to-batch consistency [23].

Compared with natural polymers, Synthetic polymers have larger scale of production and better controlled properties of strength, degradation rate, impurities and microstructures. Saturated poly-α-hydroxy esters including poly (lactic acid) (PLA), poly (glycolic acid) (PGA) and poly (lactic-*co*-glycolide) (PLGA) copolymers are the most widely used synthetic polymers for the scaffolds [23]. Food and Drug Administration (FDA) has approved the use of these polymers as degradable materials for human use for a long time. These polymers are known to degrade by non-enzymatic hydrolysis and the degradation products could be removed from the body in the form of carbon dioxide (CO_2). By changing compositions, crystallinity and molecular weight, the degradation rate of these polymers can be controlled. However, they may present problems regarding biocompatibility in the adjacent area of the defect site [16]. Polyhydoxyalkanoates (PHA) are also aliphatic polyesters and have application in medical devices and tissue

engineering, such as bone marrow scaffolds and wound dressings, due to their good biodegradable and thermo properties [24]. The blending of PHA polymers with each other or other components (polymers, ceramics or enzymes) is a common strategy to improve the mechanical strength and bio-properties [25]. However, the complicated extraction procedure of PHA from bacteria is a major problem for its further applications [26]. Polypropylene fumarate (PPF) is an unsaturated linear polyester. Its main degradation products are fumaric acid and propylene glycol which can be easily removed from human body [27]. PPF has shown acceptable biological results and has been recommended for being used as matrix for guided tissue regeneration [28] and bone cell cultures [29].

Ceramics have also been broadly used in bone substitution and regeneration. Since hydroxyapatite is the major inorganic component in natural bone and it has excellent biocompatibility, HA and other related calcium phosphates have been experienced extensive studies [30]. Calcium phosphates have osteoconductive properties and could bind to bone under some circumstances [31]. Several forms of calcium phosphates (bulk, coating, powder and porous) have been investigated and they all have shown supports to cell attachment, proliferation and differentiation, in both crystalline and amorphous phases [32]. As the best candidate among calcium phosphates, HA is usually obtained by sintering of ceramic powders at high temperature. One problem of pure HA is its low degradation rate in *vivo*. For crystalline HA, the degradation rate is related to the degree of porosity, defect structure, and the type of other phases. The dissolution rate for calcium phosphates is in the following order [32]:

AmorphousHA> α-TCP> β-TCP> crystalline HA

Stainless steel and titanium or titanium alloys are the common metals involved in implants for bone regeneration. For example, the titanium fiber meshes have been used for the culture of rat bone marrow cells under certain conditions [33]. Although metals have the advantages of excellent mechanical properties, they tend to be less adhesive to the cells [34] and they may bring the problem of toxicity because of the metal ions accumulating in the body [35].

As discussed above, various types of materials have been applied in bone tissue engineering and show some good properties. However, each material has its own good characteristic advantages and a single material cannot fulfill all the requirements as a perfect scaffold. On the other hand, composite materials often show the balance of their components just like the natural bone and therefore become promising to be a bone scaffold. The polymer constituent could provide the composite toughness and the inorganic counterpart contributes the compressive strength to the system. The combination of the inorganic and organic could make the composites have enhanced mechanical and biological properties to mimic the natural human bone. Furthermore, nano-sized component has been recognized having improved bio-activities than its micro-sized counterpart. Some typical combinations of materials in bone tissue engineering and their applications are summarized in Table 2 [36].

Table 2 Composite scaffolds for bone engineering [36]

Composite	**Fabrication technique**	**Pore size (μm)**	**Applications**
Hydroxyapatite/ poly(ε-caprolactone)	Sintering	150–200	
Hydroxyapatite/ chitosan-gelatin	Freeze-drying	300–500	Rat calvarial osteoblasts in vitro
Hydroxyapatite/β-tricalcium phosphate/chitosan	Sintering	300–600	
Collagen/hydroxyapatite	Freeze-drying	30–100	Rabbit periosteal cells in vitro
	Freeze-drying	50–300	MC3T3-E1 osteoblasts in vitro
Titanium/calcium phosphate	Sintering	50–200 (surface coating)	Femoral defects in rabbits
	Sintering	250 (porous meshes)	Ectopic bone formation in rats
	Soaking		Human osteoblasts in vitro
Titanium/polyvinyl alcohol	Sintering	170	Femoral condyles in dogs
Titanium/boron	Self-propagating high temperature synthesis		Cranial defects in rats
Poly(l-lactide-*co*-d,l lactide)/β-tricalcium phosphate	Salt-leaching	125–150	Cranial defects in rabbits
Poly(propylene fumarate)/ β-tricalcium phosphate	Salt-leaching	150–300	
Poly(l-lactide)/bioglass	Phase separation	50–200	
Silica/ceramic	Sintering	10–300	Femoral defects in rabbits
Poly(lactide-*co* glycolide)/collagen/apatite	Salt-leaching	355–425	

1.1.3.2 Fabrication techniques for scaffolds

To fulfill all the requirements for an ideal bone tissue scaffold, several important aspects should be considered. The scaffold must have enough porosity to accommodate the cells and support the cells to proliferate and differentiate. High interconnectivities between pores are also key character for cell distribution and nutrients transfer. The scaffold should have enough mechanical strength and suitable degradation rate.

Solvent casting/particulate leaching which was first described by Mikos [19] is possibly most commonly used technique for the preparation of bone tissue engineering scaffolds. This method dissolves mineral or organic particles in a polymer solution. The dispersion is then cast into the mold of a certain shape. Then the solvent is evaporated or lyophilizated and the particles will be leached out to form the porous structure. The porosity and intersection depend on the composition of the solution and the particle size and property. More than 90% porosity scaffolds can be created by this method [37]. The method is easy to operate but has some drawbacks like the limited cubic shape of the pores and limited interpore connectivity. These may cause the cell growth and mineralization limited to the outside of the scaffolds, due to no adequate internal nutrient and oxygen transport [38].

Melt based technologies such as melt moulding/particulate leaching is also a way to produce bone scaffolds. The raw polymer is beforehand mixed with a porogen and put into a mold. The mixture is heated above the glass transition temperature of the chosen polymer and the polymer-porogen composite is immersed in a solvent to dissolve the porogen [39]. All kinds of shapes of 3D structured scaffolds can be made by simply changing the mold shape [40].

Freeze drying process uses thermal phase separation. The temperature drop will

cause a phase separation of a homogeneous polymer solution. Then the solvent-rich phase is eliminated by vacuum sublimation and the “dried” polymeric foam forms. This method has been employed to produce scaffolds from both natural and synthetic origin [41]. Low mechanical stability and sensitivity of the technique are the limits of this method [42].

3D rapid-prototyping techniques are based on computer-aided design (CAD) model. 3D printing is first introduced for tissue engineering [43]. A printer head prints a liquid binder onto thin layers of powder and the structure will be built consequently layer by layer based on the instructions of CAD file [44]. The parameters of the process can be accurately controlled by the computer and other agent like biological components can also be incorporated. However, the porosity of the scaffolds is low and the week mechanical properties could also be a problem.

Electrospinning technique is a novel fabrication process using an electronic field and forming the resulted polymer fibers on a substrate [45]. Electrospinning has the ability to produce polymer fibers with diameters from several micros to hundreds of nanometers. Electrospnning will be further discussed next.

CHAPTER 2: EXPERIMETAL DETAIL

2.1 Materials processing and characterization

2.1.1 Hydroxyapatite

Hydroxyapatite is a bio-ceramic in the category of calcium phosphate. Stoichiometric hydroxyapatite has the formula $Ca_{10}(PO_4)_6(OH)_2$. Its main properties are listed in Table 3 [5]. HA is osteoconductive, non-toxic, non-immunogenic and as mentioned before, HA could form strong bond to the host bone. Therefore it is recognized as one of the best candidates in bone tissue regeneration. HA already has a wide range use in clinical fields such as the repair of long bone defects, ununited bone fractures, middle ear prostheses [5]. Nano-scale HA has received a lot of attention recently due to its unique functional properties than its micro-scale counterpart. Intensive efforts have been made to explore the HA based nano-composites to mimic the functions of the natural bone. Various processing techniques have been applied to manufacture nano-HA as summarized in Table 4.

Table 3 Properties of hydroxyapatite [5]

Properties	**Experimental data**	**Properties**	**Experimental data**
Chemical composition	$Ca_{10}(PO_4)_6(OH)_2$	Fracture toughness (MPa $m^{1/2}$)	0.7–1.2
Ca/P molar	1.67	Hardness (HV)	600
Crystal system	Hexagonal	Decomposition temperature (°C)	>1000
Space group	$P6_3/m$	Melting point (°C)	1614
Cell dimensions (Å)	$a = b = 9.42$, $c = 6.88$	Dielectric constant	7.40–10.47
Young's modulus (GPa)	80–110	Thermal conductivity	0.013

		(W/cm K)	
Elastic modulus (GPa)	114	Biocompatibility	High
Compressive strength (MPa)	400–900	Bioactivity	High
Bending strength (MPa)	115–200	Biodegradation	Low
Density (g/cm^3)	3.16	Cellular-compatibility	High
Relative density (%)	95–99.5	Osteoinduction	Nil
		Osteoconduction	High

Table 4 Synthetic techniques of nano-HA [5]

Methods	**Grain size (nm)**	**General remarks**
Solid state [46]	500	Inhomogeneous, large grain size (micro to nano), irregular shapes, reaction condition 900–1300 °C
Wet chemical [47]	20–200	Nanograin size, low crystallinity, homogeneous, reaction condition: room temperature to 100 °C
Precipitation/hydrothermal [48]	10–25	Homogeneous, ultra-fine particles, low crystallinity, reaction condition: room temperature to 200 °C (1–2 MPa)
Hydrothermal [49]	10–80	Homogeneous, fine crystals, high temperature, and high-pressure atmosphere
Mechanochemical [50]	<20	Easy production, semi-crystallinity, ultra-fine crystals, room temperature process
pH shock wave [51]	20–100	High-energy dispersing, nonporous, monocrystalline particles with Ca/P molar ratio 1.43–1.66
Microwave [52]	100–300	Uniformity, nanosize particles, time and energy saving

HA powder in this work is provided by Prof. Cs. Balazsi. The powder was prepared from raw eggshells sintered at 900°C. 30 min heat treatment was to remove the organic components. Powders were then crushed in an agate mortar and reacted with phosphoric acid powder. The received powders were milled in ethanol for 10 h by ball mill and polyethylene glycol (PEG) was added. HA green compacts were produced by dry pressing at 220 MPa. Samples were then calcinated in air at 900°C for 2 h. The HA was crushed to powder form in order to introduce HA into the electrospinning solution.

2.1.2 Cellulose acetate

As an acetate ester of cellulose, cellulose acetate (CA) has been prepared and used in a long history. Cellulose acetate fibers are used for textiles, tools, film media and spectacle frames. Recently, CA has shown potential applications as a biodegradable polymer. CA is electrospun using various solvents (acetone, acetic acid, dimethylacetamide et. al.) and smooth fibers of uniform diameters from 100 nm to 1 μm could form [53]. Electrospinning of CA and silver nitrate could form antimicrobial cellulose acetate fibers with silver nanoparticles along the fibers [54]. CA scaffolds have also been used for building structurally stable and functionally active cardiac cell networks [55].

2.1.3 Electrospinning

Electrospinnin is a process capable of making polymer fibers with nano-scale diameters and controlled morphology. The setup of electrospnning is very simple which consists of three major components: a high-voltage power supply, a syringe controlled by a programmable pump and a metallic collector. A polymer solution is loaded into the

syringe and a syringe pump puts the solution out from the needle tip with constant rate. When a voltage is applied to the needle, the droplet coming from the tip is stretched into a liquid jet. The jet is driven by the electric field toward the grounded collector and the solvent evaporates leaving a membrane of fine fibers. The resulted electrospun fibers have small diameters, good mechanical performance, large surface area and small pore size. Fiber properties such as fiber diameter, morphology, porosity, and surface functionality can be adjusted by various parameters [56]. Synthesis of composite nano-fibers by combining the inorganic particles in the polymer solution was recognized as one of the biggest breakthroughs in the electrospinning field [57].

Cellulose acetate powder with an acetyl content of 40% (Fluka Chemie CH-9471, Buchs, Switzerland) was mixed with acetone at room temperature and sonicated for 1 h to form a 15 wt/vol % solution for electrospinning. CA powder was mixed with acetone at room temperature to form a 15 wt/vol % solution and 9.38% w/v HA(hydroxyapatite) nano-particles was added to acetic acid. This mixture was combined to prepare a solution containing 71-29 vol% Acetone/Acetic acid, respectively. This mixture was sonicated for 1 hour before electrospinning.

The electrospinning setup consists of a high-voltage power supply that provides up to 40 kV, two electrodes, a programmable pump, a disposable syringe attached with a 22 gauge conducting needle and an aluminum foil as a collector. One of the two electrodes is attached to the syringe needle and the other is attached to the foil. For electrospinning parameters, a flow rate of 9.6 ml/hr and voltage of 19 kV were used. The distance from the collector to the needle was 10 cm. The whole process is shown in Figure 1.

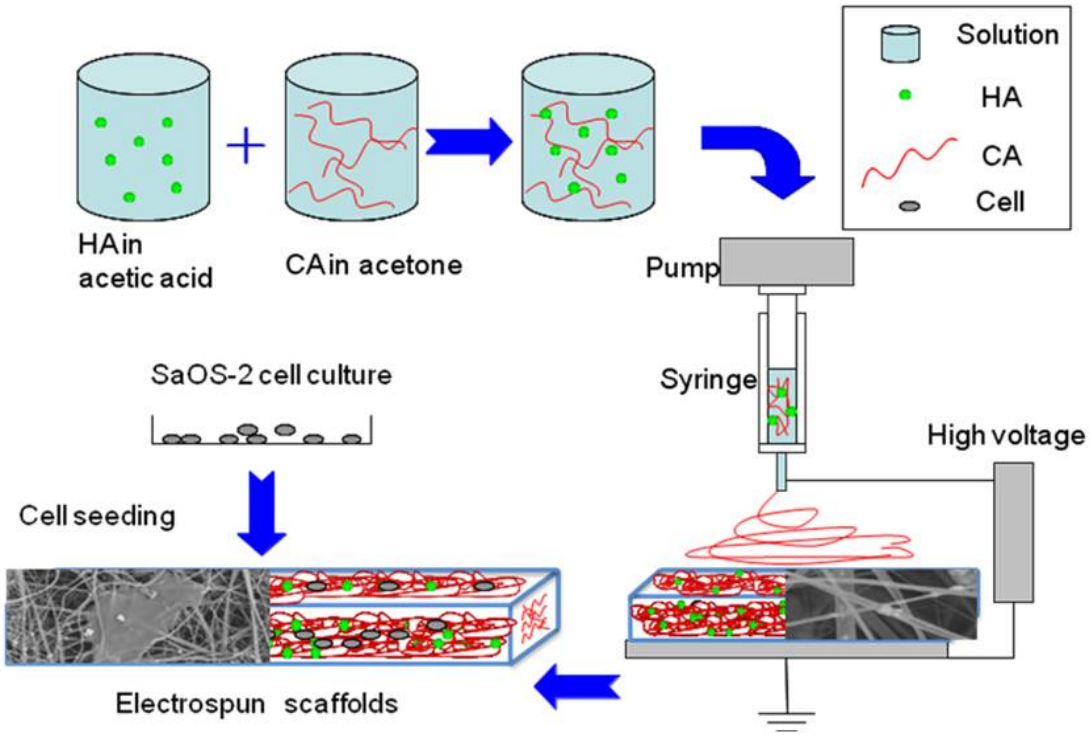


Figure 1 Schematic of the electrospinning and cell culture process used to obtain 3D cell seeded scaffolds. Insets are SEM micrographs revealing cross sectional views of the scaffolds before and after the cell seeding process.

2.1.4 Scanning Electron Microscopy (SEM)

Scanning electron microscope is a type of electron microscope capable of create high-resolution images of a sample surface using electrons rather than light. In an SEM, a beam of electrons is generated at the top of the microscope by heating of a metallic filament or by field emission. The electron beam goes a vertical path through the column of the microscope. Then electromagnetic lenses focus and direct the beam down to the sample. The electrons interact with the nucleus of the samples. Detectors collect the secondary or backscattered electrons, and convert them to a signal that is sent to a viewing screen which is similar to the one in a television to display an image [58].

2.2 Cell culture

2.2.1 Cell culture conditions

Scaffolds were seeded into wells of a 24-well tissue culture plates. Stainless steel retaining rings (snap rings) [McMaster-Carr #9158OA161] were used to secure the scaffolds in the bottom of the wells. Scaffolds were sterilized by incubating with 70% ethanol for 30 min. The ethanol was removed and the scaffolds were washed twice with sterile water. The wells were then filled with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic / antimycotic (DMEM complete medium) [All from Sigma] and allowed to equilibrate overnight.

Human osteoblast-like cells (SaOS-2) were grown at 37^{o}C, 5% CO2 in a humidified incubator in DMEM complete medium. The cells were trypsinized with 0.25% trypsin-EDTA [Sigma] and seeded on the scaffolds at a density of 50,000 cells per well in a volume of 1.5 ml. Samples were set up in triplicate wells. On day 3 the medium was changed to DMEM complete medium supplemented osteogenic factors 50 µg/ml ascorbic acid, 10 mM glycerolphosphate and 10^{-8} M dexamethasone (All from Sigma). The cells were cultured for up to 14 days and the medium was changed every 3 days.

2.2.2 MTS Assay

The CellTiter 96® AQueous Assay (MTS Assay – Promega #G3580) uses the novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and the electron coupling reagent, phenazine methosulfate (PMS). MTS is chemically reduced by cells into formazan and it is then dissolved in tissue culture medium. The measurement of the absorbance of the

formazan can be carried out in 96 well microplates at 492nm. The assay measures dehydrogenase enzyme activity found in metabolically active cells (cell viability).

2.2.3 Picogreen Assay

Quant-iT™ PicoGreen® dsDNA reagent [Invitrogen # P7581] is an ultra sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA) in solution (quantitation of relative cell number). After cells were cultured for 1, 7 and 14 days, the scaffolds were transferred to fresh wells and all wells were rinsed with PBS 2X. 300 µl of TE buffer was added to each well and samples were freeze-thawed 3 times to release the DNA from the cells. For quantitation, a standard curve was prepared with DNA in range of 1-1000 ng/ml. Background signal was evaluated using the scaffolds without cells. A 100 µl aliquot of each sample was mixed with 100 µl of working reagent and incubated for 5 min. at room temperature. The samples were read on a fluorescence plate reader with an excitation of 488nm and emission of 520 nm.

2.2.4 Alkaline Phosphatase Detection

Alkaline Phosphatase (ALP) is an osteoblast phenotypic marker. After cells were cultured for a particular period, the scaffolds were transferred to fresh wells rinsed with PBS 2X. Samples were lysed in 200 µl CellLytic M (Sigma). The samples were assayed using the Alkaline Phosphatase Detection Kit (Sigma #APF). A 20 µl aliquot of each sample was mixed with 180 µl of assay mixture and incubated for 2 hours in the dark. The samples were read on a fluorescence plate reader at an excitation of 360 nm and emission of 440 nm. To compare the ALP on a per cell basis, ALP activity was normalized by dividing by protein concentration of the cell lysates (Pierce – BCA assay).

2.2.5 Von Kossa Staining – Mineralization

This technique is for demonstrating deposits of calcium or calcium salt so it is not specific for the calcium ion itself. Samples were fixed with 100% ethanol for 30 min. and rinsed with water. Samples were treated with a solution of 1% Silver Nitrate and incubated under a UV light for 20 min. The wells were washed with water and destained with 5% sodium thiosulfate for 2 min.

CHAPTER 3: RESULTS

3.1 Viability and Proliferation

The effects of the scaffolds on cell viability and proliferation were evaluated by the MTS and Picogreen assays, respectively. In the viability assay, MTS is converted to formazan by metabolically active cells. The relative cell numbers were measured using the Fluorometric double-Stranded DNA quantitation kit, Picogreen. There was a significant increase on both cell viability and number from day 1 to day 7 for the control wells (cells on plastic). Both of the scaffolds, CA and CA/HA, had lower levels of cell viability and cell number compared to the plastic controls. Cells grown on the scaffolds exhibited little growth between day 1 and 7, but appear to show a trend toward cell growth by the day 14 time point as shown in Figure 2 and Figure 3

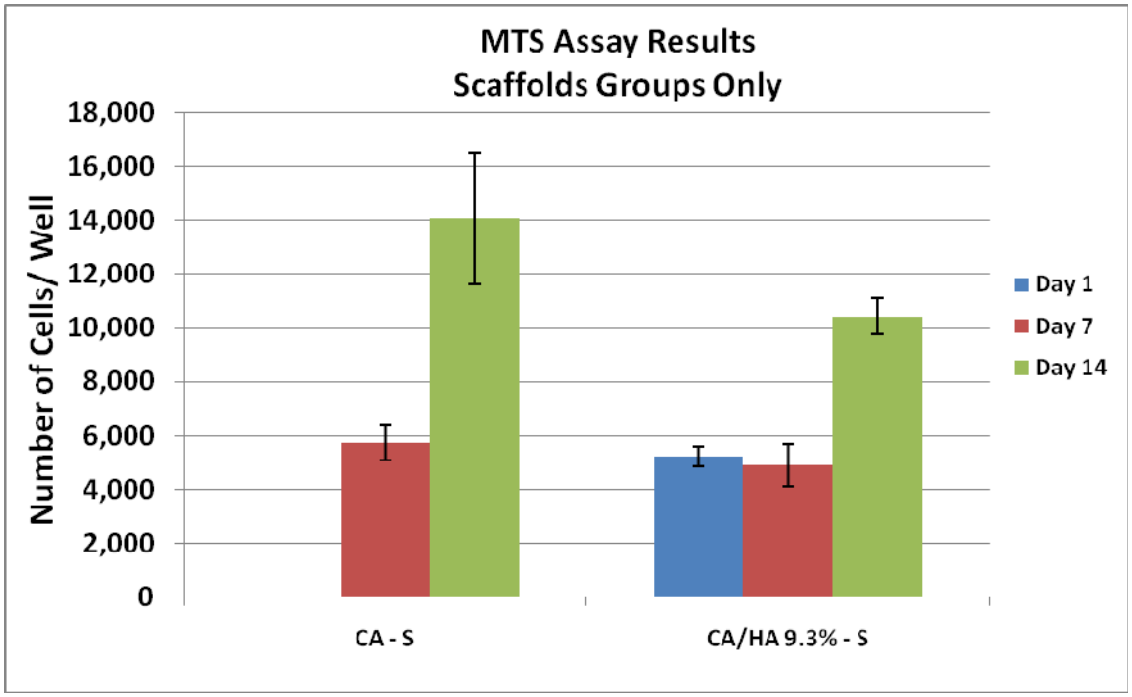


Figure 2 The MTS activity SaOS-2 cells seeded on CA and CA/HA scaffolds

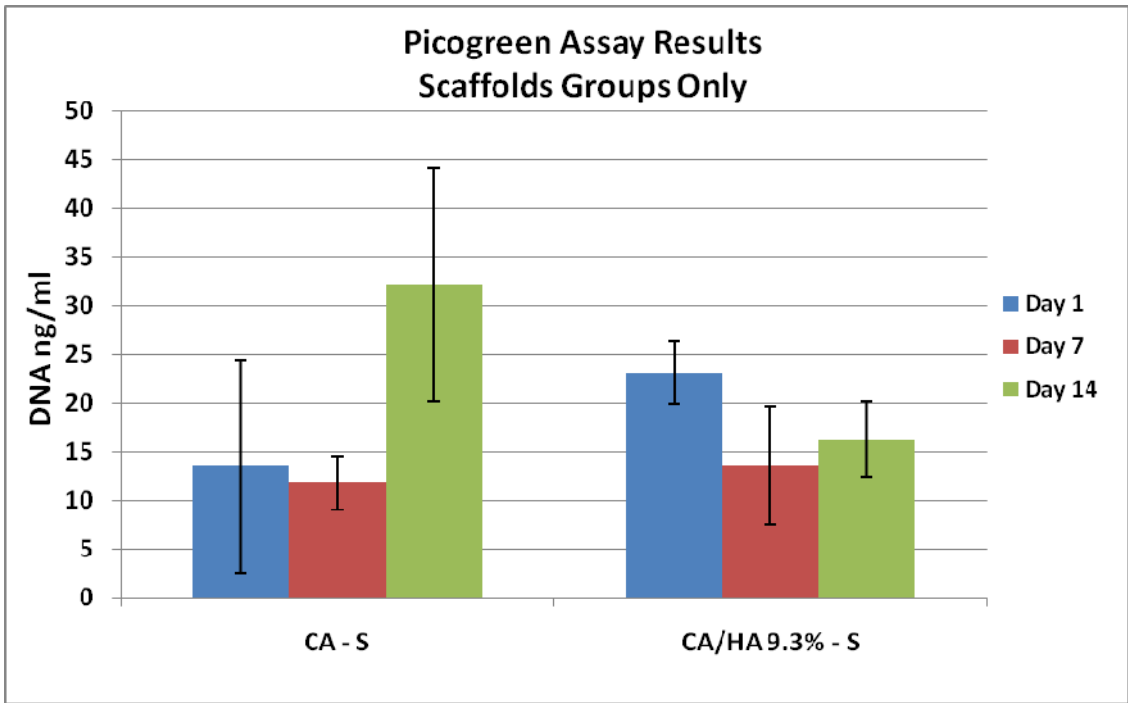


Figure 3 The Picogreen activity SaOS-2 cells seeded on CA and CA/HA scaffolds

3.2 ALP Activity

ALP activity is a marker of an osteogenic phenotype. All of the samples showed an increase in signal from day 1 to day 14. The cells grown on the CA and CA/HA scaffold had lower levels of ALP than the cells grown on plastic. When comparing the CA to CA/HA scaffolds, the ALP activity levels were of a similar magnitude (Figure 4)

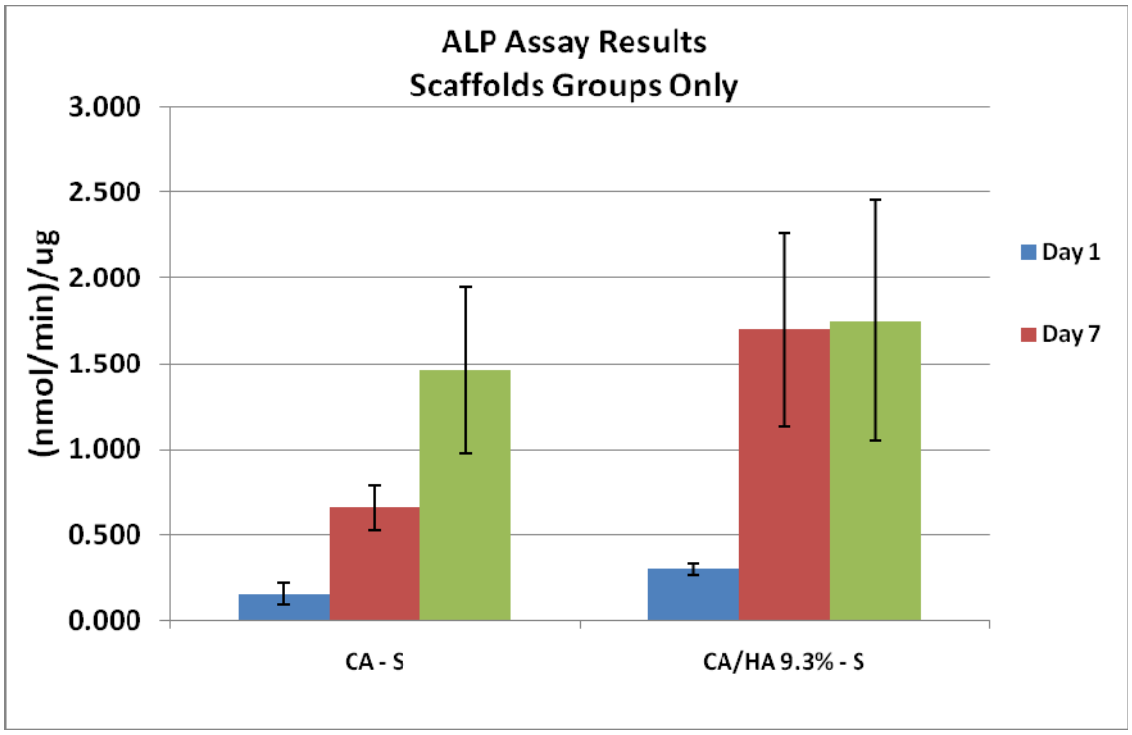


Figure 4 The ALP activity SaOS-2 cells seeded on CA and CA/HA scaffolds

3.3 Von Kossa - Mineralization

The detection of mineralization was performed on days 1, 7, and 14. Staining was also done on scaffolds without cells to evaluate the background staining. The Scaffolds without cells showed only a slight background staining even though some of the scaffolds contained hydroxyapatite which contains calcium. There was a clear increase in mineralization for all of the samples between day 1 and day 14. The CA/HA showed the

most intense staining at each time point with the day 14 samples being the most intense as shown in Figure 5. One observation made during the staining was that during the washes and staining procedures, some of the mineralization that was observed in the control samples appeared to be washing away.

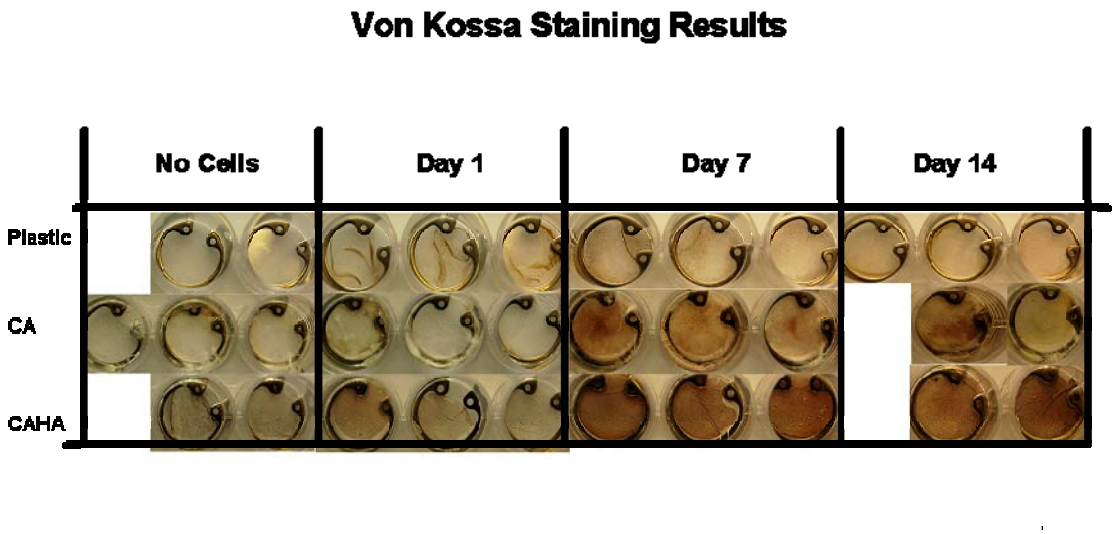
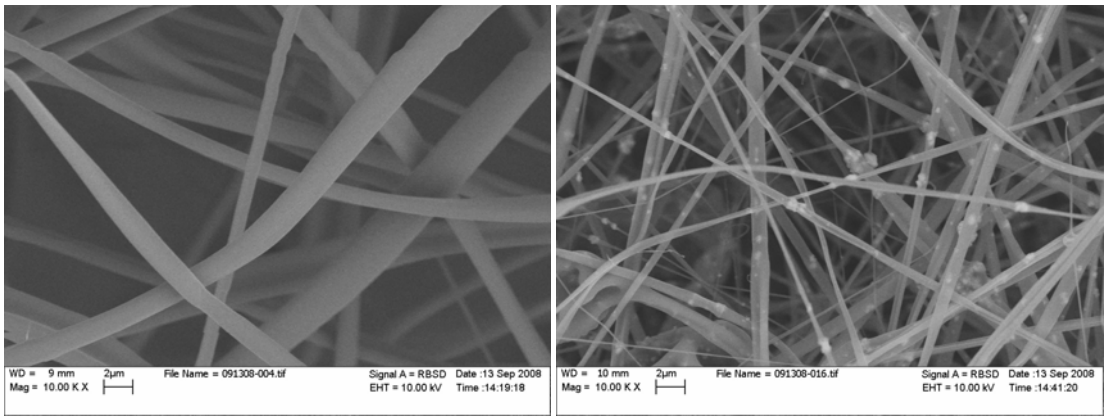


Figure 5 The mineralization (Von Kossa Staining) results of SaOS-2 cells seeded scaffolds

3.4 SEM of the scaffolds

The as-received electrospun scaffolds showed uniform distribution, porosity and ultrafine fiber diameters (Figure 6). Pure CA scaffolds have a larger fiber size at diameter of 2.9 μm on average. By contrast, due to lower polymer concentration, the CA/HA composite electrospun fibers have a 300 nm average diameter. The average size of hydroxyapatite nano-clusters on the fibers is 35 nm (Figure 7). The average pore diameter in the CA scaffolds is about 5.82 μm and in CA/HA scaffolds it is about 3.19 μm. The relative porosity of the as-spun scaffolds is 43% for the CA and 30% for the CA/HA based on calculations within a layer.



(a) (b)

Figure 6 Compare of as spun scaffolds (a) CA 10kx (b) CA/HA 10kx

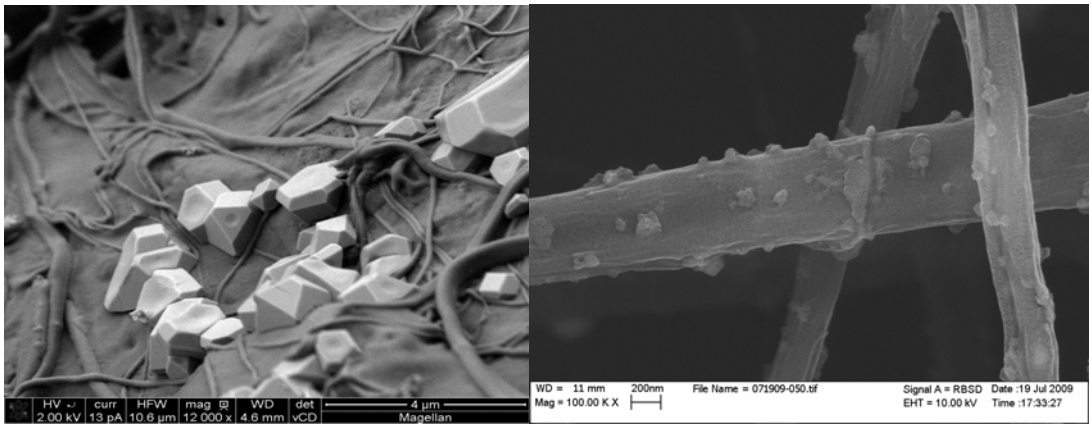
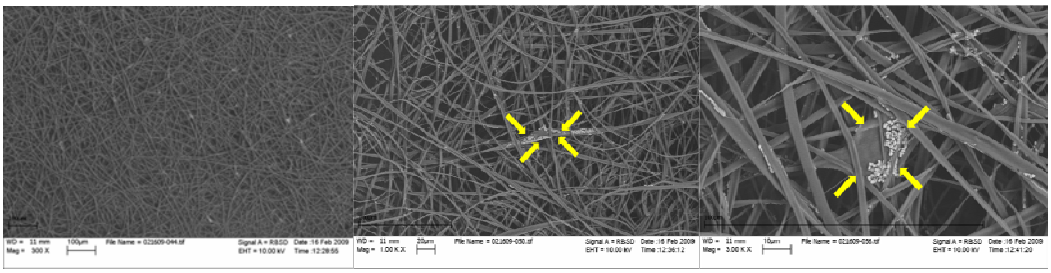


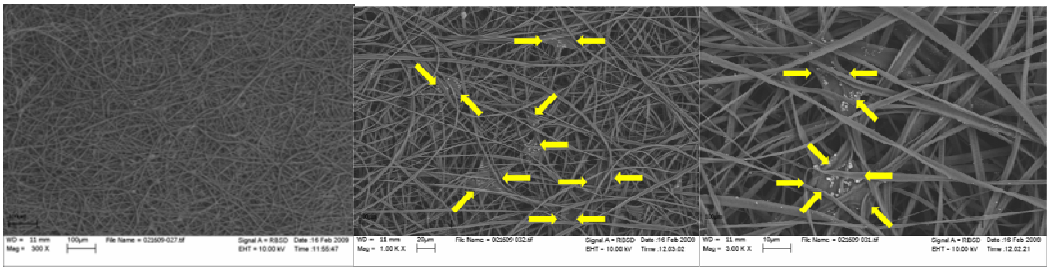
Figure 7 Distribution of HA particles along polymer fibers

Figure 8 and Figure 9 present the morphology and structure of cells cultured on the CA and CA/HA scaffolds. For CA scaffolds, the average diameters of cells for 1, 7, and 14 days culture are 15.14 µm, 17.33 µm and 26.47 µm respectively. For CA/HA composites, the amount of cells is greater than CA scaffolds in each culture period and the cell size is larger than the ones on pure CA scaffolds. The average diameters are 14.77 µm, 29.47 µm and 31.17 µm for 1, 7, 14 days culture.



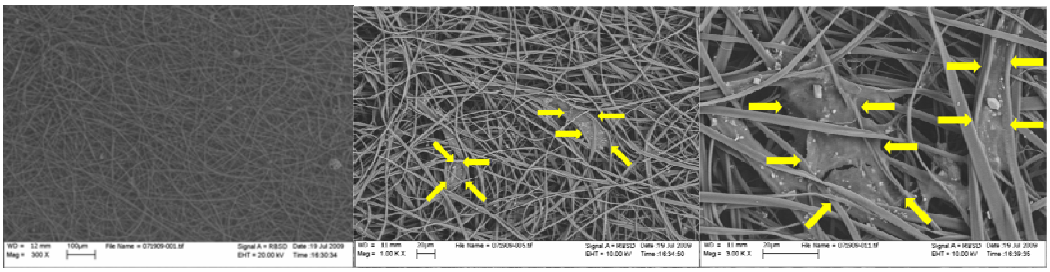
300x 1kx 3kx

(a)



300x 1kx 3kx

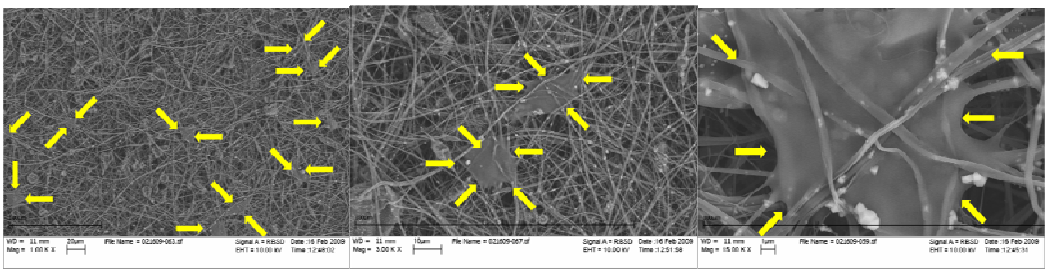
(b)



300x 1kx 3kx

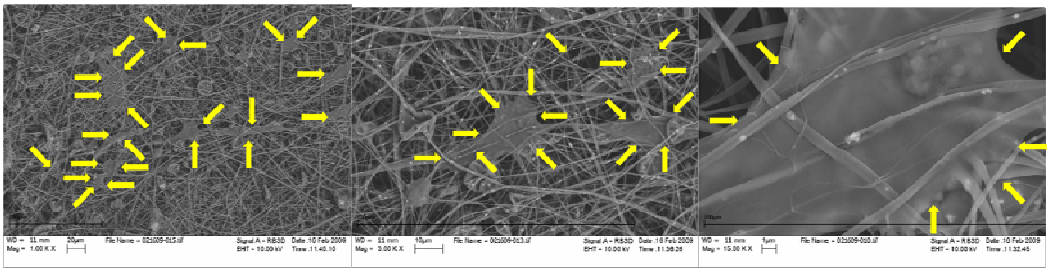
(c)

Figure 8 Cell culture on CA scaffolds (a) 1 day (b) 7 days (c) 14 days



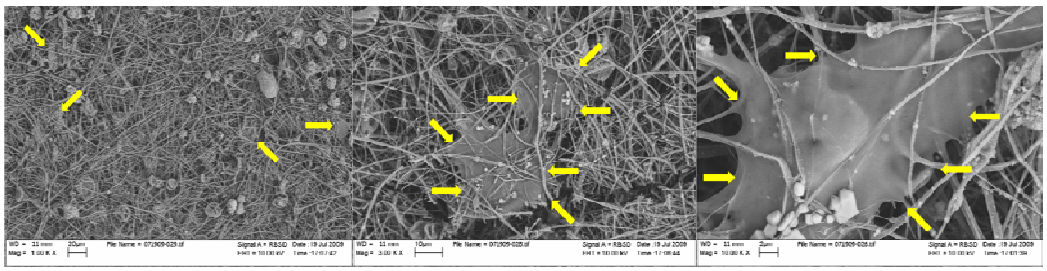
1kx 3kx 15kx

(a)



1kx 3kx 15kx

(b)



1kx 3kx 10kx

(c)

Figure 9 Cell culture on CA/HA scaffolds (a) 1 day (b) 7days (c) 14 days

CHAPTER 4: DISCUSSION

This work is based on the use of uniformly dispersed nano-hydroxyapatite clusters on the surface of fibers in non-woven mats of biocompatible polymers to form a composite with 3D structure. To manufacture excellent quality and performance ceramic polymer composites, the particle aggregates or agglomerates must be broken down during the manufacturing process into tiny particles which are adequately dispersed in the polymer medium [59]. The properties of composites are mainly influenced by particle shape, particle size and size distribution, volume percentage of particles, matrix molecular weight, dispersion of particles in the polymer matrix and the condition of the filler/matrix interface [59]. Four kinds of mutual arrangements of nano-particles to polymer chain have been classified by Kickelbick [60]: (1) inorganic particles embedded in inorganic polymer, (2) incorporation of particles by bonding to the polymer backbone, (3) an interpenetrating network with chemical bonds, (4) an inorganic-organic hybrid polymer. Bhowmik et al. [61] have shown that particle-polymer interactions have a key role in the mechanical reaction of the polymer. The load-deformation behavior at the interfaces in a polyacrylic acid/hydroxyapatite composite has been studied. It was found that the energy needed to move the polymer to its proximity with HA is significantly higher than in the absence of HA [59]. Polymers alone usually show insufficient cell adhesion; their surfaces are hydrophobic or weakly hydrophilic, which are avoiding cell growth in a three-dimensional structure [62]. Suchy et al. [63] have studied the wettability of glass fibers/siloxane composites in a tissue culture medium and found that

it is dependent on open pore size. The values of the contact angles for pore sizes 200–400, 400–600 and more than 600 μm were 33º, 64º and 67º, respectively. These values mean that the surfaces are wettable i.e. suitable for bone cells in-growth [59]. To overcome the problem of the agglomeration of HA particles during the processing, 12-hydroxystearic acid is introduced to the system of the hydrophilic HA and the hydrophobic PLA fibers and is served as a mediator. These mediators are compounds that are amphiphilic and usually a long chain hydrocarbon fragment [64]. In our work, observed from the SEM images, the HA nano-clusters have small size (37nm) and have been well dispersed along the electrospun mats. This will allow a higher open surface for the particles to interact with the cells.

Most mammalian cells are anchorage-dependent and require biocompatible scaffolds to attach, migrate and differentiate forming new tissues. The choice of bone cells (osteoblasts) in this study was meant to establish their affinity to nano-HA as opposed to nano-CA. The MTS viability assay displayed that the cells exposed to these scaffolds keep the ability to proliferate for up to 14 days. These findings were supported by measuring the amount of dsDNA (e.g. PicoGreen assay), which also showed quantifiable increases in the number of viable cells exposed to either scaffold. These findings show that these scaffolds are able to support and maintain cell proliferation.

The mechanisms of the interactions between cells and the engineered scaffolds are in part understood. Some studies have revealed that cell attachment and survival could be modulated by protein pre-adsorption on the scaffolds. Thus protein adsorption can be critical in evaluating a scaffold for bone tissue engineering [65]. Relatively abundant proteins contained in serum-supplemented culture medium such as fibronectin, laminin

and vitronectin could be adsorbed to a material. It has been shown osteoblasts have significantly improved adhesion if scaffold surface is pre-coated or the culture medium is supplemented with fibronectin or vitronectin [66]. Once such proteins coat the material's surface, cells are able to interact with the proteins through specific molecules on the surface of the cell. Therefore, it is essential for scaffold materials capable of adsorbing active proteins from serum to support cell adhesion and spreading [67]. When antibodies are used, the cell attachment will decrease. This phenomenon could prove that the main interaction between cells and adhesion proteins takes place via integrins (heterodimeric receptors in the cell membrane) [68]. Sinha and Tuan [69] conclude that differences in integrin expression observed on different materials may be the reason of variations happened in cell attachment for different biomaterials. Integrins are also reported to have various cell functions such as intracellular signaling [70]. It is reported that HA is deeply involved in the initial protein adsorption, such as fibronectin and vitronectin. It has been proved by incorporating HA in poly(l-lactic acid) (PLLA) scaffolds that fibrous scaffolds have better protein adsorption capacity than pore structured scaffolds due to much higher surface to volume ratio [71]. Therefore, the adhesion proteins in the serum, which are then transported to the cells, favor the CA-HA scaffolds and cause a better cell growth for CA-HA scaffolds.

The attachment of osteoblasts to scaffolds in this work depends on the relatively high surface area and the porosity of the electrospun scaffolds. The electrospinning process is capable of producing fibers with small diameters and relatively high surface area-to-volume ratios that are ideal for osteoblast attachment and migration. Cellulose and its derivatives, though biocompatible, are not osteoinductive materials, and

osteoblasts were not expected to interact strongly with them. Evidence for this fact is given by the micrographs of cell morphology on the scaffolds. Cells would grow only between two CA fibers and along their length in a "panel-looking" configuration in the pure polymeric scaffolds, whereas they were seen forming elongated, widely-spread, complex, "tent-like" networks involving multiple fibers at different layers. The hydroxyapatite nano-clusters were mainly located at the edge of cells, which may provide additional anchoring sites for cell to attach to the CA-HA scaffolds.

Furthermore, all evidence of mineralization on the CA scaffolds is on the surface of the cell, away from the fibers, whereas the hybrids exhibit mineral formation near anchoring sites, presumably at the interfaces with HA. There has been a large amount of studies on the mineralization treatment of electrospun mats by immersion in different fluids [72]. The apatite formation has been shown influenced by the surface functional groups of the polymer nano-fibers [73]. One example is that a high rate of nucleation of apatite crystals during mineralization could be obtained by some collagen fibers due to the large number of negatively charged carboxyl and carbonyl chemical groups on them which could bind with Ca^{2+}. [73] Moreover the formation of apatite is likely happened non-uniformly on fibers with small diameters and little mineralization is found on thicker fibers due to the lower surface area of the fibers with large diameter [74].

Finally, CA scaffolds have cells strongly adhering to individual fibers. CA/HA scaffolds had few adhesion points but an overall free surface separate from the scaffold. Although the assays used here have confirmed the retention of bone phenotype for the cultured cells on both scaffolds, there was no quantitative measurement of their relative degree of differentiation, primarily due to the presence of Ca containing HA in the hybrid

mats. The elongated shape of the cells on CA/HA scaffolds, though, suggests that there are more likely to have maintained their functionality.

CHAPTER 5: CONCLUSION

By employing the electrospinning technique, the ceramic-polymer nano-composites were successfully manufactured and applied in bone tissue engineering. CA-HA composite scaffolds were proved to promote favorable adhesion and growth of osteoblasts as well as to stimulate the cells to exhibit functional activity of bone cells. Based on our observations, the electrospun CA-HA nano-composite scaffolds are considered as a promising candidate for bone tissue engineering application.

CHAPTER 6: FUTURE WORK

High density of cells may provide more cell to cell interactions and benefit the whole tissue. Continuing the culture time out several more weeks could further evaluate the cell attachment to the scaffolds. In the MTS, Picogreen and ALP assays, there were significant amounts of cells that passed through the scaffolds and seeded in the bottom of the wells. Making the scaffolds thicker or perhaps making multi-layer make allow better attachment to the scaffold. The evaluating the biodegradability of cellulose acetate and cellulose acetate-hydroxyapatite composite is necessary for future application of the scaffolds in *vivo*. Effects of fiber diameter and scaffold morphology on cell attachment and proliferation should be considered to find optimal structure for the cell growth. The animal in *vivo* study could be conducted to prove the potential clinical application of the studied scaffolds in clinical fields.

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