CHAPTER VII

ELECTROSPUN POLYCAPROLACTONE FIBERS FILLED WITH HYDROXYAPATITE NANOPARTICLES AS BONE TISSUE ENGINEERING SUBSTRATE

ABSTRACT

Electrospun fiber mats of polycaprolactone (PCL; M_n = 80,000 g·mol^{-1}) with or without the presence of hydroxyapatite (HAp) nanoparticles (at 1% w/v based on the volume of the PCL solution) were successfully fabricated. The potential for use of these electrospun fiber mats (i.e. E-PCL and E-PCL/HAp) as bone tissue engineering substrate was assessed by human fetal osteoblasts, hFOB, in terms of the proliferation, differentiation, and mineralization. Osteoblasts were grown on the E-PCL and E-PCL/HAp surfaces. Control surfaces were tissue culture (TC) dishes and porous poly(DL-lactic-*co*-glycolic-acid) (PLGA) disc. Scanning electron microscope (SEM) showed osteoblasts grew on all the tested biomaterials. Compared to E-PCL and E-PCL/HAp, cell proliferation at Day 7 was significantly higher on TC, and significantly lower on PLGA. Between E-PCL and E-PCL/HAp, cell proliferation was significantly higher on E-PCL/HAp. ALP activity at Day 7 and calcium content at 4 weeks of culture were significantly lower on TC compared to the all other materials but there were no differences among the tested materials.

(**Key-words**: electrospinning; polycaprolactone; hydroxyapatite; osteoblast)

1. INTRODUCTION

Electrostatic spinning or electrospinning is an interesting method for producing non-woven fibers with the average diameters in the range of sub-micrometers down to nanometers. In this process, a continuous filament is drawn from a polymer solution or melts through a spinneret by high electrostatic forces and later deposited on a grounded conductive collector. Upon increasing the electrostatic field strength up to a critical value but not exceeding it, charge species accumulated on the surface of a pendant drop destabilize the hemispherical into a conical shape (commonly known as Taylor's cone). Beyond a critical value, a charged polymer jet is ejected from the apex of the cone and carried to the collector screen by the electrostatic force. The Coulombic repulsion force is responsible for the thinning of the charged jet during its flights to the collector. The charged jet travels linearly for a short distance before undergoing a bending instability which is thought to be another factor contributing to the decrease in the diameter of the jet during its flight to the collector.

Due to high surface area to volume ratio of the electrospun fibers and high porosity in sub-micrometer length scale of the obtained non-woven mat, ultrafine electrospun fibers have been proposed as ideal materials in tissue scaffolding applications.[1, 2] The challenge in tissue engineering is the design of scaffolds that can mimic the structure and biological functions of the natural extracellular matrix (ECM). The important aspects of electrospun fibers in scaffolding applications are the 3D structure with interconnected pores and high porosity, which is similar to fibrous collagen in the natural ECM.

Selection of the materials for fabrication into scaffolds is also important. The scaffolds have to replace the natural ECM until the host cells can regenerate and synthesize a new matrix. The most commonly used synthetic polymers in scaffolding application are polylactide (PLA), polyglycolide (PGA), and poly(lactide-*co*-glycolide) (PLGA), due to their biodegradability and biocompatibility. Many published works reported on successfulness of culturing tissues using PLGA as scaffold material.[3-8] For examples, Kang and co-worker demonstrated the feasibility of using PLGA microspheres as an injectable scaffold for cartilage regeneration in

vivo in subcutaneous space of athymic mice[3] and rabbit knees[4] which evidence of forming of cartilage. Karp and co-worker reported in vitro bone growth on 2D and 3D PLGA films and the formation of a mineralization matrix.[5] However, PLGA has a disadvantage of fast degradation. The in vitro and in vivo half-lives of 3 types of porous poly(DL-lactic-*co*-glycolic acid) (PLGA) (85:15) foam with different porosities were about 11.6 and 12.5 weeks, respectively.[9] This degradation study used porous PLGA foam from salt leaching process with ratio of monomer of 85:15, salt particles of 80% weight fraction and particle size of 106-150 micron. The in vitro degradation was done in pH 7.4 phosphate-buffered saline (PBS) at 37° C and in vivo degradation was studied in rat mesentery. Due to this disadvantage, polycaprolactone (PCL), a biodegradable polymer with slow degradation rate comparing to PLGA, is the promising material for using in bone regeneration application which require a period of time for tissues regeneration before scaffold is degraded.

For the design of materials in term of biological functions, hydroxyapatite (HAp) is the most interesting compound to be used as substitute[1,10] or substrate[11] in bone scaffolding applications. Since inorganic phase of bone consists of mainly calcium phosphate compounds in form of HAp, HAp is considered to be biocompatible and osteoconductive. There were many reports found that the presence of HAp help improve the proliferation and differentiation of osteoblasts.[12, 13]

In a previous related work, successful fabrication of electrospun mats of polycaprolactone (PCL) fibers and its composite with hydroxyapatite (HAp) nanoparticles was reported and characterized.[14] Cell response *in vitro* of human osteoblasts (SaOS2) cultured on these mats was evaluated.[1] It was found that electrospun PCL fibers mats filled with nanoparticles HAp contributed good attachment, proliferation, and alkaline phosphatase (ALP) activity.

The purpose of this study was to evaluate the potential use of these electrospun mats as scaffolding materials for bone regeneration. Human fetal osteoblasts (hFOB) were used in this study, in which *in vitro* proliferation, alkaline phosphatase activity, and mineralization were investigated. The results were compared against the surface of tissue culture dish (TC) and porous PLGA scaffold.

PLGA was selected to be as comparing material because it was commonly used in scaffolding application.

2. EXPERIMENTAL

2.1. Materials

Materials used in the fabrication of the electrospun PCL fibrous scaffolds and its composite with HAp nanoparticles were polycaprolactone (PCL; $\overline{M}_n$ = 80,000 g/mol; Aldrich, USA), dichloromethane (Carlo Erba, Italy), *N,N*-dimethylformamide (DMF; Lab-Scan (Asia), Thailand), and hydroxyapatite powder (HAp; synthesized following the method proposed in reference.[15]). After hydrolysis from dicalcium phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$; Fluka Chemika, Switzerland), the HAp powder was annealed at 800°C for 4 hr in air. The obtained HAp powder was characterized by a Rigaku Rint2000 wide-angle X-ray diffractometer (WAXD) with a monochromated CuK_α radiation (λ = 1.54 Å), which confirmed the formation of HAp.[15] The mean particle size of the as-synthesized HAp powder as analyzed by a JEOL JSM 5410LV scanning electron microscope (SEM) was 234 ± 68 nm.[14]

Materials used in the fabrication of the porous PLGA scaffold were poly (DL-lactic-*co*-glycolic acid) (PLGA; MW = 66,000 – 107,000 g/mol; lactide:glycolide = 75:25; Sigma, MO, USA), Chloroform (FisherBiotech, New Jersey, USA), Sodium Chloride (NaCl; Mallinckrodt, Kentucky, USA), and SigmaCoat (Sigma-Aldrich, MO, USA)

2.2. Preparation and characterization of scaffolds

Electrospun PCL fibrous scaffold (denoted as E-PCL) and its composite with HAp nanoparticles (denoted as E-PCL/HAp) were prepared by electrospinning from neat 12% w/v PCL solution in 50:50 v/v dichloromethane and DMF or PCL solution loaded with HAp powder at a concentration of 1.0% w/v. The mixture was stirred until the pellets completely dissolved and it was subsequently sonicated prior to

electrospinning.

Each of the spinning dope was contained in a glass syringe, the opening end of which was connected to a gauge 20 stainless steel needle [outside diameter (OD) = 0.91 mm] used as the nozzle. A rotating drum (width and OD of the drum = 14 and 15 cm, respectively; rotational speed = 50 rpm) was used as a collector. The outer surface of the rotating drum was set 10 cm from the tip of the needle. A Gamma High Voltage Research D-ES30PN/M692 power supply was used to generate a high DC potential. The applied potential and the collection time were 21 kV and 10 hr, respectively, while the polarity of the emitting electrode was positive. A Kd Scientific syringe pump was used to control the feed rate of the polymer solution at about 1 ml/hr. Morphological appearance of the as-spun fibers was examined by SEM and analyzed using SemAphore 4.0 software, from which the arithmetic mean values of fiber diameter of E-PCL and E-PCL/HAp scaffolds were 0.95 μm and 1.26 μm, respectively.[14] Thickness of E-PCL and E-PCL/HAp scaffolds was 130 μm. Fibrous scaffold were dried in vacuo at 40°c overnight to remove as much solvent as possible.

Porous PLGA scaffold was fabricated by solvent casting-salt leaching method. NaCl particles (range of particles size = 250 – 425 μm) 1.45 g was put into a beaker of 30 mm in diameter followed by 960 μl of 5% wt of PLGA solution in chloroform. The beaker was coated with SigmaCoat overnight before use. After solvent completely evaporated, the obtained film was immersed and washed several times with de-ionized water to remove NaCl.

2.3 Cell culture and Cell Seeding

Human Fetal Osteoblasts (hFOB)[16] are a gift from Dr. Thomas Spelsberg (Mayo Clinic, Rochester, MN). Cells were cultured as monolayer in Minimum Essential Medium Alpha Medium (with L-glutamine, ribonucleosides and deoxyribonucleosides, without sodium bicarbonate) (GIBCO, NY, USA) supplemented by 10% fetal bovine serum (FetalPlex, animal serum complex, GEMINI, BIO-PRODUCTS, USA), and 1% Penicillin-Streptomycin (GIBCO, NY, USA). For mineralization studies, cells were cultured in media containing of 5 mM

β-glycerophosphate (Sigma, MO, USA) and 50 μg/ml of L-ascorbic acid (Fisher Scientific, NJ, USA). The medium was replaced once in every 3 days and the cultures were maintained at 34°C in a humidified atmosphere containing 5% CO_2. E-PCL, E-PCL/HAp (35 mm in diameter) and PLGA scaffold (30 mm in diameter) were placed in 6-well dishes. Control wells did not contain any polymer scaffolds (only surface of tissue culture dish; denoted as TC; Corning, NY, USA). The scaffold specimens were washed with de-ionized water and sterilized by exposing under ultra violet light for 30 min. To ensure a complete contact between E-PCL and E-PCL/HAp scaffolds and the wells, E-PCL and E-PCL/HAp scaffolds were pressed with a metal ring (about 32 mm in diameter). hFOB from the cultures were trypsinized with 0.05% trypsin-EDTA (GIBCO, NY, USA), counted by a Z1 Coulter Particle Counter (BECKMAN COULTER, Coulter Electronics, Hialeah, FL) and seeded at a density of about $5x10^4$ cells/well on the scaffold specimens and TC.

2.4 Cell Proliferation

Cell proliferation was determined on Day 7 of culture. Cells were washed with phosphate buffer saline (PBS) to remove non-adherent cells and trypsinized with 0.05% trypsin-EDTA and then counted by a Z1 Coulter Particle Counter.

2.5 Alkaline phosphatase (ALP) activity assay

On Day 7 of culture, ALP was harvested by scrapping cells with 500 μl of harvesting buffer per well consisting of 10 mM Tris Cl (Fisher Biotech, NJ, USA), 0.2% NonidetP40 (Roche Diagnostics Corporation, IN, USA), and 2 mM phenylmethylsulfonyl fluoride (MP Biomedicals, OH, USA). The extracted solution was sonicated and centrifuged at 2,000 rpm, 10 min, and 4° C. The 8 μl of supernatant was mixed with 192 μl of reagent of Alkaline Phosphatase kit (STANBIO LABORATORY, 0900-151, TX, USA). The OD at 405 nm was measured with a spectrophotometer (BECKMAN COULTER, AD340). The obtained ALP value was normalized with DNA content.

DNA assay

The remaining samples left over from ALP assay was mixed with equal amount of 0.8 N perchloric acid (PCA) (Sigma-Aldrich, MO, USA) and vortex until the solution was cloudy. It was put on ice for 15 min and then centrifuged at 10,000 RPM for 10 min. The supernatant was removed. The 500 µl of 0.4 N PCA was added. The solution was again vortex and centrifuged for 10 min at 10,000 RPM then the supernatant was removed and 1 ml of 0.4 N PCA was added. The solution was boiled for 30 min then cool on ice and centrifuged at 10,000 RPM for 10 min. The 500 µl of supernatant was mixed with 1 ml of acetic acid solution (Alfa Aesar, MA, USA) containing of 0.015 g of diphenylamine (Aldrich, MO, USA), 5 µl of 1.6% acetaldehyde (Sigma-Aldrich, MO, USA), and 1.5% v/v of sulfuric acid (Fluka, Germany). The solution was vortex and left overnight before measuring OD at 600 nm with a spectrophotometer. The concentration of DNA of unknown was calculated from standard curve which plot between the OD and standard CTDNA concentration.

2.6 Morphological Observation of Cultured Cells

At 1h, 24 h, 72h, and 4 weeks of culture, morphological of cultured cells were observed by a scanning electron microscope (SEM). Cells were fixed with 3% formaldehyde solution, which was diluted from 37% formaldehyde solution (Fisher Scientific, NJ, USA). After 30 min, they were rinsed with phosphate buffer saline (PBS). After cell fixation, the specimens were dehydrated in an ethanol solution of varying concentration (i.e. 30, 50, 70, 90%, and absolute ethanol, respectively) for about 2 min at each concentration. The specimens were then dried in air. After completely dried, the specimens were coated with platinum, and observed by a SEM (JEOL6500F).

2.7 Calcium content assay

Calcium deposition from cultured cells was evaluated at 4 weeks of culture. Cells on each scaffold were incubated twice for 30 min in 5% trichloroacetic acid

(TCA) (LabChem Inc., PA). Calcium extract was mixed with reagents of a calcium kit (Calcium Liquicolor: Arsenazo, STANBIO Laboratory, TX, USA) and measured colorimetrically at 550 nm using a spectrophotometer (BECKMAN COULTER, AD340).

2.8 Energy Dispersive Spectroscopy (EDS)

Cells were cultured on each material for 4 weeks then were fixed with 3% formaldehyde solution. After 30 min, they were rinsed with phosphate buffer saline (PBS). After cell fixation, the specimens were dehydrated in an ethanol solution of varying concentration (i.e. 30, 50, 70, 90%, and absolute ethanol, respectively) for about 2 min at each concentration. The specimens were then dried in air. After completely dried, the specimens were coated with carbon. The mapping of calcium element was observed by EDS (Noran System Six Software).

2.9 Statistical analysis

All values were expressed as mean ± standard deviation. Statistical analysis was done by one-way ANOVA followed by SNK test, and the significance was considered when $p < 0.05$.

3. RESULTS

3.1 Cell Proliferation

Figure 1 shows cell proliferation on TC, E-PCL, E-PCL/HAp and PLGA at Day 7 of culture. Cell proliferation on TC was significantly higher than that on E-PCL/HAp, E-PCL and PLGA ($p<0.05$). Comparing among polymer scaffolds, E-PCL/HAp allowed higher proliferation than E-PCL ($p<0.05$) and E-PCL allowed higher proliferation than PLGA ($p<0.05$). All values shown in Figure 1 were significantly different from each other.

3.2 Alkaline phosphatase (ALP) activity

The ALP activity of hFOB cultured on TC and each scaffold was evaluated at Day 7 (see Figure 2). The ALP activity was significantly lower on TC compared to all type of scaffolds ($p<0.05$). However, there was no significant difference among E-PCL, E-PCL/HAp and PLGA ($p<0.05$).

3.3 Morphological observation of cultured cells

To observe cell morphology and interaction between cells and scaffolds, the specimens with cell cultures were visualized by SEM at different times of culture i.e. 1, 24, 72 h and 4 weeks (see Table 1). At early time points (1 h and 24 h), cells spread wider on E-PCL and E-PCL/HAp compared to PLGA. On all substrates, cells continued to proliferate. By 4 weeks, the surfaces were fully covered with cells and matrices.

3.4 Calcium content

To study the amount of mineralization of hFOB cultured on scaffolds, calcium content assay was used. Figure 3 shows calcium content of hFOB cultured on TC, E-PCL, E-PCL/HAp and PLGA at 4 weeks. Calcium content at 4 weeks from hFOB cultured on E-PCL, E-PCL/HAp, and PLGA was significantly higher than that on TC ($p<0.05$). However, there was no significant difference among E-PCL, E-PCL/HAp, and PLGA.

3.5 Energy Dispersive Spectroscopy (EDS)

Not only calcium content assay that used to evaluate the amount of calcium. Calcium deposition was confirmed by EDS. The specimens with and without cell culture were observed with EDS to show if there were calcium on surface. As seen in Table 2, column (A), there was no calcium peaks shown whereas there were calcium peaks (at about 3.692 and 4.038 kV) appeared in the spectra in column (B) for all

scaffolds. It was cleared that calcium deposition was occurred for all scaffolds. It might be questionable why there was no calcium peak shown for E-PCL/HAp surface without cell culture even though HAp is calcium phosphate compound. It could be because the amount of calcium from HAp was not high enough for detecting.

The calcium mapping were also performed which locations of calcium appearance represented by red dots. Calcium was appeared on surface of all tested materials (see column (C), (D) and (E)). The images in column (C) were SEM images of scaffold with cell culture at 4 weeks. The images in column (D) were calcium mapping for the SEM images from column (C). The overlaid images of (C) and (D) are shown in column (E). Evidently, calcium deposition appeared on all scaffolds with 4 weeks of culture.

4. DISCUSSIONS

In a previous related work,[1, 14] successful fabrications of electrospun mats of polycaprolactone (PCL) fibers and its composite with hydroxyapatite (HAp) nanoparticles was reported and characterized. Indirect cytotoxicity evaluation of the E-PCL and E-PCL/HAp mats revealed that these fibrous mats posed no threats to the cells. Cell responses *in vitro* in terms of attachment, proliferation, and alkaline phosphatase (ALP) activity of human osteoblasts (SaOS2) cultured on theses mats were evaluated. In the present work, the potential use of these electrospun mats as scaffolding materials for bone regeneration was evaluated *in vitro* with hFOB cells.

In this study, the potential of E-PCL and E-PCL/HAp as candidates for bone scaffold was investigated. The proliferation, alkaline phosphatase activity, and mineralization were investigated with human fetal osteoblasts (hFOB) cultured on TC (i.e., control), electrospun PCL fibrous scaffold (E-PCL), electrospun PCL/HAp fibrous scaffold (E-PCL/HAp), and porous PLGA scaffold (PLGA) (i.e., another control).

For the proliferation, the result showed that all scaffolds supported cell growth. Cell proliferation on TC was the highest ($p<0.05$). However, E-PCL/HAp contributed the highest proliferation comparing among all scaffolds ($p<0.05$)

whereas PLGA showed the lowest proliferation ($p<0.05$). The good proliferation of osteoblasts on E-PCL/HAp might be due to the presence of HAp. Since the inorganic phase of bone is calcium phosphate compounds mainly in form of HAp, HAp is known to be osteoconductive. There were many reports found that HAp help increase proliferation of osteoblasts.[12-13, 17-19] The ability for a scaffold to support differentiation and mineralization, in addition to proliferation, is another important aspect suggesting actual applicability of the scaffold. The ALP activity was investigated due to ALP is one indicator for early differentiation process. For evaluation of mineralization or calcium deposition, calcium content assay and EDS for detection of calcium element were carried out. From the results of ALP activity and calcium content study, all scaffolds promoted differentiation and mineralization better than that of TC ($p<0.05$). However, there was no significant difference among each scaffold ($p<0.05$). The 3D structure of each scaffold might be the reason of good differentiation and mineralization of hFOB cultured on them.

The obtained results showed that E-PCL/HAp is a good candidate to be as a bone scaffold because it supported cell proliferation, differentiation, and mineralization of hFOB. E-PCL/HAp showed superior property in contribution of proliferation than the other scaffolds as well.

5. CONCLUSION

In the present contribution, electrospinning was used to fabricate electrospun fiber mats of polycaprolactone (PCL; M_n = 80,000 g·mol^{-1}) with or without the presence of hydroxyapatite (HAp) nanoparticles from the neat PCL solution (i.e., 12% w/v in 50:50 v/v dichloromethane/*N*,*N*-dimethylformamide) or the PCL solution that contained 1% w/v of HAp nanoparticles. These fiber mats (i.e., E-PCL for the neat electrospun PCL fiber mat and E-PCL/HAp for the electrospun PCL fiber mat containing HAp nanoparticles) were used as substrates for the culture of human fetal osteoblasts, hFOB. Control surfaces were tissue culture (TC) dishes and porous poly(DL-lactic-*co*-glycolic-acid) (PLGA) disc. E-PCL and E-PCL/HAp have a potential to be used in bone tissue engineering with the evidence that E-PCL and E-PCL/HAp supported growth, differentiation and mineralization of human fetal

osteoblasts (hFOB). These substrates allowed increased differentiation and matrix mineralization of hFOB compared to TC. In addition, E-PCL/HAp showed higher cell proliferation than that of E-PCL and PLGA.

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CAPTION OF FIGURES

Figure 1 Proliferation of hFOB on TC, E-PCL, E-PCL/HAp and PLGA at Day 7.

Figure 2 ALP activity of hFOB cultured on TC, E-PCL, E-PCL/HAp and PLGA at Day 7. *Significant at $p < 0.05$ with respect to TC.

Figure 3 Calcium content of 4 weeks cultures of hFOB on TC, E-PCL, E-PCL/HAp and PLGA. *Significant at $p < 0.05$ with respect to TC.

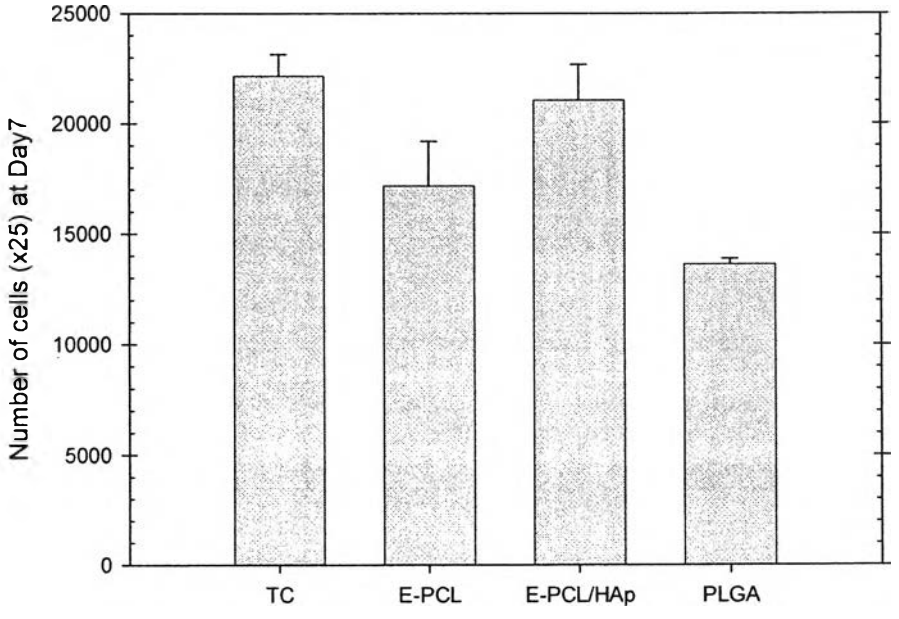


Figure 7.1 Proliferation of hFOB on TC, E-PCL, E-PCL/HAp and PLGA at Day 7.

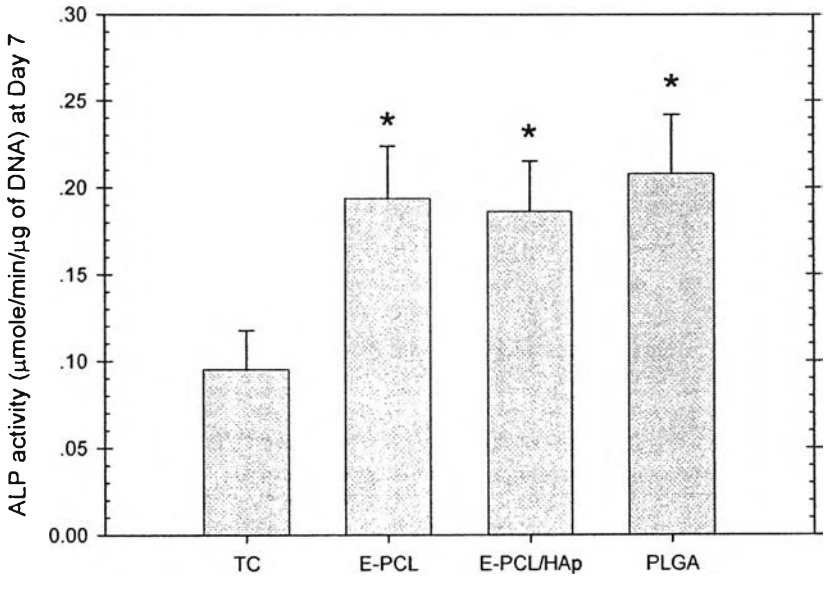


Figure 7.2 ALP activity of hFOB cultured on TC, E-PCL, E-PCL/HAp and PLGA at Day 7. *Significant at $p < 0.05$ with respect to TC.

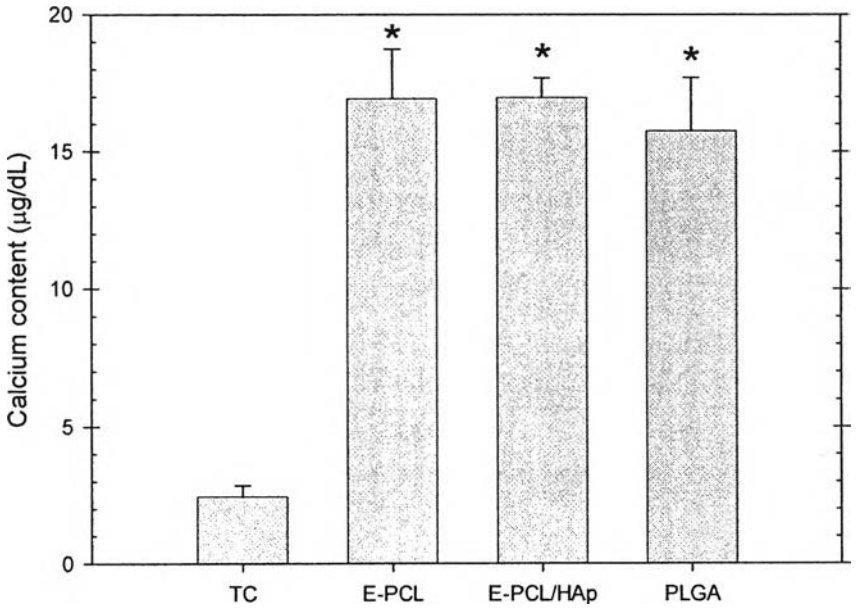


Figure 7.3 Calcium content of 4 weeks cultures of hFOB on TC, E-PCL, E-PCL/HAp and PLGA. *Significant at $p < 0.05$ with respect to TC.

Table 7.1 Selected SEM images of hFOB cultured on E-PCL, E-PCL/HAp and PLGA at different times of culture. A magnification was 2000x

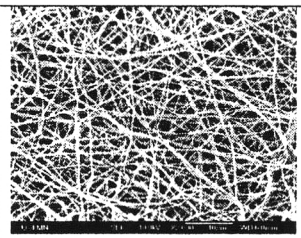
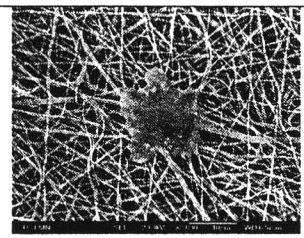
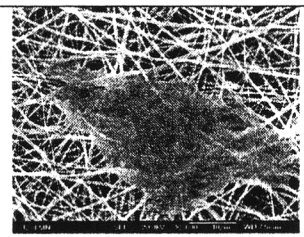
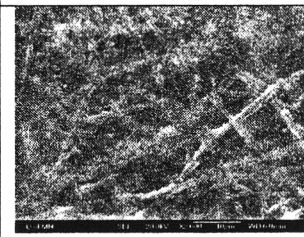
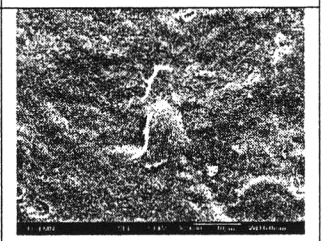
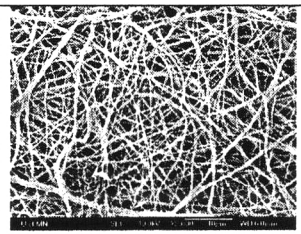
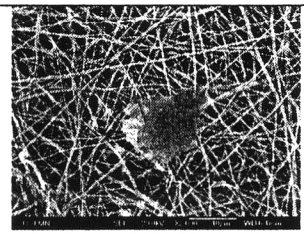
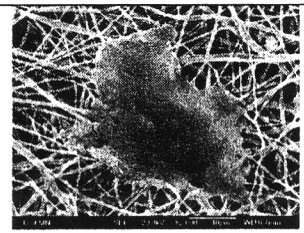
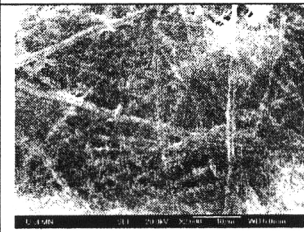
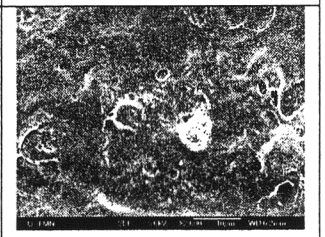
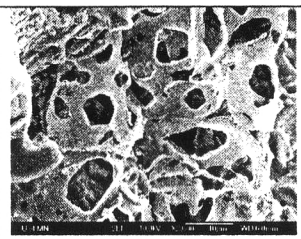
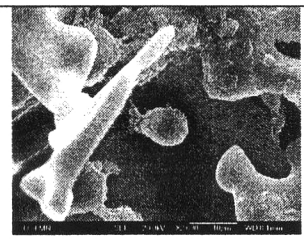
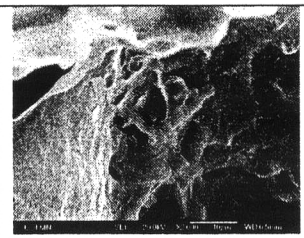
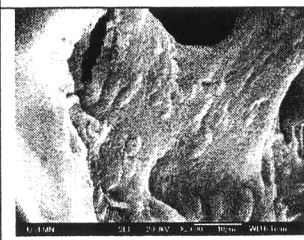
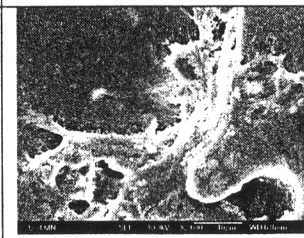
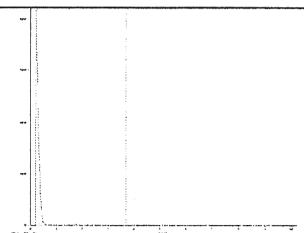
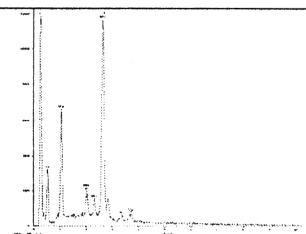
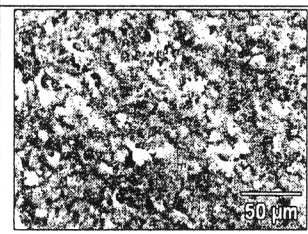
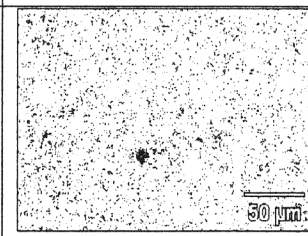
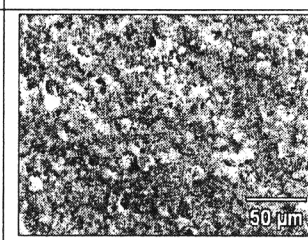
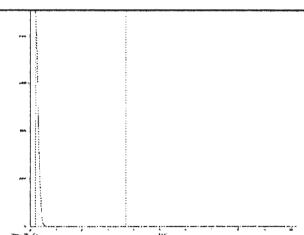
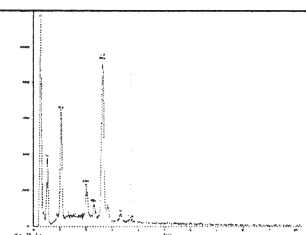
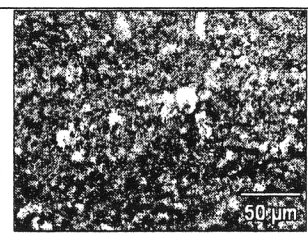
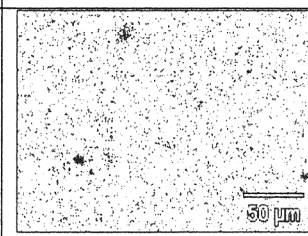
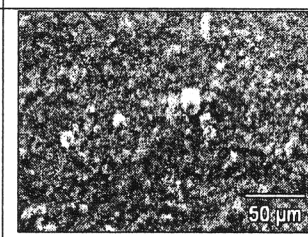
Substrate	Time in culture (h)				
	No cell	1 h	24 h	72 h	4 weeks
E-PCL					
E-PCL/HAp					
PLGA					

Table 7.2 Energy dispersive spectroscopy (EDS) to show amount of calcium

(A) Spectra of samples without cell (B) Spectra of samples with cultured cells (4 weeks) to show calcium peaks

(C) SEM images of hFOB cultured on each scaffold for 4 weeks (D) Images of calcium mapping of images (C)

(E) Overlaid images of (C) and (D)

Substrate	A	B	C	D	E
E-PCL					
E-PCL/HAp					
PLGA