CHAPTER V
NOVEL BONE SCAFFOLDS OF ELECTROSPUN POLYCAPROLACTONE FIBERS FILLED WITH NANOPARTICLES

ABSTRACT

Novel bone scaffolding materials were successfully fabricated by electrospinning from polycaprolactone (PCL) solutions containing nanoparticles of calcium carbonate ($CaCO_3$) or hydroxyapatite (HA). The potential use of the electrospun fibrous scaffolds for bone regeneration was evaluated *in vitro* with human osteoblasts (SaOS2) in terms of attachment, proliferation, and alkaline phosphatase (ALP) activity of the cells that were cultured directly on the scaffolds. The results were compared with those on corresponding solution-cast film scaffolds and tissue-culture polystyrene plate (TCPS). It was found that all of the fibrous scaffolds promoted much better adhesion and proliferation of cells than the corresponding film scaffolds and TCPS. Interestingly, the cells that were seeded on all of the fibrous scaffolds appeared to be well-expanded and attach on the fiber surface very well even only about 1 hr in culture, while those seeded on all of the film scaffolds and the glass substrate were still in round shape. Among the various fibrous scaffolds investigated, the one that was filled with 1.0% HA showed the highest ALP activity. Finally, all of the fibrous scaffolds exhibited much greater tensile strength at yield than all of the corresponding film scaffolds.

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

1. INTRODUCTION

Electrostatic spinning or electrospinning is a process by which ultra-fine fibers with diameters in the range of nanometers to sub-micrometers can be fabricated. The basic principles of the process involve an application of a high electrical potential to a polymer solution or melt across a finite distance between a conductive nozzle and a collector. When an electrostatic field is applied, charges are accumulated on the surface of a pendant droplet of the polymer solution or melt at the tip of the nozzle. The charges destabilize the partially-spherical shape of the droplet into a conical shape at a critical value of the applied electrostatic field. Further increase in the applied electrostatic field causes a charged stream of the polymer solution or melt (i.e. the charged jet) to be ejected from the apex of the cone when the repulsion force between mutual charges (i.e. Coulombic repulsion force) overcomes the surface tension. 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 their physical uniqueness, ultrafine electrospun fibers have been proposed as ideal materials for many biomedical applications, including drug delivery,[1,2] wound healing,[3,4] and tissue scaffolding.[5,6] The important advantages of electrospun fibers in scaffolding applications are the morphology and size of the fibers that can be easily controlled by carefully adjusting the solution properties and/or the process parameters, the very high surface area-to-volume or mass ratio, and the high porosity of the electrospun fiber mats that could promote better cell incorporation. An important aspect of electrospun fibrous scaffolds is to mimic the fibrillar structure of the natural extracellular matrix (ECM). ECM is composed of grounded substance (i.e. proteoglycans) and fibrous proteins (collagens). Collagens embedded as a three-dimensional (3-D) fibrous network linking with proteoglycans. Therefore, electrospun fibrous scaffolds may be an ideal structure that provide a 3-D structure similar to the natural system.

Despite such promises, selection of the materials for fabrication into scaffolds is very important. The scaffolds have to replace the natural ECM until the host cells can regenerate and synthesize a new matrix. Successful *in vitro* cell

culture on ultrafine fibrous scaffolds have been demonstrated on a number of synthetic, biodegradable polymers, e.g. polylactide (PLA), polyglycolide (PGA), polycaprolactone (PCL), and co-polymers of these polymers.[6,7] PCL, from various fabrication techniques, has been tested as scaffolding materials for cartilage,[5] skin,[8] and bone cells.[9-11] Moreover, improvement in the proliferation and differentiation of fibroblasts within a scaffold has been done by incorporating a type of calcium phosphate species such as hydroxyapatite within the scaffold.[9,12,13] The electrospun PCL/calcium carbonate composite membranes have been tested with osteoblasts and exhibited good cell attachment and proliferation.[10] The presence of either calcium carbonate or hydroxyapatite nanoparticles in the scaffolds also help improve their mechanical stability.[14]

In the present contribution, composite fiber mats of polycaprolactone (PCL) and calcium carbonate ($CaCO_3$) or hydroxyapatite (HA) nanoparticles were prepared by electrospinning. HA nanoparticles were synthesized by hydrolysis following a method proposed recently by Shih et al.[15] The potential use of the electrospun composite fiber mats as scaffolding materials for bone regeneration was evaluated *in vitro* with human osteoblasts (SaOS2), in which the attachment, proliferation, and alkaline phosphatase (ALP) activity of the cells were analyzed. The mechanical integrity in terms of the tensile strength at yield of the scaffolds was also investigated. Both biological and mechanical properties of the as-prepared fibrous scaffolds were tested in comparison with those of the corresponding solution-cast films.

2. EXPERIMENTAL DETAILS

2.1 Materials

Materials used in the fabrication of the scaffolds were polycaprolactone (PCL; $\overline{M}_n$ = 80,000 g/mol; Aldrich, USA), dichloromethane (Carlo Erba, Italy), *N,N*-dimethylformamide (DMF; Lab-Scan (Asia), Thailand), calcium carbonate nanoparticles ($CaCO_3$; cubic form; average particle size = 40 nm; NanoMaterials

Technology, Singapore), hydroxyapatite powder (HA; synthesized following the method proposed in reference[15]). After hydrolysis from dicalcium phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$; Fluka Chemika, Switzerland), the HA powder was annealed at 800°C for 4 hr in air. The obtained HA 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 HA.[14] The mean particle size of the as-synthesized HA powder as analyzed by a JEOL JSM 5410LV scanning electron microscope (SEM) was 234 ± 68 nm, while a bimodal distribution of the particle sizes with the lowest mean value of about 500 nm was suggested by a MALVERN MastersizerX particle size analyzer.[14]

2.2 Preparation and characterization of scaffolds

For qualitative comparison, two types of scaffolds were prepared: electrospun fibrous mats and solution-cast films. Fibrous scaffolds were prepared by electrospinning from neat 12% w/v PCL solution in 50:50 v/v dichloromethane and DMF or the PCL solution loaded with either $CaCO_3$ or HA powder at a concentration of 0.5 or 1.0% w/v. In order to ensure good dispersion of the particles within the PCL solution, the particles and PCL pellets were first dispersed under mechanical stirring in DMF. After a certain period of time, dichloromethane, which is a good solvent for PCL, was added. The mixture was stirred until the pellets completely dissolved and it was subsequently sonicated prior to electrospinning. Five types of fibrous scaffolds were hereafter denoted as FB (neat as-spun PCL mat), FB_$CaCO_3$0.5 (as-spun mat from 12% w/v PCL/0.5% w/v $CaCO_3$), FB_$CaCO_3$1.0 (as-spun mat from 12% w/v PCL/1.0% w/v $CaCO_3$), FB_HA0.5 (as-spun mat from 12% w/v PCL/0.5% w/v HA), and FB_HA1.0 (as-spun mat from 12% w/v PCL/1.0% w/v HA).

Each of the spinning dopes 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 and size of the as-spun fibers was examined by SEM. At least 100 readings of the fiber diameters from at least 5 SEM images were statistically analyzed using a SemAphore 4.0 software, from which the arithmetic mean values were found to range between 0.95 and 1.26 μm.[14]

The fabrication of the solution-cast scaffolds was to investigate how the surface topography of the scaffolds affected the cell response. The film scaffolds were also fabricated from the PCL solution and the spinning dopes prepared for electrospinning. Five types of film scaffolds were hereafter denoted as CF (neat as-cast PCL film), CF_$CaCO_3$0.5 (as-cast film from 12% w/v PCL/0.5% w/v $CaCO_3$), CF_$CaCO_3$1.0 (as-cast film from 12% w/v PCL/1.0% w/v $CaCO_3$), CF_HA0.5 (as-cast film from 12% w/v PCL/0.5% w/v HA), and CF_HA1.0 (as-cast film from 12% w/v PCL/1.0% w/v HA). Both of the as-spun fiber mats and the as-cast films (about 130 μm in thickness) were dried *in vacuo* at 40°C overnight to remove as much solvent as possible. Mechanical integrity in terms of the tensile strength at yield for both types of the scaffolds was investigated using a Lloyd LRX universal testing machine (gauge length = 50 mm and crosshead speed = 10 mm/min) on the samples that were cut into a rectangular shape (10 mm × 70 mm).

2.3 Cell culture and cell seeding

Human osteoblasts (SaOS2) were cultured as monolayer in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA), supplemented by 10% fetal bovine serum (FBS; BIOCHROM AG, Germany), 1% L-glutamine (Invitrogen Corp., USA) and 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate, and amphotericin B (Invitrogen Corp., USA)]. The medium was replaced once in every 3 days and the cultures were maintained at 37°C

in a humidified atmosphere containing 5% CO_2. Each scaffold was cut into circular discs (about 15 mm in diameter) and the disc specimens were placed in wells of a 24-well tissue-culture polystyrene plate (TCPS; Biokom Systems, Poland), which were later sterilized in 70% ethanol for 30 min. The specimens were then washed with autoclaved de-ionized water and subsequently immersed in DMEM overnight. To ensure a complete contact between the specimens and the wells, the specimens were pressed with a metal ring (about 12 mm in diameter). SaOS2 from the cultures were trypsinized [0.25% trypsin containing 1 mM EDTA (Invitrogen Crop., USA)], counted by a hemacytometer (Hausser Scientific, USA), and seeded at a density of about 36,000 cells/cm^2 on the scaffold specimens and empty wells of TCPS that were used as controls. The cultures were maintained in an incubator.

2.3.1 Cell attachment and preliferation

For attachment study, SaOS2 were allowed to attach to the scaffold specimens and TCPS for 30 min, 1, 4, and 16 hr, respectively. At each specified seeding time, the number of the attached cells was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich, USA) assay. Each sample was rinsed with phosphate buffer saline (PBS; Sigma-Aldrich, USA) to remove unattached cells prior to MTT assay. For proliferation study, the cells were first allowed to attach on the specimens for 16 hr. The proliferation of cells on the specimens was determined after 1, 2, and 3 day(s), respectively. After attachment for 16 hr, the cells were starved by serum-free medium (SFM; containing the same composition as DMEM, but without FBS) twice (i.e. the medium was changed with SFM once after the 16 hr-attachment period and again after 3 hr). The number of cells was, again, quantified by MTT assay. Morphological appearance of the cells during attachment and proliferation period was observed by SEM.

2.3.2 Quantification of viable cells (MTT assay)

The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals

formed is proportional to the number of viable cells. First, each sample was incubated at 37°C for 1 hr with 250 μl/well of MTT solution at 0.5 mg/ml without phenol red. After incubation, MTT solution was removed. A buffer solution containing dimethylsulfoxide (DMSO; Carlo Erba, Italy) (900 μl/well) and glycine buffer (pH = 10) (125 μl/well) was added into the wells to dissolve the formazan crystals. After 10 min of rotary agitation, the solutions were then transferred into a cuvette and placed in a Thermospectronic Genesis10 UV-visible spectrophotometer, from which the absorbance at 540 nm representing the number of viable cells was measured.

2.3.3 Morphological observation of cultured cells

After removal of the culture medium, the cell-cultured scaffold specimens were rinsed with PBS twice and the cells were then fixed with 3% gluteraldehyde solution, which was diluted from 50% glutaraldehyde solution (Electron Microscopy Science, USA) with PBS, at 500 μl/well. After 30 min, they were rinsed again with and kept in PBS at 4°C. After cell fixation, the specimens were dehydrated in an ethanol solution of varying concentration (i.e. 30, 50, 70, 90, and 100%, respectively) for about 2 min at each concentration. The specimens were then dried in 100% hexamethyldisilazane (HMDS; Sigma, USA) for 5 min and later let dry in air after removal of HMDS. After completely dried, the specimens were mounted on an SEM stub, coated with gold, and observed by SEM.

2.3.4 Production of characteristic protein of cultured cells

SaOS2 were cultured on scaffold specimens for 5 or 10 days to observe the production of alkaline phosphatase (ALP). The specimens were rinsed with PBS after removal of culture medium. Alkaline lysis buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, 0.1% Triton-X100, pH 10) (100 μl/well) was added and the samples were scrapped and then frozen at -20°C for at least 30 min prior to the next step. An aqueous solution of 2 mg/ml *p*-nitrophenyl phosphate (PNPP; Zymed Laboratories, USA) mixed with 0.1 M amino propanol (10 μl/well) in 2 mM $MgCl_2$ (100 μl/well) having a pH of 10.5 was prepared and added into the specimens. The specimens

were incubated at 37°C for 2 min. The reaction was stoped by adding 0.9 ml/well of 50 mM NaOH and the extracted solution was transferred to a cuvette and placed in the UV-visible spectrophotometer, from which the absorbance at 410 nm was measured. The amount of ALP was then calculated against a standard curve. In order to calculate for the ALP activity, the amount of ALP had to be normalized by the amount of total protein synthesized. In the protein assay, the samples were treated in the same manner as the ALP assay up to the point were the specimens were frozen. After freezing, bicinchoninic acid (BCA; Pierce Biotechnology, USA) solution was added into the specimens. The specimens were incubated at 37°C for 2 min. The absorbance of the medium solution was then measured at 562 nm by the UV-visible spectrophotometer and the amount of the total protein was calculated against a standard curve.

3. RESULTS AND DISCUSSION

In a previous related work,[14] successful fabrication of electrospun mats of smooth and bead-free polycaprolactone (PCL) fibers with or without the presence of calcium carbonate ($CaCO_3$) or hydroxyapatite (HA) nanoparticles was reported and characterized. The diameters of the as-spun fibers were found to increase with the addition and increasing amount of the nanoparticles. Specifically, they were about 0.95 μm for FB (neat as-spun PCL mat), about 1.02 μm for FB_$CaCO_3$0.5 (as-spun mat from 12% w/v PCL/0.5% w/v $CaCO_3$), about 1.12 μm for FB_$CaCO_3$1.0 (as-spun mat from 12% w/v PCL/1.0% w/v $CaCO_3$), about 1.24 μm for FB_HA0.5 (as-spun mat from 12% w/v PCL/0.5% w/v HA), and about 1.26 μm for FB_HA1.0 (as-spun mat from 12% w/v PCL/1.0% w/v HA). The porosity of the fiber mats was also estimated from the difference between the densities of the mats and the bulk PCL to range between about 82 and 90%, while the average pore size ranged between 4.3 and 5.6 μm. Indirect cytotoxicity evaluation of the electrospun mats of PCL, PCL/$CaCO_3$, and PCL/HA fibers based on human osteoblasts (SaOS2) and mouse fibroblasts (L929) revealed that these fibrous mats posed no threats to the cells. In the present work, the potential use of these electrospun mats as scaffolding materials

for bone regeneration with respect to that of the corresponding solution-cast films was evaluated *in vitro* with SaOS2 in terms of the attachment, proliferation, and ALP activity of the cultured cells. The mechanical integrity of the fibrous scaffolds in comparison with that of the corresponding film scaffolds was also investigated.

3.1 Cell attachment and proliferation

Attachment of cells is one of the most important aspects of a scaffold. The number of cells attached on a scaffolding substrate could be quantified by the UV absorbance from the MTT assay. In the present work, two types of scaffolding materials, i.e. electrospun fibrous and solution-cast film scaffolds, were investigated. Figure 1 shows attachment of SaOS2 on TCPS (i.e. controls), neat and $CaCO_3$-filled film and fibrous scaffolds. Clearly, the attachment of SaOS2 on the fibrous scaffolds was significantly better than that on the film scaffolds and TCPS. On a given substrate, the attachment of SaOS2 increased significantly during the 1 hr in culture, while it either increased moderately or started to level off afterwards. Interestingly, the attachment of SaOS2 on the film scaffolds was practically equivalent to that on TCPS. At 16 hr in culture, the number of cells attached on the film scaffolds were lower than that on TCPS, with the number of cells attached on CF_$CaCO_3$1.0 being greater than that on CF_$CaCO_3$0.5 and CF, respectively. On the other hand, the number of cells attached on the fibrous scaffolds at 16 hr in culture can be ranked as follows: FB_$CaCO_3$0.5 > FB_$CaCO_3$1.0 ≈ FB.

Figure 2 shows attachment of SaOS2 on TCPS (i.e. controls), neat and HA-filled film and fibrous scaffolds. Similarly, the attachment of SaOS2 on the fibrous scaffolds was significantly better than that on the film scaffolds and TCPS and the attachment on a given substrate increased significantly during the 1 hr in culture, while it either increased moderately or started to level off afterwards. The attachment of SaOS2 on the film scaffolds was again equivalent to that on TCPS. At 16 hr in culture, the number of cells attached on the film scaffolds were again lower than that on TCPS, with the number of cells attached on CF_HA0.5 being greater than that on CF_HA1.0 and CF, respectively. On the other hand, the number of cells attached on the fibrous scaffolds at 16 hr in culture can be ranked as follows: FB >

FB_HA0.5 > FB_HA1.0. Comparison of the cell attachment on TCPS, FB, FB_$CaCO_3$1.0, and FB_HA1.0 is illustrated in Figure 3. Evidently, the attachment of SaOS2 on all of the fibrous scaffolds shown was far better than that on TCPS, with unfilled FB generally exhibiting much better attachment of cells than filled FB. At 16 hr in culture, the attachment of SaOS2 on these scaffolds can be ranked as follows: FB_$CaCO_3$1.0 ≈ FB > FB_HA1.0 > TCPS.

Proliferation of SaOS2 on TCPS (i.e. controls), neat and $CaCO_3$-filled film and fibrous scaffolds at day 1, 2, and 3 after being allowed for cell attachment for 16 hr (i.e. the attachment at 16 hr was taked as the proliferation at day 1) is shown in Figure 4. Apparently, the proliferation of SaOS2 on the fibrous scaffolds was significantly better than that on the film scaffolds and TCPS, while the proliferation of SaOS2 on the $CaCO_3$-filled film scaffolds was only better than that on TCPS at day 3. All of the substrates investigated exhibited a very high proliferation rate between day 1 and 2. Between day 2 and 3, all of the film scaffolds exhibited an increase in the proliferation rate, while all of the fibrous scaffolds showed a decrease in the proliferation rate, in comparison with that between day 1 and 2. The proliferation rate of TCPS, however, reached a plateau after day 2. The increased proliferation rate of the film scaffolds between day 2 and 3 in comparison with that between day 1 and 2 could be due to the low number of SaOS2 attached on them after 16 hr in culture, hence greater area for cell proliferation. This is in contrast to the observed decrease in the proliferation rate of the fibrous scaffolds. Fujihara et al.[10] showed that the proliferation of human osteoblasts (hFOB1.19; ATCC, USA) on $CaCO_3$-filled PCL scaffold (i.e. the ratio between PCL:$CaCO_3$ = 75:25 w/w) was very comparable with TCPS at day 1, 3, and 5, respectively and the proliferation rate was moderate between day 1 and 3, while, between day 3 and 5, the proliferation rate was found to increase.

Figure 5 illustrates proliferation of SaOS2 on TCPS (i.e. controls), neat and HA-filled film and fibrous scaffolds at day 1, 2, and 3 after being allowed for cell attachment for 16 hr. Evidently, the proliferation of SaOS2 on the fibrous scaffolds was considerably better than that on the film scaffolds and TCPS, while the proliferation of SaOS2 on the HA-filled film scaffolds was only better than that on TCPS at day 3. All of the substrates investigated exhibited a very high proliferation

rate between day 1 and 2. Between day 2 and 3, all of the film scaffolds exhibited an increase in the proliferation rate in comparison with that between day 1 and 2. Interestingly, HA-filled fibrous scaffolds exhibited a steady increase in the proliferation of the cells throughout the culture period investigated. This observation is in contrast to $CaCO_3$-filled fibrous scaffolds in which, between day 2 and 3, the proliferation rate was found to decrease in comparison with that between day 1 and 2. Comparison of the cell proliferation on TCPS, FB, FB_$CaCO_3$1.0, and FB_HA1.0 is illustrated in Figure 6. Apparently, the proliferation of SaOS2 on all of the fibrous scaffolds shown was far better than that on TCPS, with filled FB generally exhibiting much better proliferation of cells than unfilled FB. At 3 days in culture, the proliferation of SaOS2 on these scaffolds can be ranked as follows: FB_HA1.0 > FB_$CaCO_3$1.0 > FB > TCPS. Clearly, the presence of HA within the scaffolds should be responsible for the steady increase in the proliferation of SaOS2 of the HA-filled fibrous scaffolds.[9,12,13]

Table 1 shows SEM images of SaOS2 that were cultured on FB, FB_$CaCO_3$1.0, and FB_HA1.0, while Table 2 shows those of SaSO2 that were cultured on CF_HA1.0 and a glass substrate, at different times in culture. SEM images of neat as-spun scaffolds were also shown in Table 1 as references. According to these images, cell morphology and interaction between cells and the scaffolds can be visualized. Due to the similarity of the cells that were cultured on CF, CF_$CaCO_3$1.0, and CF_HA1.0, only the images of the cells that were cultured on CF_$CaCO_3$1.0 and CF_HA1.0 were shown. These images confirm that the phenotype of SaOS2 was maintained after they were seeded on different types of substrates. Interestingly, the cells that were cultured on all of the fibrous scaffolds were already expanded with evidence of anchoring ligands stretching out to help attach themselves on the fiber surface (especially for cells that were cultured on FB_HA1.0), while those cultured on all of the film scaffolds and the glass substrate were still in round shape. At longer times, the cells on all of the fibrous scaffolds expanded even more. For film scaffolds, most of the seeded cells were still in round shape after about 4 hr in culture, but became well expanded at long times in culture (i.e. after about 1 day). On the contrary, the cells that were cultured on the glass substrate were well expanded after about 4 hr. The obtained results suggest that the

fibrous scaffolds promote the attachment of SaOS2 and help maintain the integrity of the cells during culture much better than the film counterparts. Figure 7 illustrates low-magnification SEM images (i.e. 500x) of SaOS2 that were cultured on FB_HA1.0 after about 1 and 48 hr in culture, respectively. Apparently, a number of rather round cells were distributed throughout the scaffold after about 1 hr in culture, while a large number of well-expanded cells were distributed throughout the scaffold after about 48 hr in culture. In addition, the area on the scaffold that the cells occupied also increased with increasing time in culture (i.e. about 80% of the scaffold was occupied by the cells after about 48 hr in culture).

3.2 Alkaline phosphatase (ALP) activity

The ability for a scaffold to support differentiation, in addition to attachment and proliferation, of cultured cells is another important aspect suggesting actual applicability of the scaffold. Among the various biological functions of osteoblasts, secretion of alkaline phosphatase (ALP) is an important indicator determining the activity of the cells on a scaffold. The ALP activity of SaOS2 on TCPS (i.e. controls), FB, FB_$CaCO_3$1.0, and FB_HA1.0 was monitored at 5 and 10 days in culture (see Figure 8). Apparently, for all of the fibrous scaffolds investigated, the ALP activity at day 5 was much greater than that at day 10, while, for TCPS, a contrast result was observed. At either 5 or 10 days, the ALP activity on different substrates can be ranked as follows: TCPS > FB_HA1.0 > FB > FB_$CaCO_3$1.0. The observed highest ALP activity of SaOS2 on TCPS in comparison with other substrates should be due to the fact that proliferation of the cells, after 2 days in culture, on TCPS was found to be constant, while that on all of the fibrous scaffolds investigated still increased (i.e. the proliferation of SaOS2 on both FB and FB_$CaCO_3$1.0 was found to decrease very slightly, while that on FB_HA1.0 increased steadily after 2 days in culture). If we are to assume that the differentiation should begin as soon as the proliferation rate starts to decrease,[16] the amount of ALP synthesized from the cells after 2 days in culture on TCPS should be the highest, which is exactly what has been observed in this work. According to Figure 8, FB_HA1.0 showed the highest ALP activity among the various fibrous scaffolds

investigated, which, in addition to the results on cell proliferation (see Figure 6), suggests that FB_HA1.0 was the best among the fibrous scaffolds that promoted both proliferation and differentiation of SaOS2 particularly well. However, additional long term experiments in order to clarify the effect of scaffolds on the differentiation of osteoblasts are currently underway.

3.3 Mechanical integrity

Mechanical integrity of a scaffold is another important aspect requiring a careful consideration. The well-designed scaffold is not only capable of promoting attachment and proliferation and maintaining biological functions of the cultured cells, but also capable of maintaining its mechanical stability during the surgical period required to repair an injured tissue. Figure 9 shows tensile strength at yield of both neat and filled film and fibrous scaffolds. Clearly, the property value of all of the fibrous scaffolds was significantly greater than that of the film counterparts. While incorporation of the nanoparticles within the film scaffolds did not affect much the mechanical property of the scaffolds, their incorporation within the fibrous scaffolds was responsible for the observed improvement in the tensile strength at yield when compared with that of the neat one. Fujihara et al.[10] reported that the tensile strength of nanofibrous PCL membranes decreased from about 4.7 to 2.8 MPa with increasing the amount of $CaCO_3$ nanoparticles.

4. CONCLUSIONS

In the present contribution, electrospinning was used to fabricate novel bone scaffolding materials from electrospun mats of polycaprolactone (PCL) filled with nanoparticles of calcium carbonate ($CaCO_3$) or hydroxyapatite (HA). The potential use of the electrospun fibrous scaffolds for bone regeneration was evaluated *in vitro* with human osteoblasts (SaOS2) in terms of attachment, proliferation, and alkaline phosphatase (ALP) activity of the cells that were cultured directly on the scaffolds. The results were compared with those on corresponding solution-cast film scaffolds and tissue-culture polystyrene plate (TCPS). The obtained results showed that all of

the fibrous scaffolds exhibited much better adhesion and proliferation of cells than the corresponding film scaffolds and TCPS. Interestingly, the cells that were cultured on all of the fibrous scaffolds after only about 1 hr appeared to be well-expanded and attach on the fiber surface very well, while those seeded on all of the film scaffolds and the glass substrate were still in round shape. Among the various fibrous scaffolds investigated, the one that was filled with 1.0% HA showed the highest ALP activity. Finally, the tensile strength at yield for all of the fibrous scaffolds was significantly greater than that for all of the film counterparts, and the incorporation of the nanoparticles within the fibrous scaffolds helped improve the property value as compared with that of the neat one.

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CAPTION OF FIGURES

Figure 1 Attachment of SaOS2 on TCPS, neat and $CaCO_3$-filled film and fibrous PCL scaffolds as a function of time in culture.

Figure 2 Attachment of SaOS2 on TCPS, neat and HA-filled film and fibrous PCL scaffolds as a function of time in culture.

Figure 3 Comparison of SaOS2 attachment on various fibrous PCL scaffolds and TCPS.

Figure 4 Proliferation of SaOS2 on TCPS, neat and $CaCO_3$-filled film and fibrous PCL scaffolds as a function of time in culture.

Figure 5 Proliferation of SaOS2 on TCPS, neat and HA-filled film and fibrous PCL scaffolds as a function of time in culture.

Figure 6 Comparison of SaOS2 proliferation on various fibrous PCL scaffolds and TCPS.

Figure 7 Selected SEM images of SaOS2 cultured on FB_HA1.0 after 1 (a) and 48 (b) hr in culture at a magnification of 500 times (scale bar = 50 μm).

Figure 8 ALP activity of SaOS2 cultured on TCPS and various fibrous PCL scaffolds after 5 and 10 days in culture.

Figure 9 Mechanical integrity in terms of tensile strength at yield of neat and filled film and fibrous PCL scaffolds.

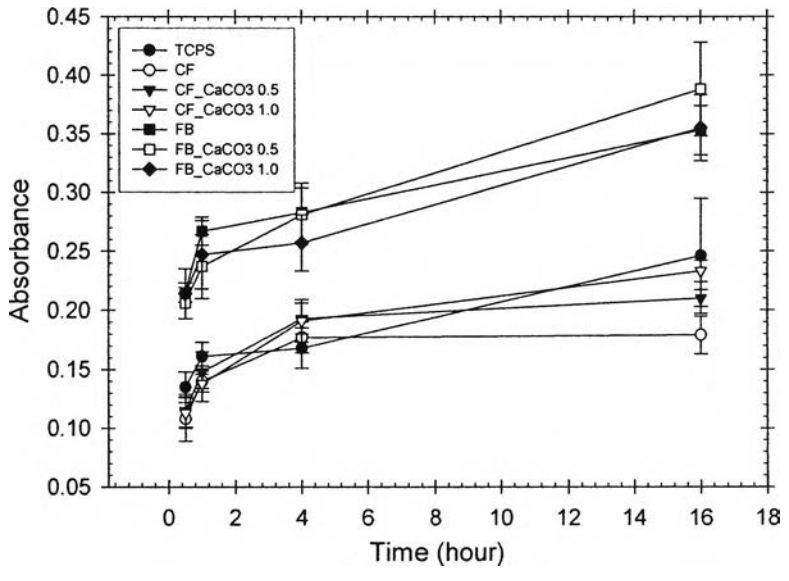


Figure 5.1 Attachment of SaOS2 on TCPS, neat and $CaCO_3$-filled film and fibrous PCL scaffolds as a function of time in culture.

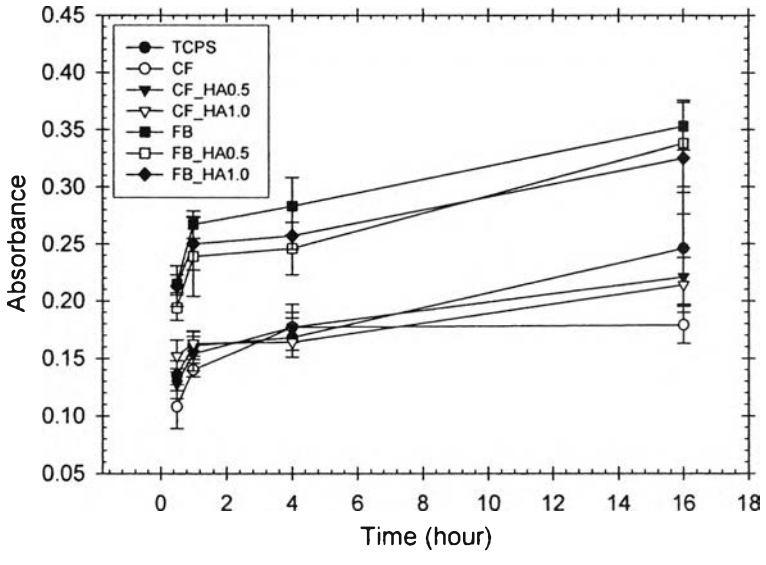


Figure 5.2 Attachment of SaOS2 on TCPS, neat and HA-filled film and fibrous PCL scaffolds as a function of time in culture.

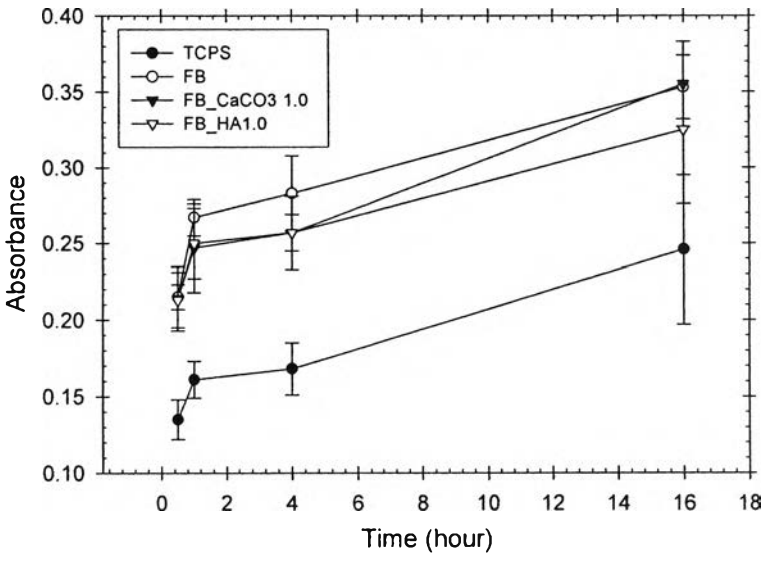


Figure 5.3 Comparison of SaOS2 attachment on various fibrous PCL scaffolds and TCPS.

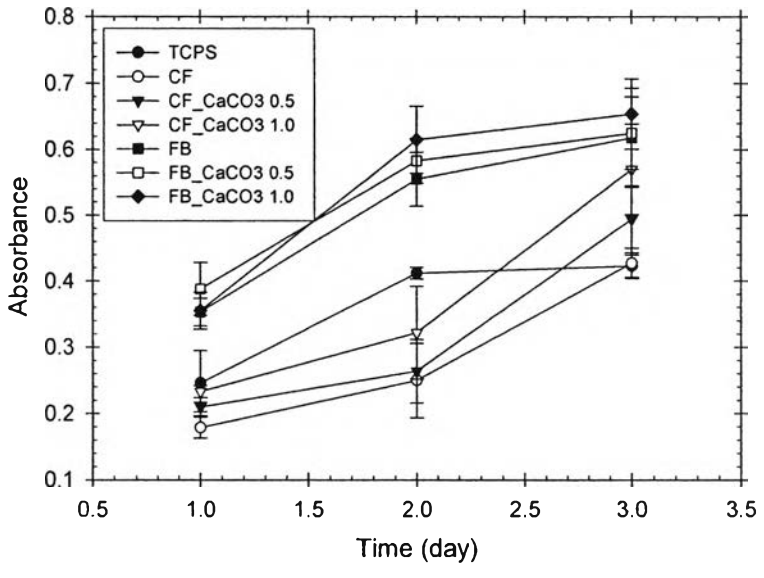


Figure 5.4 Proliferation of SaOS2 on TCPS, neat and $CaCO_3$-filled film and fibrous PCL scaffolds as a function of time in culture.

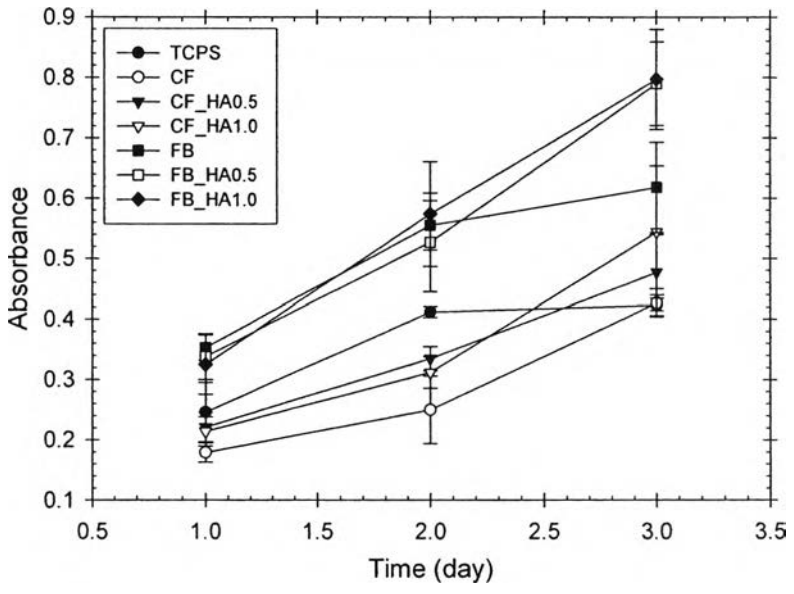


Figure 5.5 Proliferation of SaOS2 on TCPS, neat and HA-filled film and fibrous PCL scaffolds as a function of time in culture.

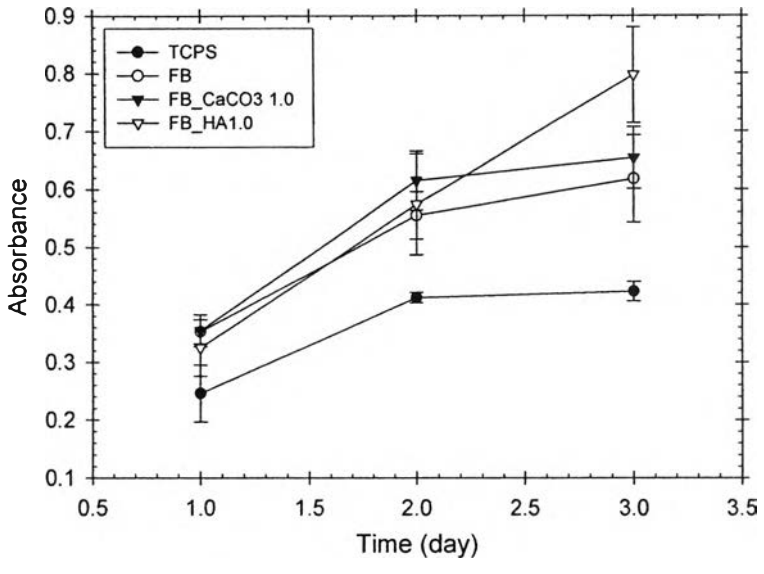


Figure 5.6 Comparison of SaOS2 proliferation on various fibrous PCL scaffolds and TCPS.

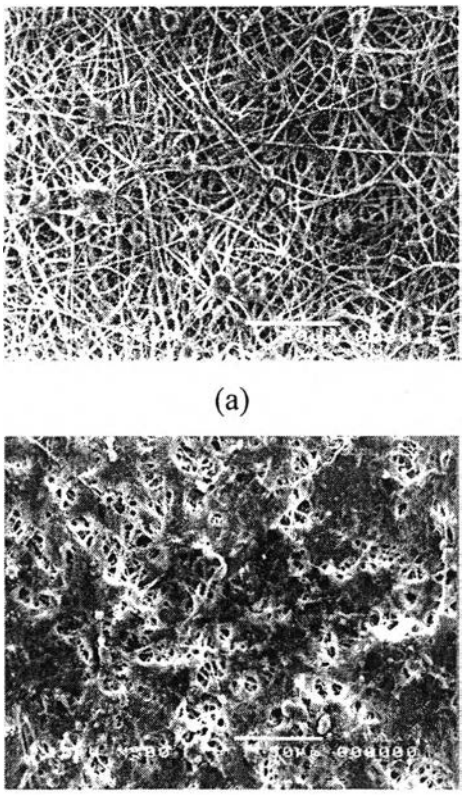


Figure 5.7 Selected SEM images of SaOS2 cultured on FB_HA1.0 after 1 (a) and 48 (b) hr in culture at a magnification of 500 times (scale bar = 50 μm).

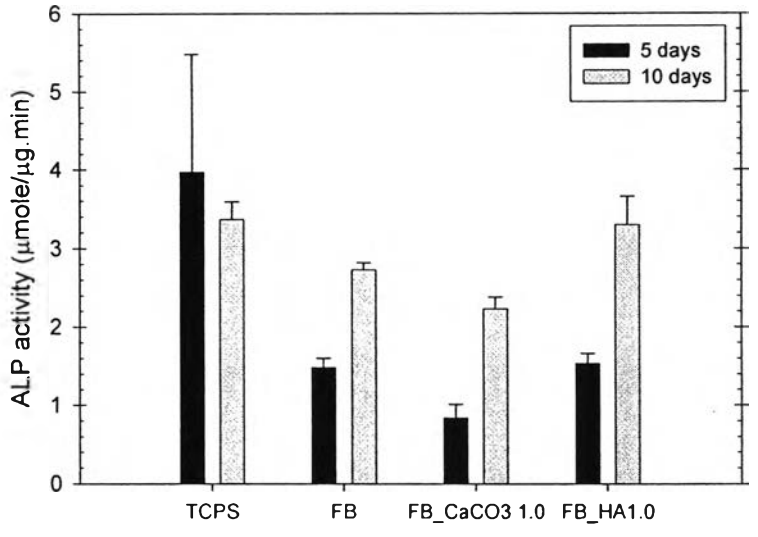


Figure 5.8 ALP activity of SaOS2 cultured on TCPS and various fibrous PCL scaffolds after 5 and 10 days in culture.

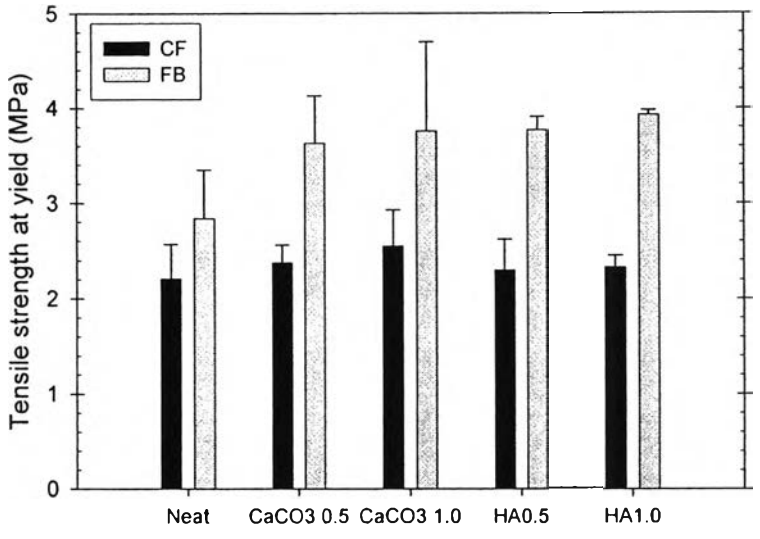


Figure 5.9 Mechanical integrity in terms of tensile strength at yield of neat and filled film and fibrous PCL scaffolds.

Table 5.1 Selected SEM images of SaOS2 cultured on various types of fibrous PCL scaffolds at different times in culture

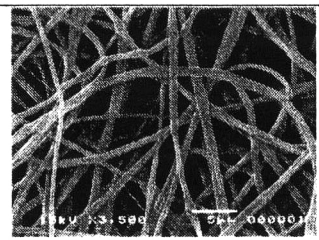
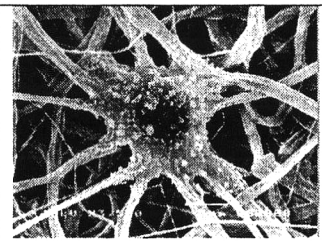
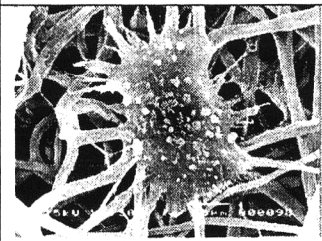
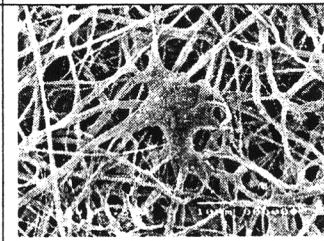
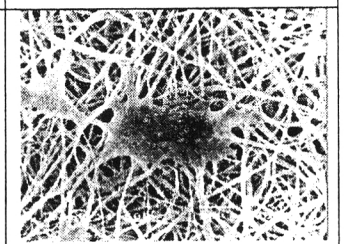
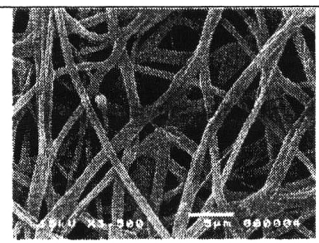
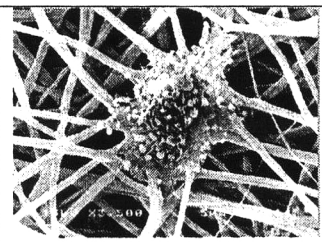
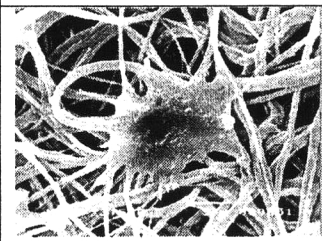
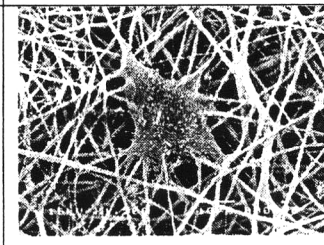
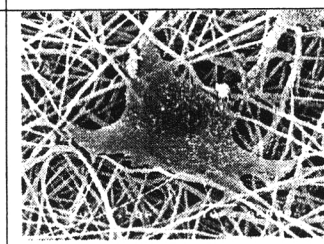
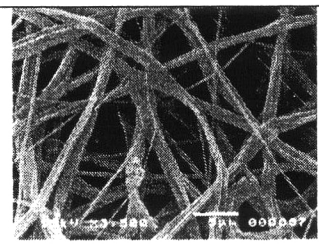
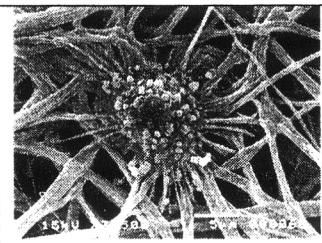
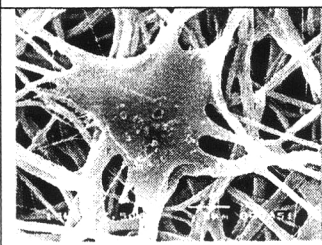
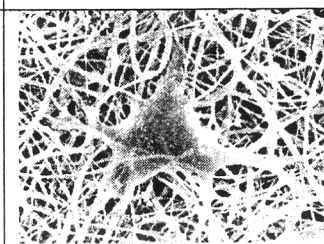
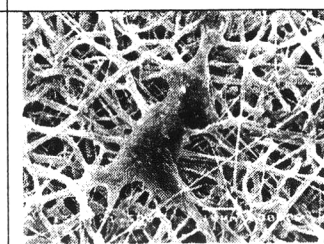
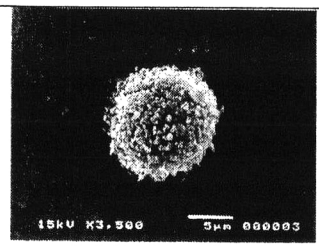
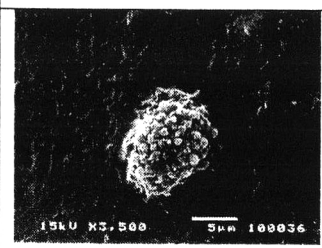
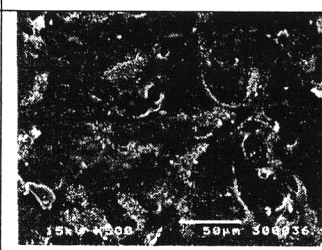
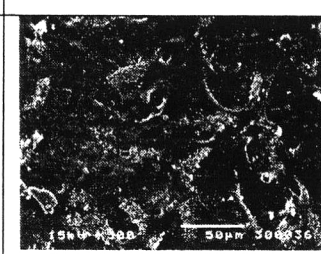
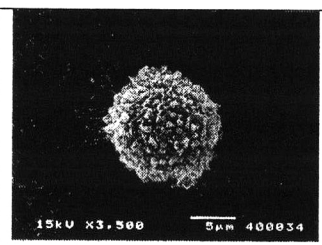
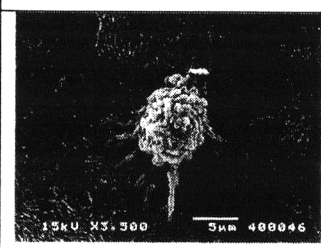
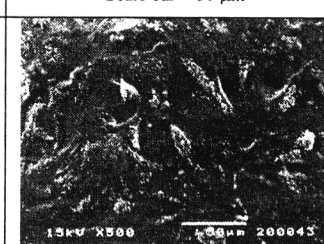
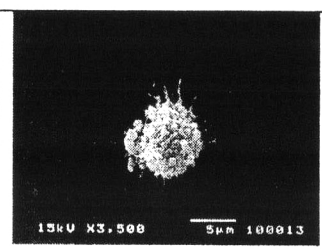
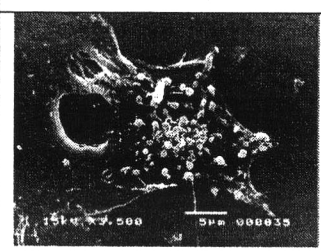
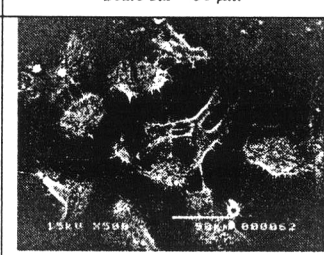
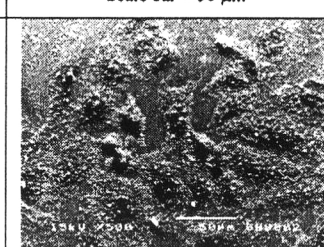
Substrate	Time in culture (hr)				
	As-spun	1	4	24	48
FB	 Scale bar = 5 μm	 Scale bar = 5 μm	 Scale bar = 5 μm	 Scale bar = 10 μm	 Scale bar = 10 μm
FB_$CaCO_3$1.0	 Scale bar = 5 μm	 Scale bar = 5 μm	 Scale bar = 5 μm	 Scale bar = 10 μm	 Scale bar = 10 μm
FB_HA1.0	 Scale bar = 5 μm	 Scale bar = 5 μm	 Scale bar = 5 μm	 Scale bar = 10 μm	 Scale bar = 10 μm

Table 5.2 Selected SEM images of SaOS2 cultured on film scaffolds filled with CaCO3 or HA and glass substrate at different times in culture

Substrate	Time in culture (hr)			
	1	4	24	48
CF_$CaCO_3$1.0	 Scale bar = 5 μm	 Scale bar = 5 μm	 Scale bar = 50 μm	 Scale bar = 50 μm
CF_HA1.0	 Scale bar = 5 μm	 Scale bar = 5 μm	 Scale bar = 50 μm	 Scale bar = 50 μm
Glass	 Scale bar = 5 μm	 Scale bar = 5 μm	 Scale bar = 50 μm	 Scale bar = 50 μm