Fabrication of porous hydroxyapatite-gelatin scaffolds crosslinked by glutaraldehyde for bone tissue engineering

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Abstract
In this study, to mimic the mineral and organic components of natural bone, hydroxyapatite[HA] and gelatin[GEL] composite scaffolds were prepared using the solvent-casting method combined with a freeze drying process. Glutaraldehyde[GA] was used as a cross linking agent and sodium bisulfite was used as an excess GA discharger. Using this technique, it is possible to produce scaffolds with mechanical and structural properties close to those of the natural trabecular bone. The prepared scaffold has an open, interconnected porous structure. It was found that the GEL/HA ratio with a 50 wt% (weight percent) HA has the compressive modulus, the ultimate compressive stress and elongation similar to those for the trabecular bone. The chemical bonding and the microstructure of the composites were investigated by FT-IR (Fourier Transform Infra Red), SEM (Scanning Electron Microscopy) and Light microscopy, indicating the presence of bonds between Ca^{2+} ions of HA and $R\text{-}COO^-$ ions of GEL in the HA-GEL composite scaffolds. It was found that the addition of HA content can reduce the water absorption and porosity of scaffold. The porosity and the apparent density of 50 wt% HA scaffold were also calculated. The biological responses of scaffolds were examined in L929 fibroblast cell culture, showed partially proliferation of cells around and on the composite surface.
Keywords: *Scaffolds; Hydroxyapatite; Gelatin; Composite; Bone tissue engineering; Glutaraldehyde*

INTRODUCTION

Tissue engineering offers a new and promising approach to the creation of biological alternatives for implants and prostheses (Langer and Vacantri, 1999). A key component in tissue engineering for bone regeneration is the scaffold that serves as a template for cell interactions and the formation of bone-extracellular bone matrix to provide structural support to the newly formed tissue. Scaffolds for the regeneration should meet certain criteria to serve this function, including mechanical properties similar to those of the bone repair site, biocompatibility and biodegradability at a rate commensurate with remodeling (Karageorgiou and Kaplan, 2005). Bone tissue is composed of minerals and proteins. The minerals are mostly apatites such as hydroxyapatite (HA: $Ca_{10}(PO_4)_6(OH)_2$, fluorapatite and carbonate-apatite (Gineste *et al*., 1999). In general HA is the main component of bone mineral, however, in some cases like dental enamel carbonate-apatite is the main hard tissue component, (Sonju Clasen and Ruyter, 1997). HA is widely accepted as a bioactive material for guided regeneration (Cerroni *et al.*, 2002). It has excellent biocompatibility with hard tissues (Wozney and Rosen, 1998; Suchanek and Yoshimura, 1998), high osteoconductivity and bioactivity despite its low degradation rate and mechanical strength (Asashina *et al.*, 1997; Chang *et al.*, 2003). Calcified tissue, such as long bone and jaw bone is considered a biologically and chemically bonded composite between HA and type-I collagen (Rose, 1985). Collagen is biocompatible, biodegradable and osteoinductive, acting as an excellent delivery system for bone morphogenetic proteins (BMPs) (Reddi, 1998; Chevallay and Herbage, 2000). Gelatins are compositionally virtually identical to the collagen from which they are derived. They have been shown to be biocompatible and resorbable. GEL is readily assimilated in the body (Cooper and Falb, 1968). A composite

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scaffold of HA and gelatin is therefore expected to show increased osteoconductivity and biodegradation with sufficient mechanical strength (Bigi *et al*., 1988). One of the major problems in practice with type-I collagen is its cost and the poor definition of commercial sources of this material. Therefore in this study, collagen type-I was replaced by GEL. Most tissue engineering approaches to the restoration and repair of damaged tissues require a scaffold material upon which cells can attach, proliferate, and differentiate, into a proper tissue (Atala and Lanza, 2001). Thanks to use of the sophisticated methods, various HA base composites have been developed with improved mechanical properties, HA/poly (ε-caprolactone) (Kim *et al.,* 2004), HA/chitosan-GEL (Zhao *et al.*, 2002), HA/collagen (Lickorish *et al*., 2004), HA/poly(lactide-co-glycolide) (Chen *et al*., 2001) etc. are examples of such HA base composites. Solvent casting is a simple method for fabricating constructs in tissue engineering. In this method, the polymer is dissolved in a suitable solvent and poured into a mold. The solvent is then removed, leaving the polymer in the desired shape. The major goal in fabricating scaffolds for tissue regeneration is to accurately control pore size and porosity. By using the freeze drying method, the liquid state structure of composite is locked and the solvent is removed (Whang *et al.*, 1995; Healy *et al.,* 1998). To mimic the mineral and organic component of natural bone, a bone like scaffold composite can be produced by dispersing particulate hydroxyapatite (HA) throughout a gelatin matrix. Our objective in this study is to characterize the prepared HA-GEL composites. Glutaraldehyde can be used as a crosslinking agent for making gelatin-tricalcium phosphate composites rendering them no longer water soluble. Although GA is a known cytotoxin, its use as a crosslinking agent in the preparation of possible large bone substitutes has been judged (Lin *et al.,* 1998), to be acceptable upon implantation evaluations. In any case residues of this agent can be removed by drying the treated material to 100°C, as well as by treatment with sodium bisulfite (Usta *et al.,* 2003).

MATERIALS AND METHODS

Materials used in this study were microbiological gelatin (Merck Inc. 4070), bioceramic grade hydroxyapatite (Merck Inc. 2196) and deionized water [DI] as GEL solvent. A 25% (V/V) solution of glutaraldehyde (Merck Inc. 2927) was used as the crosslinking agent. The KH_2PO_4 (BDH Chemicals Inc. 10203), $Na_2HPO_4.12H_2O$ (Merck Inc. 106573), KCl (Merck Inc. 4936) and NaCl (Merck Inc. 106400) were purchased for the preparation of phosphate buffer saline (PBS).

The slurry composites were prepared using the solvent casting method, a definite amount of GEL (12.33 wt%) was dissolved in DI at 45°C (Usta *et al.,* 2003). As dry GEL is essentially intractable material, they can readily become castable or shapeable when transformed into a sol-gel state by dissolution in water up to approximately 5-30 wt% (Usta *et al*., 2003). In order to have a homogenous and stronger composite the HA particles finer than 75 μm were obtained using a sieve with mesh No. 200. The 30 wt%, 40 wt% and 50 wt% of HA were added to 12.33% (V/V) gelatin then. The reinforced slurry composite was then stirred and incubated at 45°C in a water bath for 1h. To avoid air bubbles, the slurry was injected immediately by using a syringe into cylindrical Teflon molds (15 mm in height and 10 mm in diameter). The molds were chosen bigger in size to compensate for shrinkage and skin occurrence. The molds were frozen at -70°C and dried in a commercial freeze-dryer for 6 h to remove the solvent. The white composites were maintained at room temperature for 24 h, and then immersed in an 8% (V/V) solution of GA for 3 h. The cross linked composites had a bright brown color. To remove the residues of the GA agent, the cylinders were immersed and rinsed in DI for 24 h, during which time the water was refreshed every 6 h. Besides, the sodium bisulfite (SBS) with a concentration 3% (V/V) was used to discharge the excess GA within 10 minutes at room temperature. Since in the freeze-drying technique a surface skin occurs, therefore to achieve a porous structure the surface of the scaffolds were sand blasted, The obtained scaffolds were 12 mm in height and 6 mm in diameter, in accordance with the compression mechanical test guidelines set in ASTM F 451-95. Four samples of each type were investigated in all analysis and the average was reported.

Infrared analysis: FT-IR spectroscopy (Thermonicholet NEXUF870) was used to estimate the conformational change in structure of the HA-GEL composites cross-linked by GA (Payne and Veis, 1988; Doyle, 1975).

Mechanical properties: one of the major problems for mechanical characterization of porous ceramic scaffolds is the difficulty in machining and gripping the specimen; hence the conventional methods of mechanical characterization such as tensile, biaxial and impact testing are usually inapplicable to porous materials (Curry, 1970). Instead, the compression test

has been widely accepted and used successfully for characterization of cancellous bone and porous HA (Hodgskinson and Currey, 1990; Hing *et al.*, 1999). The compressive strength, Young's modulus and elongation of composites were measured at 25°C. Scaffolds were tested with an Instron materials testing machine (model 1195, Instron Corp., UK) using a cross-head speed of 1 mm min^{-1} with 1000 N load cell. As mentioned before the samples were cylinders of approximately 6 mm in diameter and 12 mm in height.

Morphology: the morphology and microstructure of the scaffolds were examined using a SEM LEO 440i at 10 or 15 kV and light microscopy. The scaffolds were cut by a razor and polished and then were gold-sputtered (POLARION SC7610) before scanning.

Porosity and density: a liquid displacement method was used to measure the porosity and density of 50 wt% HA scaffold. The density measurements provided information about pore size and distribution, permeability, and presence of structural faults in sintered ceramic structures (Hodgskinson and Currey, 1990). A scaffold with measured weight (W) was immersed in a graduated cylinder containing a known volume (V_1) of ethanol. The cylinder was placed in a vacuum to force the ethanol into the pores of the scaffold until no air bubble emerged from the scaffold. The total volume of the ethanol and scaffold was then recorded as V_2. The difference volume (V_2-V_1) represented the volume of the skeleton of the scaffold. The scaffold was removed from the ethanol and the residual ethanol volume was measured as V_3. The total volume of the scaffold, *V*, was then

$$V = V_2 - V_3$$

The apparent density of the scaffold, ρ was evaluated as,

$$\rho = \frac{W}{(V_2 - V_3)}$$

The porosity of the open pores in the scaffold ε was evaluated as (Hodgskinson and Currey, 1990),

$$\varepsilon = \frac{(V_1 - V_3)}{(V_2 - V_3)}$$

Cytotoxicity evaluation: the mouse L929 fibroblast cells were used as a test mode in this study. The cells were maintained in RPMI-1640 growth medium supplemented with 100 IU ml^{-1} penicillin, 100 μg ml^{-1}, streptomycin (Gibco BRL Laboratories) and 10% (V/V) fetal calf serum (FCS, Gibco BRL). A routine subculture was used to maintain the cell line. The cells were incubated in a humidified atmosphere of 5% (V/V) CO_2 at 37°C. A cell suspension of 4×10^5 cells/ml was prepared before seeding. The samples were sterilized in 70% (V/V) alcohol and washed in culture medium before the cell seeding procedure. 1 ml cell suspensions were then placed in a polystyrene multi well tissue culture plate with 24 wells (Nunc, Denmark). 4 wells kept as a negative control and the plate was then maintained in the incubator for 48 h. After incubation the samples were removed from the incubator and washed immediately in PBS. The attached cells were determined using the image processing system (×100 magnification, Nikon ECLIPSE TE2000-U).

RESULTS

Infrared analysis: The FT-IR spectrum of the GEL and 30 wt%, 40 wt%, 50 wt% HA-GEL composites were shown in Figure 1. The composite spectrum is very similar to the spectra of real bone (Paschalis *et al.*, 1996; Evans *et al.*, 1992; Socrates, 2001). The band at 1337 cm^{-1} in the GEL spectrum is attributed predominantly to the wagging vibration of proline side chains. The 1337 cm^{-1} peak of the GEL spectrum is one of a number of bands in the range of 1400-1260 cm^{-1} which are attributed to the presence of type-I GEL (Chang *et al.*, 2003; Socrates, 2001). The amide A band arising from N-H stretching was distributed at 3270-3370 cm^{-1} with the degree of cross-linking, C-H stretching at ~2920 cm^{-1} for the amide B, C=O stretching at 1670-1650 cm^{-1} for the amide I, N-H deformation at 1500-1550 cm^{-1} for the amide II (Epaschalis *et al.*, 1997; Socrates, 2001). The appearance of an amide I mode indicated that the HA-GEL composites adopt a predominantly α-helical configuration and this is confirmed by the appearance of amide II at ~1540 cm^{-1} (Payne and Veis, 1988; Doyle, 1975; Socrates, 2001). As HA related bands, there are OH stretching (4000-3200 cm^{-1}) and liberational (~600 cm^{-1}) bands, and phosphate contours. There are carbonate CO_3 ν3 bands at 1580-1400 cm^{-1} and 1530-1320 cm^{-1}, the CO_3 ν2 band is located at 890-800 cm^{-1} (Myung and Junzo, 2002).

The phosphate band is between 900 and 1200 cm^{-1}. The shift of the 1337 cm^{-1} band in the GEL spectrum has been effectively used to confirm the chemical bond formation between carboxyl ions in the GEL and HA phases (Kikuchi *et al.*, 1999; Chang *et al.*, 2002; Socrates, 2001).

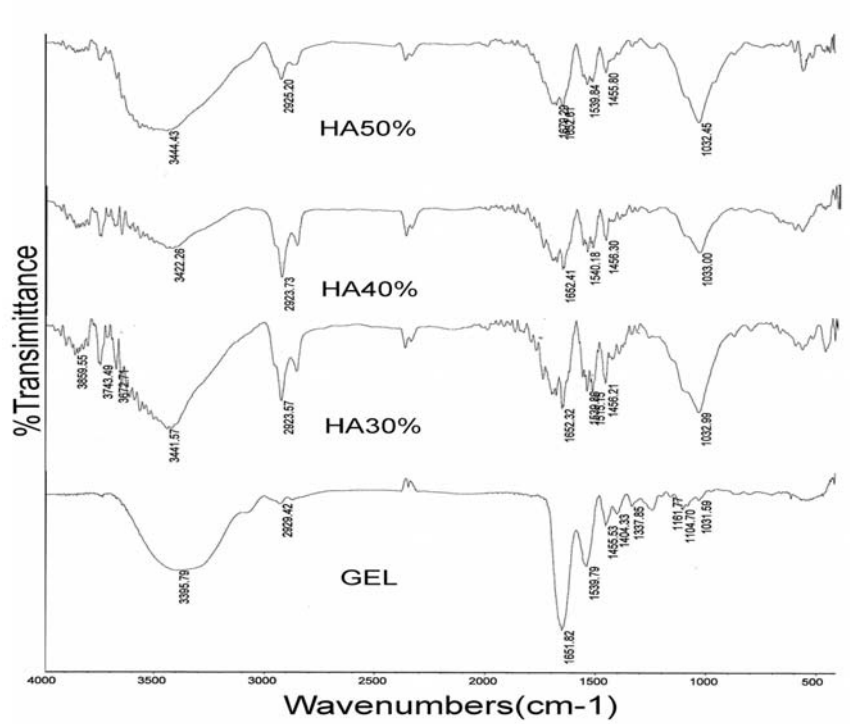

Figure 1. FT-IR spectra for GEL and 30 wt%, 40 wt%, 50 wt% cross-linked HA-GEL composites.

Mechanical properties: The Young's module was defined by the slope of the initial linear portion of the stress-strain curve. The ultimate compressive strength was determined from the stress-strain curve by applying the load until the scaffold was cracked. The compressive modulus of HA-GEL scaffolds increased with HA content (Fig. 2 and 3). The 30 wt%, 40 wt% and 50 wt% composites had compressive modulus of 2.3, 7.3 and 10.2 GPa respectively and the ultimate compressive strengths were 9.7, 25.6 and 32.1 MPa respectively. The obtained ultimate elongation also varied from 3.1-4% which decreased with HA content.

Morphology: It is observed in Figures 4 and 5 that the pores in the scaffolds are interconnected and range from 80 to 400 μm.

Porosity and density: The apparent density of a porous scaffold can influence its mechanical strength, permeability, and presence of structural defects (Spulveda *et al.*, 2000). The measured density and porosity of HA-GEL composite scaffolds prepared with slurries of different HA concentrations using the method shown before, were less than 1.17 g/cm^3 and higher than 70% respectively. The apparent density of

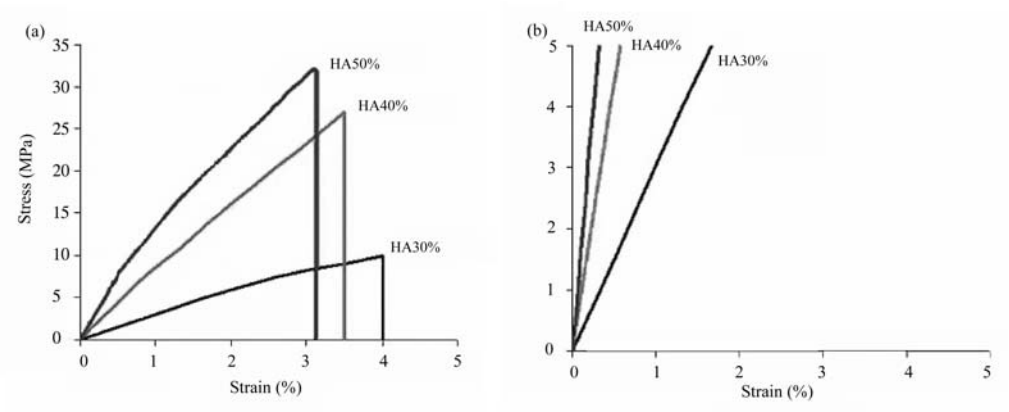

Figure 2. (a) Typical stress-strain curves recorded from HA30%, HA40% and HA50% composites (b) initial regions of the stress-strain curves.

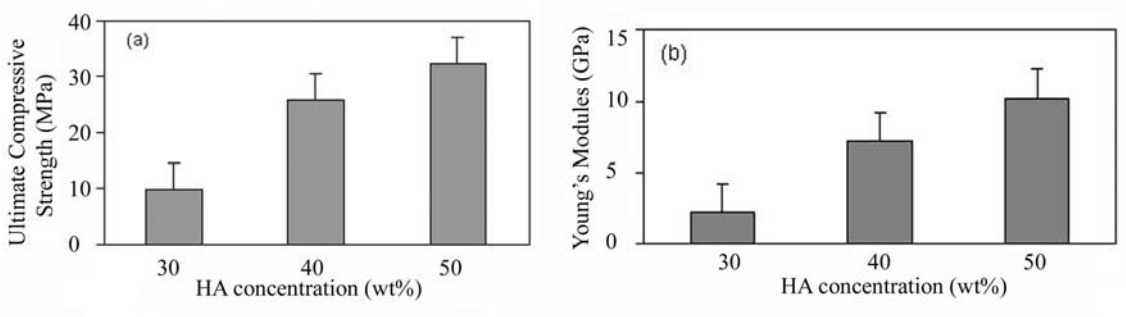

Figure 3. Comparison of (a) ultimate compressive strength and (b) Young's modulus of different HA contents composites.

trabecular bone ranges from 0.14 to 1.10 g/cm^3 (average: 0.62 ± 0.11 g/cm^3) (Liebschner, 2004).

Cytotoxicity evaluation: Cell culture experiments were carried out to test the biocompatibility of scaffolds. By using sodium bisulfite which is safe for biomedical use, the problem of residual cross-linking agent (GA) can be resolved. Rarely cellular degeneration or death are observed in the standard cytotoxicity assays of the biomaterial samples studied. Cells exhibited rather good proliferation and partially covered the composite surface (Fig. 6).

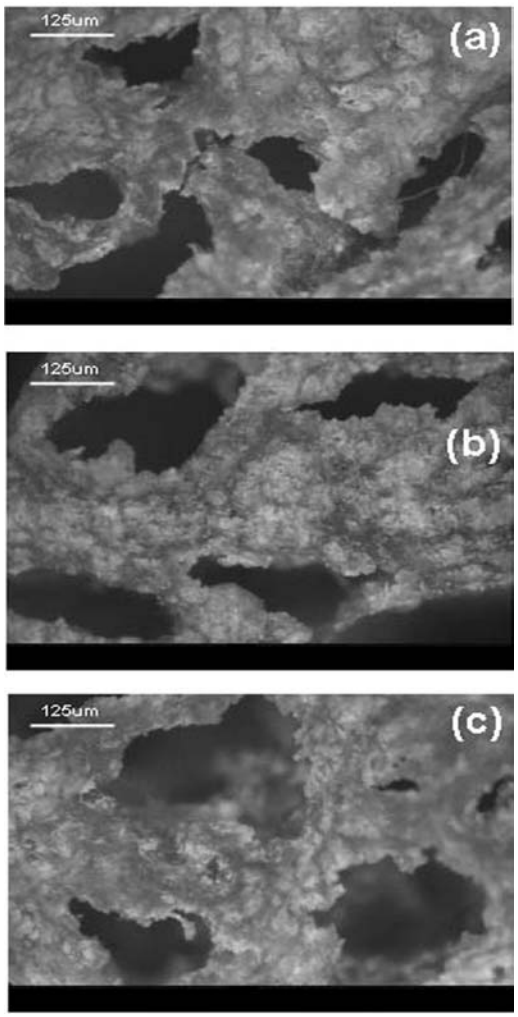

Figure 4. Morphology of the composite scaffolds under light microscopy (a) HA30% (b) HA40% and (c) HA50%.

DISCUSSION

In this work a method of producing a three dimensional, open-cells composite scaffold of HA-GEL was developed. The technique involves the solvent-casting method combined with a freeze drying process. A compressive Young's modulus of 10.2 GPa, ultimate compressive strength of 32.1 MPa and an apparent density of 1.17 g/cm^3 for the scaffold with HA concentration of 50 wt% were achieved which are comparable to that of trabecular bone. For human trabecular bone, the maximum Young's modulus (E) belongs to the femoral head which is 900 ± 710 MPa with a 9.3 ± 4.5 MPa compressive strength. For the cortical bone the maximum compressive E belongs to Tibia ranges using from 24.5-34.3 GPa, with a 166 MPa compressive strength. Scaffolds Ultimate elongation varied from 3.1-4%, for human bone, compressive strain rates range from -7000 to -34000 με/s which are normally about 2.9%, (Liebschner, 2004). The FT-IR spectrum for the cross-linked HA-GEL composite indicates chemical bond formation between the carboxyl ions in the GEL and HA phases. Moreover, the cross-linking induces the shortening of the distance between HA-GEL fibrils within the critical length and so a higher concentration of the Ca^{2+} ions on HA will have a chance to bind with $R\text{-}COO^-$ ions of GEL molecules (Chang *et al.*, 2002; Socrates, 2001). The prepared scaffolds are porous, with porosity higher than 70% and have pores ranging from 80 to 400 μm. Since one osteoblast occupies an area of approximately 700 μm^2 (Thomson *et al.*, 1998), the pore size of 500 μm is compatible for osteoconduction (Flatley *et al.*, 1983),

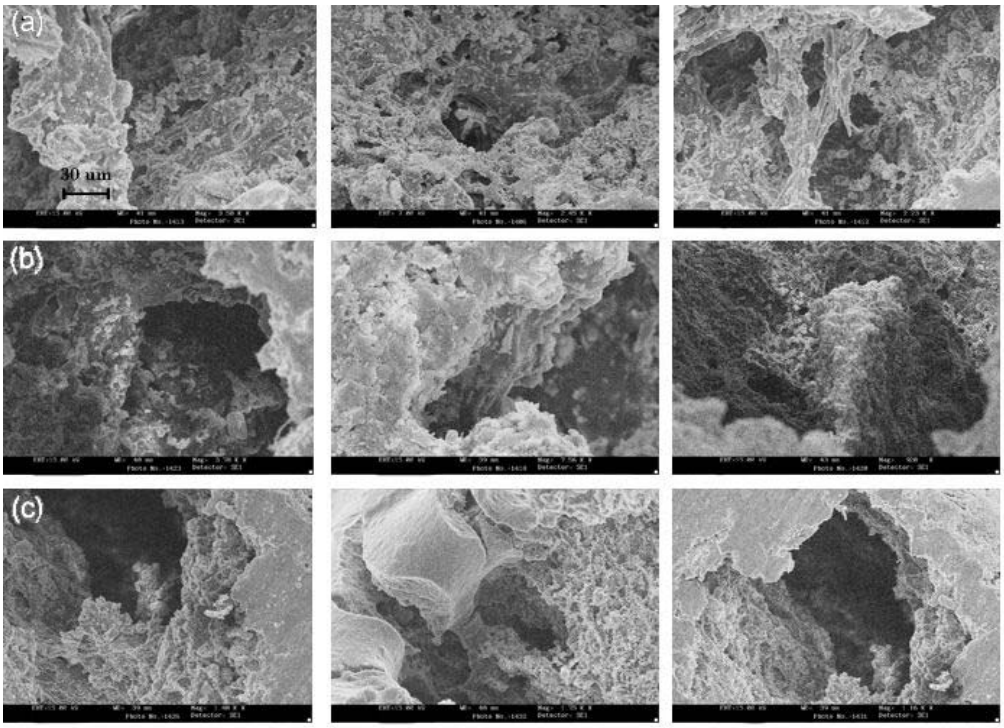

Figure 5. SEM micrographs of pores from the cross-section of different HA-GEL scaffolds. Raw (a) 30 wt% HA, raw (b) 40 wt% HA, raw (c) 50 wt% HA.

however the optimum pore size for osteoconduction is 150 μm (Hulbert *et al.*, 1972). This shows that the scaffold pores are sufficiently large to accommodate the cells. Porosity characterization is based on the presence of open pores which are related to properties such as permeability and surface area of the porous structure. It was found that the addition of HA result in more dense and thicker pore walls with lower porosity, therefore addition of HA content improves the mechanical properties. Since a higher density usually leads to higher mechanical strength while a high porosity provides a favorable biological environment, a balance between the porosity and density for a scaffold must be established for the specific application.

In cytotoxicity evaluation, the scaffolds exhibited good tissue compatibility to the L929 fibroblast cell culture. It was observed that cells cultured in scaffolds could partially attach, spread, and proliferate in Petri dish containing samples, and the scaffold surface were covered by fibroblast cells as well.

Acknowledgments

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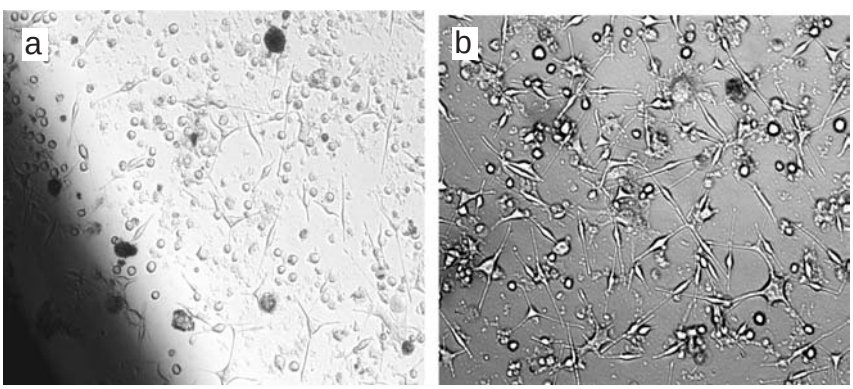

Figure 6. Fibroblast cells partially proliferated and covered (a) the scaffold surface and (b) in a Petri dish containing sample 48 h after seeding.

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