Surface-Engineered Hydroxyapatite Nanocrystal/Poly(ε-caprolactone) Hybrid Scaffolds for Bone Tissue Engineering

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ABSTRACT: To achieve novel polymer/bioceramic composite scaffolds for use in materials for bone tissue engineering, we prepared organic/inorganic hybrid scaffolds composed of biodegradable poly(ε-caprolactone) (PCL) and hydroxyapatite (HA), which has excellent biocompatibility with hard tissues and high osteoconductivity and bioactivity. To improve the interactions between the scaffolds and osteoblasts, we focused on surface-engineered, porous HA/PCL scaffolds that had HA molecules on their surfaces and within them because of the biochemical affinity between the biotin and avidin molecules. The surface modification of HA nanocrystals was performed with two different methods. Using Fourier transform infrared, X-ray diffraction, and thermogravimetric analysis measurements, we found that surface-modified HA nanocrystals prepared with an ethylene glycol mediated coupling method showed a higher degree of coupling (%) than those prepared via a direct coupling method. HA/PCL hybrid scaffolds with a well-controlled porous architecture were fabricated with a gas-blowing/particle-leaching process. All HA/PCL scaffold samples exhibited approximately 80–85% porosity. As the HA concentration within the HA/PCL scaffolds increased, the porosity of the HA/PCL scaffolds gradually decreased. The homogeneous immobilization of biotin-conjugated HA nanocrystals on a three-dimensional, porous scaffold was observed with confocal microscopy. According to an *in vitro* cytotoxicity study, all scaffold samples exhibited greater than 80% cell viability, regardless of the HA/PCL composition or preparation method. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 1921–1929, 2011

Key words: biocompatibility; biomaterials; polyesters

INTRODUCTION

There is a growing need for bone regeneration because of various clinical bone diseases such as bone infections and bone tumors and because of bone loss due to trauma.[1–7] Current therapies for bone defects include autografts, allografts, xenografts, and artificial substitutes such as metals, synthetic cements, and bioceramics.[1–8] However, these substitutes are far from ideal, and each has specific problems and limitations. Autografts have limitations due to the necessity of additional surgery, a limited donor bone supply, anatomical and structural problems, and an inadequate resorption rate during healing. Allografts have the disadvantages of a potential immune response, disease transmission, and the possible induction of osteo-induction loss. Metals alone or coated with bioactive and bio-inert ceramics have been used for load-bearing orthopedic applications, but problems may be experienced because of metal corrosion, ceramic–metal interface wear, and dense fibrous tissue formation on the bone–implant interface.[8]

To address these issues, recent research has been devoted to bone tissue engineering, in which a three-dimensional (3D), porous scaffold is loaded with specific living cells/and or tissue-inducing factors to initiate natural tissue regeneration or replacement.[1,2,4–10] These materials should maintain adequate mechanical strength, should be osteoconductive, and should degrade at a controlled rate to provide space for the formation of new bone.[8] There has been widespread use of calcium phosphate bioceramics, such as hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$ or HA] and tricalcium phosphate, for bone regeneration applications.[11–17] In particular, HA is one of the most frequently used bioceramics for bone and dental tissue reconstitution. This material has excellent biocompatibility with hard tissues and high osteoconductivity and bioactivity despite its low degradation rate, mechanical strength, and osteo-inductive potential.[11,12] The exceptional biocompatibility is thought to be due to the chemical and structural similarities of HA and the mineral phase of

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native bone. The interactions of osteogenic cells with bioceramics are important for bone regeneration. Bioactive ceramics are known to enhance osteoblast differentiation as well as osteoblast growth. Bioactive ceramics have been used in dental and orthopedic surgery to fill bone defects and to coat metallic implant surfaces to improve implant integration with the host bone. However, their clinical applications have been limited because of brittleness, difficulty with shaping, and an extremely slow degradation rate in the case of HA.[1–3,8]

To overcome these limitations associated with bioceramics, the use of biodegradable polymer/bioceramic composites as materials in bone grafts is promising.[1–3,18–20] The addition of biodegradable polymers to calcium phosphate ceramics would allow for better manipulation and control of both the macrostructures and microstructures in shaping composites and for better fitting into bone defects.[18–20] In addition, biodegradable polymers can be used as binders for HA to reduce the brittleness of the ceramics. However, the polymer coating on ceramics created by polymer solutions may hinder the exposure of the ceramics to the scaffold surfaces, and this could decrease the probability of contact between the osteogenic cells and the bioactive ceramics.[19]

In this study, we prepared organic/inorganic hybrid composite scaffolds composed of biodegradable poly(ε-caprolactone) (PCL) and HA. PCL is a Food and Drug Administration approved bioresorbable polyester with potential applications in bone and cartilage repair. It is semicrystalline and has high thermal stability and a degradation time of approximately 2 years. The slow degradation and resorption kinetics of PCL might limit its application to drug delivery and resorbable sutures. However, this property could be beneficial for bone tissue engineering. Because the degradation time exceeds 1 year, the human bone cells have sufficient time to replace the entire scaffold before its complete degradation.[1,9]

To improve the interactions between scaffolds and osteogenic cells, we focused on surface-engineered, porous HA/PCL scaffolds, which have HA nanocrystals on their surfaces and within them because of the biochemical binding affinity between biotin and avidin molecules.[21–24] The surface of HA was modified and then characterized with Fourier transform infrared (FTIR) spectroscopy, powder X-ray diffraction (XRD), and thermogravimetric analysis (TGA) measurements. HA/PCL composite scaffolds with a well-controlled porous architecture were prepared with a gas-blowing/particle-leaching method. The structure, porosity, cytotoxicity, and HA distribution of the porous HA/PCL scaffolds were also investigated.

EXPERIMENTAL

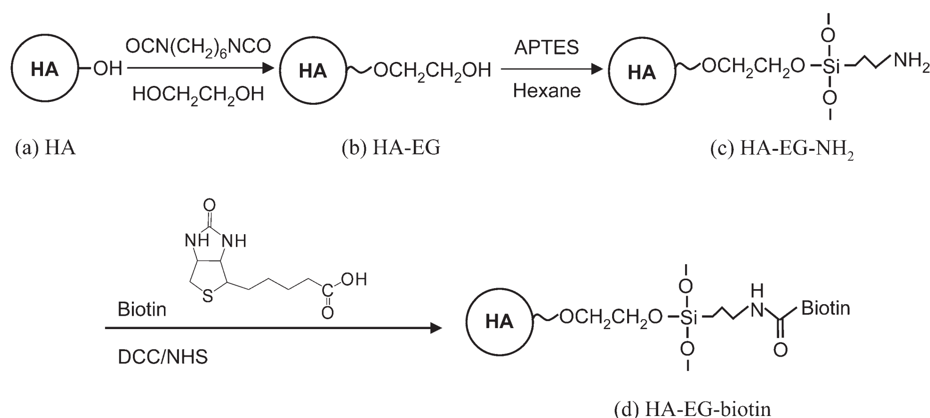
Materials

HA (nanopowder; particle size < 200 nm), hexamethylene diisocyanate (HMDI), dibutyl tin dilaurate, and ethylene glycol (EG) were purchased from Aldrich Chemical, Inc. (Milwaukee, WI), and were used without further purification. 3-Aminopropyl triethoxysilane (APTES), *N,N′*-dimethylformamide (DMF), methylene chloride, and *n*-hexane were also purchased from Aldrich Chemical. Biotin and avidin were obtained from Sigma Chemical Co. (St. Louis, MO). Texas red avidin was purchased from Vector (Burlingame, CA). DMF was dried over magnesium sulfate and vacuum-distilled. *N,N′*-Dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) were purchased from Fluka (Buchs, Switzerland). Dul (DCC)becco's modified Eagle medium, Dulbecco's phosphate-buffered saline (PBS), penicillin/streptomycin (100U/mL), trypsin/ethylene diamine tetraacetic acid (0.5% trypsin and 5.3 m*M* ethylene diamine tetraacetic acid tetrasodium), and fetal bovine serum were purchased from Gibco BRL (Rockville, MD). A solution of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) was obtained from Promega Corp. (Madison, WI). Distilled and deionized water was prepared with a Milli-Q Plus system (Millipore, Bedford, MA). All other chemicals were reagent-grade and were used as purchased without further purification.

Methods

Surface modification of HA

The biotin molecules were used to modify the surfaces of HA nanocrystals with two different methods, as shown in Figure 1. In method I, an EG-mediated coupling reaction, HMDI (1.5 mL, 8.92 mmol) and dibutyl tin dilaurate (0.045 g, 0.07 mmol) were added to a suspension of HA (3 g) in dry DMF (45 mL), and the mixture was stirred at 50°C under nitrogen for 8 h. EG (5.86 g, 0.094 mol), which was dissolved in DMF (5.86 mL), was added to the reaction mixture, and the mixture was stirred overnight at 60°C. After the reaction, EG-conjugated HA (HA–EG) was purified and collected by repeated washing with methylene chloride and centrifugation at 2500 rpm for 20 min (× 5). The resulting HA–EG was dried at 60°C *in vacuo* for 24 h. Then, HA–EG was silanized via immersion in a solution of APTES (0.1*M*) in anhydrous hexane for 4 h with stirring, and this was followed by three hexane washes. The product was dried *in vacuo* for 24 h. The amine groups of the resulting modified HA (HA–EG–NH_2) were coupled with the carboxyl groups of the biotin molecules, as shown in Figure 1(A). Biotin (0.5 g)



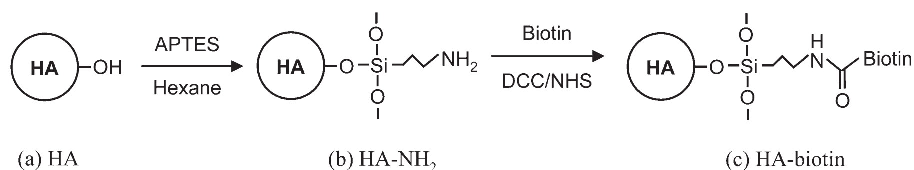


Figure 1 Synthetic scheme for surface-modified HA: (A) method I and (B) method II.

was dissolved in a suspension of HA–EG–NH_2 (0.5 g) in dry DMF, and then, *N,N′*-dicyclohexylcarbodiimide and NHS were added to a solution of amine-conjugated HA (HA–NH_2) and biotin. The reaction was continued at room temperature for 24 h under a nitrogen atmosphere. The product was purified via three hexane washes. The resulting biotin-conjugated HA (HA–EG–biotin) was dried *in vacuo* for 24 h.

For method II, the surface hydroxyl groups on HA were directly used for silanization without a reaction with EG [HA–NH_2; Fig. 1(B)]. Further synthesis procedures were the same as those of method I [Fig. 1(A)].

Preparation of the porous HA/PCL composite scaffolds with a gas-blowing method

Porous inorganic/organic hybrid scaffolds composed of HA and PCL were prepared with a gas-blowing/particle-leaching method. Briefly, PCL was dissolved in methylene chloride at a 10% (w/v) concentration, and HA and NaCl (diameter = 100–200 μm) were added to the PCL solution. The mass ratio of PCL to HA/NaCl was 1 : 0–1 : 9. Nitrogen gas (gas flow rate = 20 mL/min) was injected into the PCL/HA/NaCl mixture solution through the bottom of the flask for 1 h while the temperature of the solution was maintained at 0–4°C. In the steady state, the injected gas bubbles were homogeneously dispersed into the mixture solution by an impeller rotating at 150 rpm. The PCL/HA/NaCl mixture was loaded into Teflon disk molds (diameter = 12.0 mm, height = 7.0 mm). After solvent evaporation, the polymer disks with entrapped salt particles were removed from the molds through immersion in distilled water for 48 h. The rinsed scaffolds were frozen at −50°C and lyophilized.

In addition, porous HA/PCL scaffolds were also fabricated with the conventional solvent-casting/particle-leaching method for use as controls. Briefly, PCL was dissolved in methylene chloride (10% w/v), and HA and NaCl were added to the PCL solution with the same sizes and ratios used for the gas-blowing/particle-leaching scaffolds. This mixture was then loaded into Teflon disk molds. After solvent evaporation, the polymer disks with entrapped salt particles were removed from the molds via immersion in distilled water for 48 h.[19]

Preparation of surface-engineered, porous HA/PCL hybrid scaffolds via the biochemical affinity of biotin and avidin molecules

Porous HA/PCL scaffolds were treated with an avidin solution (2 mg/mL in PBS) for 6 h. The avidin-immobilized scaffolds were rinsed with deionized water to remove any loosely adsorbed protein and then were lyophilized. The lyophilized samples were stored at −20°C before use. The subsequent attachment of the biotin-conjugated HA (HA–biotin)

molecules onto the surfaces of the porous scaffolds was achieved via binding interactions between avidin and biotin molecules. Briefly, the avidin-immobilized scaffolds were placed in the HA–EG–biotin suspension (2 mg/mL in PBS) with gentle shaking. The reaction was allowed to proceed for 12 h, and the resulting scaffold samples were rinsed with deionized water and lyophilized.

Characterization of the HA nanocrystals

To demonstrate the synthesis of HA and the surface modification of HA, FTIR spectra were recorded on an FT/IR-460 Plus spectrometer (Jasco, Tokyo, Japan) over the range of 4500–650 cm^{-1} with a resolution of 2 cm^{-1} and with 64 scans. XRD measurements were performed with a Rigaku (Tokyo, Japan) D/max RB apparatus powder diffractometer and image-plate photography with graphite-monochromatized Cu K α radiation (λ = 1.542 Å). Data were collected from 20 to 60° with a step size of 0.05° and a step time of 5 s. The number of surface-modified molecules on HA was measured via TGA with a Mettler-Toledo (Columbus, OH) TGA/SDTA 851^e apparatus. Approximately 20 mg of surface-modified HA powder was placed in an alumina sample pan for TGA and was heated from room temperature to 1000°C at a rate of 5°C/min under a nitrogen atmosphere so that we could examine the thermal degradation of the organic components on HA (n = 3). The number of surface-modified molecules was determined as a weight-loss percentage during heating.

Characterization of the HA/PCL scaffolds

The morphologies of HA/PCL and surface-engineered HA/PCL scaffolds were investigated with field-emission scanning electron microscopy (FESEM; 6700F, JEOL, Kyoko, Japan) at an operating voltage of 15 kV. The samples were coated with platinum on a Cressington Scientific Instruments 108 auto sputter coater (Watford, UK). The specific surface area of the porous HA/PCL scaffolds was determined by Brunauer–Emmett–Teller nitrogen gas adsorption with a Nova 4000e surface area and pore size analyzer Quantachrome Instruments (FL, USA).

In addition, to investigate the immobilization of avidin molecules and the binding of biotin via an affinity interaction, Texas red conjugated avidin molecules were incorporated onto the surface-engineered HA/PCL scaffolds with immobilized biotin-conjugated nanocrystals. Then, the incorporation of fluorescent avidin onto the scaffolds was observed with a confocal microscope (Fluoview FV500, Olympus, Tokyo, Japan).

In vitro cytotoxicity study

The *in vitro* cytotoxicity of the HA/PCL scaffolds was evaluated with an indirect extraction method.[25–27] The extracts were obtained by the immersion of fragments of each scaffold (5 cm^2/mL) in a culture medium at 37°C. After incubation for 3 days, the extracts of the scaffolding products were collected. Human fibroblasts were seeded in 96-well plates at a density of 2.0×10^4 cells per well and were incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h. The fibroblasts were incubated with a culture medium composed of Dulbecco's modified Eagle medium, 10% fetal bovine serum, and 100 U/mL penicillin/streptomycin. After the cells had attached to the wells, the initial culture medium was removed and replaced with the scaffold-extract medium, and the cells were incubated for 3 days at 37°C. The extract medium was changed every 2 days. At the end of the incubation period, the extract medium was discarded, and the cell viability was determined with a tetrazolium compound (MTS inner salt) assay (n = 4). The untreated cells served as positive controls and were recorded to have 100% viability.

RESULTS AND DISCUSSION

Surface modification of nano-HA

To prepare the biotin-conjugated HA nanocrystals, the surface modification of HA was performed with two different methods, as shown in Figure 1. In method I, the surface hydroxyl groups were first reacted with HMDI and then with EG in a one-pot reaction. Then, the silanization of HA–EG was carried out by a reaction with APTES in anhydrous hexane. The amine groups of the resulting modified HA (HA–EG–NH_2) were coupled with the carboxyl groups of biotin molecules, as shown in Figure 1(A) (HA–EG–biotin). In method II, the surface hydroxyl groups on HA were directly used for silanization without a reaction with EG, and then the amine groups of the resulting modified HA (HA–NH_2) were conjugated with biotin molecules [HA–biotin; Fig. 1(B)].

The FTIR spectra of the resulting products at each step were used to confirm the modification of the surfaces of HA nanocrystals, as shown in Figure 2. Figure 2(A) shows the FTIR spectra of HA, HA–NH_2, and HA–biotin prepared with method II. The FTIR spectra of the resulting products at each step and HA–EG–biotin prepared with the indirect conjugation method using EG (method I) are shown in Figure 2(B).

Compared with the unmodified HA [Fig. 2(B-a)], HA–EG [Fig. 2(B-c)] exhibited absorption bands for

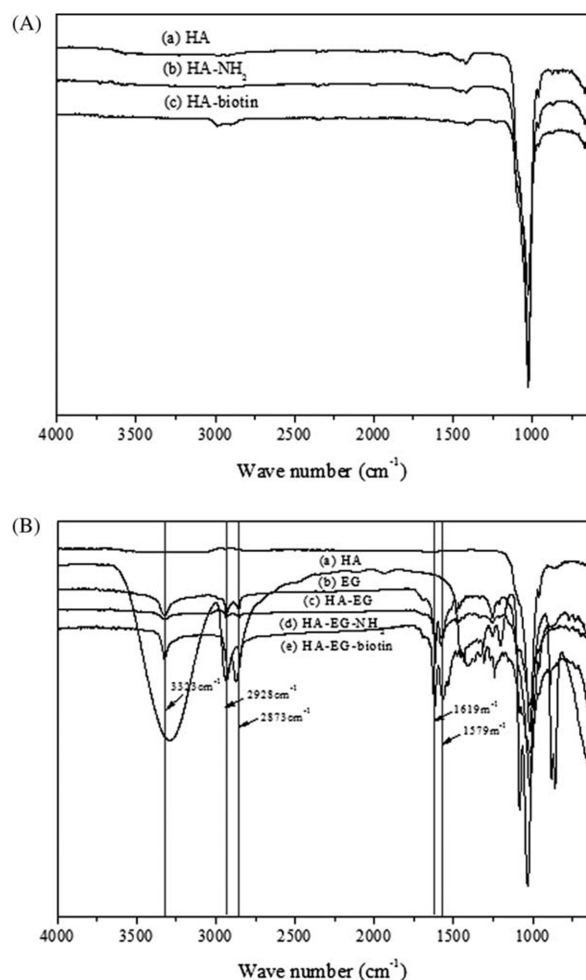


Figure 2 FTIR spectra of surface-modified HA prepared by (A) the direct coupling reaction (method II) and (B) the EG-mediated coupling reaction (method I).

CH stretching vibrations in EG at 2873 and 2928 cm^{-1} and an absorption band of the terminal hydroxyl groups in EG at 3323 cm^{-1}. The FTIR spectrum of HA–EG also showed the presence of —NHCO— between the HA surface and EG with two peaks at 1619 and 1579 cm^{-1}. For HA–EG–NH_2 [Fig. 2(B-c)], the absorption bands due to the deformation vibration of N—H in NH_2 overlapped that of the amide group. HA–EG–biotin exhibited new characteristic peaks of biotin, as shown in Figure 2(B-d). However, the FTIR spectra of HA–NH_2 and HA–biotin prepared with the direct coupling reaction (method II) did not show significant changes in comparison with that of the unmodified HA [Fig. 2(A)]. This indicated that the EG-mediated coupling reaction (method I) enhanced the coupling efficiency onto HA surfaces in comparison with that of surface hydroxyl groups on unmodified HA (method II). The hydroxyl groups of HA–EG were linked to the HA surface via a relatively long chain spacer [HA–OCONH$(CH_2)_6$NHOCOCH_2CH_2—OH]. Therefore, the steric hindrance of the hydroxyl group seemed to be very low and possibly allowed more accessibility for the coupling reaction.

Figure 3 shows the XRD patterns of unmodified HA, HA–EG–NH_2, and HA–EG–biotin. As shown in Figure 3(a), the characteristic peaks in the 2θ regions of 26, 29, 32–34, 40, and 46–54° indicated the crystalline nature of the HA nanocrystals. From the XRD patterns of HA–EG–NH_2 and HA–EG–biotin, we found that the coupling reactions did not significantly affect the crystalline phases of the HA nanocrystals, and the intrinsic properties of HA were maintained.

To quantitatively determine the incorporation of biotin molecules onto the surface of HA, TGA measurements were taken. Inorganic HA and surface-modified HA were completely dried for 48 h, and then TGA measurements were taken from room temperature to 1000°C. Therefore, the weight loss on the TGA curve was exclusively ascribed to the organic substances incorporated onto HA.

The results are summarized in Table I. Typical TGA curves are also shown in Figure 4. From the results of the TGA measurements of unmodified HA, HA–NH_2, and HA–biotin prepared with the direct coupling reaction (method II), the percentages of coupling for HA–NH_2 and HA–biotin were 10.9 and 31.6%, respectively. The percentages of coupling for HA–EG, HA–EG–NH_2, and HA–EG–biotin prepared with the EG-mediated coupling reaction (method I) were 19.8, 34.1, and 58.7%, as shown in Table I. This suggests that the EG-mediated coupling reaction was more effective than the modification of hydroxyl groups on the HA surface. These results were in good agreement with the results of FTIR measurements.

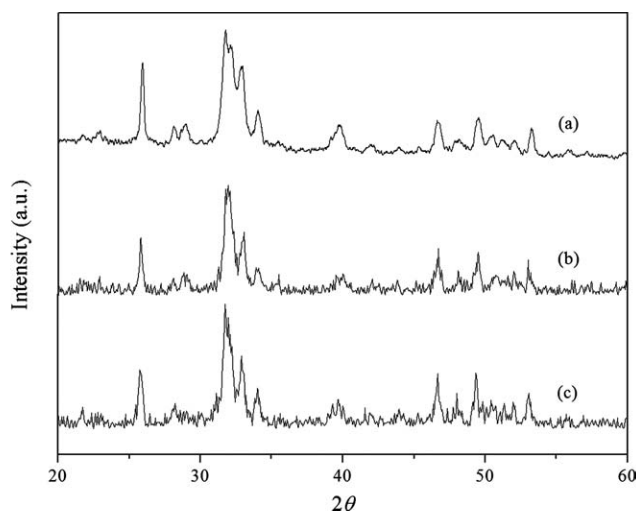


Figure 3 XRD patterns of surface-modified HA prepared by method I: (a) HA, (b) HA–EG–NH_2, and (c) HA–EG–biotin.

TABLE I
Degree of Coupling of Modified HA Nanocrystals

No.	Sample	Preparation method	Degree of coupling (%)
1	HA–EG	Method I	19.8 ± 0.26
2	HA–EG–NH_2	Method I	34.1 ± 1.02
3	HA–EG–biotin	Method I	58.7 ± 0.78
4	HA–NH_2	Method II	10.9 ± 0.60
5	HA–biotin	Method II	31.6 ± 0.53

The degree of coupling was determined from TGA measurements of the original HA and the modified HA and was calculated as follows (n = 3):

$$\text{Degree of coupling (\%)} = \frac{\text{Weight of modified organic molecules on HA (g)}}{\text{Weight of modified HA (g)}} \times 100$$

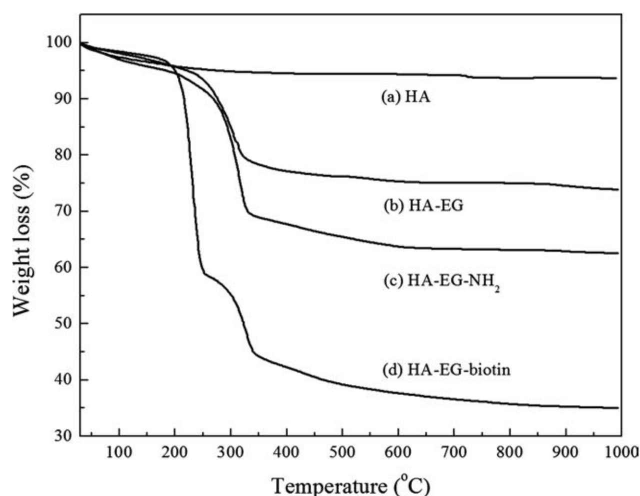


Figure 4 Typical TGA curves of surface-modified HA prepared by the EG-mediated coupling reaction (method I): (a) HA, (b) HA–EG, (c) HA–EG–NH_2, and (d) HA–EG–biotin.

Morphologies of the HA/PCL scaffolds

To prepare a biocompatible matrix for bone tissue engineering, porous inorganic/organic hybrid scaffolds composed of HA and PCL were prepared via a gas-blowing/particle-leaching method. As controls, porous HA/PCL scaffolds were also fabricated with a conventional solvent-casting/particle-leaching method. In addition, to improve the interactions between scaffolds and osteoblasts, we fabricated surface-engineered HA/PCL scaffolds that had HA nanocrystals on their surfaces and within them because of the biochemical binding affinity between biotin and avidin molecules. As shown in Table II, HA/PCL scaffolds were prepared by the variation of the HA/PCL feed ratio, the preparation process, and the surface modification due to the biochemical binding affinity.

The morphologies of the porous HA/PCL scaffolds prepared with the novel gas-blowing/particle-leaching method and the conventional solvent-casting/particle-leaching method were characterized with FESEM measurements. Figure 5(A) shows the FESEM images of the surfaces and cross sections of the HA/PCL scaffolds prepared with the solvent-casting/particle-leaching process; a closed-pore structure is demonstrated. On the other hand, the HA/PCL scaffolds prepared with the gas-blowing/particle-leaching method possessed an interconnected-pore structure, as shown in Figure 5(B). The pore size gradually decreased with increasing HA composition within the HA/PCL scaffolds, regardless of the preparation process.

In addition, the specific surface areas of the porous HA/PCL scaffolds were also investigated with Brunauer–Emmett–Teller nitrogen gas adsorption measurements. Table III shows the porosity (%) of the HA/PCL scaffolds according to the fabrication method and the HA/PCL composition. All HA/PCL scaffold samples exhibited approximately 78–86% porosity. As the HA concentration of the HA/PCL scaffolds increased, the porosity of the HA/PCL scaffolds gradually decreased. For HA/PCL

TABLE II
Preparation Processes and Feed Compositions of Porous PCL Scaffolds, Porous HA/PCL Scaffolds, and Surface-Engineered, Porous HA/PCL Scaffolds

No.	Sample	Formulation	PCL : HA feed weight ratio	Preparation process	HA immobilization on the surface[b]
1	HA/PCL-SP-0.0	PCL[a]	1.00 : 0.0	Solvent casting/particle leaching	×
2	HA/PCL-SP-0.5	HA/PCL	1.00 : 0.5	Solvent casting/particle leaching	×
3	HA/PCL-SP-1.0	HA/PCL	1.00 : 1.0	Solvent casting/particle leaching	×
4	HA/PCL-GP-0.0	PCL	1.00 : 0.0	Gas blowing/particle leaching	×
5	HA/PCL-GP-0.5	HA/PCL	1.00 : 0.5	Gas blowing/particle leaching	×
6	HA/PCL-GP-1.0	HA/PCL	1.00 : 1.0	Gas blowing/particle leaching	×
7	S-HA/PCL-GP-0.5	HA/PCL	1.00 : 0.5	Gas blowing/particle leaching	○
8	S-HA/PCL-GP-1.0	HA/PCL	1.00 : 1.0	Gas blowing/particle leaching	○

[a] Molecular weight = 80,000.
[b] Biotin-modified HA nanocrystals were incorporated onto the surfaces of porous HA/PCL scaffolds as a result of the biochemical binding affinity between avidin and biotin molecules; X = not immobilized; O = immobilized.

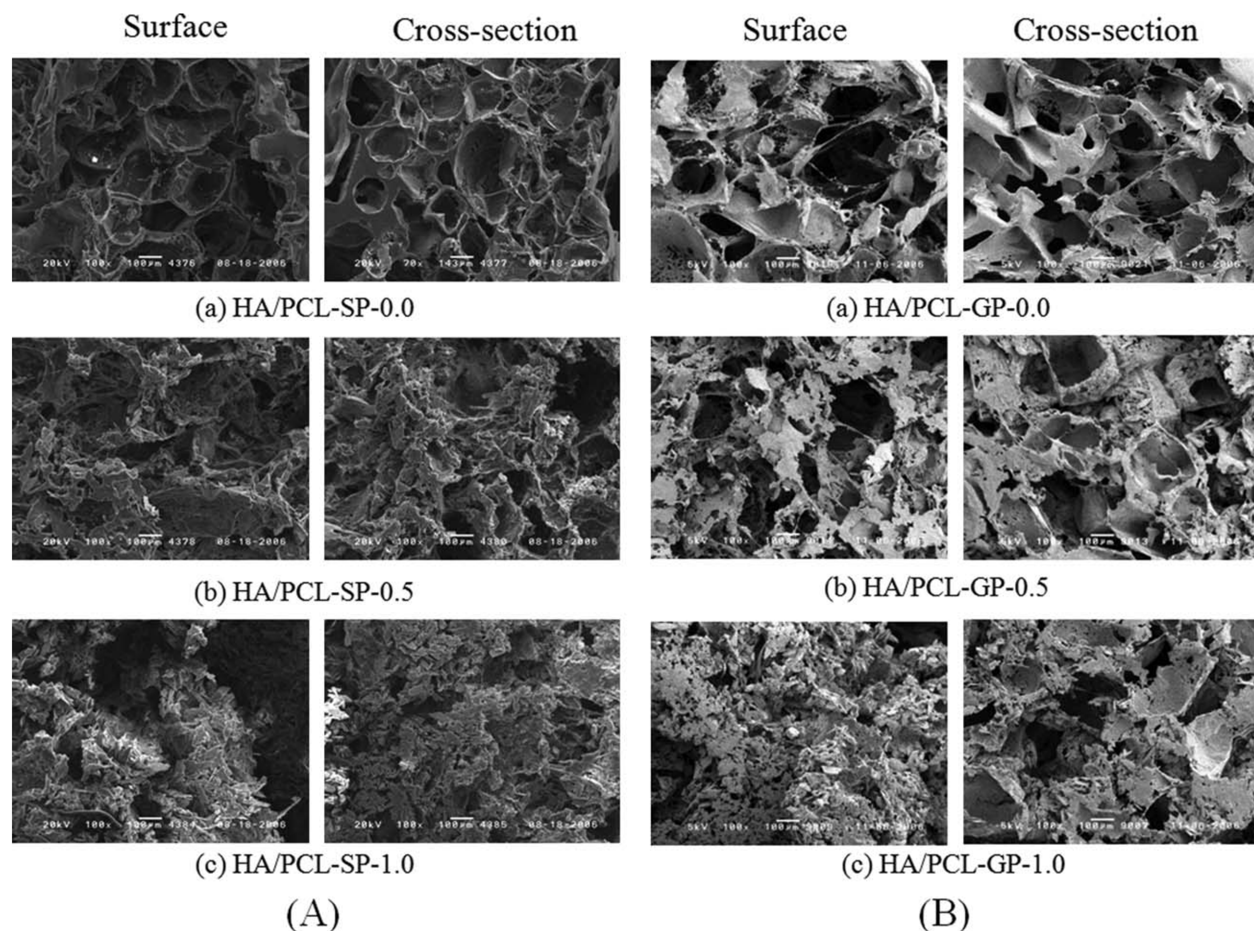


Figure 5 Surface and cross-sectional images of porous HA/PCL scaffolds observed with FESEM: (A) HA/PCL scaffolds prepared with the solvent-casting/particle-leaching method and (B) HA/PCL scaffolds prepared with the gas blowing/particle-leaching method.

scaffolds with the same HA/PCL composition, the scaffolds prepared with the solvent-casting/particle-leaching method had lower porosity than those fabricated with the gas-blowing/particle-leaching process, possibly because of the closed-pore configuration of the solvent-casting/particle-leaching method. Furthermore, surface-engineered HA/PCL scaffolds with HA molecules on their surface and within them exhibited lower porosity (80.7% for S-HA/PCL-GP-0.50) in comparison with other scaffolds with no HA nanocrystals on their surface and with the same HA/PCL composition (81.9% for HA/PCL-SP-0.5 and 84.5% for HA/PCL-GP-0.5). This indicated that HA–EG–biotin nanocrystals were incorporated onto the HA/PCL scaffolds through the biochemical binding affinity between biotin and avidin molecules, and this resulted in the decreased porosity of the scaffolds.

TABLE III
Porosity of the HA/PCL Scaffolds with Respect to the Preparation Processes and Formulations

No.	Sample	Preparation process	Porosity (%)
1	HA/PCL-SP-0.0	Solvent casting/particle leaching	78.6 ± 0.48
2	HA/PCL-SP-0.5	Solvent casting/particle leaching	81.9 ± 1.06
4	HA/PCL-GP-0.0	Gas blowing/particle leaching	86.6 ± 0.13
5	HA/PCL-GP-0.5	Gas blowing/particle leaching	84.5 ± 0.57
6	HA/PCL-GP-1.0	Gas blowing/particle leaching	82.3 ± 0.24
7	S-HA/PCL-GP-0.5	Gas blowing/particle leaching	80.7 ± 0.46
8	S-HA/PCL-GP-1.0	Gas blowing/particle leaching	79.8 ± 0.21

Immobilization of the biotin-conjugated HA nanocrystals on the porous HA/PCL scaffolds

Avidin and biotin are broadly used in biological analysis techniques such as immunoassays because they form a highly specific and stable complex. Avidin (molecular weight ≈ 68 kDa) is a glycoprotein with four subunits, each of which can bind a biotin molecule (also known as vitamin H; molecular weight = 244.3). To investigate the feasibility of using the avidin–biotin binding system for the immobilization of HA nanocrystals on scaffolds, avidin molecules were physically incorporated onto the surfaces of porous HA/PCL scaffolds, and biotin-conjugated HA was subsequently immobilized on the basis of its specific binding affinity. To detect a specific avidin–biotin reaction, the incorporation of fluorescent avidin molecules onto the surface-engineered HA/PCL scaffolds was observed with a confocal microscope. Figure 6 shows fluorescence photomicrographs of HA/PCL scaffolds with various HA/PCL compositions. Detectable fluorescence intensity in the image of HA/PCL-GP-0.5 without biotin-conjugated HA nanocrystals was not observed [Fig. 6(A)], whereas surface-engineered HA/PCL scaffolds (S-HA/PCL-GP-0.5 and S-HA/PCL-GP-1.0) exhibited high fluorescence intensity in the cross section and on the surfaces of the scaffolds [Fig. 6 (B,C)]. These results suggest that HA nanocrystals can be successfully homogeneously immobilized onto 3D porous scaffolds because of the binding affinity between avidin and biotin molecules.

Probably the most important driving force behind the development of polymer/bioceramic composite scaffolds for bone tissue engineering is the need to confer bioactive behavior to the polymer matrix, which is achieved through the use of bioactive inclusions or coatings.[1,4,28] It has been shown that the degree of bioactivity can be adjusted by changes in the volume fraction, size, shape, or arrangement of the inclusions.[1] Most previous research on the fabrication of polymer/bioceramic composite scaffolds used solvent-casting/particle-leaching and phase-separation methods.[29–31] The composite scaffolds prepared with these methods may reduce the exposure of the ceramics to the scaffold surfaces, and this could decrease the probability of osteogenic cells coming into contact with the bioactive ceramics.[1,19] Therefore, surface-engineered, porous HA/PCL hybrid scaffolds with HA molecules on their surface and within them because of the biochemical affinity between biotin and avidin molecules could enhance the interaction between bioceramics and osteogenic cells in comparison with those fabricated with conventional methods.

In vitro cytotoxicity

The biocompatibility of porous HA/PCL scaffolds and surface-engineered, porous HA/PCL scaffolds

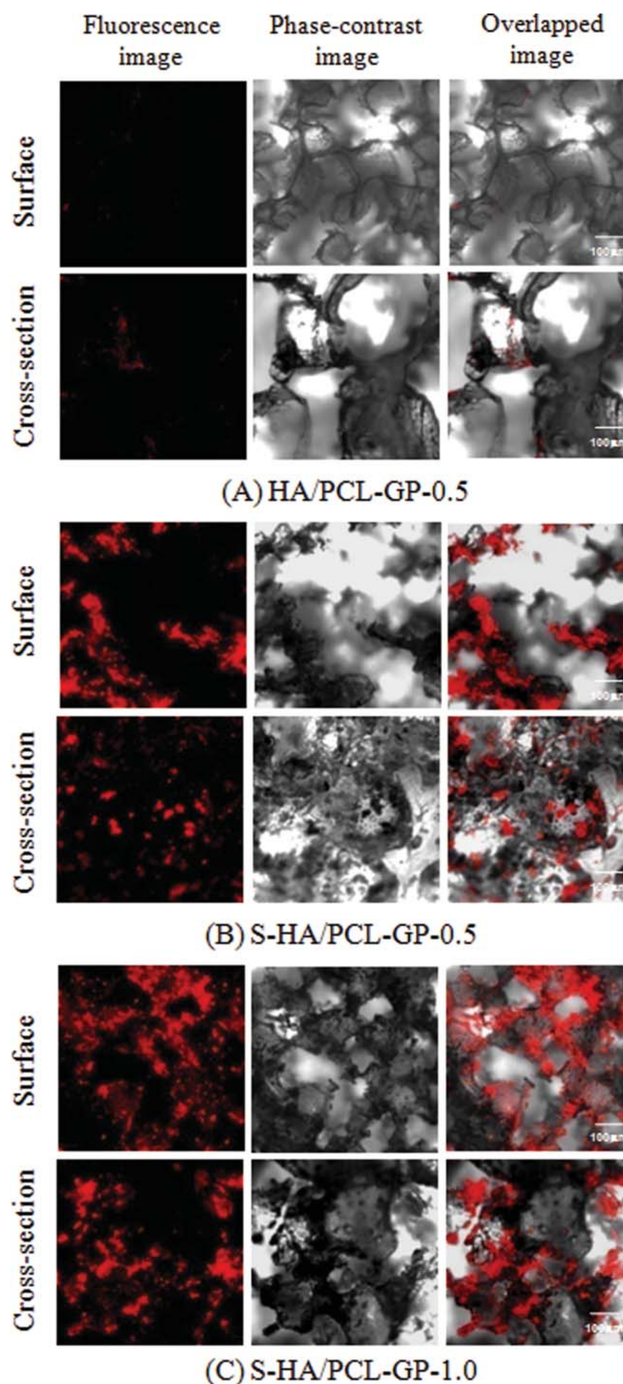


Figure 6 Confocal fluorescence microscopy images of HA/PCL scaffolds of various compositions: (A) HA/PCL-GP-0.5, (B) S-HA/PCL-GP-0.5, and (C) S-HA/PCL-GP-1.0. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

was evaluated with *in vitro* cytotoxicity tests using normal human fibroblasts. Figure 7 shows the cytotoxicities of HA/PCL scaffolds of different compositions. The viability of human fibroblasts cultured with extracts of scaffolds for 72 h was determined with the MTS assay. The viability was expressed as a percentage of the living cells with respect to the number of positive control cells grown on a tissue culture plate

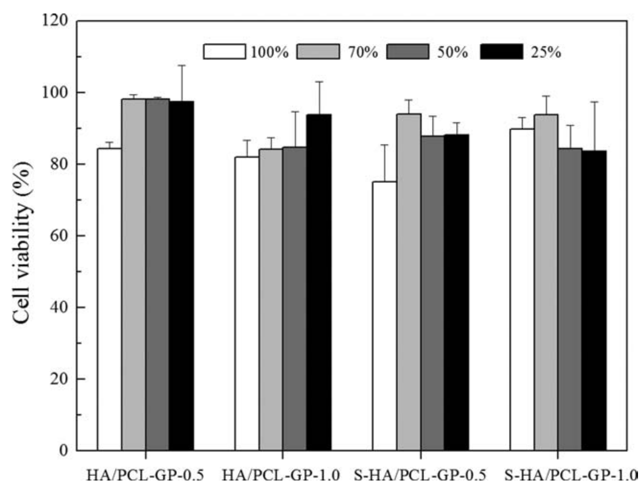


Figure 7 Viability of human fibroblasts cultured with extracts of HA/PCL as determined by the MTS assay. The viability is expressed as a percentage of the living cells in the samples (n = 4) with respect to a positive control cell group grown on tissue culture plates with no scaffold material.

without any extracts of scaffold material. All scaffolds tested showed relatively high cell viability. Regardless of the HA/PCL composition or preparation method, all scaffold samples exhibited greater than 80% cell viability. As shown in Figure 7, cell viability was also not significantly influenced by the extract concentration. Future studies will investigate the growth, proliferation, and functional activity of osteoblasts on surface-engineered, porous HA/PCL scaffolds.

CONCLUSIONS

Polymer/bioceramic composite scaffolds composed of PCL and HA were designed to produce promising, novel scaffold materials for bone tissue engineering. To improve the interactions between scaffolds and osteoblasts, we focused on surface-engineered, porous HA/PCL scaffolds that have HA molecules on their surfaces and within them because of the biochemical affinity between biotin and avidin molecules. It was found that the surface-modified HA nanocrystals prepared with the EG-mediated coupling method had a higher degree of coupling (%) than those prepared with the direct coupling method, as determined from the results of FTIR, XRD, and TGA measurements. In addition, HA/PCL hybrid scaffolds with a well-controlled porous architecture were fabricated via a gas blowing/particle-leaching process. All HA/PCL scaffold samples exhibited approximately 80–85% porosity. As the HA composition within the HA/PCL scaffolds increased, the porosity of the HA/PCL scaffolds gradually decreased. Using confocal microscopy measurements, we found that biotin-conjugated HA nanocrystals were incorporated homogeneously onto 3D porous HA/PCL scaffolds. All scaffold samples exhibited relatively high cell viability (>80%), regardless of the HA/PCL composition or preparation method. Therefore, these porous, surface-engineered HA/PCL hybrid scaffolds could be used as biomimetic matrices for bone tissue engineering.

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