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Effects of the surface modification of poly(amino acid)/hydroxyapatite/calcium sulfate biocomposites on the adhesion and proliferation of osteoblast-like cells

Xiaoxia Fan,[1] Haohao Ren,[1] Pengzheng Liu,[2] Peng Wang,[1] Hong Li,[1] Yonggang Yan,[1] Guoyu Lv[1]

[1]College of Physical Science and Technology, Sichuan University, Chengdu 610064, China

[2]Sichuan National Nano Technology Company, Chengdu 610041, China

Correspondence to: Y. Yan (E-mail: yan_yonggang@vip.163.com) and G. Lv (E-mail: lgy929@126.com)

ABSTRACT: In this study, we focused on the surface modification of a novel poly(amino acid) (PAA)/hydroxyapatite/calcium sulfate composite and the effect of its surface modification on cellular responses. The surface modification was performed by sandblasting (sample S2), calcium chloride ethanol saturated solution etching (sample S3), and formic acid etching (sample S4) followed by *in vitro* culturing of osteoblast-like cells. The obtained results indicate that a new interface of the composite was formed during the modification, and the modified surface was changed with respect to its surface morphology by physical abrasion. The calcium chloride ethanol saturated solution etchant etched PAA selectively whereas forming rich calcium-phosphate (Ca–P) apatite on the surface of S3. The formic acid etchant attacked the inorganic component without changing the PAA state. Cell attachment and cell proliferation were improved by the treatments of S2 and S3 in comparison with no treatment and the treatment of S4 © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42427.

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INTRODUCTION

Surface modification to adjust or control the surface characteristics, such as the surface chemistry, topography, and mechanical properties, has been proven to be effective in improving the biological functions of materials, and thus, it has become one of the most attractive branches in the field of biomedical research.[1,2] Modification, by either chemical or physical methods, is very important to achieve new uses and different properties.[3–5] Modification takes place only on the very surface layer of the substance, and the results of modification can well be controlled. Because of the different composition of the composite, it is possible to preferentially etch one component of a composite with the other one intact. A conventional composite of calcium phosphate compounds (Ca–P apatite) and an organic polymer results in the reduced functionality of Ca–P apatite because most of the inorganic particles are normally embedded in polymer matrices.[6] The surface of Ca–P apatite must be exposed to the surrounding environment for its valuable properties, that is, the biological properties, to be seen.[7] However, most methods of surface modification require long processing times or complicated equipment; this makes them time-consuming and expensive.[3,7] Therefore, an etching strategy was introduced into this study for producing a rich Ca–P apatite layer on the surface of the composite, and in contrast, another etching strategy was used in this study to selectively produce a polymer layer on the surface of the composite. Also, a relatively rough surface of the same composite was made by mechanical abrasion to favor the cells interacting as another control of the biological properties of a composite; this also promoted cell attachment and maintenance.

Poly(amino acid)s (PAAs) are ubiquitous, naturally occurring polymers. A modified PAA copolymer was synthesized with ε-aminohexanoic acid as the main framework and other natural amino acids as the block group, and this copolymer had molecular groups similar to those of collagen protein in natural bone.[8–10] Also, PAA was biocompatible and bioactive when combined with inorganics of Ca–P ceramic, as we found in a previous study.[11] Calcium sulfate (CS) is recognized as a well-tolerated material in bone regeneration.[12] Reports in the dental and orthopedic applications demonstrated that CS was degradable,[13,14] and the release of Ca from CS may produce a Ca-rich environment that might be responsible for stimulating cell proliferation and differentiation;[15] this affects conductive bone formation.[16,17] Synthetic hydroxyapatite [HA; $Ca_{10}(PO_4)_6(OH)_2$] was clinically used as a bioceramic for bone replacement over decades because of its similarity to the inorganic component of hard tissues (e.g., of bone and tooth) and its excellent

biocompatibility and bioactive nature.[18,19] However, compared with alumina and zirconia,[20] this group of ceramics is mechanically weak and exhibits poor crack growth resistance; this restricts its uses to non-load-bearing applications. Thus, the combination of the bioactivities of CS and HA and the reasonable mechanical properties of polymers is of interest for the development applicable composites with improved properties for bone regeneration. Furthermore, our previous work about PAA illustrated that some certain PAAs possessed excellent mechanical properties and biocompatibility, and the addition of inorganic ceramics improved its bioactivity and other properties.[11,21–23]

Therefore, in this study, a dense biocomposite containing PAA, HA, and CS was prepared with an *in situ* melting polycondensation technique, which was different from most of the previous methods used for fabricating polymer/bioceramic composite scaffolds, such as particulate leaching, solvent casting, and phase separation, with organic solvents.[24,25] The modification strategies of mechanical abrasion and selective etching were used to create different surface conditions for cellular responses as compared to an untreated composite with an *in vitro* cell culture of MG-63 as a potential tool for improving the biological performance of the (amino acid) copolymer-based composite for their use in biomedical applications for bone tissue engineering.

EXPERIMENTAL

Materials

HA particles 100–200 μm in diameter used in this study were supplied by Sichuan National Nano Technology Co., Ltd. Calcium sulfate hemihydrate was obtained from Sigma-Aldrich Co., LLC., and was ground and sieved in the diameter range 100–200 μm. Absolute ethyl alcohol was from Sichuan Xilong Chemical Agent Co., Ltd. (China, analytical-reagent grade). ε-Aminocaproic acid, L-proline, L-lysine, L-alanine, L-phenylalanine, and L-hydroxyproline were purchased from Hebei Kairuijie Amino Co., Ltd. (China).

Preparation of the PAA/HA/CS Composites and Surface Modification

The PAA/HA/CS composite was synthesized with the *in situ* melting polycondensation method.[21] In short, 245.00 g (1.87 mol) of ε-aminohexanoic acid, 2.30 g (0.02 mol) of L-proline, 3.60 g (0.02 mol) of L-lysine, 2.80 g (0.03 mol) of L-alanine, 3.10 g (0.02 mol) of L-phenylalanine, and 8.00 g (0.06 mol) of L-hydroxyproline were added to the reaction flask, and 0.5 mL of phosphorous acid was added as a catalyst. To prevent oxidation, the system was held under a continuous flow of nitrogen. The mixture was kept at 200°C until the water was evaporated; this was followed by melting at 210°C for 2 h. Then, 20.5 g CS (25.93 g of calcium sulfate hemihydrate) and 166.5 g of HA powder were added to the previous melt with stirring (500 rpm) for 2 h at 220°C. After reaction, the melt was kept under a nitrogen environment until it cooled down to room temperature. Finally, a composite with an inorganic component of 42.32 ± 0.546 wt % was obtained. (This result was obtained by the burning of the composites at 700°C under an air atmosphere and expressed as mean plus or minus the standard deviation, $n = 3$).

The composites were cut into small discs with a diameter of 5 mm and a thickness of 2 mm by a numerically controlled lathe for surface modification. The original sample without any treatment was marked as S1. The method of surface modification included mechanical abrasion (sample S2) with a grit-blasting process to produce a microrough surface with a sand-blasting machine (CS-960W, Chongqing Chuangshuo Co., Ltd.) and chemical methods of saturated calcium chloride ethanol solution etching (sample S3) and acid etching in formic acid (sample S4). For S3, the etchant was prepared by the addition of calcium chloride powder into boiling ethanol until it was saturated; the samples were then immersed into the boiling solution for 10 min. However, for S4, the samples were etched in formic acid at room temperature for 1 min. After this treatment, the samples were ultrasonically washed several times with demineralized water in a container and were then dried at 80°C. The samples were sterilized with ethylene oxide gas.

Characterization of the Modified PAA/HA/CS Composites

Fourier transforms infrared (FTIR) spectra were collected with a PerkinElmer 6000 FTIR spectrometer (Nicolet PerkinElmer Co.). X-ray diffraction (XRD) analysis of the samples was conducted with a Philips diffractometer (X'Pert Pro-MPD) with Cu Kα radiation ($\lambda = 1.5405$Å) over the 2θ range 10–70°. The surface morphology of the samples was observed by scanning electron microscopy (SEM; JEOL JSM 5600LV, Japan) at 20kV with gold coating before observation. Corresponding to SEM, energy-dispersive X-ray spectroscopy (EDS) was performed to analyze the weight rates of the elements (C % + O % + Ca % + P % = 100%) on the surface, and the results represent the mean plus or minus the standard deviation ($n = 5$).

Cell Cultures

MG-63 Cells. MG-63 cells, which were previously used as an *in vitro* test system for assessing the effects of many types of biomaterials, were used in this study.[26] At 37°C in a humidified 5% CO_2 atmosphere, MG-63 osteoblast-like cells were cultured in dulbecco's modified eagle medium (DMEM) (SH30022.01B, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum plus 100 U/mL penicillin and 100 μg/mL streptomycin sulfate. After the cells grew nearly confluent, they were trypsinized, collected, and adjusted to 2×10^4 cells/mL for the following experiment.

Cell Cultures with Materials. MG-63 cells were seeded on the samples at a volume of 1 mL/well (2×10^4 cells/mL) loaded into 48-well tissue culture plates (TCPs) with TCP as a control. Cells were allowed to adhere for 1 h before each well was gently flooded with 1 mL of medium. The cell-sample constructs were maintained at 37°C and 5% CO_2 under humidified atmosphere conditions with a refreshment of culture medium once in 2 days. Cells were cultured on the materials for 4 h, 1 day, 3 days, 5 days, and 7 days, respectively, as was reported before.[27,28]

3-(4,5-Dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) Assay. MTT assay was used to determine the cells on the materials. At the preset time, 500 μL of MTT solution (1 mg/mL, dissolved in RPMI 1640 medium) was added to each well and incubated for 3 h ($n = 3$ per condition). At the end of incubation, the supernatant in the 48 wells was discarded; 100 μL of isopropyl alcohol was then added and vortexed for 5 min to allow total color release. The absorbency was measured at 570 nm (reference wavelength = 650 nm) with a microplate

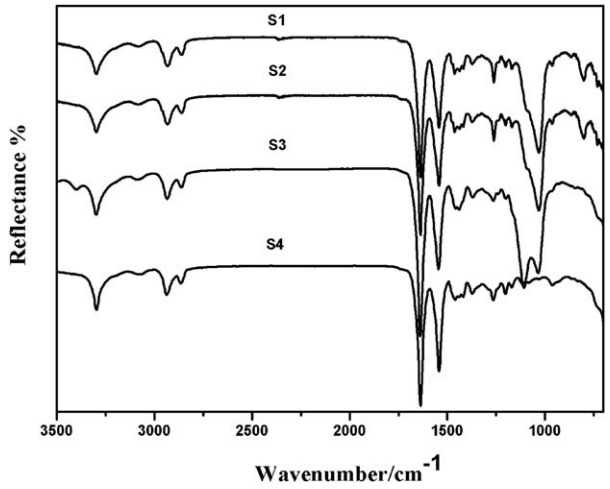

Figure 1. FTIR patterns of samples S1, S2, S3, and S4.

reader (Bio-Rad, iMark). The results are reported as optical density (OD) units and expressed as mean plus or minus the standard deviation ($n = 6$).

Cell Morphology. The morphology of the MG-63 cells cultured on the samples was observed by SEM. At a preset time, the samples were washed twice with warm PBS solution, fixed with 2% glutaraldehyde buffer (pH 7.4) at room temperature for 30 min, subsequently washed twice with phosphate buffer solution (PBS), and dehydrated by an increase in the concentration of alcohol (30, 50, 70, 80, 90, 95, 99, and 100%) for 3 min each. Finally, the samples were critical-point dried with liquid CO_2 and sputter-coated with gold and examined with SEM observation.

Statistical Analysis

Statistical analysis was performed with a one-way analysis of variance followed with a Tukey *post hoc* test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Physicochemical Characteristics of the Material Surface

FTIR Analysis. Figure 1 shows the FTIR spectra of all of the samples. The characteristic absorption peaks at 3300 cm^{-1} (N—H stretching), 2935 and 2860 cm^{-1} (—CH_2— absorption), 1637 cm^{-1} (C═O stretching), 1543 cm^{-1} (C—N stretching and N—H bending), and 1261 cm^{-1} (—CHN— absorption in amide bond III) were attributed to the characteristic peaks of S1. The peaks at 1029 cm^{-1} were assigned to the characteristic stretching vibration peaks of PO_4^{3-}. The characteristic peak of carboxyl at 1740 cm^{-1} did not appear obviously, and specific peaks of amide linkages appeared with slight shifts for all of the samples. This indicated that most of the —COOH groups reacted with —NH_2 groups during the synthetic process while leaving bits of monomer unreacted. Furthermore, we found that the spectrum of S2 was the same as those of S1; we deduced that physical treatment did not change the chemical structure of the samples. Compared with S1, S3 showed no obvious change but showed the characteristic peak of SO_4^{2-} at 1161 cm^{-1}. The surface modification of saturated calcium chloride ethanol solution did not change the chemical nature of the composite. S4 was significantly different from others, in which characteristic stretching vibration peaks of PO_4^{3-} at 1029cm^{-1} disappeared, and the characteristic absorption peaks belonged mainly to amide linkages. These transformations of characteristic absorption peaks were caused by the surface modification of formic acid, and these changes indicated that formic acid created a new interface with mainly organic remains.

XRD Analysis. XRD of all of the samples are shown in Figure 2. The two peaks at $2\theta = 20.0$ and 23.2° (S1) belonged to the characteristic diffraction pattern of the amide linkages of the composites from the PAA polymer. The characteristic peaks of HA appeared at $2\theta = 25.7$, 31.6, 32.8, 33.8, 39.5, and 46.4° in the composite (S1). The two peaks at $2\theta = 11.5$ and 28.9° belonged to the diffraction pattern of CS ($2H_2O$) in Figure 2 (S4). We observed that S2 was the same as S1 in Figure 2. The crystallinity of the amide linkages of the composite decreased, whereas the crystallinity of HA increased in S3 after the surface treatment. However, the intensity of these two diffraction peaks of amide linkages and the peaks of CS ($2H_2O$) of the composite increased. Also, the intensity of the characteristic diffraction pattern of HA dropped in S4 as compared to S1. The results indicate that the surface modification by chemical methods disrupted the surface structure of the composite. The surface of the organic component was destroyed by the saturated calcium chloride ethanol solution etchant. As a result, the crystallinity of the inorganic component increased. As shown in Figure 2 (S4), the modification made the crystallinity of HA decrease, and the crystallinity of CS and PAA increased with the formic acid etching of inorganics on the surface of the composite.

SEM Analysis. Figure 3 shows the typical SEM images of the surface morphology of the samples. Many little particles were shown on the surface of S1, but the surface was almost smooth. Many irregular cracks and pits were visible on the modified surface of S2; these had an average value in the range between the peak and trough of 12.37 μm (observed by an optical profiling system, Contour GT-K, Veeco). Because these defects were not observed on the original surface (of S1), we concluded that these defects were formed during the mechanical abrasion. The numerous cavities on the surface shown in Figure 3 (S3) were

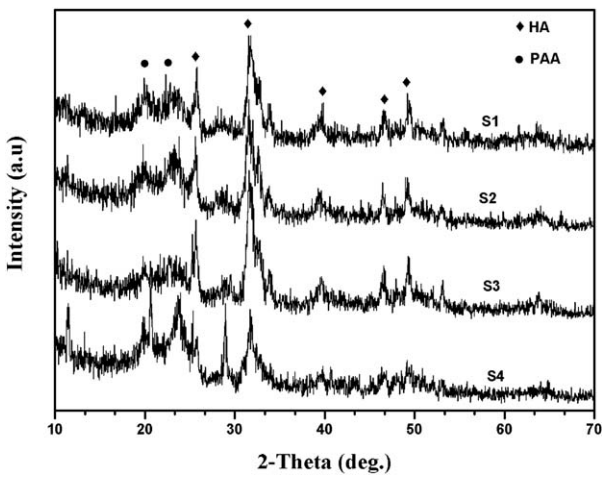

Figure 2. XRD patterns of samples S1, S2, S3, and S4.

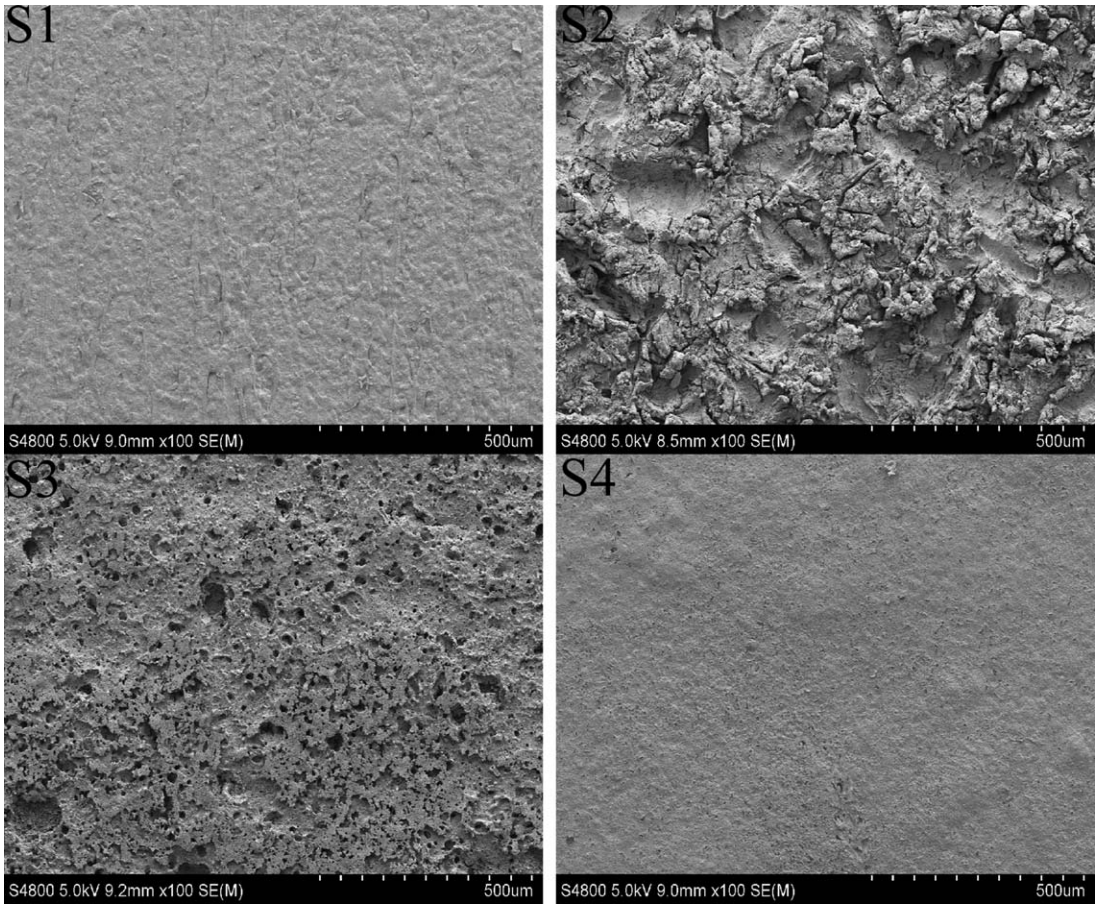

Figure 3. SEM photographs of samples S1, S2, S3, and S4.

regarded as the sites of the Ca–P apatite fillers after etching. They showed a very rough surface caused by the optional etching by the calcium chloride ethanol saturated solution etching. S4 showed a smooth surface in comparison with the other sample surfaces because of the disappearance of inorganic components on the surface of the composite.

EDS Analysis. The EDS profile of the samples is displayed in Figure 4 and Table I. We observed that the weight rate of calcium (18%) and phosphorous (5%) on the surfaces of S1 and S2 were the same. However, it was obvious that the content of calcium and phosphorus on S3 (23 and 10%, respectively) was higher than those in S1 and S2. On the contrary, calcium and phosphorus disappeared in S4, and the peak in Figure 4 (S4) at about 2KeV belonged to the gold coating.

As a summary of the foregoing, the results show that the modified surface of the composite showed an obvious change in morphology after physical abrasion. The calcium chloride ethanol saturated solution etchant etched PAA selectively while forming a rich Ca–P component on the surface of the composite. The formic acid etchant attacked the inorganic component without changing the PAA state.

Cell Responses to Samples

Cell Adhesion. Cell-material hybrids harvested at 4 h were used to analyze the influence of the surface treatment on the cell adhesion (the number of cells on TCP as a control for 100%). The result of the cell adhesion ratio is profiled in Figure 5. We found that the cell adhesion ratio for S1 and S2 were 108 and 110%, respectively. The cell attachment ratio of S3 (115%) was higher than that of S1 and S2; this indicated that the cell attachment on S3 was better than those on S1 and S2. On the other hand, the results show that the adherence of MG-63 cells on S4 (92%) was worse than that on S1.

Cell Proliferation. To evaluate the influence of the modified surface on cell proliferation, samples harvested at days 1, 3, 5, and 7 for MTT assay are shown in Figure 6. The OD values of S3 were higher than those of S2 at 1, 3, 5, and 7 days; this indicated that the cell proliferation on S3 was enhanced as compared to S2. In addition, both the OD values of S3 and S2 remained higher than those of S1 at 1, 3, 5, and 7 days. The OD of S4 was improved gradually at 1, 3, 5, and 7 days but remained worse than those of other samples. No significant differences appeared all of the time for S4 and TCP because of the absence of Ca and P.

Cell Morphology. Samples harvested at day 5 were used to observe the influence of the surface treatment on MG-63 regarding the cell morphology under SEM. As shown in Figure 7, the cells were well spread over the surface of S2 and S3 with intimate contact with the material surface. Meanwhile, the cells maintained physical contact with each other. A higher cell

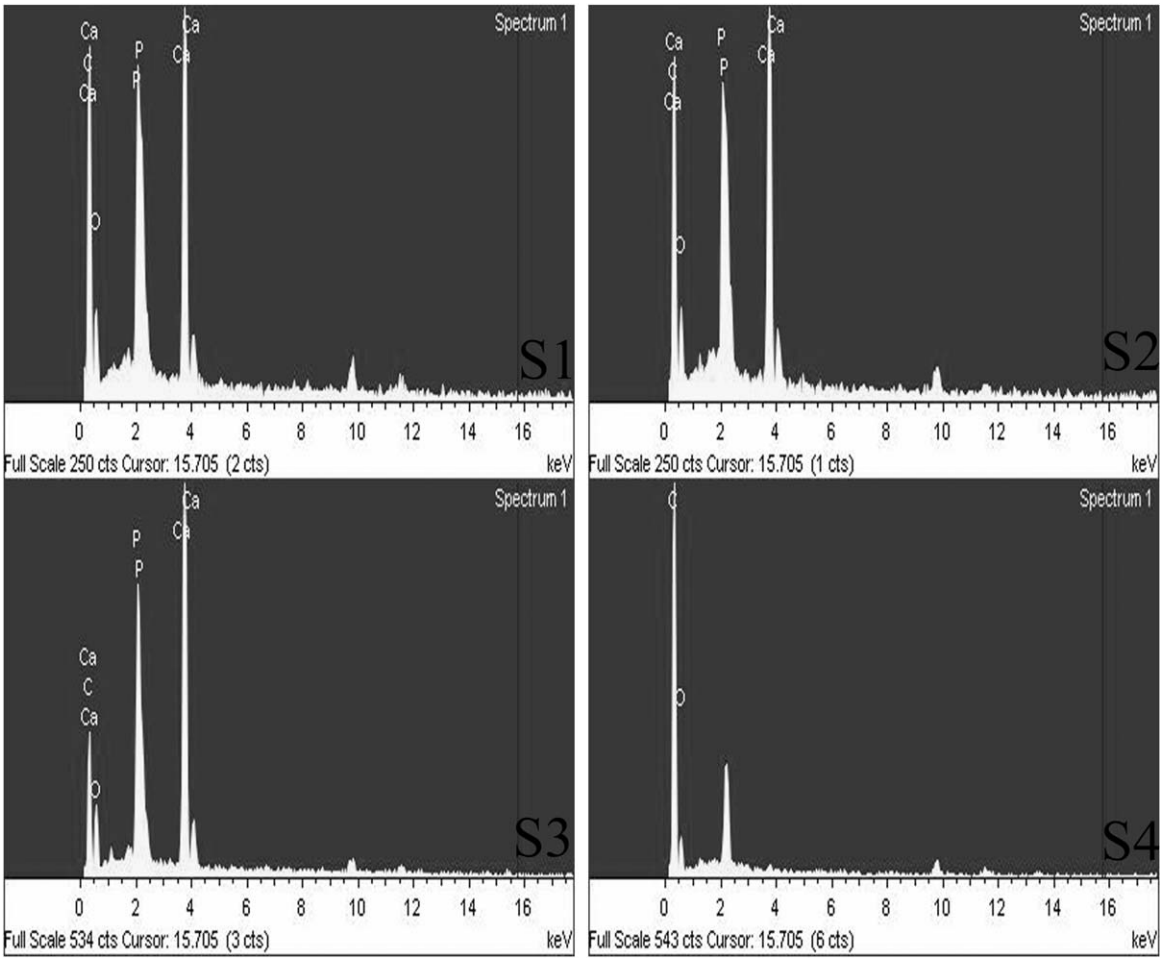

Figure 4. EDS of samples S1, S2, S3, and S4.

density could be seen on S3 in comparison with S1. By contrast, cells cultured on the S4 scaffold appeared flat and partially covered the material surface.

DISCUSSION

Bone can be considered a nanocomposite material made of collagen fibers threading through the mineral phase.[29] On the basis of a biomimetic mechanism, PAA with random amino acid copolymers was prepared with the similar molecular groups to collagen in natural bone. Furthermore, the incorporation of inorganic materials of HA and CS were introduced uniformly into this biomimetic PAA macromolecular network to mimic the inorganic and organic components of normal bone.[30,31] In addition, it is well-known that when there are higher contents of inorganic (e.g., HA and apatite) in the composite, better bioactivity can be achieved.[32,33] Thus, a novel multi(amino acid) copolymer/HA/CS composite with *in situ* melting polycondensation techniques was prepared for to maintain the best mechanical properties and excellent bioactivity.

The challenge in developing biomaterials for tissue engineering is to create surfaces that can direct new tissue regeneration.[34] In this study, three different methods were introduced to change the surface physicochemical properties of the biocomposite to improve the *in vitro* effect of cell behavior; the biocomposites

Table I. EDS Values of the Samples

	Weight rate of element (C % + O % + Ca % + P % = 100%)			
Sample	C	O	Ca	P
S1	51.84 ± 0.07	24.56 ± 0.06	18.03 ± 0.06	5.58 ± 0.07
S2	51.72 ± 0.07	24.50 ± 0.04	18.13 ± 0.07	5.66 ± 0.02
S3	34.25 ± 0.02	32.32 ± 0.22	23.11 ± 0.06	10.32 ± 0.22
S4	73.30 ± 0.02	26.70 ± 0.03	0	0

The data represent the mean plus or minus the standard deviation ($n = 5$).

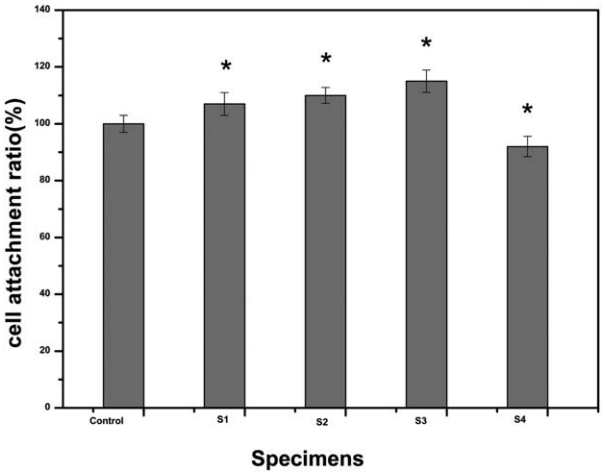

Figure 5. MG-63 attached on the surfaces of the samples at 4 h with TCP as a control and samples S1, S2, S3, and S4. Asterisks indicate that the cell attachment ratio seeded on the samples was significantly higher or lower than that of TCP ($p < 0.05$)

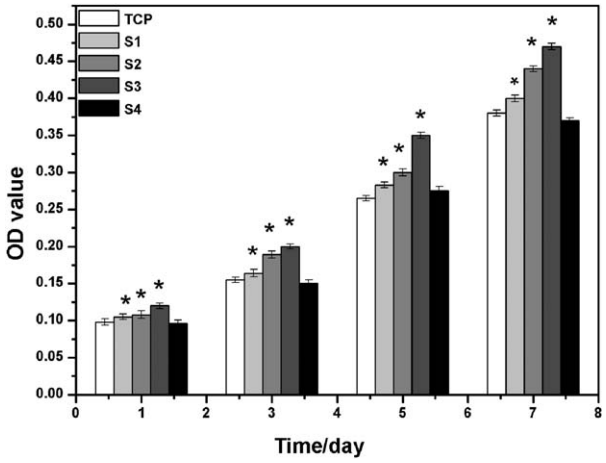

Figure 6. Proliferation of the MG-63 cells on samples for 1, 3, 5, and 7 days with TCP as a control and samples S1, S1, S2, S3, and S4. Asterisks indicate that the proliferation rate of the cells seeded on the samples was significantly higher than that of TCP ($p < 0.05$)

were treated by sandblasting, formic acid etching, and saturated calcium chloride ethanol solution etching. The obtained result indicated that the physical treatment of sandblasting did not change the chemical characterization of surface but made the surface rougher (sample S2). Although the chemical treatment changed the material surface both physically and chemically.

The evaluation of the etching solution for the composite showed that PAA were preferentially etched in the calcium chloride ethanol saturated solution, and the inorganic component was preferentially attacked in the formic acid. Ethanol solution was a kind of protic solvent; this combined with calcium chloride by the formation of a complex and facilitated the permeability of the solvent. Then, the polymer in the surface of the

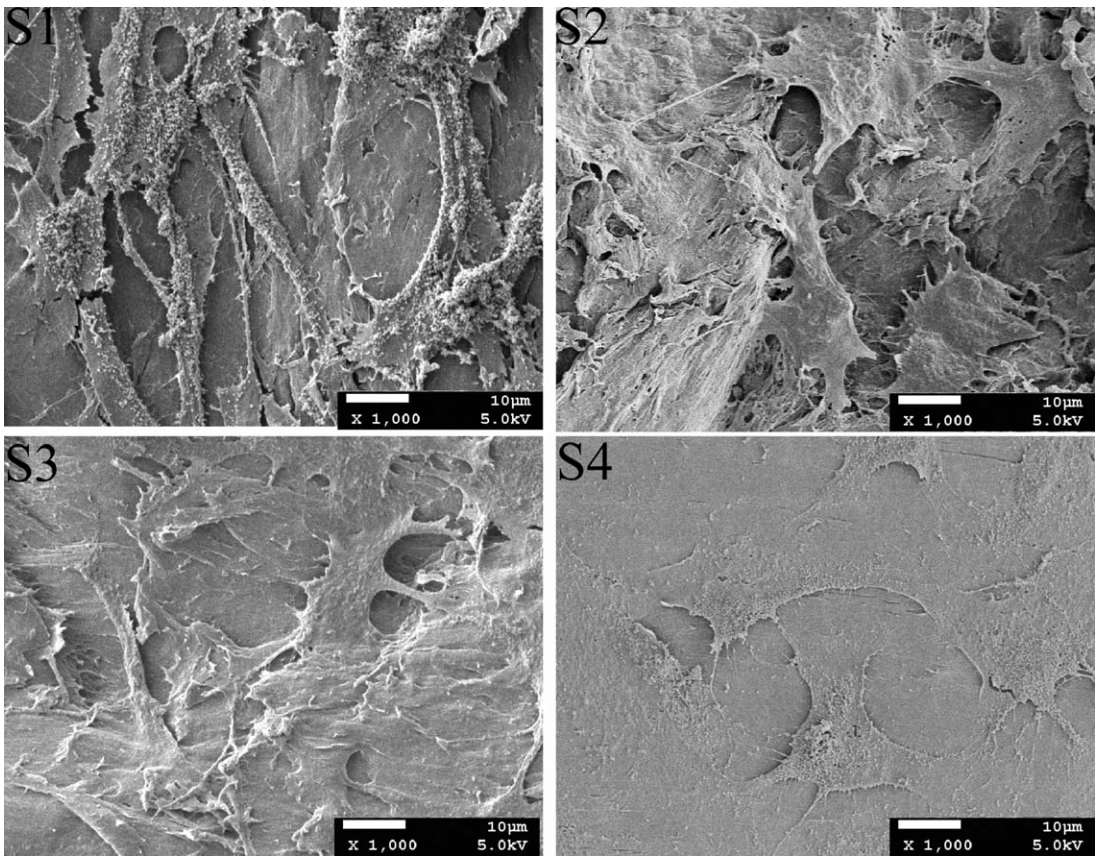

Figure 7. Morphology of the MG-63 cells on the surfaces of samples at day 5 under SEM for samples S1, S2, S3, and S4.

composite might have been disrupted by this kind of solution. As a result of this effect, the crystallinity of the inorganic component increased, and the crystallinity of the organic component decreased in XRD (sample S3). Formic acid had a strong corrosivity, and the surface of the samples could be corroded in the short run when the material was immersed into it. The inorganic component disappeared, and the organic component remained in the surface of sample. These transformations might have been caused by the stickiness of polymer and the runoff of inorganics. As a result, formic acid did not change the surface morphology notably but it dissolved the inorganics on the surface of the composite, leaving organics (S4).

It is known that the cellular responses to a material, such as attachment, proliferation, and differentiation, depend on not only physical status but also on the surface chemical composition of the material.[35–39] It is suggested that a relatively rough surface could be more in favor of cell interaction; this could promote cell attachment and maintenance. Compared with S1, the treatment of S2 improved cell attachment and proliferation. The enhancement was likely associated with the microrough surface of the composite because S1 and S2 were chemically the same. The cell adhesion and proliferation were further improved on S3 as compared to S2. Given the fact that both S2 and S3 had rougher surfaces compared to S1 and the fact that there were richer calcium and phosphate contents on the surface of S3, we concluded that the improvement of cell adhesion and the cell proliferation of S3 occurred because of the presence of rich Ca–P apatite and the rough surface. The results suggest that the adhesion and proliferation of osteoblast-like cells were not only affected by surface roughness but also by the chemistry. The influence of the surface chemistry on the cell adhesion and proliferation was further confirmed when S1 and S4 were compared. With the absence of calcium and phosphate on the surface after formic acid etching (S4), cell proliferation was inhibited compared to that in S1, even though the two samples had similar surface topographies. However, the proliferation result shows no significant differences at any time for S4 and TCP. Also, further *in vitro* and *in vivo* study should be considered.

Collectively, the results presented herein suggest that the adhesion and proliferation of osteoblasts could be influenced by the physicochemical properties of the materials. In the previous study, researchers tried to create a rich Ca–P surface by plasm spraying, high-velocity oxyfuel spraying, the sol–gel method, and so on. Nevertheless, all of these technologies require a long processing time or complicated equipment; this makes them time-consuming and expensive. Furthermore, it is difficult to ensure the strong bonding between the coating and matrix because of their different natures. In our research, we found that saturated calcium chloride ethanol solution etching generated a rough and rich Ca–P apatite surface of the composite in comparison with the original samples; this improved the cell adhesion and proliferation. We expected that this specific surface treatment could improve the performance of PAA/HA/CS composites for bone regeneration. Also, the formic acid etching of organic and inorganic composites is a suitable method for selectively changing its surface properties, and this will improve the application of this composite as a biomaterial or for other applications. However, further studies need to be conducted to confirm the application of these kinds of surface modifications for other composites, especially for polyamide-based composites.

CONCLUSIONS

By introducing HA and CS to PAA, we developed PAA/HA/CS composites. The surface modifications of mechanical abrasion and selective etching significantly changed the surface structure and/or surface chemistry. As a result, the samples modified by grit-blasting and saturated calcium chloride ethanol solution etchant facilitated MG-63 adhesion and supported cell proliferation over time, whereas the cell adhesion and proliferation were inhibited because of the absence of calcium and phosphate by the selective etching of formic acid. The results suggest that modification with mechanical abrasion and selective chemical etching may constitute an effective way of improving the biological performance of (amino acid) copolymer-based composites for their use in biomedical applications for bone tissue engineering or other applications.

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