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Fluor-hydroxyapatite sol–gel coating on titanium substrate for hard tissue implants

Hae-Won Kim[a], Hyoun-Ee Kim[a,*], Jonathan C. Knowles[b]

[a] School of Materials Science and Engineering, Seoul National University, Seoul 151-742, South Korea
[b] Department of Biomaterials and Tissue Engineering, Eastman Dental Institute, University College London, 256 Gray's Inn Road, London WC1X 8LD, UK

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Abstract

Hydroxyapatite (HA) and fluor-hydroxyapatite (FHA) films were deposited on a titanium substrate using a sol–gel technique. Different concentrations of F^- were incorporated into the apatite structure during the sol preparation. Typical apatite structures were obtained for all coatings after dipping and subsequent heat treatment at 500°C. The films obtained were uniform and dense, with a thickness of ~5 μm. The dissolution rate of the coating layer decreased with increasing F^- incorporation within the apatite structure, which demonstrates the possibility of tailoring the solubility by a functional gradient coating of HA and FHA. The cell proliferation rate on the coating layer decreased slightly with increasing F^- incorporation. The alkaline phosphatase (ALP) activity of the cells on all the HA and FHA coated samples showed much higher expression levels compared to pure Ti. This confirmed the improved activity of cell functions on the substrates with the sol–gel coating treatment.

Keywords: Hydroxyapatite/fluor-hydroxyapatite films; Ti substrate; Sol–gel method; Biological properties; Functionally gradient coating

1. Introduction

Titanium (Ti) and Ti-based alloys are widely used in dental and orthopaedic implants because of their good biocompatibility to such applications [1]. However, the bone ingrowth properties and implant fixation behaviour need to be improved in order to shorten the implant-tissue osseointegration time [2]. Therefore, a great deal of research has been carried out to improve the physical and chemical properties of the surface structure of Ti [3–5]. Among the different solutions which have been proposed, hydroxyapatite [HA, $Ca_{10}(PO_4)_6(OH)_2$] coatings on Ti have attracted a lot of attention over the last few years [6–8]. The excellent biocompatibility of HA is closely related to its chemical and biological similarities with human hard tissues [9]. In vivo studies of HA coatings on Ti implants have revealed good fixation to the host bones and an increased amount of bone ingrowth into the implants [10].

Along with HA, fluorapatite [FA, $Ca_{10}(PO_4)_6F_2$] coatings on metallic substrates have also attracted considerable attention in areas that require long-term chemical and mechanical stability of the coating layer [11]. Pure FA has a lower bio-resorption rate than HA, and has a level of biocompatibility comparable to that of HA, demonstrating such properties as fixation to bone and bone ingrowth [12,13]. Moreover, FA forms a fluor-hydroxyapatite [FHA, $Ca_{10}(PO_4)_6(OH,F)_2$] solid-solution with HA by replacing F^- with OH^- over a wide range of concentrations [14]. In practice, F^- itself has been widely investigated in dental restoration areas, because it prevents dental caries in a bacteria containing, acidic environment [14,15]. Furthermore, F^- promotes the mineralization and crystallization of calcium phosphate in the bone forming process [16].

Currently, most HA and FHA coatings are obtained using a plasma-spraying technique [6,7]. However, there are some problems associated with the plasma-spraying process, such as poor adherence to the substrate, chemical inhomogeneity and high porosity. Most of

*Corresponding author. Tel.: +82-2-880-7161; fax: +82-2-884-1413.
E-mail address: kimhe@snu.ac.kr (H.-E. Kim).

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these problems are associated with the excessively high fabrication temperature [6,7]. In comparison, the sol–gel technique offers certain advantages, because of the high chemical homogeneity, fine grain structure, and low crystallization temperature of the resultant coating, as well as its being both an economical and technically simple procedure to perform [17,18].

In spite of these advantages, there are few reports on the fabrication and properties of HA and FHA films using the sol–gel method. The purpose of this study was to deposit HA and FHA films on Ti substrates using the sol–gel approach and to evaluate the biological performance of these films in terms of their dissolution behaviour and in vitro cell responses.

2. Materials and methods

2.1. Preparation of sols and coating process

The preparation of the HA sol is described in detail elsewhere in the literature [19]. Briefly, controlled amounts of triethyl phosphite (TEP $[P(C_2H_5O)_3]$, Aldrich, USA) and distilled water were mixed in ethanol. The FHA sols were prepared using various amounts of ammonium fluoride [NH_4F, Aldrich, USA] in the P containing solution. The [P]/[F] molar ratios were 12, 6, and 4 in order to have the corresponding compositions of $Ca_{10}(PO_4)_6(F_{0.5}\cdot OH_{1.5})$, $Ca_{10}(PO_4)_6(F\cdot OH)$, and $Ca_{10}(PO_4)_6(F_{1.5}\cdot OH_{0.5})$, by replacing the OH group with F ions in molar ratios of 0.25, 0.5 and 0.75, respectively. After stirring for 24 h, the solutions were added slowly to a solution containing a stoichiometric amount ($Ca/P\sim1.67$) of calcium nitrate [$Ca(NO_3)_2\cdot 4H_2O$, Aldrich, USA]. The mixture was stirred at room temperature for 72 h and then for a further 24 h at 40°C.

As the substrate for the coating, a commercially pure Ti (c.p. Ti, grade 2) disc was prepared after polishing with sand paper (#1800) and cleaning in acetone and ethanol. The HA and FHA films were obtained by dip coating at a withdrawal speed of 5 mm/min. The obtained films were dried in an oven at 80°C for 12 h, and then heat-treated at 500°C for 1 h in air. The specimens with FA/HA ([F]/[OH]) ratios of 0, 0.25, 0.5, and 0.75 were designated as HA, 25FHA, 50FHA, and 75FHA, respectively.

2.2. Characterization and dissolution test

The phase change during the heat treatment was characterized using X-ray diffraction (XRD; M18XHF-SRA, Mac Science Co., Japan). The surface and cross section morphologies of the coating layer were observed using scanning electron microscopy (SEM; JSM-5600, JEOL, Japan).

In order to observe the dissolution behaviour of the films, the coating layers on Ti were immersed in a physiological saline solution (0.9% NaCl) and aged for predetermined periods of time. At the end of each incubation period, the sample was removed and the Ca^{2+} ion concentration dissolved from the film was measured using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES; ICPS-100IV, SHIMADZU, Japan).

2.3. Cell proliferation

The MG63 cell line was cultured in flasks containing Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc., MD, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc., MD, USA). Each specimen for the cell test was prepared in a disc shape with dimensions of $\sim 11\times 1$ mm (diameter × thickness) followed by sterilization at 121°C for 20 min. A Thermanox cover slip (NUNC, IL, USA) was used as a control, and pure Ti was also tested for the purpose of comparison. In order to measure the effect of each material on the cell growth, the cell lines were plated at a density of 3×10^4 cells/ml on all the specimens and the Thermanox control in individual wells of a 24-well plate. The cells were cultured for up to 7 days in an incubator humidified with 5% CO_2/95% air at 37°C. Following the incubation, the cells were detached with a trypsin-EDTA solution (trypsinization) and the living cells were counted using a haemocytometer (Superior Co., Germany). Each set of tests was performed in triplicate, and the data was normalized by taking the surface area into consideration. The cell morphology after proliferation was observed with SEM after fixing the cells with 2.5% glutaraldehyde, dehydrating them with graded ethanol (70%, 90%, and 100%), and critical point drying with CO_2.

2.4. Alkaline phosphatase activity

The isolated human osteosarcoma (HOS) cell line was used to determine the alkaline phosphatase (ALP) activity. The cells were plated at a density of 1×10^4 cells/ml on the specimens and cultured for 10 days. At harvest, the culture media was decanted, and the cell layers were washed with Hank's Balanced Salt Solution (HBSS), which was then followed by a trypsinization procedure, as described above. After centrifugation at 1200 rpm for 7 min, the cell pellets were washed with phosphate-buffered saline solution (PBS) and resuspended by vortexing in 200 μl of 0.1% Triton X-100. The pellets were disrupted by 7 cycles of sequential freezing and thawing, each for 2 min. After centrifugation at 13,000 rpm in a microcentrifuge for 15 min at 4°C, the cell lysates were quantified using the BioRad DC protein assay kit (BioRad, Hercules, CA)

and assayed colorimetrically for ALP activity using *p*-nitrophenyl phosphate as the substrate at pH 10.3 (Sigma, St. Louis, MO, USA). Each reaction was initiated with *p*-nitrophenyl phosphate, and allowed to proceed for 60 min at 37°C, and then quenched on ice. The *p*-nitrophenol produced was measured at 410 nm using a spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan).

3. Results

3.1. Phase and morphology

The XRD patterns of the apatite coatings on the Ti substrate after heat treatment at 500°C for 1 h are shown in Figs. 1(A)–(D). As shown in these patterns, typical apatite peaks were observed in all cases. The peak intensity appeared to increase with increasing F content, but the difference was minimal.

The surface morphologies of the Ti, before and after coating with the apatite films, were observed with SEM, and are represented in Figs. 2(A)–(D). Machining

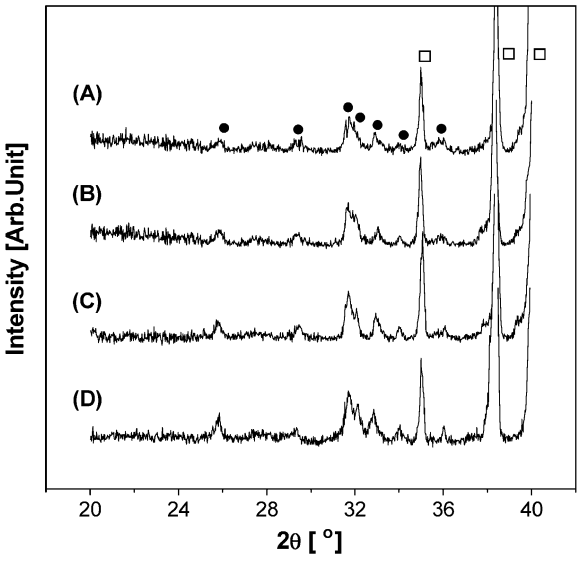

Fig. 1. XRD patterns of the sol–gel HA and FHA coatings on Ti after heat treatment at 500°C for 1 h: (A) HA, (B) 25FHA, (C) 50FHA, and (D) 75FHA. Legends are (□) Ti and (●) HA.

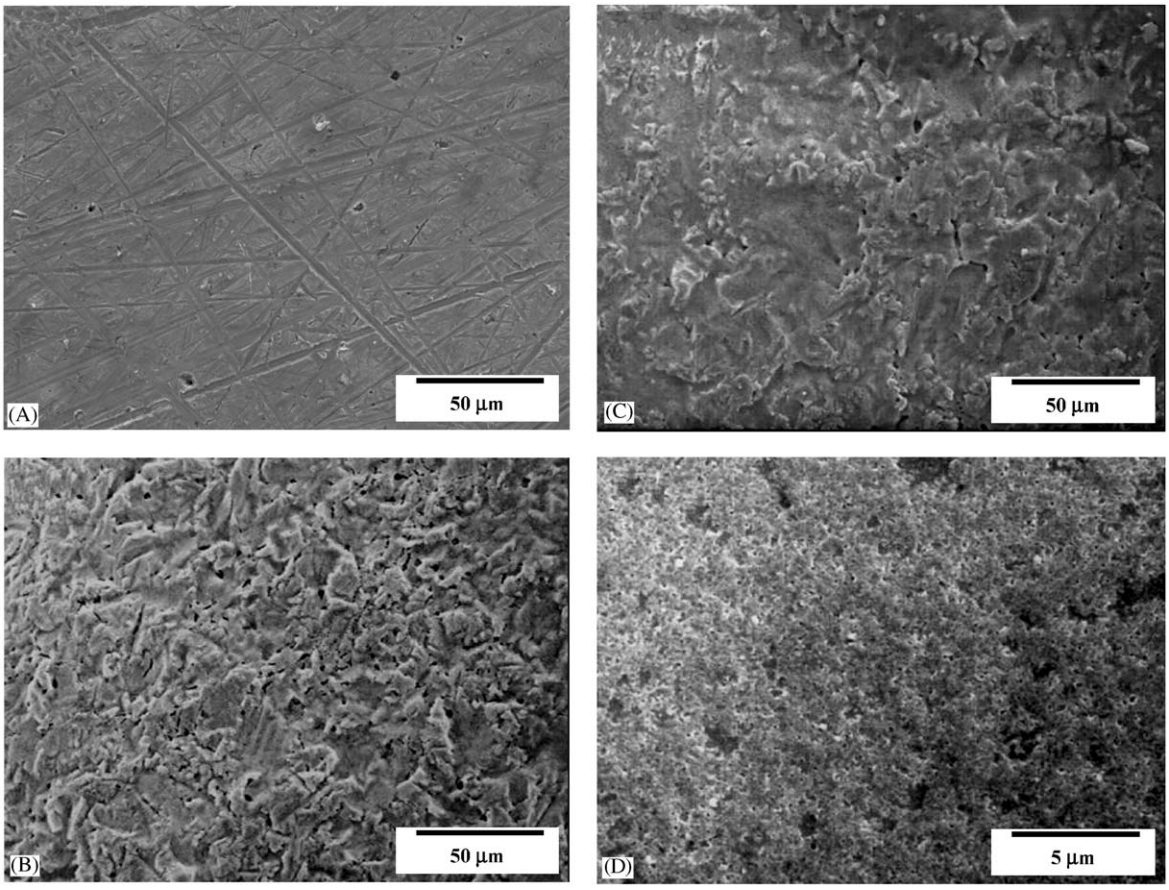

Fig. 2. SEM surface morphologies of the pure Ti and apatite coated Ti after heat treatment at 500°C for 1 h: (A) pure Ti, (B) HA coating, (C) 50FHA coating, and (D) 50FHA coating at higher magnification.

scratches were observed on the pure Ti surface (Fig. 2(A)). When a pure HA film was coated on Ti, the surface was rather porous and rough (Fig. 2(B)). The morphology of the 50FHA layer appeared to be much denser and smoother than the HA layer (Fig. 2(C)). However, at high magnification, very small nano-pores were observed in the 50FHA coating surface (Fig. 2(D)).

The fractured cross-sectional morphologies of the 50FHA films are shown in Figs. 3(A) and (B). A smooth, uniform, approximately 5 μm thick layer was formed on the Ti substrate (Fig. 3(A)). At high magnification, the coating structure was observed to be rather nano-porous (Fig. 3(B)). The morphologies of the other apatite films were similar to that of the 50FHA specimen (data not shown here).

3.2. Dissolution behaviour

The dissolution behaviours of the various apatite coatings on the Ti substrate after heat treatment at 500°C for 1 h are shown in Fig. 4. The concentration of Ca^{2+} ions released from the coating layer was monitored after incubation in the physiological saline solution for up to 14 days. For all coating samples, Ca^{2+} ions began to dissolve after a very short period of time (3 h), and the dissolved amounts increased with increasing incubation time. After 24 h, the dissolution rates differed markedly one another. The pure HA coating dissolved much faster than all the other FHA coatings, and this trend became increasingly obvious as the length of the incubation period increased. As expected, the dissolution rate of the 75FHA sample was the lowest.

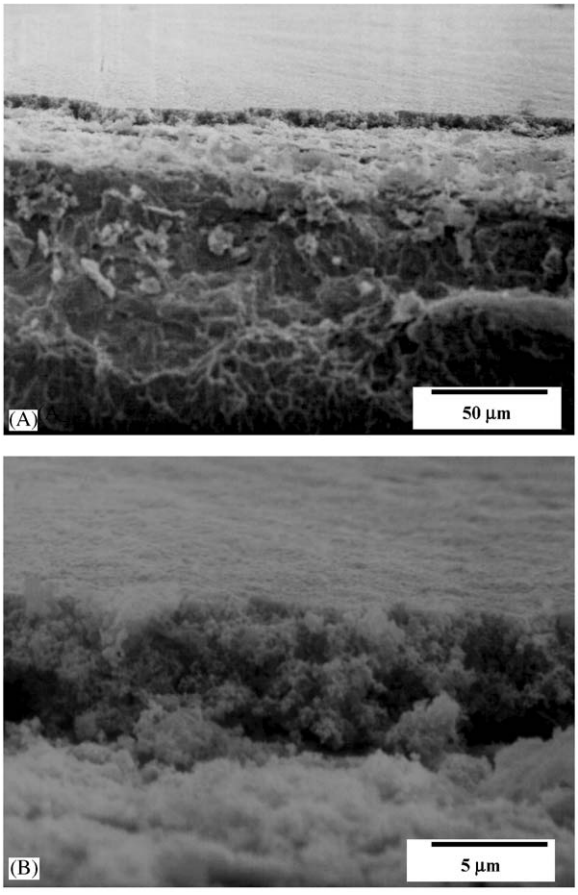

Fig. 3. SEM cross sectional morphologies of the sol–gel 50FHA coatings on Ti after heat treatment at 500°C for 1 h at (A) low and (B) high magnification.

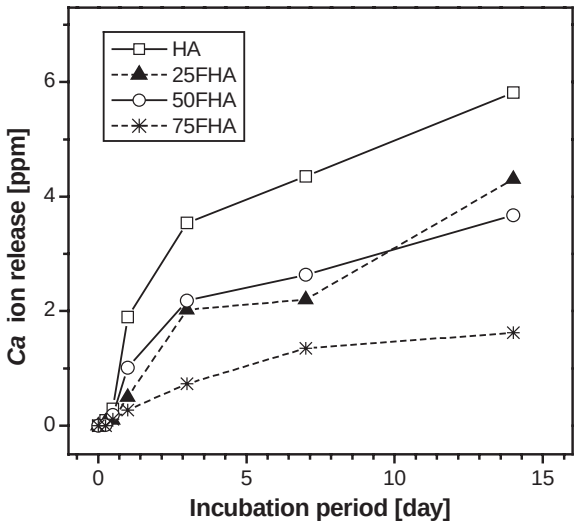

Fig. 4. Dissolution behaviour of the sol–gel HA and FHA coatings on Ti after heat treatment at 500°C for 1 h.

Figs. 5(A)–(D) show the surface morphologies of the coating layers after dissolution for 1 week. A large portion of the HA coating dissolved (denoted by 'Ti') leaving only a small portion of the original HA coating (denoted by 'C') as shown in Fig. 5(A). At high magnification, the remaining coating layer was observed to be highly porous. When the 25FHA specimen was dissolved under the same conditions, the dissolved area was much lower, as shown in Fig. 5(B). At high magnification, the remaining coating surface exhibited a spike-like pattern, suggesting the selective resorption of the coating surface. Similar patterns were observed in the 50FHA sample (Fig. 5(C)). The 75FHA film was barely dissolved, as shown in Fig. 5(D). However, very small spike-like dissolution patterns were observed at high magnification.

3.3. Cellular responses

The in vitro cell responses to the apatite coatings on Ti were assessed using osteoblastic MG63 and HOS cells. The MG63 cell morphologies on the pure Ti and coated samples after culturing for 5 days are shown in Figs. 6(A)–(E). On the pure Ti surface, the cells were highly elongated having a rod-like shape (Fig. 6(A)). On

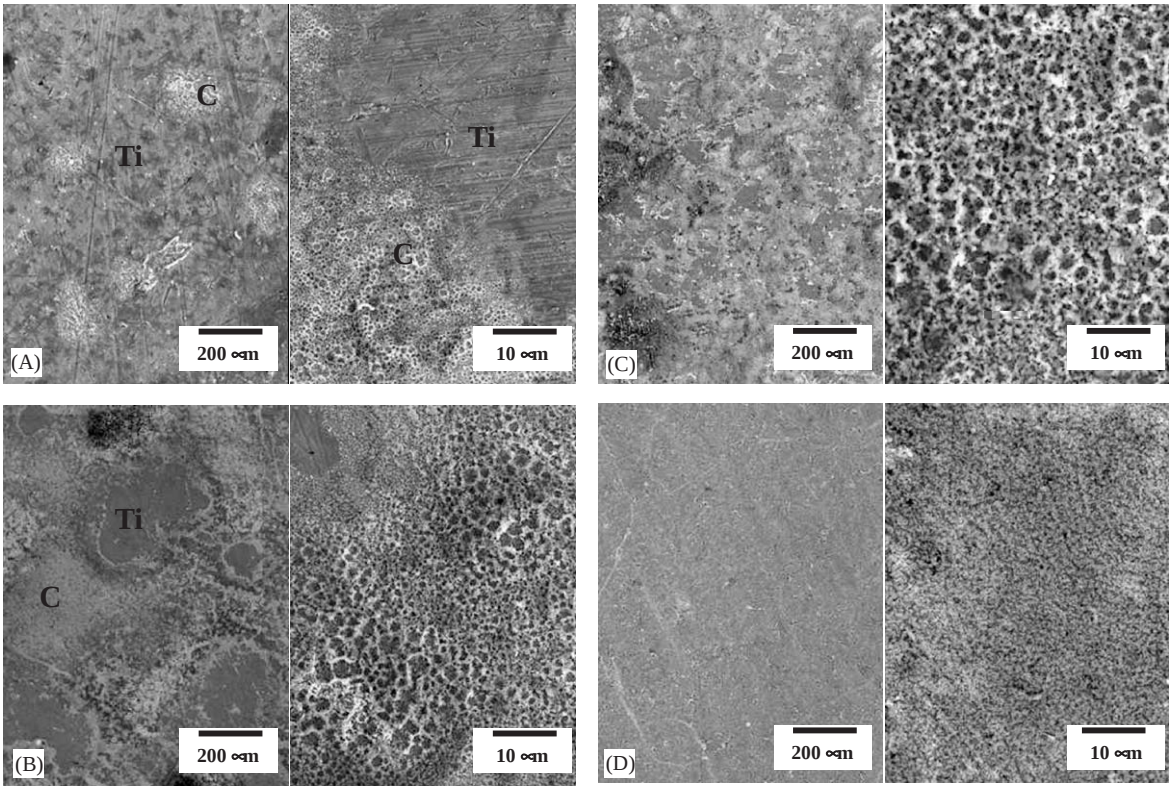

Fig. 5. SEM of the dissolved surface of the various sol–gel coatings on Ti after heat treatment at 500°C for 1 h. The dissolution test was performed for 7 days in a 0.9% NaCl saline solution: (A) HA, (B) 25FHA, (C) 50FHA, and (D) 75 FHA. Dissolved surface and remaining coating areas are marked by 'Ti' and 'C', respectively.

the HA coating surface, the cell spreading direction was much more diverse (Fig. 6(B)). On all the FHA coating samples, the cell morphologies were not much different from that on the pure HA coating (Figs. 6(C)–(E)). The degree of dissolution of the coating layers is also well illustrated in these micrographs. For the pure HA coating, almost all the HA dissolved, leaving only the underlying Ti metal (Fig. 6(B)). On the other hand, the FHA coating surface became rough and porous as a result of the selective resorption of the coating layer. Even though the extent of dissolution of the coating differed from one sample to another, the cells grew favourably on all the specimens and the membrane extended in intimate contact with the surface. The degree of cell proliferation was quantified after culturing for up to 7 days, as shown in Fig. 7. A plastic culture dish was also tested for comparison purposes. After 7 days, the cell numbers on all samples increased by factors of ~2–3 compared to those after 5 days. There was little difference in cell numbers among the HA and FHA coating samples compared to the pure Ti, although slight decreases were observed in the 50FHA and 75FHA coatings.

Fig. 8 shows the ALP activity of the cells used to assess the functional activity of the proliferated cells. The HOS cell was used to determine the ALP expression level, since it is known to have a high ALP activity at a specific culture period and cell density [20]. Moreover, the expression of ALP has been recognized as an index for detecting the activity of osteoblastic cell functions in the bone forming process [21,22]. Compared to the pure Ti and culture dish, the coating samples showed much higher ALP expression levels after culturing for 10 days. Interestingly, there was no difference between the HA and FHA coating series.

4. Discussion

The purpose of this study was to improve the biocompatibility of pure Ti through surface coatings with HA or FHA. Up to now, most HA and FHA coatings have been deposited using a plasma-spraying technique. However, many problems, such as low bonding strength or chemical inhomogeneity due to the formation of CaO, TCP, and TTCP, have limited the efficiency of plasma-sprayed coating when used for biological applications [6,7]. Among the possible alternatives, the sol–gel method was chosen for the homogeneous composition and uniform and fine microstructure of the resultant coating, as well as for the simplicity and low cost of this procedure [17,18].

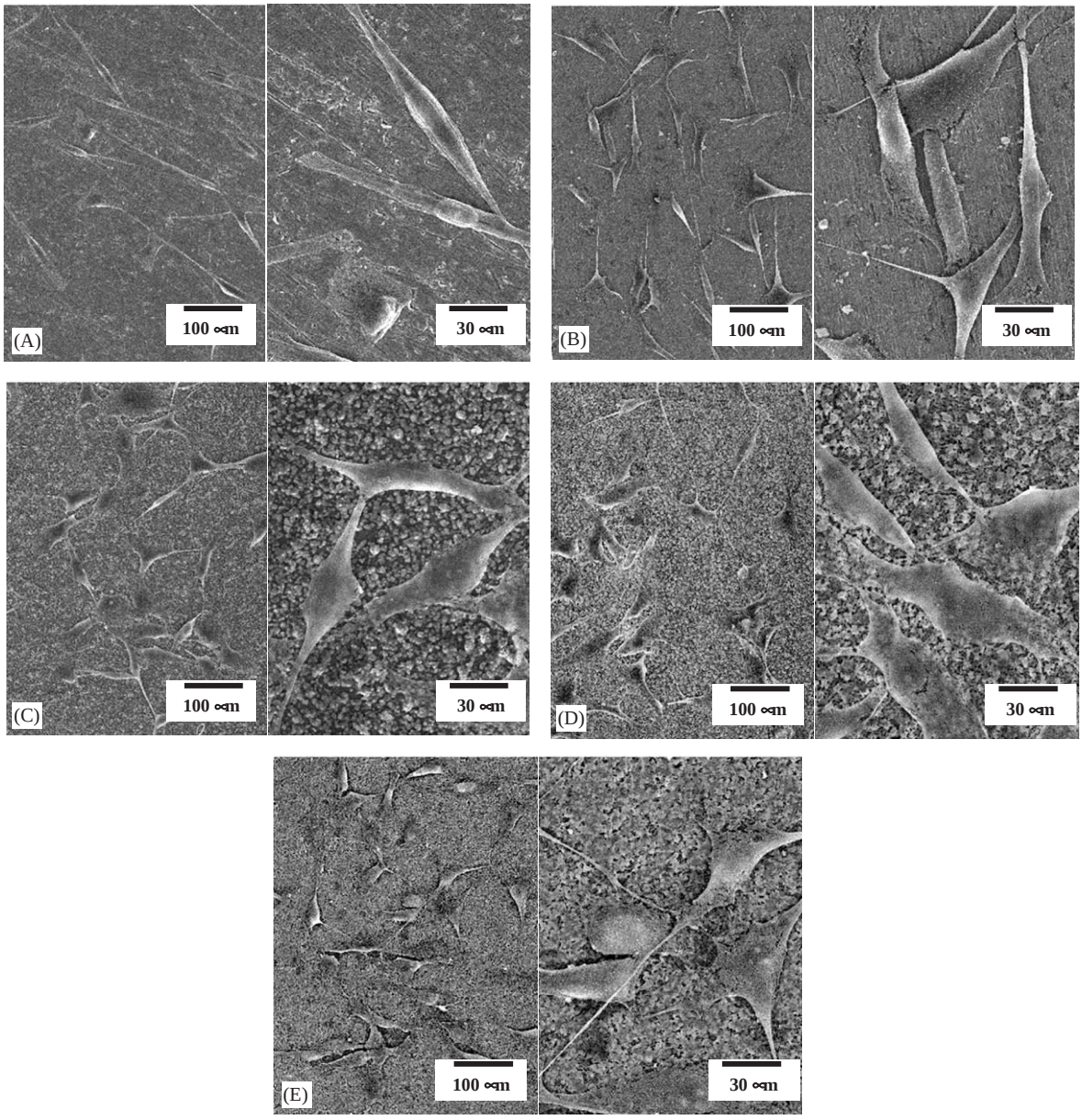

Fig. 6. SEM of the MG63 cells growing on pure Ti and various sol–gel coatings after culturing for 5 days: (A) pure Ti, (B) HA, (C) 25FHA, (D) 50 FHA, and (E) 75FHA.

One of the most important issues when considering HA for biological coatings is the dissolution rate in an environment where human body fluids are present. It has been reported that plasma-sprayed HA coatings dissolve and degrade quickly, resulting in the weakening of the coating-substrate bonding or the implant fixation to the host tissues [23–25]. For this reason, a FHA solid solution coating, which has a higher chemical stability than pure HA, was employed to improve the integrity and longevity of the coating layer.

As expected, the dissolution rate of the FHA coatings was considerably lower than that of the pure HA coating (Fig. 4). The observed dissolution rates (HA > 25FHA–50FHA > 75FHA) suggest the possibility of tailoring the solubility of the coating layers through F ion incorporation. Moreover, when deposited layer by layer (HA to FHA; from outer to inner layer), the coating system possesses a gradient dissolution rate that may be potentially useful for applications requiring a specific degree of bioactivity.

As in the case of the dissolution rate, the cell behaviours on the coating system were quite different from those on the pure Ti, in terms of cell growth, proliferation and ALP activity. The cell morphologies on the coatings were relatively isotropic and star-shaped compared to those on the pure Ti, where the cells were highly elongated, having a rod-like shape. The number of proliferated cells on the sol–gel coatings was slightly

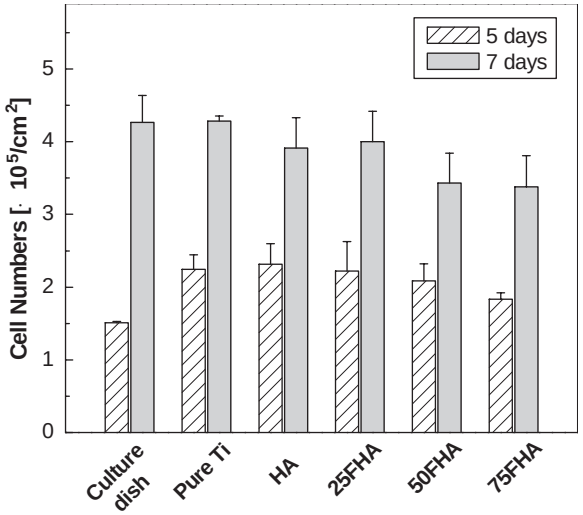

Fig. 7. Proliferation rate of the MG63 cells on each sample after culturing for up to 7 days. A cell culture dish was used as a control.

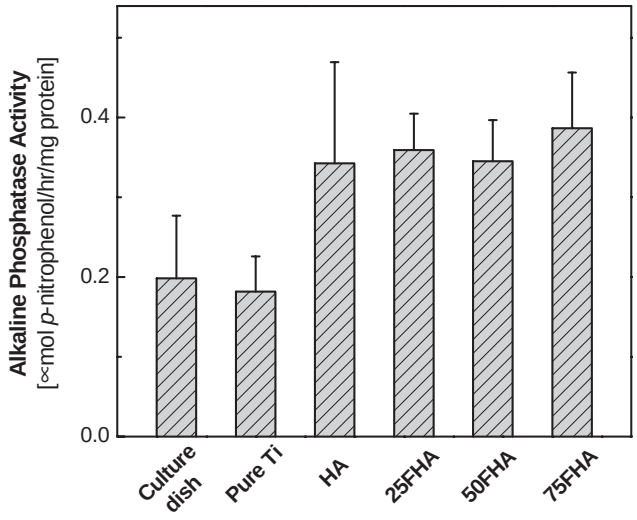

Fig. 8. ALP activity of HOS cells on each sample after culturing for 10 days. A cell culture dish was used as a control.

lower than that on the pure Ti. However, the ALP activity was significantly up-regulated with the sol–gel coatings (both HA and FHA), confirming the improved activity and functionality of the proliferated cells. The levels of cell attachment, proliferation and differentiation are affected by the physical and chemical properties of the material surface [26,27]. In contrast to pure Ti, where the surface properties are not supposed to change significantly during the culture period, the apatite coated surfaces were continuously changing due to the dissolution of the coating layer. For example, the surface morphology and roughness changed and the ion concentrations (Ca, P, and F) around the cells increased as the coating layers dissolved. In this case, dynamic interactions between the cells and the coating material are inevitable, which explains the higher level of cell functionality. Among the apatite coatings (HA and FHAs), comparable improvements in ALP activity were observed, despite the differences in surface properties driven by the dissimilar dissolution behaviours. At this point, the exact effect of F^- ion release on the cell responses has not yet been elucidated, because the required cell differentiation tests regarding bone-related proteins, such as osteocalcin, bone sialo-protein, and collagen, were not yet carried out in this study. However, the similar degree of cell proliferation and the higher ALP activity on the HA and FHA coatings compared to those on the pure Ti suggest the promising performance of sol–gel HA/FHA coatings on Ti in biomedical applications. Furthermore, the capability of tailoring the solubility of the coating layer is quite useful for the long-term stability of the implants in host tissues.

5. Conclusions

To improve biocompatibility of Ti, hydroxyapatite (HA) and fluor-hydroxyapatite (FHA) sol–gel layers were coated. The coating layers were phase-pure, dense, and uniform, and had a thickness of $\sim 5\,\mu m$ after heat treatment at 500°C. The FHA layer showed much lower dissolution rate than pure HA, suggesting the tailoring of solubility with F^- incorporation within the apatite structure. The osteoblast-like MG63 and HOS cells grew and proliferated favourably on all the HA and FHA coatings and pure Ti. Especially, the HA- and FHA-coated Ti exhibited higher ALP expression levels as compared to pure Ti, confirming the improved activity and functionality of cells on the substrate via the coatings.

Acknowledgements

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