Initial attachment of osteoblastic cells onto sol-gel derived fluoridated hydroxyapatite coatings

Yongsheng Wang,1 Sam Zhang,1 Xianting Zeng,2 Lwin Lwin Ma,3 Khiam Aik Khor,1 Min Qian2
1School of Mechanical and Aerospace Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798
2Singapore Institute of Manufacturing Technology, 71 Nanyang Drive, Singapore 638075
3School of Materials Science and Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798

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Abstract: Initial cell attachment and spreading of anchorage-dependent cells onto the material surface are crucial concerns for the development of more effective implants. In this study, MG63 cells were employed to investigate the initial cell response to sol-gel derived fluoridated hydroxyapatite (FHA) coatings. Along with that, surface roughness, wettability, and protein adsorption were also characterized for those FHA coatings, respectively. It was observed that both the surface roughness and contact angle have a slight increase in response to the incorporation of more fluorine ions. All FHA coatings showed similar amount of adsorbed proteins (~1.6 μg/cm²) upon testing in culture medium. Cell counting showed that no significant difference was observed for the amount of initially attached cells between HA and fluoridated HA coatings during the first 4 h culture. On the other hand, the well-spread cell on all prepared coating surface indicates that the incorporated fluorine ions have no adverse effect on cell spreading process. Therefore, it was suggested from this study that the prepared fluoridated hydroxyapatite coatings have comparable bioactivity to that of pure hydroxyapatite coating, and these results are meaningful for further investigation for application of FHA coatings. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 84A: 769–776, 2008

Key words: cell attachment; fluoridated hydroxyapatite; osteoblastic; protein adsorption; spreading

INTRODUCTION

Hydroxyapatite (HA) has been long recognized as substitute materials for repairing/replacement of human bones and teeth in orthopaedics and dentistry due to its chemical and biological similarity to human hard tissues and also the direct bonding capability to the surrounding tissues.1 Being intrinsically brittle and poor in strength, HA has been developed as a coating on metallic implants to shorten the implant-tissue osteointegration time and enhance fixation of the implants to the surrounding tissues.2,3 The HA-coated implant combines with the superior mechanical properties of metallic substrate with the unique biological properties of HA coating. However, the long-term clinical success of HA-coated implants is still questionable due to the high bioresorption rate of HA coatings in physiological environment,4,5 which could cause detrimental effect on adhesion properties, resulting in the generation of undesirable debris and even delamination of HA coating, which eventually lead to the failure of the implant.4,6

As the most promising substitute for HA as a coating, fluoridated hydroxyapatite (FHA, Ca₁₀(PO₄)₆(OH)₂ₓFx) has attracted much attention due to its biodegradation resistance and biocompatibility.7,8 It is well-known that fluorine is an essential trace element required for dental and skeletal development as well as the prevention of dental caries formation.9,10 On the other hand, it is reported that the presence of fluorine ions can also enhance the proliferation and differentiation of osteoblastic cell11 and promote bone regeneration.12 Until now, many methods had been employed for the preparation of FHA coatings on metallic substrates. The sol-gel method is thought to be the most promising method for its composition homogeneity, low cost as well as ease in operation and doping of desired ions.13–15

As a biological coating, the coating’s biological performance is of fundamental concern. In vitro study of FHA coated implants soaked in simulated body fluid
(SBF) and organic-modified SBF has shown that fluoride improves apatite deposition, thus is osteoinductive. However, the actual interaction between implant and bone is a cell-mediated (especially osteoblastic cell) regeneration process. In vitro cell response provides valuable indications of in vivo behavior of the coating. For anchorage-dependent cells, for example, osteoblastic cells, initial cell attachment, and spreading are crucial prerequisite in determination of long-term viability of cells on the implant surface, involving cell proliferation, differentiation, mineralization, and successful osteointegration. Although some studies have reported initial osteoblastic cell attachment and spreading on bulk HA surface, it is still an open question for FHA, either in the form of bulk material or coating. The present work aims to investigate the initial cell response on the sol-gel derived FHA coatings, that is, cell attachment and spreading. Along with this, surface roughness, surface energy, and protein adsorption are also characterized.

MATERIALS AND METHODS

Coating preparation and characterization

The preparation of dipping-sols and deposition of FHA coatings were described in details in our previous work. In brief, the selected precursors, that is calcium nitrate tetrahydrate (Ca(NO₃)₂·4H₂O, Sigma-Aldrich, AR), phosphorous pentoxide (P₂O₅, Merk, GR), and hexafluorophosphoric acid (HPF₆, Sigma-Aldrich, GR) were dissolved in absolute ethanol respectively for preparation of the dipping-sols. The designed degree of substitution of OH⁻ by F⁻ was indicated by the x value in the general formula of FHA, (Ca₉₋ₓ(PO₄)₆(OH)ₓ·Fₓ)₃, where x was selected as 0/3, 2/3, 3/3, 4/3, and 6/3, the subsequent coatings obtained were labeled as F0 (HA), F2, F3, F4, and F6 respectively. Titanium alloy (Ti₆Al₄V) slab of 20 × 30 × 1.2 mm³ polished to grade no. 1200 of SiC sandpaper were used as substrate. The dipping run was repeated four times for a final coating thickness of ~1.5 micron.

The surface morphology of FHA coatings was characterized using scanning electron microscopy (SEM, LEICA S360). Surface roughness (Rₛ) of the prepared FHA coating was determined with a noncontact optical profiler (Wyko NT2000, Vecco Instruments, USA). Results of X-ray diffraction analysis, coating surface morphology as well as mechanical properties were reported in our previous work. Contact angle was measured using the sessile drop technique with First 10 Angstroms 200 Goniometer (using deionized water for the measurement).

Cell attachment assay

Human osteosarcoma MG63 cells obtained from American Type Culture Collection (ATCC. Rockville, USA) were used to assay the osteoblastic cell attachment onto FHA coating surface. Cell culture was conducted at 37°C in a humidified 5% CO₂ atmosphere in a standard culture medium containing Eagle’s Minimum Essential Medium (EMEM, ATCC) supplemented with 10% fetal calf serum (FCS, ATCC) and 1% Penicillin/Streptomycin (ATCC). For cell attachment assay, the FHA coated samples were cut into small size (10 × 10 mm²) and sterilized in an autoclave at 121°C for 20 min and cultured in 24-well tissue-culture test plates. Cells were seeded on the sterilized coating surface at a density of 4 × 10⁵ cells/mL and cultured for up to 4 h. At each culture period (0.5, 1, 2, and 4 h), the samples were taken out and removed to new 24-well tissue-culture plates. After being washed two times with phosphate buffered saline solution (PBS), cells were detached with trypsin/EDTA and stained with trypan blue followed by counting the living cells using a haemocytometer (Becton Dickinson, Germany). Statistical analysis was carried out on cell counting using one-way analysis of variance (ANOVA) at an average of 3–5 replicates and significance was considered at p < 0.05.

Protein adsorption onto FHA coatings

To evaluate protein adsorption onto FHA coatings, smaller samples (10 × 20 mm²) were cut from the prepared coatings, and ultrasonically washed in acetone for 20 min followed by sterilizing in an autoclave at 121°C for 20 min. Then the samples were soaked in culture medium at a constant temperature of 37°C for 20 min. After that, the samples were gently rinsed in deionized water for three times and then immersed separately in 4 mL 0.1M EDTA-dissodium salt solution for 5 h at 37°C. Each sample received two more washes in the same EDTA solution (4 mL) for 1 h at 37°C. The solution was saved separately. The quantitative measurement of adsorbed proteins was conducted using a commercial Micro BCA protein assay kit (PIERCE, USA). All the experimental processes were carried out strictly according to protocol. By using the microplate assay mode, the measurement was read at 570 nm in a microplate reader and the amount of adsorbed proteins was calculated from a standard curve. Each set of tests was performed in triplets, statistical analysis was carried out using one-way analysis of variance (ANOVA) and statistically significant was considered at p < 0.05.

Cell Morphology

Scanning electron microscopy (SEM) was employed to examine the morphology of MG63 cell attachment and spreading. Cells were seeded on the prepared FHA samples (10 × 10 mm²) at cell density of 3 × 10⁵ cells/mL and incubated for 0.5, 1, 2, and 4 h. At each culture period, cells were fixed with 2.5% glutaraldehyde for 1 h at room temperature followed by dehydrating with a series of graded ethanol/water solutions (50%, 70%, 80%, 95%, and 100%, respectively). Then hexamethyldisilazane was added in each well to preserve the original morphology of the cells. The test plates were kept in a fume hood to dry at room temperature. The samples were coated with gold before observation under SEM (JEOL, JSM-5600LV) operating at 10 kV.
RESULTS AND DISCUSSION

Surface analysis

According to our previous results,25 fluorine ion has been incorporated into HA lattice structure with Ca/P molar ratio of 1.63–1.67 in FHA coatings. The surface morphologies of different FHA coatings are shown in Figure 1. The substrates have been completely covered by the coatings and the coatings look uniform and generally smooth. The fluorine (HPF₆) concentration in the dipping sols plays an important role on the surface morphologies of the prepared FHA coatings. As can be seen in Figure 1(a–d), the coatings tend to gain the rougher surface with the increasing fluorine ion content. Coating surface roughness ($R_a$ and $R_q$) measured with optical profiler indicates a slight increase with increasing fluorine ion concentration in the dipping sols (as shown in Table I), attributing to the formation of nc-CaF₂ in the dipping sols during coating preparation process.²⁸ On the other hand, with the increase in fluorine, the contact angles on FHA coatings gradually increases from 50.5° on F0 to 55.5° on F6 (as shown in Table I). Though the surface roughness may have influence on the coating wettability, the increase in contact angle should be attributed mainly to the incorporation of fluorine, as fully fluoridated HA ($x = 2$, fluorapatite) has a much higher contact angle (62°) than that of HA (49°).²⁹ This opinion is also agreed by recent studies.³⁰ According to Vogler’s definition, a hydrophobic surface is that with a contact angle $>65^\circ$,³¹ so defined, all the FHA coatings in this study are hydrophilic.

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<td>Measured Surface Roughness ($R_a$ and $R_q$) and Contact Angle of Different FHA Coatings</td>
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<td>Surface Roughness (nm)</td>
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Figure 1. SEM micrographs of FHA coating surfaces: (a) F0, (b) F2, (c) F4, (d) F6.
Proteins exist in blood plasma or serum in great numbers; once in touch with an implant, they quickly adhere to the implant surface because they are intrinsically highly surface active. It is through this adsorbed protein layer that cells sense foreign surfaces\(^{24,32}\). The adsorption of proteins plays a pivotal role for the subsequent cell attachment and the eventual cell morphology. Figure 2 summarizes the amount of adsorbed proteins on FHA coatings measured by the Micro-BCA method. No significant difference \((p > 0.05)\) is observed in the number of adsorbed proteins between different FHA coatings immersed in the culture medium for 20 min (all at the level of \(\sim 1.6 \mu g/cm^2\)). But this does not mean that fluorine incorporation has no influence on protein adsorption. It has been demonstrated that greater contact angle results in reduction of protein adsorption\(^{30,33}\). At the same time, however, larger surface area results in more adsorption. As a result of the competition between increase of contact angle and increase of surface roughness (thus surface area), the net change in protein adsorption seems negligible.

### Quantitative assay of cell attachment

The initial cell attachment onto FHA coatings was directly monitored by quantifying cell numbers at each culture period (0.5 h, 1 h, 2 h, and 4 h). The results were shown in Figure 3. Similar amounts of cells were attached onto different FHA coatings at each time slot. This indicates that the incorporated fluorine ions have no significant influence on initial cell attachment. In other words, all FHA coatings show comparable cell attachment, and there is no significant difference from that of the pure HA (see sample F0). This is understandable since cells attach to the coating surface through attachment on a layer of protein which in turn attach to the coating\(^{24,34}\). As the number of protein attached to the surface is the same on FHA and on pure HA, it follows that there is no difference in number of cells attached on these coatings. Since FHA coatings are hydrophilic and a similar amount of adsorbed proteins is observed, naturally, about the same amount of anchorages would be provided for cell attachment. Besides protein adsorption, surface roughness could also exert some influence on initial cell attachment. Deligianni et al.\(^{35}\) reported that the number of cells attached onto HA surface experienced insignificant increase when the surface roughness \((R_a)\) increased from 0.733 \(\mu m\) to 2.856 \(\mu m\); however, a significant difference was observed when the surface roughness...
reached 4.68 μm. In our study, the roughness variation (max ~33 nm in Ra) is too small to cause any significant increase in cell attachment.

With increase of incubation time, a slight increase in the cell attachments is observed. The increase becomes significant (p < 0.05) after 4 h incubation compared with that of 0.5 h. Most cells are believed to arrive at the coating surface in 0.5 h due to the exertion of gravity,36 but a large amount of weakly adhered cells are removed during the washing. After longer incubation, most cells attach well on the coating surface thus an increase in cell attachment is seen.

Cell morphology

The individual cells spreading process and the corresponding morphologies were studied with SEM. Since the cells do not reach the coating surface at the same time or spread at the same rate, the morphology may not be the same at a culture interval for individual cells (typically, c.f. Fig. 4). Therefore, the cell morphologies presented here are the typical ones at each corresponding culture period.

The cell spreading process as monitored by the change of cell morphologies was almost the same on all FHA coatings. Typically, Figures 5 and 6 show the morphological sequences of osteoblastic cells spreading on sample F0 (HA, without incorporation of fluorine ions) and F4, respectively. After having cultured for 0.5 h, the osteoblastic cells still appeared spherical with a rough surface texture containing numerous blebs [Figs. 5(a) and 6(a)]. Those blebs are believed to increase the total surface area of the cells to accommodate the excess membrane as the cells round up from the flattened state in response to trypsin.36 At the base of cells, filopodia were developed and extended on coating surface. The filopodia enabled the cells to contact and attach onto the surface. That was the first step. With increase of incubation time, the filopodia gradually extended outward for spreading/webbing of cytoplasm. After 1 h incubation [Figs. 5(b) and 6(b)], though the main body of cells was still almost spherical, the cells were well attached to the surface with...
extended filopodia as well as lamellipodia formed by the spreading of cytoplasm. As time passed, cytoplasm continued to spread outwards leading to flattening of the cells. After 2 h [Figs. 5(c) and 6(c)], the main body of the cells became cone-shaped and surrounded by cytoplasm. At this stage, a high adhesion strength is expected due to the increase of contact area between the cells and the surface. The progression of cytoplasm webbing finally resulted in a fully flattened cell, as shown in Figures 5(d) and 6(d), where even the nucleus could be seen. The cells attached to the surface tightly and their shape changed from spherical to polygonal, similar to that after 72 h (as shown in Fig. 7). As such, the cell attachment is completed within 4 h of incubation.

On the basis of the above observations, the process of cell attachment and spreading happens in the following steps, similar to that observed with osteoblast or other cells on HA, alumina, bone, and tissue culture polystyrene samples:

1. Adsorption of proteins on coating surface (surface roughness plays a positive role);
2. Contact and attachment of cells onto the protein layer (surface roughness does not have effect unless very rough-more than a few microns difference);
3. Centrifugal growth of filopodia;
4. Cytoplasm spreading and

![Figure 6](image1.jpg)
![Figure 7](image2.jpg)

**Figure 6.** Morphological changes of osteoblastic cells after incubating on F4 for up to 4 h: (a) 0.5 h, (b) 1 h, (c) 2 h, (d) 4 h.

**Figure 7.** A typical cell morphology after incubation for 72 h on FHA coatings.
finally; (5) cell flattening. Fluorine incorporation in HA causes an insignificant increase of surface roughness (∼33 nm $R_a$) and decrease in wettability (increased contact angle), it does not affect protein adsorption or initial cell attachment). In other words, fluoridated HA coatings are the same for cell attachment and spreading as pure HA coating (Figs. 5 and 6). Cellular adhesion strength increases in the spreading process resulting in a slight increase in the numbers of attached cells (Fig. 3). According to Folkman and Moscona,22 well-spread cells after 4 h incubation are more favorable for cell proliferation and differentiation that follows. Consequently, incorporation of fluorine ions in HA has no adverse effect on cell activity.

CONCLUSIONS

Incorporation of fluorine in HA from $x = 0$ to $x = 2$ (where $x$ is the molar value of F in Ca$_{10}$(PO$_4$)$_6$(OH$_2$)$_2$F$_x$) causes only insignificant increase in surface roughness (up to 33 nm $R_a$) and a slight increase in contact angle (from 50.5° for HA to 55.5° for Fe6). Incorporation of fluorine in HA does not affect the number of proteins adsorbed on the coating surface: about 1.6 μg/cm$^2$ for all FHA and HA coatings alike (even after 20 min in culture medium). This, in turn, results in no effect of fluorine incorporation on initial cell attachment. Incorporation of fluorine in HA has no adverse effect on cell spreading process. In summary, fluoridated hydroxyapatite coatings have comparable bioactivities to that of pure hydroxyapatite coating: fluorine does not exert adverse effect in the initial bio-processes.

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References