Osteoblastic cell response on fluoridated hydroxyapatite coatings

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Abstract

Fluoridated hydroxyapatite (FHA) coatings were deposited onto Ti6Al4V substrates by sol–gel dip-coating method. X-ray photoelectron spectroscopy results showed that fluoride ions were successfully incorporated into the hydroxyapatite (HA) lattice structure. The dissolution behavior in Tris-buffered physiological saline indicated that all fluoridated HA coatings had lower solubility than that of the pure HA coating. The lowest solubility was obtained at fluoride ion concentrations of 0.8–1.1 M. In vitro cell responses were evaluated with human osteosarcoma MG63 cells in terms of cell morphology, proliferation and differentiation (alkaline phosphatase activity and osteocalcin level). For all coatings tested, similar cell morphologies and good cell viability were observed. Coatings fluoridated to 0.8–1.1 had a stronger stimulating effect on cell proliferation and differentiation activities. The influences on cell phenotypes were attributed mainly to a combined ion effect of Ca, P and F released from the coating during dissolution. For the best dissolution resistance and cell activities, it is recommended that the molar level of fluoride ion be from 0.8 to 1.1, such that the coating takes the form of Ca10(PO4)6(OH)1.2–0.9F0.8–1.1.

Keywords: Cell response; Fluoridated hydroxyapatite; Proliferation; Differentiation

1. Introduction

The purpose of orthopedic implants is to restore the structural integrity and functionality of damaged hard tissues, and to minimize complications such as implant structural failure, loosening, etc., so as to improve the quality of life of a patient [1]. Hydroxyapatite (HA)-coated metallic implants combine the advantages of the bioactivity of HA with the excellent mechanical properties of metallic substrates, and thus are most attractive in clinic applications. In addition, the HA coating also serves as a protective layer for the metallic substrate against corrosion in the biological environment. However, pure HA has a long-term stability problem: the high rate of its bioresorption results in loosening and implant failure [2–4]. Fluoride exists in human bones and teeth as an essential element against dissolution. Recent research and development of fluoridated hydroxyapatite (FHA) has attracted much attention as a promising replacement for HA because FHA demonstrates significant resistance to biodegradation [5–7].

With the incorporation of fluoride ions in HA, the adhesion of the coating to the metallic substrate is also enhanced [8–10] and toughness increases at the coating/substrate interface [8]. As a biological coating, of course, mechanical properties are only part of the story. Biological performance – cytotoxicity, osteoinductivity, biodegradation, etc. – is another fundamental concern. Although in vivo testing is the most direct and reliable method to evaluate these properties, in vitro testing minimizes the number of live animals

The dissolution behavior of the FHA coatings was investigated by soaking in a Tris-buffered physiological saline solution (0.9% NaCl, pH 7.4) at a constant temperature of 37 °C for fixed periods of time. At the end of the period, the sample was taken out and the concentration of Ca^{2+} in the solution was analyzed with an inductively coupled plasma atomic emission spectrometer (ICP-AES, Perkin-Elmer Optima 2000).

2.2. Dissolution test

The dissolution behavior of the FHA coatings was investigated by soaking in a Tris-buffered physiological saline solution (0.9% NaCl, pH 7.4) at a constant temperature of 37 °C for fixed periods of time. At the end of the period, the sample was taken out and the concentration of Ca^{2+} in the solution was analyzed with an inductively coupled plasma atomic emission spectrometer (ICP-AES, Perkin-Elmer Optima 2000).

2.3. Cell and culture conditions

Human osteosarcoma MG63 cells obtained from American Type Culture Collection (ATCC, Rockville, MD) were used to assay the osteoblastic cell response on the coating surface. Cell culture was conducted at 37 °C in a humidified 5% CO_2 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 assay, the FHA-coated samples were cut into small pieces (10 mm × 10 mm × 1 mm) and sterilized in an autoclave at 121 °C for 20 min, before being cultured in 24-well tissue culture test plates to observe cell morphology, proliferation and differentiation.

2.4. Cell morphology

After incubation for 3 days, cells at a density of 5.3 × 10^3 cells ml^{-1} were fixed with 2.5% glutaraldehyde for 1 h at room temperature followed by dehydration with a series of graded ethanol/water solutions (50%, 70%, 80%, 95% and 100%, respectively). Then 0.5 ml hexamethyldisilazane was added to each well to preserve the original morphology of the cells; the test plates were kept in fume hood to dry at room temperature. The samples were coated with gold before observation under a scanning electron microscope (SEM, Leica S360) to determine their morphology.

2.5. Cell proliferation

Cells were seeded on the sterilized coating surface at a density of 4.8 × 10^3 cells ml^{-1} and cultured for up to 7 days. At each culture period (1, 2, 3, 5 and 7 days), the samples were taken out and removed to new 24-well tissue culture plates. After being washed twice with phosphate-buffered saline (PBS) solution, cells were detached with trypsin/EDTA and stained with trypan blue, after which the living cells were counted with a hemocytometer (Becton Dickinson, Germany).

2.6. Differentiation assay

To evaluate the functionality of the cultured cells on FHA coatings, intracellular ALP activity and osteocalcin (OC) expression were analyzed. Cells were seeded on each
sample at a density of $6 \times 10^6$ cells ml$^{-1}$ and cultured for 7, 14 and 21 days with a weekly change of culture medium. At harvest, all cell layers were washed twice with PBS and then detached from the coating surface with trypsin/EDTA. After centrifugation, the cell pellets were washed twice with deionized water and finally resuspended by vortexing in 0.6 ml lysis buffer solution (1% Triton X-100 in Tris–HCl buffer, pH 7.4). Following one cycle of freezing/thawing, cells were further lysed by sonicating them for 5 min at 4°C. Finally, after being centrifuged at 10,000 rpm for 5 min at 4°C, the supernatants were collected as cell lysates for the assay of ALP activity, OC concentration as well as total protein synthesis.

The ALP activity was measured using the phosphate detection kit (BIOMOL GREEN, USA). All the experimental processes were conducted strictly according to the provided protocol. By using the microplate assay mode, the measurement was read at 620 nm in a microplate reader and the ALP activity was calculated from a standard curve.

The quantitative measurement of OC in the cell lysates was conducted using an immunoenzymatic assay (EASIA ELISA kit, BioSource, USA). The experimental process was carried out strictly according to the directions of the manufacturer. The absorbance was measured in a microplate reader at 405 nm against a reference filter set at 620 nm and the OC expression level was evaluated based on a standard curve.

The measured ALP activity and OC concentration were finally normalized to the total protein content, which was determined using a commercial Micro BCA protein assay kit (Pierce, USA).

2.7. Statistical analysis

Statistical analysis was carried out on cellular tests using one-way analysis of variance (ANOVA) at an average of 3–5 replicates. Differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Incorporation of fluoride ion

The $F^-$ concentration in the coating determined by XPS is shown in Fig. 1. The dashed line shows the ideal situation where all $F^-$ in the dipping sol completely incorporates into the HA lattice. The data points are measured $F^-$ concentrations in the prepared FHA coatings. A discrepancy is observed between the intended $X$ value and the measured $F^-$ incorporation ($x$ value), especially at high concentrations, which is attributed to the loss of fluorine in the form of HF during the sol–gel coating process [16]. XPS narrow scan reveals only one peak located at 684.3 eV belonging to F1s (as a typical example, the inset in Fig. 1 shows that of F4 or $X = 4/3$), which is the fingerprint of $F^-$ in the FHA or FA structure [17], indicating that fluoride ions have been successfully incorporated into the HA lattice structure.

3.2. Dissolution behaviors

The dissolution behaviors in Tris-buffered physiological saline solution are shown in Fig. 2. The concentrations of Ca$^{2+}$ released from the coatings into the solution increase with soaking time and the dissolution rates decrease gradually, especially after a soaking duration of 7 days (Fig. 2). At equilibrium, dissolution decreases as $F^-$ is incorporated, with F3 and F4 reaching a minimum (see inset of Fig. 2 for all coatings after 21 days). All the fluoridated coatings have a lower Ca$^{2+}$ concentration compared with that of pure HA, but the dissolution rates follow the order $F_0 > F_2 > F_6 > F_3 \approx F_4$ (cf. Fig. 2). This can be explained on the basis of the disordered structure of HA and the hydrogen bonding in the hydroxyl group to $F^-$ occurring in the structure [18–20]: in the HA crystal structure, H+ in a hydroxyl group can bond with O$^2-$ from different directions along the c-axis; once an OH group is substituted by a $F^-$, a stronger hydrogen bond forms between
the F⁻ and the nearby OH group, forming a more ordered structure with enhanced chemical and thermal stability [20]. As such, the greatest contributions to the stability of the crystal structure will be located at a 50% substitution of OH by F⁻ (or at \( x = 1 \)). In our present study, from Fig. 1, F3 (\( X = 3/3 \)) and F4 (\( X = 4/3 \)) corresponds to measured \( x \) of 0.84 and 1.04, respectively. These coatings have lower solubility than others (cf. inset of Fig. 2). Taking into consideration the random nature of the substitution of F for OH, it is reasonable to assume that our current study indicates a probable optimum range of 0.8–1.1 as the degree of fluoridation giving minimum solubility.

3.3. Cell responses on FHA coatings

Fig. 3 shows typical SEM micrographs of MG63 cells after 3 days of culture on FHA coatings. No significant difference is observed in cell morphology on different FHA coatings. All cells spread well and grew favorably across the coating surface. As shown in Fig. 3(a)–(c), typically, the cells are seen flattened and attach tightly on coating surfaces with their filopodium and lamellipodium, suggesting good cell viability on all FHA coatings.

Cell proliferation was directly monitored by quantifying cell numbers at each culture period, and the results are shown in Fig. 4. Within the first 2 days, cell number increased slightly and no significant \( (p < 0.05) \) difference was observed between the coatings. A greater proliferation rate occurs between 2 and 5 days followed by a slow-down until 7 days. The cell numbers on F3 and F4 were significantly \( (p < 0.05) \) higher than those on F0, F2 and F6 after 5 days. No significant difference was observed among F0, F2 and F6 throughout the entire evaluation period. Comparing with the initial cell density, a significant increase is observed in the amount of cells after 7 days of culture on all coating surfaces (at least four times as many as the seeding density). That means all coatings, regardless of degree of fluoridation, have favorable viability with MG63 cells and thus have comparable bioactivities in vitro. Meanwhile, the cell numbers on F3 and F4 coatings were significantly higher than on other coatings after 7 days (Fig. 4), this indicates that F3 \( (x = 0.84) \) and F4 \( (x = 1.04) \) coatings stimulate cell proliferation more. Similarly, Harrison et al. [21] reported that a higher number of mouse embryonic stem cells were obtained with a fluoride level of 0.3–1.1 on FHA discs. Qu and Wei [22] reported that after 7 days, osteosarcoma cells (SAOS-2) on FHA discs with fluoride

![Fig. 3. Morphology of MG63 cell after 3 days on: (a) F0; (b) F4 and (c) F6.](image-url)
content of 0.3–0.567 (mol F⁻/mol apatite) had a significantly higher total amount of protein than that on pure HA, indicating a higher proliferation rate on those discs. Therefore, a certain amount of F⁻ in the coatings is believed to benefit cell proliferation.

As an early differentiation marker, it is reported that ALP is associated with calcification and an enhanced expression of this enzyme is apparently needed just before the onset of matrix mineralization, providing localized enrichment of inorganic phosphate, one of the components of apatite, the mineral phase of bone [23, 24]. In our present study, Fig. 5 reveals the intracellular ALP activity of MG63 cells on FHA coatings over the culture period. There was a highly significant increase (p < 0.01) in ALP level for F0 and F6 in the first 2 weeks, followed by an insignificant (p > 0.05) increase in the third week. In contrast, although a significant increase (p < 0.01) was achieved for F2 and F3, and an insignificant change for F4 within the first 2 weeks, they all presented a significant decrease (p < 0.01) in ALP activity in the following week (down-regulation), indicating that more cells cultured on F2, F3 and F4 stepped into the next differentiation stage [23]. F4 displayed the highest ALP level and no significant difference with other samples within the first week. At week 2, the ALP levels in F2, F3 and F4 were significantly higher than others (p < 0.05), suggesting that the cells shifted to a more differentiated stage. Although no significant difference was observed for different degrees of fluoridation, after 3 weeks, F2, F3 and F4 did enter the stage of down-regulation of ALP activity. Even though some researchers [13, 14] reported that the incorporation of F⁻ had an insignificant effect on ALP activities, the present study contradicts these findings: a significant increase was obtained on F4 after 1 week, and on F2, F3 and F4 at week 2 followed by significant decrease at week 3. This confirmed that a certain amount of F⁻ stimulates early cell differentiation [22, 25].

Along with ALP activity, OC is specifically synthesized by osteoblasts at the late stage of differentiation and has the ability to chelate Ca²⁺ to form bone minerals [26, 27], thus it also serves as a marker of mineralization. In the present study, the levels of intracellular OC secreted by MG63 cells on different coatings, as illustrated in Fig. 6, were similar to each other at week 1, then a significant increase (p < 0.05) was observed for F3 and F4 compared with other samples between weeks 1 and 2. Finally, all of the samples reached a similar peak value at week 3. On the late differentiation marker, although all the coatings presented comparable levels of OC after 3 weeks (Fig. 6), significantly higher amounts of OC were obtained on F3 (x = 0.84) and F4 (x = 1.04) at week 2, suggesting that F⁻ incorporation in HA for x 0.8–1.1 can stimulate the entry of cells into the late differentiation stage. Therefore, given the positive influence of F⁻ on ALP activities and OC levels, HA with a F⁻ content in the range of 0.8–1.1 will strongly stimulate the cell differentiation process, consistent with Ref. [28].

3.4. Combination of ion effects on cell responses

Based on our in vitro cell culture studies, the difference observed in cell responses (including cell proliferation, ALP activity and OC level) for different FHA coatings suggests that the incorporated F⁻ ions play a critical role in influencing cell behavior. Even though the chemical or physical properties of the fluoridated HA coatings do affect cell response, the main influence should be the combined ionic effects along with the dissolution behavior. Fluoride ion concentration in the range of ~10⁻⁷–10⁻⁵ M (released from NaF) stimulates proliferation and increases the ALP level of osteoblastic cells [29, 30]. In vivo tests also indicate that the presence of low doses of NaF could stimulate the osteoblastic activity, resulting in a higher OC concentration [31]. Ca is found to enhance osteoblast differentiation [32], whereas the presence of inorganic phosphate (Pi) slows down osteoblast differentiation and mineralization. Meleti et al. [33] concluded, in addition, that cells display a
profound loss of mitochondrial membrane potential in the presence of Pi, suggesting that Pi activates cell apoptosis through induction of a mitochondrial membrane transition. Understandably, the results of the present study are the net result of the ion effect of Ca, F and P. Assuming a stoichiometric dissolution behavior in FHA coatings, the F\textsuperscript{−} released can be estimated as being in the range of \(10^{-7} - 10^{-5}\) M in all of the fluoridated HA coatings; this falls perfectly into the above-mentioned effective range. The stimulating effects of Ca and F were compromised by the adverse effects of P; consequently, the most positive outcomes were observed on coatings with F\textsuperscript{−} concentration (x value) between 0.8 and 1.1 (F3 and F4).

4. Conclusions

Incorporation of F\textsuperscript{−} into the hydroxyapatite (HA) lattice structure lowers the solubility of HA in physiological saline solution. The lowest solubility can be achieved at a fluoridation degree of 0.8–1.1 (the x value in Ca\textsubscript{10}(PO\textsubscript{4})\textsubscript{x}(OH)\textsubscript{2−x}F\textsubscript{x}\textsuperscript{−}). Cells adhere and spread well across the entire coating surface regardless of F\textsuperscript{−} content. Fluoride ion has a positive stimulating effect on cell proliferation, alkaline phosphatase activities and, osteocalcin levels, especially at fluoridation degrees in the range of 0.8–1.1. The influence on cell phenotypes comes from the combined ion effects of Ca, P and F released from the coating. For the best resistance to dissolution and the highest cell activities, it is recommended that the fluoridated hydroxyapatite take the form of Ca\textsubscript{10}(PO\textsubscript{4})\textsubscript{y}OH\textsubscript{1−y}F\textsubscript{y}0.8–1.1.

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