In vitro behavior of osteoblast-like cells on fluoridated hydroxyapatite coatings

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Abstract

In this work, fluoridated hydroxyapatite (Ca\textsubscript{10}(PO\textsubscript{4})\textsubscript{6}F\textsubscript{x}(OH)\textsubscript{2−x}, or FHA) coatings are prepared by sol–gel method for study of the influence of F content on the behavior of osteoblast-like cells. The results show that the cells well attach and proliferate on the FHA coatings studied (Ca\textsubscript{10}(PO\textsubscript{4})\textsubscript{6}F\textsubscript{0.67−2.00}(OH)\textsubscript{0−1.33}). With increasing F content in the FHA coatings, percentage of cell in S period increases, indicating F in the coating favors the proliferation process of the cells. On the other hand, the proliferation rate increases inversely with zeta potential of the coating surface. As tested from the MTT of the cells cultured in the leaching out solution, increase of F content in the FHA coatings results in a slight decrease in cell proliferation rate, which is most probably due to reduction in release of Ca\textsuperscript{2+} ions. As a compromise among cell attachment, cell proliferation, apatite deposition and ability to resist dissolution, it is suggested that FHA coatings (Ca\textsubscript{10}(PO\textsubscript{4})\textsubscript{6}F\textsubscript{x}(OH)\textsubscript{2−x}) with x in the range of 0.67–1.48, from the results of this study, may be most suitable for real case implantation.

Keywords: Fluoridated hydroxyapatite; Surface fluorine content; Osteoblast-like cells

1. Introduction

Synthetic hydroxyapatite, Ca\textsubscript{10}(PO\textsubscript{4})\textsubscript{6}(OH)\textsubscript{2}, HA, can directly bond to bones without infection and fibrous encapsulation, thus is regarded as bioactive and biocompatible [1]. However, its brittleness and high Young’s modulus limit its application as load bearing implants. One solution is to coat HA on the surface of a biomedical metal, so that the bioactivity and biocompatibility are utilized along with the good mechanical properties of the metal [2].

However, the long-term effectiveness is still questionable because HA coatings have a relatively large solubility in body fluid [3,4]. The long-term effectiveness can be improved if the solubility of HA in body fluid is lowered without sacrificing bioactivity and biocompatibility. The solubility of fluoridated hydroxyapatite (Ca\textsubscript{10}(PO\textsubscript{4})\textsubscript{6}F\textsubscript{x}(OH)\textsubscript{2−x}, FHA) is lower than that of HA [5], and in the mean time, the bioactivity and biocompatibility maintain comparable [6,7]. This makes FHA a promising coating material.

Although many methods have been developed to synthesize FHA [8–13], the most prevalent method in preparation of FHA coating is still sol–gel method because of its ease in operation and tailoring of composition [14–16], as well as in scaling up for mass production.

The soaking experiment in SBF shows FHA coatings with proper F content have a faster apatite deposition than pure HA [14,16]. Recently, cell culture assay on FHA coating has been carried out with exciting results: cell attachment on FHA and the cell’s alkaline
phosphatase expression are comparable to that on HA [14,15,17]. However, these reports are based on FHA with a fixed F content like Ca\(_{10}(PO_4)_6F_1.0(OH)_{1.0}\), sometimes, the composition is just nominal.

This work attempts to understand the influence of different F content in FHA coatings on the behavior of the cells cultured, trying to know which F content range is suitable for future clinical applications. The FHA coatings with different F content were prepared via sol–gel method based on previous work [18]. The dissolution behavior, cell attachment and proliferation on FHA coatings, and cell culture in leaching out solutions were measured and assayed, the relations between the cell culture results with physical chemistry properties of FHA were established and discussed.

2. Experimental

2.1. Coating preparation and characterization

Calcium nitrate tetrahydrate (Ca(NO\(_3\))\(_2\)-4H\(_2\)O, GR, Merck) was dissolved in ethanol (GR, Merck) to form the 2 mol/L Ca-precursor; ethanol was gradually poured into phosphor pentoxide (P\(_2\)O\(_5\), GR, Merck) to form 2 mol/L solution, which was refluxed for 24 h to become the P-precursor. Then, the Ca- and P-precursor were mixed with various amount of HPF\(_6\) (GR, Merck) for solutions of 5 ratios of HPF\(_6\)/Ca (0 through 4/90). These solutions were further refluxed for 24 h to be the “dipping-sols”.

Titanium alloy (Ti-6Al-4 V) substrates were polished down to #1200 grade SiC paper. The substrates were rinsed in double distilled water and ultrasonically washed in acetone for 10 min. After drying, the substrates were dipped in the sols and slowly drawn out at a speed of 3 cm/min. The sol-coated substrates were then immediately transferred into an oven at 150 °C and held for 15 min to dry before transferring to a firing oven at 600 °C. The firing occurred at 600 °C for 15 min. This dipping–drawing–drying–firing process was repeated for desired coating thickness. For zeta potential of the coatings, the “dipping-sols” with different F contents were dried on a hot plate held at 150 °C, followed by further firing at 600 °C. The powders were soaked in TRIS solution buffered at pH 7.25 for different times before zeta potential measurement using Analyzer (Delsa 440X). These values were regarded as the zeta potential of the corresponding coatings.

The coatings obtained were characterized by XRD (RIGAKU, D-Max, RA, 2°/min, 0.02° per step) for the identification of crystalline phase. The surface morphology of the coatings was observed by SEM (HITACHI, S-4100). The F contents of the coatings were determined by XPS (AXIS HSi 165 Ultra, Aluminum mono).

2.2. Dissolution of the coating

The coatings were immersed in covered containers with TRIS solution buffered at pH 7.25, and then the containers were transferred into water bath held at 37 °C for up to 2 weeks. The concentration of Ca\(^{2+}\) ions in the soaked solution was monitored by ICP-OES (JOBY-VON 70 plus) analysis.

2.3. Cell culture

The osteoblast-like cells were extracted from adult rabbit back shank. The cells were washed twice in phosphate buffer solution (PBS), and then collected via centrifugation. In the standard incubation condition (5%CO\(_2\), 37 °C), the cells were incubated for about one week in different cultural media containing Dulbecco’s Modified Eagle’s Medium (DMEM), dexamethasone, vitamin C and β-sodium glycerol-phosphate. The coatings were cut into small pieces with a dimension of 10 mm × 10 mm × 1 mm, and sterilized in 120 °C water steam for 20 min. The samples were then placed in 24-well plates for osteoblast-like cells implantation at a set density of 1 × 10\(^4\) cells/cm\(^2\). The osteoblast-like cells were further incubated in DMEM solution supplemented with 10% FBS for 1–7 days in the standard culture condition.

In characterization of cell attachment, the coatings were taken out every day for digestion with 0.25% trypsin EDTA solution, and then the cell numbers were evaluated by haemacytometer. Eight replicate samples were processed for each coating composition.

For cell cycle analysis, the DMEM solution was replaced by 2.5% trypsin EDTA solution at the 4th and 7th day of culture. After further incubation for 3–5 min at 37 °C, the solution was centrifugated at a speed of 1000 r/min. The centrifugate was washed with PBS and alcohol (70 vol%) before addition of PI fluorescence. After 30 min, the cells were analyzed by flowcytometry (Coulter EPICSXL).

For effect of leaching out of the coating, the samples were immersed in DMEM solution at 37 °C for 4 days at a ratio of 10 ml/cm\(^2\) (DMEM volume/coating area). The immersed solution was used for incubation of osteoblast-like cells. These cells were seeded in 96-well plates at a density of 5000 cells/well. Untreated DMEM solution was also processed as the control. After 1 and 3 days of culture, the cell viability was evaluated by MTT-assay using a microplate reader (BIO-RAD Model550) operating at 570 nm. The tests were performed on 3 replicate samples.

For cell morphology observation, the osteoblast-like cells attached on the coatings were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 4 h at 4 °C, 1% osmium tetroxide in Veronal buffer, and critical
point dry with CO₂. Finally the cells were gold coated by sputtering and observed in a PHILIPS XL30E SEM.

2.4. Statistics

For both cell culture assays on the coating and in DMEM with the leaching out, the Student’s t test was carried out in comparing data between different samples as well as the different period of culture. Statistical significance was considered at \( p < 0.05 \).

3. Results

3.1. FHA coating

As shown in Fig. 1, the coatings contained apatite phase, no trace of CaO, CaF₂ or other calcium phosphate phase were presented. The shift of the X-ray diffraction peaks to higher \( \theta \) with the increasing F occurred. The above facts meant that FHA had been formed [18]. Based on the quantitative XPS results, the exact FHA molecular formula were obtained and tabulated in Table 1. The morphology of the coatings observed by SEM are shown in Fig. 2. For coating F0 (pure HA), the surface was smooth (Fig. 2a), while with the presence and further increase of F, the surface became increasingly rougher (Fig. 2b and c, F067 and F148, respectively); however, with further increase in F (F190 and F200), the coatings became smooth again (Fig. 2d, e). Such effect was attributed to the promotion of HPF₆ addition on the gelation behavior of the system [19]. High HPF₆ contents facilitate the formation of homogeneous intermediate gel coating, leading to smooth coating surfaces. While low HPF₆ contents will favor the formation of some rather large gel clusters in the intermediate gel coating, resulting in rough coating surfaces.

3.2. Dissolution of the coating

After 14-day immersion in TRIS buffer solution, all the coatings exhibited dissolutions, as confirmed by the presence of Ca²⁺ ions in the soaked solution. In Fig. 3a, the Ca²⁺ concentration of the soaked TRIS solution decreased with the increasing F content, indicating the solubility of the coatings decreased with increasing F. The average reduction in the coating thickness was estimated to be from about 170–68 nm, according to dissolution amount, soaked coating area and coating density (using density value of hydroxyapatite [20], 3.16 g/cm³).

Fig. 3b depicts the typical Ca²⁺ concentration evolution in the TRIS solution. At the beginning of immersion, the Ca²⁺ concentration increased rapidly, while after about 100 h, such changes tapered off. After about 150 h, the Ca²⁺ concentration tended to be saturated.

3.3. Cell culture test

The cells used in the culture test were the osteoblast progenitor cells derived from adult rabbit bone marrow. With inductive agent [21], the cells developed into osteoblast-like cells. After implantation, as shown in the typical SEM micrographs in Fig. 4, the cells firmly attached on the coating surface. They spread well, and the pseudopods were clearly seen, indicating good livelihood. Fig. 5 plots the cell density as a function of culture time for samples of different F in the coating but the same initial cell implanting density of \( 1 \times 10⁴ \) cell/cm². In pure HA coating (F0), the cell concentration reached about \( 5 \times 10⁴ \) cell/cm²; for F067 and F148, only about \( (3–4) \times 10⁴ \) cell/cm²; for F190, it also reached about \( 5 \times 10⁴ \) cell/cm², which is similar to pure HA; as for F200, the concentration reached \( 11 \times 10⁴ \) cell/cm².

Table 1

<table>
<thead>
<tr>
<th>HPF₆/Ca ratio</th>
<th>F0</th>
<th>F067</th>
<th>F148</th>
<th>F190</th>
<th>F200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular formula</td>
<td>Ca₁₀(PO₄)₆(OH)₂</td>
<td>Ca₁₀(PO₄)₆(OH)₁₃₃F₀₆₇</td>
<td>Ca₁₀(PO₄)₆(OH)₁₃₃F₀₆₇</td>
<td>Ca₁₀(PO₄)₆F₁₉₀</td>
<td>Ca₁₀(PO₄)₆F₂₀₀</td>
</tr>
<tr>
<td>Intensity (a.u.)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 1. XRD pattern of coatings with different F contents.
The cell cycles of these cells were further characterized by flow cytometry. Table 2 gives the percentage of cells in different cytoidesis phase (G0G1: pre-DNA synthesis or resting; S: DNA synthesis phase; G2M: post-DNA synthesis and mitosis). The percentage of the cells in every phase is different: for all the coatings and culture time involved, more cells were found in S period when they are cultured on the coatings than those on the control. Compared to the control, the difference is significant.

Fig. 6 contained the MTT statistics (at $p < 0.05$) of the cells cultured for 1–5 days. At first, all the cells showed relatively low absorbance indicating the cells were adapting to the new environment. Thus the number of living ones fell below that of the control. After 3 days, the absorbance increased drastically on all the coatings, meaning the number of the living cells increased, i.e., the cells proliferated. At this moment, leaching out from coating with different F content showed significant difference (considering at $p < 0.05$) in cell proliferation: pure HA and FHA with less F content (F0, F067 and F148) showed even better cell proliferation than control (higher absorbance). On coating with very high F content or pure FA (F190 and F200), less cell proliferation was seen than the control.

4. Discussion

All the coatings showed a pure apatite phase. With the increasing F, the X-ray diffraction peaks shifted to higher 2θ direction in Fig. 1, indicating lattice parameter reduction as a result of substitution of OH by F [10,22]. In Table 1, the F contents in the coatings increase with increasing HPF$_6$ content in the starting solution. As a result, the incorporation of F into hydroxyapatite does reduce the dissolution of the coating in the TRIS solution (Fig. 3a).

4.1. Coating depth involved in cell culture

When the osteoblast cells are cultured on the FHA coating, it is possible for F to have two ways to affect the behavior of the cultured cell. First, changes in
surface properties arising from the F existence in FHA coating could have a direct influence on interactions between the cell and the coating; second, changes in the leaching-outs of the coatings due to the F incorporation could have an influence on cell growth by means of ion concentration changes in the culture medium. As such, the cell behavior will be affected. Considering the above two factors, it is obvious that only the surface layer of the coating is involved.

Based on the dissolution results obtained in a pH condition similar to that of the cell culture media (Fig. 3a), the maximum depth involved can be estimated through dividing the weight loss by the density and the soaked areas. The depth is calculated to range from about 170 to 68 nm, inversely proportional to F content. This range is exactly what XPS can characterize precisely. Therefore, it is believed that the F content from XPS can well represent one involved in the cell culture experiments.

Fig. 3. Dissolution behavior of the coating (F0): (a) dissolution amount after 14-day immersion in TRIS solution and (b) typical Ca²⁺ concentration evolutions during immersion.

4.2. Effect of F content in the coating on the direct interaction between the cell and the coating

Apparently, all the coatings show good biocompatibility because osteoblast-like cells could attach and grow well on the surface of the coatings. However, the interaction between the cell and the coating depends on the F content in the coating. The cell morphology and growth rate are affected by the F content in the coating. The typical morphology of cells attached on the coatings is shown in Fig. 4. The cell density on the coatings with different F contents is shown in Fig. 5. The cell growth curve is shown in Fig. 5.

Fig. 4. Typical morphology of cells attached on the coatings: (a) F148 and (b) F0.

Fig. 5. Cell growth curve of cells attached on coatings with different F contents.

4.2. Effect of F content in the coating on the direct interaction between the cell and the coating

Oct. 31, 2005
spread well on the coatings, as shown in the SEM results (Fig. 4). In the cell growth curve (Fig. 5), when the coatings have low F content (F067 and F148), less cell attachment is seen as compared to pure HA. Only when the concentration is high (to the extent of F190), does it show positive effect on cell attachment: F190 has a cell attachment similar to that of pure HA while F200 produces the maximum.

Further cell cycle analysis results are tabulated in Table 2. With the increasing F content in the coating, the percentage of cells in S period increases, indicating the cells attached on the coating seem to become more active in DNA replication process. From both the cell attachment and cell cycle results, We can conclude that it is quite clear that the variations of cell behaviors are greatly affected by the F content in the coatings.

As widely accepted, rough surfaces promote cell attachment. However, even F067 and F148 have rougher surfaces than all the other coatings (Fig. 2) and the number of the cells attached is still less (Fig. 5). The same situation happened to F190 and F200, where F190 seems to have rougher surface than F200, but the cells attached on F190 are significantly less than F200. Obviously, the cells attachment differences here are not mainly induced by the roughness variations.

The above cell behavior variations could have resulted from the surface property change owing to F incorporation. Fig. 7 gives the zeta potential results for 3-day and 6-day soaking. The tendency of the zeta potential against the F content coincides with the trend of the cell attachment as a function of F content: roughly lower potential corresponds to higher number of attachment. The variation of zeta potential may influence the adsorption of Ca$^{2+}$ ions, which, in turn, affect the adsorption of extracellular matrix (ECM, including osteocalcin, osteopontin, etc.) adsorption [23]. Consequently, the attachment of the cells is affected because osteoblast-like cells actually attach on the ECM preadsorbed on the surface of the sample.

The F influence on surface potentials can be attributed to FHA coating’s different dissolution behavior. According to apatite self-inhibition dissolution model [24,25], after the initial dissolution of a small portion of apatite, a steady calcium-rich layer consist of Ca$^{2+}$ ions and their anionic counter parts will firstly form and then control the further dissolution of apatite.

Table 2
The cell cycles of cells on different coatings at different time

<table>
<thead>
<tr>
<th>Period of cell cycle</th>
<th>G0G1 (%)</th>
<th>S (%)</th>
<th>G2M (%)</th>
<th>Proliferation index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F190, 4-day</td>
<td>50.2 ± 4.8</td>
<td>49.8 ± 3.2</td>
<td>0</td>
<td>50.2 ± 4.5</td>
</tr>
<tr>
<td>F148, 4-day</td>
<td>60.0 ± 5.2</td>
<td>34.4 ± 2.6</td>
<td>5.6 ± 0.3</td>
<td>65.6 ± 5.4</td>
</tr>
<tr>
<td>F0, 4-day</td>
<td>86.5 ± 6.8</td>
<td>11.2 ± 1.3</td>
<td>2.3 ± 0.2</td>
<td>88.8 ± 5.7</td>
</tr>
<tr>
<td>F190, 7-day</td>
<td>32.9 ± 2.1</td>
<td>67.1 ± 5.3</td>
<td>0</td>
<td>32.9 ± 2.9</td>
</tr>
<tr>
<td>F067, 7-day</td>
<td>56.4 ± 4.3</td>
<td>34.8 ± 2.6</td>
<td>9.7 ± 0.6</td>
<td>66.1 ± 6.5</td>
</tr>
<tr>
<td>F0, 7-day</td>
<td>88.2 ± 5.6</td>
<td>11.8 ± 1.3</td>
<td>0</td>
<td>88.2 ± 5.1</td>
</tr>
<tr>
<td>Control</td>
<td>99.5 ± 6.3</td>
<td>0</td>
<td>0.5 ± 0.1</td>
<td>100 ± 6.4</td>
</tr>
</tbody>
</table>

Fig. 6. MTT results of cells cultured in coating leaching out solutions (considered at $p<0.05$).

Fig. 7. Zeta potential of FHA coatings after different times of immersion in TRIS solution.
If this “calcium-rich layer” is regarded as the “compact layer” in the double layer theory of colloidal, the exact content of the “calcium-rich layer” will affect the zeta potential of apatite. With the F existence in the surface layer of apatite, more Ca$^{2+}$ ions will be attracted in the “calcium-rich layer” due to the strong eletronegativity of F, leading to increases of zeta potential (F067 and F140). However, even higher F content will result in solubility decrease, which will in turn produce a low Ca$^{2+}$ concentration in the solution, leading to decrease of zeta potential (F190 and F200).

4.3. Effect of leaching out on behavior of cultured cells

When a coating exists in body, the dissolution of the coating will affect surface potential as mentioned above, as well as the long-term stability [3,4]. The dissolution also produces ion concentration variations around the vicinity of the coating in the initial time after implantation. This could influence new bone formation. One approach to getting insight into the influence could be an in vitro test through observing behavior of osteoblast-like cells in a culture medium with leaching out, which is prepared by soaking the coating in the medium.

In this work, the 4-day soaked culture media are used for cell culture, because the media are considered to soak long enough to saturate with HA (in Fig. 3b) as well as FHA [26], and able to show differences in ion (Ca$^{2+}$ and PO$_4^{3-}$, etc.) concentration for different FHA coatings. The MTT measurement demonstrates that the leaching out of the different coatings do have different effects on cell proliferation. The number of the living cells (or percentage of absorbance) for pure HA (F0) is rather similar to that of FHA with low F concentration (F067 and F148), while for coating with high F concentration, the number of the living cells is significantly lower than pure HA (compare F0 with F190 and F200).

Based on the relation between F content and dissolution (Fig. 3a), the maximum concentration of F ions is estimated to be $\sim 6.3 \times 10^{-8}$ mol/L, which falls below the determination limit of normal measurement methods such as ion-selective potentiometry. In such a low concentration, it is impossible for F ions in the culture medium to play a significant role in affecting the behavior of osteoblast-like cells [27]. Comparing the Ca$^{2+}$ dissolution (Fig. 3a) with that of the MTT result (Fig. 6), it is seen that as the concentration of Ca$^{2+}$ increases, the number of living cells increases in cell culture experiment. This is in agreement with the report where the existence of Ca$^{2+}$ benefits osteoblast cell proliferations [28]. The above variation of the F content with cell proliferation could be attributed to Ca$^{2+}$ ion concentration changes that depend on F content in FHA coating, i.e. an increase in F content in the coating leads to a decrease in Ca$^{2+}$ release, which, in turn, decreases cell proliferation.

Although the situation for the cell growth in body is different from that in culture media, the above analysis may help us to understand influences of the coatings as comprehensive as possible.

4.4. Optimization consideration of F contents

The cell culture results demonstrate that the F content in FHA coatings affects the behaviors of cultured cells in different ways: high F content produces a low surface potential which favors cell attachment but at the same time, a reduction in Ca$^{2+}$ release in culture medium which could inhibit cell proliferation. Our previous work shows that high F content in FHA coatings have low solubility in SBF which is good to guarantee long-term stability; also, certain F content promotes apatite deposition in SBF [16]. From the coating design point of view, all these effects of F content do not necessarily function in line with one another. However, all these effects are important in clinical applications, thus need to be taken into consideration simultaneously and holistically. Base on our experimental data, FHA coatings with moderate F content, Ca$_{10}$(PO$_4$)$_6$ F$_{0.67}$−1.48(OH)$_{0.52}$−1.33, may have a more promising potential. Savarino [29] showed that Ca$_{10}$(PO$_4$)$_6$ F$_{1.0}$(OH)$_{1.0}$ coating in vivo test was better than both Ca$_{10}$(PO$_4$)$_6$(OH)$_{2.0}$ and Ca$_{10}$(PO$_4$)$_6$F$_{2.0}$ coating. Our analysis above on F influences and optimized F content serves well in explaining Savarino’s results.

5. Conclusion

Fluoridated hydroxyapatite (FHA) coatings with different F content prepared by sol–gel method show good cytocompatible. High F content in the FHA coatings results in low zeta potential that promotes cell attachment. High F content also results in decrease in Ca$^{2+}$ release in culture medium due to reduced solubility and as a result, cell proliferation is inhibited. For clinical application, it is suggested that a moderate content of F, such as Ca$_{10}$(PO$_4$)$_6$F$_{0.67}$−1.48(OH)$_{0.52}$−1.33, is most suitable as a compromise among cell attachment, cell proliferation, apatite deposition and dissolution resistance.

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