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# 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 $Ca_{10}(PO_4)_6(OH)_{1.2-0.9}F_{0.8-1.1}$.


*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

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required and provides useful insights as to whether an implant/device needs further evaluation in much more expensive in vivo experiments [11]. In vitro study of FHA-coated implants soaked in simulated body fluid (SBF) and organic-modified SBF has shown that fluoride improves apatite deposition, and is thus osteoinductive [7,12]. In in vitro cell culture studies, there is no consensus regarding effect of $F^-$ incorporation in HA. Some researchers [13,14] reported that the incorporation of $F^-$ could enhance cell proliferation rate but produced no significant difference in alkaline phosphatase (ALP) activity. Our previous work [15] observed FA promoting cell proliferation but no significant difference on other sol–gel derived FHA coatings, while Lee et al. [10] saw lower proliferation rates on FHA prepared via electron-beam deposition ($p < 0.05$) with no significant difference in ALP activity. With respect to current documented results on cell response to FHA coatings, these reports are only focused on cell proliferation and a few of them only evaluated the early differentiation marker, i.e., ALP levels. More importantly, to date, no published papers have given the optimal degree of fluoridation in terms of cell response.

This work investigates the osteoblastic cell response on sol–gel derived FHA coatings deposited on Ti6Al4V substrates. Cell proliferation rate is determined and cell differentiation (ALP, and osteocalcin levels) evaluated. The influence of fluoride ions on cell behavior is discussed.

## 2. Materials and methods

### 2.1. Coating preparation

The preparation of dipping-sols and the deposition of FHA coatings were described in detail in our previous work [8]. In brief, calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$, Sigma–Aldrich, AR), phosphorous pentoxide ($P_2O_5$, Merck, GR) and hexafluorophosphoric acid ($HPF_6$, Sigma–Aldrich, GR) were selected as Ca-precursor, P-precursor and F-precursor, respectively. Absolute ethanol was used as solvent for preparation of the dipping-sols. The intended degree of substitution of $OH^-$ by $F^-$ was indicated by the $X$ value in the general formula of FHA, $Ca_{10}(PO_4)_6(OH)_{2-X}F_X$, where $X$ was selected as 0/3, 2/3, 3/3, 4/3 and 6/3 (i.e., 0, 2/3, 1, 4/3 and 2), the coatings subsequently obtained were named F0, F2, F3, F4 and F6, respectively. A titanium alloy (Ti6Al4V) slab 20 mm × 30 mm × 1.2 mm, polished with #1200 grade silicon carbide sandpaper, was used as substrate. The final substrate surface roughness was ~0.32 μm. The dipping run was repeated four times for a final coating thickness of ~1.5 μm.

The $F^-$ concentration in the coating was determined by X-ray photoelectron spectroscopy (XPS, Kratos-Axis Ultra System) using monochromatic Al $K_\alpha$ X-ray radiation (1486.7 eV); the measured $F^-$ concentration is indicated by $x$ to distinguish this from the intended value, $X$. The results of X-ray diffraction analysis, coating surface morphology as well as mechanical properties were reported in our previous work [8,9,12].

### 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 $5.3 \times 10^3$ 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 $4.8 \times 10^4$ 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

| ID | Title | Pages |
|---|---|---|
| 2399 | Osteoblastic cell response on fluoridated hydroxyapatite coatings | 7 |