Introduction

Hydroxyapatite (HA) has been developed as a coating for metallic implants in the field of dentistry due to its chemical and biological similarity to human bone, as well as its capability to directly bond to surrounding tissues\(^1,2\). HA is a bioactive implant material that enhances early bone formation\(^3,4\). However, pure HA coatings are subject to a relatively high dissolution rate in biological environments\(^5\).

A thin-layer coating of HA on the surface of a metal implant may promote osseointegration and increase mechanical stability of the implant\(^6\). Although various techniques for coating titanium-based implant devices with HA have been reported\(^7,8\), plasma-spray is currently the only method that is commercially available\(^9\). However, plasma-sprayed HA films have several drawbacks, including poor surface adherence and crystallinity\(^10\). Many other methods, such as magnetron sputtering\(^11\), ion beam-assisted deposition\(^9\), chemical vapor phase deposition\(^12\), sol-gel\(^13\), biomimetic\(^14\), and pulsed excimer laser deposition (PLD), exist for coating surfaces. PLD\(^15,16\) enables the deposition of very thin films, control of surface roughness, and ablation of any material. As a result, PLD facilitates strong bonding between film and substrate. A previous study\(^15\) reported that PLD produces an ultra-thin HA film with improved mechanical properties, including increased tensile strength and decreased film thickness.

Improving the biological and physicochemical properties of HA can be achieved by doping HA with ions that are usually present in natural apatites of vertebrate skeletal systems\(^17\). Fluorine exists as a trace element in the mineral phase of bone and teeth. Fluoridated HA (FHA) has a lower solubility than pure HA, while maintaining comparable bioactivity and biocompatibility\(^5,18\). FHA coatings have been produced using different methods, including thermal spray deposition, electro-deposition, and the sol-gel process\(^19\). In general, these coating techniques produce micron-thick FHA coatings; however, there have been few published studies focusing on the production of thin films from partially FHA, especially using PLD.

In this study, an ultra-thin FHA film was applied to titanium substrates using PLD, and dissolution resistance and cell attachment of human mesenchymal stem cells (HMSCs) were examined.

Materials and Methods

Starting materials

HA and fluoroapatite disks were used as targets for PLD. The raw HA and fluoroapatite (Ca\(_{10}\)\(\text{PO}_4\)\(_6\)F\(_2\)) powder (Taihei Chemical Industrial, Osaka, Japan) was pressed at 19.6 MPa in a cylindrical steel die to form “compacts” 16 mm in diameter and 1 mm in thickness, and then annealed by heating at 750°C for 10 h.

Preparation of titanium surfaces

The titanium disks were 15 mm in diameter and 1 mm thick, and were certified according to Japanese Industrial Standard Grade II specifications. The discs

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Fluoridated hydroxyapatite (FHA) was investigated for application as an implant coating for titanium bone substitute materials in dental implants. A KrF pulsed excimer deposition technique was used for film preparation on a titanium plate. The compacts were ablated by laser irradiation at an energy density of 1 J/cm\(^2\) on an area 1×1 mm\(^2\) with the substrate at room temperature. Energy-dispersive spectrometric analysis of the FHA film revealed peaks of fluorine in addition to calcium and phosphorus. X-ray diffraction revealed the presence of crystalline FHA on the FHA film after a 10 h post annealing treatment at 450°C. The FHA film coating exhibited significant dissolution resistance to sodium phosphate buffer for up to 21 days, and favorable cell attachment of human mesenchymal stem cells compared with HA film. The results of this study suggest that FHA coatings are suitable for real-world implantation applications.

Keywords: Fluoridated hydroxyapatite, Thin film coating, Krypton fluoride pulsed laser deposition

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were prepared by manual parallel polishing using silicon carbide papers (grade 80 to 4000) and automatic mirror polishing using 3- to 0.05-μm Al₂O₃ particles.

Deposition of HA and fluoroapatite onto the targets was performed in water vapor generated by bubbling O₂+H₂O gas through a water bath at 1 Pa. The compacts were ablated by laser irradiation at an energy density of approximately 1 J/cm² within an area of 1×1 mm². An krypton fluoride excimer laser (COMPexPro 205; wavelength λ=248 nm and pulse width τ=20 ns) operating at a repetition rate of 10 Hz was used for deposition. The substrate temperature (Ts) was room temperature and the deposition rate was 10 nm/min. Film thickness was estimated on the basis of the deposition rate. Film thicknesses of 1 μm were prepared and annealed by heating at 400, 450, and 500°C for 10 h, at heating and cooling rates of 1.5°C/min in water vapor generated by bubbling O₂ gas through a water bath. Before analysis and in vitro experiments, test samples were cleaned using a series of 10-min ultrasound treatments in pure acetone, pure alcohol, and distilled water. For cell culture experiments, test samples were sterilized in an autoclave. Four samples from each group were used for testing.

Coating characterization
Surface morphology and composition of the FHA film were analyzed using scanning electron microscopy coupled to an energy-dispersive spectrometer (KRA8800, Ametek, Tokyo, Japan). After annealing, the crystal structure and the lattice parameters of the thin films were evaluated using an X-ray diffraction (XRD) apparatus (Ultima IV, Rigaku, Tokyo, Japan) and conventional 2θ/θ measurements. Fourier-transform infrared (FT-IR) spectra were obtained using a Spectrum One (Perkin-Elmer, Waltham, MA, USA) equipped with a diffuse reflectance unit at a resolution of 4 cm⁻¹ with 16 scans.

Dissolution testing
A sodium phosphate buffer (0.5 M, pH 5.8, temperature 37°C) was used to study the dissolution behavior of the HA and FHA films. The HA and FHA film coating on the titanium disc samples were soaked into the solution (without Ca²⁺ ions) for 1, 3, 10, and 21 days. At the end of each immersion, the samples were removed and the concentration of Ca²⁺ released from the HA and FHA films into the solution was examined using inductively coupled plasma mass spectrometry (Agilent 8800, Agilent technologies, Tokyo, Japan).

Cell culture
Because most bone implant materials are implanted into adult bone that is in direct contact with bone marrow tissue, HMSCs from adult human are useful for investigating the effect and safety of new implant materials. HMSCs proliferate and differentiate into a phenotype that expresses bone cell markers that form mineralized nodules in vitro. HMSCs were provided by RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Science, Sports Culture and Technology, Japan. HMSCs were maintained in continuous culture at 37°C in a humidified atmosphere containing 5% CO₂. The HMSCs were expanded for 7–10 days in Dulbecco’s Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum, 3 ng/mL fibroblast growth factor-2 (Reprocell, Kanagawa, Japan), and a 1% antibiotic/antimycotic in 75-mm² flasks. After a sufficient number of HMSCs were obtained, they were seeded at a density of 3×10⁴ cells/plate onto HA and FHA films and coated onto the HMSCs in the wells of 24-well tissue culture plates. The cells were incubated for 3 h in a CO₂ incubator at 37°C. The cells were fixed using 4% paraformaldehyde solution for 20 min at room temperature. Following fixation, the cells were permeabilized using 0.2% Triton-X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 2 min and incubated for 30 min with Alexa-Fluor 488 phalloidin and DAPI solution (BD Biosciences, San Jose, CA, USA), which specifically bind to actin filaments and nuclei, respectively. The cells were then washed five times with phosphate-buffered saline. The cells were analyzed using automated fluorescence microscopy (BZ-9000, Keyence, Osaka, Japan). Three cell counts per area (nuclei/mm²) in each of four plates were measured using threshold imaging of nuclei (10× magnification, DAPI) in Image J. The mean and standard deviation for each parameter were compared between groups using the Student’s t test.

RESULTS

Structure of FHA films
Figure 1 presents scanning electron microscopy (SEM) images of the HA and FHA films. There were negligible differences in the surface structure of the two films. Energy-dispersive spectrometry revealed peaks of fluorine apart from those of Ca, titanium and phosphorus on the FHA film coating Titanium plate (Fig. 2).

The XRD profiles of the coatings are shown in Fig. 3. After annealing at 400, 450 and 500°C, several peaks in the XRD profiles of the coatings corresponded with FHA film under all post-annealing conditions tested. The shape of the peak after annealing at 450 and 500°C was altered from broad to sharp compared with annealing at 400°C, reflecting the increased crystallinity of the FHA film in the coatings. However, several CaF₂ peaks were observed in the XRD profile after annealing at 500°C. Lee et al.²² reported lattice parameters of the powders to confirm the formation of fluor-hydroxyapatite and fluorapatite from the XRD profile. In the present study, an a-axis length of FHA film and FHA target, calculated from XRD profiles, were 9.389 and 9.373 Å, respectively. Therefore, the composition of FHA film is Ca₁₀[PO₄]₁₀[OH]₁₀F₁.₄ according to the lattice parameters reported by Lee et al.²².

The FTIR spectra for the FHA film is presented in Fig. 4. The vibration bands exhibited typical apatite characteristics, with PO₄²⁻ bands at around 1,085, 1,040, 590, and 564 cm⁻¹. The hydroxyl stretching peaks at 3,570 cm⁻¹ for FHA film coatings split, and an additional OH–F stretched band appeared at a lower frequency,
Fig. 1  Surface structure analysis of HA films and FHA and on a titanium (Ti) substrate according to SEM.

Fig. 2  Compositional analysis of FHA film on a titanium (Ti) substrate according to energy-dispersive spectrometry (EDS). EDS revealed peaks of F other than calcium (Ca), phosphorus (P), and Ti on the FHA film.

Fig. 3  XRD profiles of FHA film on titanium (Ti) substrate after annealing at 400, 450, and 500°C.

signifying the substitution of F$^-$ for OH$^-$. Furthermore, a new peak for F$^-$, appearing at approximately 667 cm$^{-1}$ in the FHA film coatings, was also found.

**Dissolution behavior analysis**
The dissolution behaviors of the HA and FHA films coating after annealing at 450°C are shown in Fig. 5. Ca$^{2+}$ ions released from the coatings were detected after soaking in sodium phosphate buffer for up to 21 days. For all specimens, the dissolved amounts of Ca$^{2+}$ ions increased with immersion time. At any time point observed, the dissolution rate of the pure HA coating occurred more rapidly than FHA coating.

**Effect of the films on cell attachment**
The ability of HMSCs to organize actin cytoskeleton during culturing was investigated. Microscopic images after actin staining with Alexa-Fluor 488-phalloidin revealed that HMSCs spread markedly further on FHA films than on HA films after 3 h incubation (Fig. 6). FHA films exhibited significantly higher cell attachment compared with HA films after 3 h incubation according to Image J ($p<0.05$) (Fig. 7).
DISCUSSION

In the current study, we used PLD to apply a thin FHA film to a highly polished titanium substrate and succeeded in identifying optimum annealing conditions. The fabricated thin FHA film exhibited high crystallinity, dissolution resistance and cell attachment.

The PLD coatings examined in this study were produced at room temperature to avoid titanium oxidation. The amorphous HA coatings applied using PLD were produced by maintaining the substrate at 290°C, while the crystalline coatings were obtained at 460°C\(^2\). The degree and rate of recrystallization were time- and temperature-dependent, with effective recovery of the crystalline structure occurring between 500 and 700°C\(^2\). Rau et al.\(^2\) demonstrated that PLD performed at temperature range of 500–600°C enables the application of FHA films with a composition nearing that of the initial target material. However, a previous study\(^1\) reported that treatment at high temperatures decrease the integrity and interfacial bond strength of HA coatings, which could increase the likelihood of cracking.

Post-deposition heat treatment has often been used to improve the crystallinity of HA coatings\(^2\). In the present study, the crystallinity of FHA film in the
coatings increased continuously with increasing the annealing temperature; however, CaF$_2$ impurities were detected in the FHA film after annealing at 500°C. In general, fluorine ions replace some of the hydroxyl ions present on the c-axis column of the apatite structure$^{27}$. The reasons for the presence of CaF$_2$ after annealing at 500°C were unclear; however, some of the fluorine ions that were not replaced with hydroxyl ions may have bonded with calcium ions formed at the time of FHA film formation under high-temperature annealing. In the present study, FHA films with high crystallinity without any impurity phases, such as CaF$_2$, were obtained on the titanium surfaces under conditions of coating at room temperature and a 10-h post-annealing treatment at 450°C.

To analyze the dissolution behavior of FHA films, concentrations of Ca$^{2+}$ ions released from the coating layer were monitored after incubation in physiological saline solution. The pure HA film coating dissolved significantly faster than the FHA film at any time point, suggesting that FHA film coating using PLD has higher in vitro stability than HA coating. Wang et al.$^{3}$ also reported high dissolution resistance of FHA deposited on titanium substrates using an electrochemical technique. Chen et al.$^{28}$ reported that the substitution of hydroxyl (OH$^-$) ions with fluorine (F$^-$) ions in the HA resulted in FHA (Ca$_{10}$[PO$_4$_6][OH$_2$]$_{2-2x}$F$_{2x}$, where 0<x<1), which exhibited increased crystallinity and higher corrosion resistance compared with pure HA.

In general, apatite formation on titanium surfaces result in increased adsorption of serum proteins compared with a titanium surface alone. Specifically, HA films induce cell attachment and proliferation$^{29}$ due to the adsorption of a high quantity of fibronectin$^{30,31}$. Our previous study$^{32}$ demonstrated that HMSCs grew faster and exhibited higher osteoblast differentiation compared with pure titanium when cultured on HA film coatings applied using PLD. In the present study, the FHA film had a significant impact on cell attachment of HMSCs compared with HA film. Zang et al.$^{33}$ showed that a low concentration of fluorine ions in the culture medium play a significant role in affecting the behavior of osteoblast-like cells. On the other hand, Lee et al.$^{27}$ reported that cells on fluoridated coatings exhibited a lower proliferation level compared with cells on pure HA coatings due to increases in cytotoxicity levels. In the present study, only cell attachment of HMSCs on FHA film was investigated, however, the reason of the advantage against HA was unclear. Further in vitro studies investigating cell proliferation and differentiation are, therefore, warranted.

Many arguments have been made both in opposition to and in favor of the use of HA coatings$^{34}$. This debate has been fueled by the failures of clinical implants, supposedly as a result of poor coating-substrate adherence and problems with the crystallinity of the coating$^{9-11}$. In this study, PLD coating of the substrate at room temperature and post-annealing at 450°C resulted in an FHA thin film with high crystallinity, dissolution resistance and cell attachment with HMSCs. It is well known that fluorine ions promote mineralization and crystallization of calcium phosphate during dental and bone formation processes$^{35}$. This suggests that integration of titanium implants in bone tissue could be improved if they are coated with a thin film of FHA. However, animal experiments and clinical studies are still needed to validate the material’s performance efficiency.

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