Preparation and characterization of fluorinated porcine hydroxyapatite

Quan LIU, Zetao CHEN, Haijing GU and Zhuofan CHEN

Guanghua School of Stomatology, Hospital of Stomatology, Institute of Stomatological Research, Guangdong Provincial Key Laboratory of Stomatology, Sun Yat-sen University, 56 Ling Yuan Road West, Guangzhou 510055, China

Corresponding author, Zhuofan CHEN; E-mail: dentistczf@163.com

Simple chemical and thermal treatments were applied to prepare fluorinated porcine hydroxyapatite (FPHA). Morphology of FPHA was observed using SEM. Physiochemical characteristics, namely crystalline phase, chemical composition, functional groups, and binding energy of fluoride were investigated using XRD, EDX, FTIR, and XPS respectively. Concentration of free fluoride ion released from FPHA in HCl solution (pH 3.0–4.0) was detected using a fluoride ion concentration meter. SEM, XPS, XRD, and FTIR results confirmed the fluorination of porcine hydroxyapatite (PHA). Significant crystal morphological difference was observed between PHA and FPHA. Concentration of free fluoride ion released from FPHA increased with rising concentration of immersion solution and length of immersion period. Fluoride was successfully incorporated into PHA by chemical and thermal processes in this study. Fluoride incorporation rate into PHA was a strong function of the fluoride concentration in the immersion solution.

Keywords: Hydroxyapatite, Porcine bone, Ion incorporation, Fluorine

INTRODUCTION

Hydroxyapatite (HA) is the main inorganic component of animal bones and teeth. It is widely used as a bone substitute for the reconstruction of bone defects. Biological HA derived from animal bones or teeth has a composition and structure highly similar to those of human bone, and a bone resorption rate match up with the bone formation rate in human bone. These superior biocompatibility properties have fueled numerous clinical studies on HA derived from biological hard tissue, such as bovine bone, cuttlefish bone, and freeze-dried bone1-3—with some even culminating in commercial applications. In particular, bovine bone-derived HA, widely recognized to promote osteogenic response, has been used clinically for decades4. However, among the frequently used animal bones, porcine bone bears the closest resemblance to human bone in terms of bone macrostructure and microstructure, bone composition, and bone remodeling rate5.

Numerous studies were carried out on ion incorporation (e.g., F, Mg2+, Sr2+, CO32−) into synthetic apatite6-12 and biological apatite (such as bovine bone, natural coral, or cuttlefish bone)13-16. In general, these studies showed that ion-incorporated hydroxyapatite composites were promising candidates for biomedical applications. On the use of fluoride in treating bone diseases, it was found that a low serum fluoride level (95–190 μg/L) was beneficial for osteoblastic adhesion and proliferation and in stimulating bone formation17. Therefore, fluoride-incorporated biological HA might be an efficient approach to promote bone formation, remodeling and reconstruction.

A numbers of studies have been carried out on fluoride-incorporated synthetic HA11,19-21, but information remains scarce on the preparation and characteristics of fluorinated biological HA14. Moreover, the preparation processes previously reported for fluorinated biological HA14 seemed too complicated on the one hand, and the characterization efforts lacking in thoroughness on the other hand. The purpose of this study was to prepare fluorinated porcine hydroxyapatite (FPHA) using straightforward chemical and thermal processes, followed by a thorough investigation into its morphological and physiochemical characteristics.

MATERIALS AND METHODS

PHA preparation

Porcine hydroxyapatite (PHA) was prepared from cancellous porcine bone. After bone samples (femoral epiphysis) were thoroughly cleaned to remove macroscopic impurities, they were dissected into blocks of 10 mm×10 mm×5 mm dimensions. Calcination was carried out at 800°C for 2 h in air (heating rate: 10°C/min) in a muffle furnace (SGM6812BK, Xigema, Luoyang, Henan, China). The thermally treated samples, known as PHA, were washed in deionized water to remove organic ashes and other mixed impurities. After drying in air overnight at 80°C, PHA blocks were stored under sealed moisture-proof condition at room temperature.

FPHA synthesis

PHA blocks were divided into three groups: Test group A, Control group B, and No-treatment group C. In test group A, FPHA was synthesized by chemical and thermal processes. First, PHA blocks were immersed in sodium fluoride (NaF, analytical grade, Guangzhou Chemical Reagent Factory, Guangzhou, China) aqueous solutions of varied fluoride concentrations (F−: 0.25, 0.50, 0.75, 1.00 mol/L) and for different immersion periods (3, 6, 12, 24 h), hence constituting the different subgroups of
Group A. After chemical treatment in NaF, calcination was carried out at 700°C for 3 h in air (heating rate: 10°C/min). Thermally treated blocks were cooled down to room temperature, washed with deionized water, and dried at 80°C for 12 h. Synthesized FPHA blocks were stored under sealed moisture-proof condition at room temperature.

In group B, PHA blocks were immersed in deionized water for 24 h. All subsequent procedures from calcination to storage were as per those carried out for Group A.

In group C, no treatments were carried out on the as-prepared PHA blocks.

**Scanning electron microscopy (SEM)**

FPHA sample from Group A (F−: 1.00 mol/L; immersion period: 24 h) and PHA samples from Groups B and C were characterized by energy dispersive X-ray spectroscopy (Quanta 400 FEG, FEI/Oxford Instruments/HKL, Eindhoven, Netherlands). After block samples were ground into powder and compressed into pellets (uniaxial compression: 80 MPa), they were cemented on copper stubs using graphite paint. An interest area of approximately 500×300 μm was analyzed for each sample and analyzed under 30× magnification.

**Energy dispersive X-ray spectroscopy (EDX)**

To determine their chemical compositions, FPHA samples from Group A (F−: 0.50 and 1.00 mol/L; immersion period: 24 h) and PHA samples from Groups B and C were characterized by energy dispersive X-ray spectroscopy (Quanta 400 FEG, FEI/Oxford Instruments/HKL, Eindhoven, Netherlands). After block samples were ground into powder and compressed into pellets (uniaxial compression: 80 MPa), they were cemented on copper stubs using graphite paint. An interest area of approximately 500×300 μm was chosen on each sample and analyzed under 30× magnification.

**X-ray diffraction (XRD)**

Changes in the crystalline phase of Group A (F−: 1.00 mol/L, immersion period: 24 h) and Group B samples before and after heat treatment at 700°C were examined using XRD (D/MAX Ultima III, Rigaku, Tokyo, Japan). The crystalline phase of untreated PHA sample in Group C was also identified using XRD and used for comparison.

Powdered samples were mounted on glass stubs. A diffracted beam graphite monochromator was used to produce Cu Kα radiation with a wavelength of 1.54056 Å at a scanning speed of 10° (2θ)/min.

**Fourier transform infrared spectroscopy (FTIR)**

The functional groups in Group A (F−: 1.00 mol/L, immersion period: 24 h) and Group B samples before and after heat treatment at 700°C were identified using FTIR (Vector 33, Bruker Optics, Ettlingen, Germany). The functional groups in untreated PHA sample in Group C were also identified using FTIR and used for comparison.

Powdered samples were mixed with KBr powder (IR grade, Merck, Giessen, Germany) using a mortar and pestle, and then compressed into pellets. IR spectra were collected in transmittance mode with a scanning range of 4000–400 cm−1.

**X-ray photoelectron spectroscopy (XPS)**

The binding energies of fluorine in FPHA (Group A, F−: 0.50 and 1.00 mol/L; immersion period: 24 h) and PHA (Groups B and C) samples were investigated using XPS (ESCALAB 250, Thermo Scientific, Barrington, IL, USA). Powdered samples were compressed into pellets and attached to a sample holder. An interest area of approximately 500×300 μm was analyzed for each sample.

**Fluoride ion activity measurement**

The concentrations of free fluoride ions released from FPHA (all subgroups in Group A) and PHA (Groups B and C) samples in HCl solution (pH 3.0–4.0) were measured using a fluoride ion concentration meter (PXJ-1C°, Fangzhou Technology, Chengdu, China). To plot the standard calibration curve, standard sodium fluoride (NaF) solutions of different fluoride concentrations (100.00 μg/mL, 10.00 μg/mL, 1.00 μg/mL, 0.10 μg/mL, 0.01 μg/mL) were prepared by diluting 10 mL of each standard NaF solution with 10 mL of a buffer solution (buffer pair: C6H5O7−/CH3COOH, pH: 5.0–5.5) before measurement. To minimize measurement errors, each standard NaF solution was measured up to 3 min each time for a total of three times. An average value was calculated for each fluoride concentration and the standard calibration curve was thus constructed.

To measure fluoride ion activities in PHA and FPHA samples, hydrochloric acid solution (5.00 mol/L, 10.00 mL) was added to dissolve 10 mg of each sample, as recommended by the ion concentration meter’s manufacturer. The pH value of this mixed solution was adjusted to 3.0–4.0 by adding 10.00 mL of buffer solution (buffer pair: C8H5O7−/CH3COOH, pH: 5.0–5.5). Fluoride ion activity in each sample was measured up to 3 min each time for a total of three times, and the average value thereby calculated.

**RESULTS**

**Morphological observation by SEM**

Interconnected porous structures were observed for both PHA and FPHA (Figs. 1a, 1c, and 1e; Fig. 2), with slight macrostructural difference between them. Heat treatment eliminated all organic impurities. Inorganic minerals with a crystal size of 0.2–0.6 μm (Fig. 1) were observed. Under high-power magnification, rod-like crystals were observed in FPHA (Fig. 1b), which were significantly different from those of PHA (Figs. 1d, 1f).

**Compositional analysis by EDX**

Ca, P, O, and C were found to be the main components of PHA and FPHA (Table 1, Fig. 3), and trace amounts of Na and Mg were also detected. No fluorine was found in PHA (Groups B and C), but appreciable amounts (2.41% and 3.67%) were detected in FPHA after 24-h immersion in 0.5 mol/L and 1.0 mol/L F− concentrations. The Ca/P
ratios of PHA (1.49) and FPHA (1.49 or 1.51) were lower than that of stoichiometric HA (1.67).

**Crystalline phase identification by XRD**

The XRD signatures of PHA (Fig. 4) were in agreement with the stoichiometric reference HA pattern (JCPDS 72-1243), indicating that PHA was crystallized in pure phase. For FPHA after heat treatment, its reflection peaks shifted toward higher diffraction angles.

**Identification of functional groups by FTIR**

Absorption peaks corresponding to the apatite phase were seen in the FTIR spectra of PHA and FPHA (Fig. 5). Apart from the characteristic phosphate (PO₄³⁻) and
hydroxyl (OH\(^{-}\)) groups, absorption peaks at around 1455, 1410, and 875 cm\(^{-1}\) due to carbonate groups indicated the substitution of CO\(_3^{2-}\) ions into PHA, thus resembling the composition of carbonate apatite in human calcified tissue. For FPHA, the absorption peak of hydroxyl at about 634 cm\(^{-1}\) disappeared after heat treatment.

**Determination of binding energies of fluorine by XPS**

Photoelectron peaks at particular binding energies (Peak BE) indicate the presence of specific elements in PHA and FPHA. Peak BE is dependent on the oxidation state and chemical environment of each element. As shown in Table 2 and Fig. 6, the decrease in the binding energy of fluorine in FPHA samples (Group A, F\(^{-}\): 0.50 and 1.00 mol/L, immersion period: 24 h) indicated a change in chemical environment from Na-F to Ca-P-O-F-C. Their fluorine amounts, 2.10% and 3.82% (Table 2), were in close agreement with the EDX results in Table 1.

**Fluoride ion activity**

Fluoride ion concentration in HCl solution (pH 3.0–4.0)
was about 54.95–245.47 μg/L at 24.50±0.20°C (Table 3), as calculated from the standard calibration curve. Table 3 also shows that fluoride ion activity generally increased with an increase in the fluoride concentration of immersion solutions and length of immersion period, although the electric potentials were slightly fluctuant.

Table 1 Chemical compositions of FPHA and PHA as determined by EDX

<table>
<thead>
<tr>
<th>Sample</th>
<th>Atomic (%)</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>Ca</td>
<td>P</td>
<td>O</td>
<td>C</td>
<td>Na</td>
<td>Mg</td>
<td>F</td>
<td>Ca/P</td>
</tr>
<tr>
<td>FPHA(A-0.5 mol/L-24 h-heated)</td>
<td>18.46</td>
<td>12.25</td>
<td>58.50</td>
<td>7.15</td>
<td>0.85</td>
<td>0.38</td>
<td>2.41</td>
<td>1.51</td>
</tr>
<tr>
<td>FPHA(A-1.0 mol/L-24 h-heated)</td>
<td>16.56</td>
<td>11.09</td>
<td>60.53</td>
<td>6.81</td>
<td>0.66</td>
<td>0.67</td>
<td>3.67</td>
<td>1.49</td>
</tr>
<tr>
<td>PHA(B-heated)</td>
<td>17.79</td>
<td>11.93</td>
<td>60.40</td>
<td>9.27</td>
<td>0.61</td>
<td>0.00</td>
<td>0.00</td>
<td>1.49</td>
</tr>
<tr>
<td>PHA(C)</td>
<td>17.71</td>
<td>12.03</td>
<td>61.29</td>
<td>7.49</td>
<td>0.59</td>
<td>0.53</td>
<td>0.00</td>
<td>1.47</td>
</tr>
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</table>

In Fig. 7, dispersion in fluoride ion activities (as measured by the electric potential) was greater among the different fluoride concentrations of the immersion solutions (Fig. 7a) than among the different immersion periods (Fig. 7b).

Fig. 4 XRD patterns of FPHA (Group A) and PHA (Group B) before and after heat treatment, with comparison against non-treated PHA (Group C) and stoichiometric reference HA pattern (JCPDS 72-1243): (a) 20°–60°; (b) 30°–35°.

Fig. 5 FTIR spectra of FPHA (Group A) and PHA (Group B) before and after heat treatment, with comparison against non-treated PHA (Group C): (a) 0–4,000 cm⁻¹; (b) 500–2,000 cm⁻¹.
DISCUSSION

Ion incorporation

Ionic substitution, which involves the incorporation of ions such as $F^{−}$, $Mg^{2+}$, and $Si^{4+}$ into the apatite lattice, can affect the crystal structure, crystallinity, surface charge, solubility and other vital properties, causing major changes in the biological performance upon implantation. While synthetic apatite is a promising candidate for ionic substitutions, biological apatite is the preferred choice as it inherently possesses marked similarities with the human bone in terms of crystallographic properties, chemical composition, porosity, and a resorption rate which matches the bone.

Table 2  Chemical states of fluorine in PHA and FPHA as determined by XPS before and after heat treatment at 700°C

<table>
<thead>
<tr>
<th>Group</th>
<th>Peak BE of fluorine (eV)</th>
<th>Atomic (%)</th>
</tr>
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<tbody>
<tr>
<td>FPHA(A-0.5 mol/L-24 h)</td>
<td>685.09</td>
<td>3.61</td>
</tr>
<tr>
<td>FPHA(A-0.5 mol/L-24 h-heated)</td>
<td>684.38</td>
<td>2.10</td>
</tr>
<tr>
<td>FPHA(A-1.0 mol/L-24 h)</td>
<td>685.71</td>
<td>4.23</td>
</tr>
<tr>
<td>FPHA(A-1.0 mol/L-24 h-heated)</td>
<td>685.05</td>
<td>3.82</td>
</tr>
<tr>
<td>PHA(B) –</td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>PHA(B-heated) –</td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>C –</td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>NaF</td>
<td>687.19</td>
<td>33.29</td>
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</table>

Table 3  Fluoride ion concentrations and activities of all FPHA and PHA samples measured using a fluoride ion concentration meter

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (mol/L)</th>
<th>Time (h)</th>
<th>E (mV)</th>
<th>$F^−$ (μg/L)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>0.25</td>
<td>3</td>
<td>-289.40</td>
<td>54.95</td>
</tr>
<tr>
<td>A</td>
<td>0.25</td>
<td>6</td>
<td>-287.10</td>
<td>66.07</td>
</tr>
<tr>
<td>A</td>
<td>0.25</td>
<td>12</td>
<td>-284.70</td>
<td>77.62</td>
</tr>
<tr>
<td>A</td>
<td>0.25</td>
<td>24</td>
<td>-286.70</td>
<td>67.61</td>
</tr>
<tr>
<td>A</td>
<td>0.50</td>
<td>3</td>
<td>-278.40</td>
<td>120.23</td>
</tr>
<tr>
<td>A</td>
<td>0.50</td>
<td>6</td>
<td>-282.20</td>
<td>93.33</td>
</tr>
<tr>
<td>A</td>
<td>0.50</td>
<td>12</td>
<td>-276.20</td>
<td>141.25</td>
</tr>
<tr>
<td>A</td>
<td>0.50</td>
<td>24</td>
<td>-276.20</td>
<td>141.25</td>
</tr>
<tr>
<td>A</td>
<td>0.75</td>
<td>3</td>
<td>-274.70</td>
<td>151.36</td>
</tr>
<tr>
<td>A</td>
<td>0.75</td>
<td>6</td>
<td>-272.00</td>
<td>177.83</td>
</tr>
<tr>
<td>A</td>
<td>0.75</td>
<td>12</td>
<td>-269.30</td>
<td>213.80</td>
</tr>
<tr>
<td>A</td>
<td>0.75</td>
<td>24</td>
<td>-267.90</td>
<td>229.09</td>
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<tr>
<td>A</td>
<td>1.00</td>
<td>3</td>
<td>-270.60</td>
<td>199.53</td>
</tr>
<tr>
<td>A</td>
<td>1.00</td>
<td>6</td>
<td>-269.70</td>
<td>208.93</td>
</tr>
<tr>
<td>A</td>
<td>1.00</td>
<td>12</td>
<td>-267.20</td>
<td>245.47</td>
</tr>
<tr>
<td>A</td>
<td>1.00</td>
<td>24</td>
<td>-267.00</td>
<td>245.47</td>
</tr>
<tr>
<td>B</td>
<td>0.00</td>
<td>24</td>
<td>-369.60</td>
<td>0.00</td>
</tr>
<tr>
<td>C</td>
<td>0.00</td>
<td>–</td>
<td>-374.70</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Temperature: 24.50±0.20°C, blank electric potential: −330.00 mV, pH: 3.0–4.0
Amongst the foreign ions used to enhance the biological performance of implanted materials, fluoride has aroused great interest since a low dose of fluoride was reported to stimulate bone formation and increase bone mass without causing any mineralization defects \(^{28}\). On the other hand, toxic effects arising from high doses of fluoride include hypomineralization of newly formed osteoid, hypermineralization of bone, and endemic fluorosis \(^{29}\). Incorporation of fluoride into biological apatite might cause diverse changes, ranging from crystallographic alterations to impact on biological performance. However, the details thereof remained unknown.

**Synthesis of fluorinated PHA**

Both chemical and thermal approaches have been used to prepare biological HA derived from animal bones \(^{14,24,30,31}\). Thermal treatment has several advantages: it is the only means to eliminate organic matter \(^{32}\) and it decreases the risk of disease transmission and immunoreactivity \(^{30}\). For biological HA prepared using the thermal approach, its characteristics depend on heating conditions such as the temperature, gas atmosphere, and heating time.

Morphologically, bovine bone heated between 800°C and 1000°C maintained the spongy structure of a natural bone with an interconnected porous network \(^{32}\). Although crystallinity increased with rising temperature, a small amount of \(\beta\)-TCP was formed at 800°C by the partial decomposition of HA \(^{32}\) when oxygen was absent during the heat treatment \(^{30}\).

In the present study, raw porcine bone blocks were calcined at 800°C for 2 h to eliminate all organic matter. Heat treatment was also carried out with the door of the muffle furnace opened to prevent possible decomposition of PHA. XRD results revealed that PHA was composed of pure hydroxyapatite only, with no organic or other crystalline phases detected. SEM images revealed that the interconnected porous structure was maintained in both PHA and FPHA after heat treatment (Figs. 1 and 2), albeit with varied pore sizes.
Characterization of fluorinated PHA

SEM revealed similar macrostructures for both PHA and FPHA in the present study, but microstructural differences could be discerned under high-power magnification (Fig. 1). The change in crystal morphology could be ascribed to the incorporation of fluoride ion into PHA, resulting in crystal lattice shrinkage and change in crystal shape.

Apart from SEM observation, fluorne incorporation into PHA was also cross-checked by comparing the results of EDX, FTIR, XPS, and XRD measurements in this study. It was previously reported that the amount of ionic substitution in human teeth-derived hydroxyapatite was below the detection limits for XRD and FTIR measurements. Therefore, a bigger suite of characterization techniques was employed in this study as there was a concern that marginal ion substitution into HA might not be detectable.

An apparent evidence of fluoride ion substitution was the presence of F element in FPHA and its stark absence in PHA (Table 1, Fig. 3). The lower Ca/P molar ratios of PHA and FPHA (1.49 or 1.51), as compared with stoichiometric HA (Ca/P=1.67), could be due to the substitution of other ions such as Na+, Mg2+, and CO32−. Consequently, Ca deficiency led to lower measured Ca/P molar ratios.

The binding energy of an element is very sensitive to its chemical state and environment. The chemical environment of fluoride changed once it was incorporated into the crystal lattice of PHA, causing a change in binding energy, i.e., a chemical shift occurred. The binding energy peak of fluorine was observed after immersion and different from that of NaF (Table 2, Fig. 6), demonstrating that immersion in NaF alone could lead to fluoride incorporation. Based on the different binding energy peaks of fluorine between 0.50 mol/L and 1.00 mol/L FPHA samples, it seemed that the fluoride concentration of immersion solution was an influencing factor of fluoride incorporation into PHA.

The X-ray photoelectron spectrometer used in the current study had an accuracy of 0.01 eV. To be more accurate, any change of binding energy lower than 0.03 eV was regarded as a measurement error. After thermal treatment, decrease in the binding energy of fluorine of 0.50 mol/L and 1.00 mol/L FPHA samples was higher than 0.03 eV (Table 2, Fig. 6). Fluorine chemical shift was caused by its incorporation into PHA, and fluoride ion substitution occurred by a partial hydroxyl group replacement. XRD results further confirmed the replacement of hydroxyl group by fluoride ion with the shift of reflection peaks of FPHA toward higher diffraction angles after heating at 700°C (Fig. 4). Similarly, the absorption peak of hydroxyl group was absent in the FTIR spectrum of FPHA after thermal treatment (Fig. 5). Taking together the results of XPS, XRD, and FTIR in this study, it was shown that thermal treatment could bring about ion incorporation, a finding which echoed that of Sogo et al.21.

Effects of fluoride concentration and immersion period on PHA fluorination

The ideal pH range for free fluoride ion detection should be 5.0–6.0, according to the instruction manual of the fluoride ion concentration meter used in this study. However, a full dissolution of FPHA could not be achieved in this condition. After several pre-experimental trials, the optimal compromise was dissolution in HCl solution of 3.0–4.0 pH range.

It was reported that the therapeutic window of serum fluoride was 95–190 μg/L, and the estimated toxic level was 950 μg/L. In the present study, the fluoride levels of all FPHA subgroups were lower than the toxic level, and some were below the therapeutic range (Table 3). However, the exact content of fluoride might be slightly higher. This was because detection pH was decreased from 5.0–6.0 to 3.0–4.0, causing more HF (and less free fluoride ions) to be formed in a more acidic condition.

Figure 7 reflects the different effects of two factors on the fluorination of PHA: fluoride concentration of immersion solution versus immersion period. The dispersion of data in Fig. 7(a) was higher than that in Fig. 7(b), a clear indication that the fluoride concentration of immersion solution contributed more to fluoride incorporation than did the immersion period.

CONCLUSIONS

A straightforward and cost-effective approach to obtaining fluorinated biological apatite from porcine bone was presented in this study. Within the limitations of this study, the following conclusions were drawn:

1. FPHA, prepared via chemical and thermal processes, possessed high similarities to the biominerals of human calcified tissue without any detectable mineralization defects.
2. Fluoride ion incorporation into PHA was mainly dependent on the fluoride concentration of the immersion solution.

Further studies on the biological properties of FPHA should be pursued for its potential application in the reconstruction and treatment of bone defects.

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