Preparation of protamine-adsorbed calcium phosphate powders and their antibacterial property

Daisuke Koizumi\(^a\), Kitaru Suzuki\(^b\), Hirogo Minamisawa\(^c\), Rie Togawa\(^b\), Kosuke Yasui\(^b\), Keishi Iohara\(^b\), Michiyo Honda\(^a\) and Mamoru Aizawa\(^a\)

\(^a\)Department of Applied Chemistry, School of Science and Technology, Meiji University, Kawasaki, Kanagawa, Japan; \(^b\)Central Research Institute, Maruha Nichiro Co, Tsukuba, Ibaraki, Japan; \(^c\)Organization for the Strategic Coordination of Research and Intellectual Properties, Meiji University, Kawasaki, Kanagawa, Japan

ABSTRACT
Calcium phosphates are key biomaterials in bone regeneration and dental treatment fields. Biomaterials must exhibit antibacterial properties to ensure protection against microbial infection in implantation frameworks. Various starting calcium phosphate and protamine-adsorbed calcium phosphate powders were prepared in this study to develop novel antimicrobial biomaterials exhibiting the biocompatibility of calcium phosphates and the antimicrobial property of protamine, and their properties were examined. Specifically, hydroxyapatite (HAp) and its precursors—amorphous calcium phosphate (ACP) and octacalcium phosphate (OCP)—were synthesized using the conventional wet process. Moreover, three types of protamine-adsorbed calcium phosphate powders (ACP-Protamine, OCP-Protamine, and HAP-Protamine) were prepared via adsorption of protamine (25.2, 5.28, and 19.2 mg g\(^{-1}\)) respectively on the starting calcium phosphate powders in water for 48 h. The antimicrobial property of the protamine-adsorbed calcium phosphate powders against Staphylococcus aureus, a representative bacterium that causes implantation-related infection of biomaterials, was evaluated. All the examined protamine-adsorbed calcium phosphate powders exhibited antibacterial properties and, therefore, show promise as effective antimicrobial biomaterials.

1. Introduction
Calcium phosphate is an inorganic substance found abundantly in human bones and teeth. Calcium phosphate is widely used as a biomaterial in bone regeneration and dental applications, and in coatings, cements, and scaffolds because of its high biocompatibility, osteoconductivity, and mechanical strength [1–3]. In general, bone contains approximately 60–70 mass% minerals, most of which pertain to hydroxyapatite (HAp, Ca\(_{10}\)(PO\(_4\))\(_6\)(OH)\(_2\)) [4], and tooth dentin and tooth enamel contain approximately 70 mass% and 97 mass% of HAp, respectively [3]. Moreover, the inorganic phase of bones and teeth comprises poorly crystalline ion-substituted calcium phosphate with a structure similar to that of HAp [5]. In the biological environment, HAp has a hexagonal structure and is the most stable calcium phosphate at room temperature and in a pH range of 4–12 [1,6]. Amorphous calcium phosphate (ACP) and octacalcium phosphate (OCP, Ca\(_8\)H\(_2\)(PO\(_4\))\(_6\)·5H\(_2\)O), which are precursors of HAp, have been reported to have superior bone regeneration ability compared to that of HAp [7–9]. Moreover, ACP has an amorphous structure consisting of ion clusters, which are known as Posner’s clusters, with a diameter of 0.95 nm and a chemical composition of Ca\(_9\)(PO\(_4\))\(_6\). ACP is the first solid phase to emerge from saturated calcium phosphate solutions and gradually transforms into OCP and HAp, which are more stable solid phases. ACP exhibits high solubility, high absorption, and high bioactivity [8,10]. The structure of OCP is similar to that of HAp, with overlapping apatite and hydrated layers. However, the properties of OCP as a substitute bone material are different from those of HAp in terms of osteoconductivity and biodegradability; for instance, a higher osteoconductivity was observed in the bone tissue response of mouse cortical bone when OCP was placed onto the calvaria in a granular form compared to that of other calcium phosphate materials, including ACP and HAp [9].

Biomaterials are crucial in the treatment of diseases and injuries. However, during their implantation, biomaterials are susceptible to infection [11]. Several cases of infection in substitute bones and dental implants have been reported. The main causative organisms of such infections are the Staphylococcus species, such as Staphylococcus aureus (S. aureus) and Staphylococcus epidermidis [11]. In this context, the development of novel biomaterials that are impervious...
to infection is critical. In particular, antimicrobial biomaterials that can inhibit microbial adhesion have been developed as drug-release-based antimicrobial coatings [12], such as chlorhexidine-, silver-, furanone-, and nitric-oxide-releasing coatings.

Protamine, a cationic protein that is rich in arginine residues, has a molecular weight of approximately 5 kDa and an isoelectric point of 12–13. This protein, which is also known as salmine and clupeine, is bound to DNA in the sperm cells of fish, birds, and mammals. Protamine exhibits a broad spectrum of antibacterial activity against gram-positive and gram-negative bacteria, and has been used as a food preservative [13–16]. Although the antimicrobial mechanisms of protamine vary among species and have not been comprehensively elucidated, two mechanisms have been proposed to date [17]: i) electrostatic interactions between positively charged protamine and negatively charged extracellular epithelium to release K+, ATP, and intracellular enzymes; and ii) targeting of the cell membrane and inhibition of the energy transmission and nutrient uptake functions. In addition, protamine can regulate the transcription of bone sialoprotein, which can form HAp crystals [18]. Moreover, synthetic chemical antibiotics face an alarming issue involving resistant bacteria. However, the issue of resistant bacteria is believed to be less severe for antimicrobial peptides, such as protamine, because of their antimicrobial mechanism involving action on the biological membranes of the pathogens, which are dynamic fluids [19].

To exploit the antimicrobial property of protamine in bone regeneration and dental applications, we previously prepared protamine-adsorbed calcium phosphate from dicalcium phosphate anhydrous (DCPA) and HAp, and confirmed the resistance of these materials to microorganisms such as Streptococcus mutans (S. mutans) and S. aureus, which are typical caries-causing and surgery site infection-causing bacteria, respectively [20,21]. Because ACP has an amorphous structure [8] and OCP has a plate-like morphology [22], they differ from HAp not only in their in vivo behavior but also in their structure, indicating that their protamine-adsorption-related properties as starting powders and the antimicrobial properties of the resulting protamine-adsorbed calcium phosphates could be superior to those of HAp. In addition, antimicrobial biomaterials, such as ACP and silver nanocomposites [23] and esthetic silver-doped octacalcium phosphate powders [24], have been developed using ACP and OCP. Considering the broad antimicrobial behavior of protamine and the antimicrobial mechanism that likely hinders the growth of resistant bacteria, protamine-adsorbed calcium phosphates prepared using ACP and OCP as starting materials could be more desirable antimicrobial materials than currently existing equivalents. However, the preparation and evaluation of protamine-adsorbed calcium phosphate using ACP and OCP as starting powders have not been reported to date. Therefore, protamine-adsorbed calcium phosphates were prepared in this study using ACP, OCP, and HAp as starting powders to create novel biomaterials with antimicrobial properties, and their properties were evaluated.

2. Materials and methods
2.1. Synthesis of calcium phosphates

Three types of starting calcium phosphate powders (ACP, OCP, and HAp) were synthesized in-house to obtain fine particles with a median diameter of the order of micrometers.

ACP was prepared using a previously reported protocol [25]. Specifically, 350 cm$^3$ of 0.25 mol·dm$^{-3}$ (NH$_4$)$_2$HPO$_4$ solution was added to 200 cm$^3$ of 0.75 mol·dm$^{-3}$ Ca(NO$_3$)$_2$·4H$_2$O solution; both solutions were maintained at 4°C, and the resulting mixture was stirred for 1 min under ice-cold conditions. The pH values of both solutions were adjusted to 10.0 using ammonia water prior to their mixing. The produced slurry was suction filtered and washed twice with distilled water (pH = 10) and three times with distilled water. A powder was obtained thereafter by freeze drying and was ground in an agate mortar to prepare the starting ACP powder, which was stored in a drying cabinet for subsequent use.

OCP was prepared using a previously reported method [26]. Specifically, 0.0335 mol of CaCO$_3$ and 0.1 mol of CaHPO$_4$·2H$_2$O (DCPD) were mixed in 1000 cm$^3$ of distilled water, sonicated for 10 min, and stirred at 60°C for 4, 6, and 15 h. The resulting slurry was suction filtered and washed three times using distilled water. A powder was subsequently obtained by freeze drying and was ground in an agate mortar to prepare the starting OCP powder, which was stored in a drying cabinet for further experiments.

HAp was prepared using a previously reported protocol [27,28]. Specifically, 1000 cm$^3$ of a 0.50 mol·dm$^{-3}$ Ca(OH)$_2$ suspension was prepared, and 1000 cm$^3$ of 0.30 mol·dm$^{-3}$ H$_3$PO$_4$ was added dropwise for 3 h with stirring. After the entire amount was added, the pH was adjusted to 8.70 using ammonia water. The solution was stirred for an additional 3 h, and the pH was adjusted thereafter to 8.70. The resulting slurry was aged in an incubator at 37°C for 3 d. After aging, the slurry was suction filtered and washed three times with distilled water. Subsequently, the slurry was dried in a drying oven at 110°C for 2 d, and the resulting powder was ground in an agate mortar to prepare HAp powder, which was stored in a drying cabinet for subsequent experiments.
2.2. Characterization of synthetic calcium phosphates

The crystalline phases of the synthetic calcium phosphates were identified using an X-ray diffractometry (XRD; MiniFlex, Rigaku Co., Japan) setup with Cu-Ka radiation operating at 30 kV and 15 mA. Moreover, Fourier transform infrared (FT-IR) spectroscopy (NICOLET iS10, Thermo Fisher Scientific K.K., Japan) was performed using the KBr method to clarify the functional groups of the synthetic calcium phosphates. The calcium:phosphorus molar ratio (Ca/P ratio) was determined by inductively coupled plasma (ICP, ICPE-9000, HORIBA Co., Japan). The specific surface area (SSA) was determined using a Brunauer–Emmett–Teller (BET) surface area analyzer based on the BET method (FlowSorb III, Micromeritics Instrument Co., USA). In the SSA analysis, degassing was performed at 70°C to prevent changes in the crystalline phase. The particle morphology was observed through i) scanning electron microscopy (SEM, TM4000Plus, Hitachi High-Tech Fielding Co., Japan) without platinum deposition and ii) transmission electron microscopy (TEM, JEM-2100 F, JEOL Ltd., Japan) at an acceleration voltage of 200 kV. The samples for TEM observation were prepared by dispersing the powders in ethanol and dropping the solution onto carbon-reinforced copper grids (400 mesh, JEOL Ltd., Japan).

Each calcium phosphate specimen was synthesized several times, and the XRD patterns, FT-IR spectra, median sizes, and SSAs shown in the figures and table are representative data obtained from multiple evaluations.

2.3. Adsorption of protamine onto synthetic calcium phosphates

The concentration of a protamine-charged solution was examined in a previous study, and the adsorption of protamine, the antimicrobial properties, and the lack of apparent cytotoxicity in vivo and in vitro of the prepared calcium phosphates were detected upon using a 500 µg·cm⁻³ protamine-charged solution [20,21]; therefore, protamine adsorption in the present study was also conducted at a concentration of 500 µg·cm⁻³. Specifically, 45 cm³ of a 500 µg·cm⁻³ protamine solution (salmon-derived protamine hydrochloride, Maruha Nichiro Co., Japan) was added to 1.0 g of various calcium phosphates (ACP, OCP, and HAp), and the mixtures were stirred in a tube rotator (RCC-100, Iwaki Glass Co., Ltd., Japan) for 48 h under room temperature conditions for adsorption. The supernatants and precipitates were collected via centrifugation at 8600 × g for 30 min. The precipitates were washed three times using ultrapure water and lyophilized to obtain protamine-adsorbed calcium phosphates, which were stored in a drying cabinet until further use.

2.4. Characterization of protamine-adsorbed calcium phosphates

The crystalline phases of the protamine-adsorbed calcium phosphates were identified by XRD and FT-IR spectroscopy. Protamine in the supernatant after the adsorption was quantified by high-performance liquid chromatography (HPLC, Alliance, Nihon Waters K.K., Japan). The amount of protamine adsorbed on the calcium phosphates was determined using a procedure described henceforth. One hundred milligrams of each protamine-adsorbed calcium phosphate powder and 0.45 cm³ of 1 mol·dm⁻³ HCl were stirred for 1 h. The supernatant was collected via centrifugation at 13,000 × g for 10 min. The protamine content in the supernatant was quantified by HPLC, and the amount of protamine adsorbed on the calcium phosphates was calculated.

2.5. Evaluation of antimicrobial property

Each calcium phosphate powder was placed in a sterile bag (HM-3002, HOGY MEDICAL CO., LTD., Japan) and sterilized using ethylene oxide gas. Phosphate buffered salts (PBS; 9 cm³, 9.57 mmol·dm⁻³; Takara Bio Inc., Japan), a slurry of each calcium phosphate powder with sterile water (100 mm³; final concentrations: 1 mg·cm⁻³ and 2 mg·cm⁻³), and S. aureus FDA209 suspended in PBS (1 cm³, final concentration: 10⁵ CFU·cm⁻³) were added to test tubes. Each test tube was incubated at 30°C for 6 h with agitation at 180 rpm, and colony counts were performed. The culture supernatant was filtered through a 0.22 µm pore size filter, and the protamine content in the supernatant was quantified using the Bradford method (FUJIFILM Wako Pure Chemical Co., Japan).

2.6. Statistical analysis

The data were statistically evaluated using one-way analysis of variance, followed by a Tukey post-hoc test, and the results were expressed as mean ± standard deviation.

3. Results and discussion

3.1. Syntheses of calcium phosphates and their powder properties

XRD and FT-IR analyses were performed to confirm the crystalline phase of the synthesized calcium phosphates (Figures 1 and 2).
The XRD results for the ACP indicated an ACP-specific broad peak (Figure 1(a)). Moreover, the FT-IR spectra showed the amorphous bands of $\nu_3$ PO$_4^{3-}$ and $\nu_4$ PO$_4^{3-}$ at approximately 1000 cm$^{-1}$ and 550 cm$^{-1}$, respectively (Figure 2(a)) [7].

OCP was prepared by the reaction of DCPD and CaCO$_3$ in water, as previously reported [26], because OCP powder with high crystallinity and purity can be reproducibly prepared using this method. A larger scale of preparation was employed in this study.

**Figure 1.** X-ray diffraction (XRD) patterns of synthetic calcium phosphates: (a) ACP, (b) OCP-4 h, (c) OCP-6 h, (d) OCP-15 h, and (e) HAp. DCPD, OCP, and HAp were identified using ICDD-PDF cards No. 01-072-0713, No. 00-026-1056, and No. 00-009-0432, respectively.

**Figure 2.** Fourier transform infrared (FT-IR) spectra of synthetic calcium phosphates: (a) ACP, (b) OCP-4 h, (c) OCP-6 h, (d) OCP-15 h, and (e) HAp.
compared to that in previous studies to obtain a sufficient amount of sample. However, insufficient dispersion of the raw materials occurred during stirring. Therefore, a 10 min ultrasonic treatment was performed prior to stirring to enhance the dispersion. In previous study [26], OCP formation was initiated within 1 h of the reaction at 60°C. However, the reaction time was also examined owing to the larger scale of the reaction in the present study.

In the XRD patterns of the synthesized OCPs (Figure 1(b-d)), diffraction peaks were observed at 2θ = 4.9°, 9.6°, and 9.9°, which are characteristic of OCPs, at all the investigated reaction times (4, 6, and 15 h). At 4 h, a diffraction peak was observed at 2θ = 11.8°, which is characteristic of DCPD, suggesting that the DCPD used as a raw material for OCP preparation remained in the synthesized OCP powder (Figure 1(b)). At 6 h and 15 h, the XRD patterns exhibited a single phase of OCP (Figure 1(c,d)). In particular, the relative intensity of the diffraction peak at 2θ = 4.9°, the strongest peak of OCP, was the highest at 15 h. As shown in Figure 2(b-d), the FT-IR spectra exhibited four P-O bands, characteristic of OCP, at approximately 1125, 1076, 1057, and 1025 cm⁻¹, and two sharp bands at 560–600 cm⁻¹ derived from the crystalline calcium phosphate of ν₄PO₄³⁻ [22,26].

In the case of HAp, typical diffraction peaks of HAp were observed at 2θ = 31.9°, 32.9°, and 39.9°, in the XRD pattern (Figure 1(e)). Moreover, as shown in Figure 2(e), the FT-IR spectra exhibited bands of ν₃PO₄³⁻ and ν₁PO₄³⁻ at approximately 1034 cm⁻¹ and 962 cm⁻¹, respectively, and those of ν₂PO₄³⁻ at approximately 604 and 565 cm⁻¹ [6,26]. In addition, a band of CO₃²⁻ was observed at approximately 1455 cm⁻¹, suggesting that a portion of the phosphate site of the synthesized HAp was replaced by carbonate ions [6]. The HAp-derived OH band was not visible owing to the presence of a broad band at ~3000–3500 cm⁻¹, which was derived from water in the calcium phosphate crystal.

The Ca/P molar ratios, median diameters, and SSAs of the calcium phosphates are listed in Table 1. The Ca/P molar ratios of ACP and HAp were similar to the theoretical values of 1.50 [8] and 1.67 [6], respectively, whereas that of OCP was greater than the theoretical value of 1.33 [26] at all examined reaction times (4, 6, and 15 h). The difference in the determined Ca/P molar ratio with the theoretical value in the case of OCP could be attributed to the partial formation of HAp converted from the OCP, according to the reactions presented in (1) and (2) [26].

$$
6\text{CaHPO}_4 \cdot 2\text{H}_2\text{O} + 2\text{Ca}^{2+} + 2\text{CO}_3^{2-} \\
\rightarrow \text{Ca}_6\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O} \downarrow + 2\text{CO}_2 \uparrow + 9\text{H}_2\text{O} \quad (1)
$$

$$
\text{Ca}_9\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O} + 2\text{Ca}^{2+} + 2\text{CO}_3^{2-} \\
\rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \downarrow + 2\text{CO}_2 \uparrow + 5\text{H}_2\text{O} \quad (2)
$$

Although the mechanism of protein binding to calcium phosphate is complex and remains unclear, multiple parameters such as the size, charge, structural stability, and unfolding rate of proteins, and the topography, composition, hydrophobicity, heterogeneity, and potential of calcium phosphate are clearly involved [29]. Because it is generally accepted that the amount of adsorbed protein increases with increasing SSA, the particle size of the material is related to SSA; therefore, particle size and SSA were measured as representative indicators of the protamine-binding ability. Moreover, the median diameter was used to represent the particle size because of a distribution in the particle size of the prepared powders. The median diameters of the synthesized ACP, OCP, and HAp ranged from 14 to 21 μm. The SSAs of ACP, OCP, and HAp ranged from 33 to 69 m²·g⁻¹ and decreased in the following order: HAp > ACP > OCP. Degassing was generally performed at temperatures above 100°C during the SSA measurements. However, degassing was conducted at 70°C for 40 min in this study to avoid the possible decrease in crystallinity of the prepared calcium phosphates at temperatures above 100°C. Additionally, the XRD patterns confirmed the modest changes in crystallinity before and after the degassing (data not shown). The reason behind the largest SSA of HAp among the prepared specimens despite its large median size could not be established; however, because SSA is related not only to the median size but also to the roughness, porosity, and pore size of calcium phosphates [29], these parameters could also be employed for SSA analysis.

As mentioned in the preceding text, we examined the reaction time (4, 6, and 15 h) for OCP. In the XRD diagram, diffraction peaks associated with DCPD, which was used as a raw material, were observed in the case with the reaction time of 4 h, and the relative intensity of the diffraction peak at 2θ = 4.9° was the highest in the case with the reaction time of 15 h. The Ca/P molar ratio, median diameter, and SSA did not differ significantly with the reaction time, and thus, the sample with a reaction time of 15 h was used in the subsequent tests.

| Calcium phosphates | Theoretical Ca/P molar ratio | Analytical Ca/P molar ratio | Median size (μm) | Specific surface area (m²·g⁻¹) |
|--------------------|-----------------------------|-----------------------------|------------------|-----------------------------|
| ACP                | 1.50                        | 1.50 ± 0.01                 | 18               | 46                          |
| OCP-4 h            | 1.33                        | 1.42 ± 0.01                 | 16               | 33                          |
| OCP-6 h            | 1.33                        | 1.40 ± 0.00                 | 14               | 34                          |
| OCP-15 h           | 1.33                        | 1.42 ± 0.01                 | 20               | 33                          |
| HAp                | 1.67                        | 1.63 ± 0.01                 | 21               | 69                          |

*All values were referred from [6,8,26]; ¹ Mean ± standard deviation (n = 3)
Figure 3. Scanning electron microscopy (SEM) photographs of synthetic calcium phosphates: (a) ACP, (b) OCP, and (c) HAp. Bars indicate 10 μm.

The SEM images of the starting calcium phosphate powders (Figure 3) confirmed the morphologies of the prepared powders. Because the examination of large particles can result in only a few particles in the field of view, areas containing particles smaller than the median diameter were viewed to observe a larger number of particles. In addition, the TEM (bright field image) and selected area electron diffraction (SAED) images were acquired (Figure 4). According to the SEM observations, the prepared ACP powder was composed of granular particles (Figure 3(a)). The TEM results (Figure 4(a)) showed the aggregation of spherical particles with a diameter less than 50 nm. The SAED image indicated the halo pattern characteristic of amorphous materials. These observations were in agreement with those reported previously for ACP [30]. Although a few points were observed in the halo pattern, these were possibly derived from ACP crystallized by the electron-beam irradiation employed during acquisition of the SAED image, based on the ACP characteristics exhibited in the XRD pattern and FT-IR spectra and the electron-beam-irradiation-induced transformation of OCP to HAp [31]. Thus, the powder synthesized in this study was determined to be ACP.

In the case of OCP (Figures 3(b) and 4(b)), needlelike and plate-like crystals were observed in the SEM and TEM images. In the SAED image (Figure 4 (b1,b2)), the (002) and (260) planes of OCP were observed in the needlelike crystals, and the (002), (201), and (T42) planes of OCP were observed in the plate-like crystals. Moreover, the needlelike crystals were polycrystalline, as concentric circles were observed in Figure 4 (b1). In contrast, concentric spots were observed in the plate-like crystal. This result indicated that the polycrystalline crystal grew into a single crystal, and the plate-like crystal was a polycrystalline body similar to a single crystal.

In the case of HAP (Figures 3(c) and 4(c)), a grainy shape was observed in the SEM image. The TEM results indicated the presence of flake-like particles with a length of approximately 50–100 nm, and the SAED image highlighted the (002), (202), (211), and (222) planes of HAP. As the spots appeared in concentric circles, it was concluded that the HAP synthesized in this study had a polycrystalline body.

3.2. Characterization of protamine-adsorbed calcium phosphates

The crystalline phase of the protamine-adsorbed calcium phosphates was confirmed through the XRD and FT-IR results (Figures 5 and 6).

As shown in Figure 5(a), the XRD patterns of the ACP-Protamine exhibited diffraction peaks at 2θ = 31.9°, 32.9°, and 39.9°, similar to those of HAP (Figure 1(e)). However, the broader diffraction peaks compared to those of HAP (Figure 1(e)) suggested that ACP-Protamine was less crystalline than HAP. In the FT-IR spectra, bands of ν3 PO4 3− at approximately 1034 cm−1, ν1 PO4 3− at approximately 962 cm−1, and ν4 PO4 3− at approximately 604 and 565 cm−1 were observed, similar to those of HAP (Figure 6(a)). Moreover, a band of CO3 2− was observed at approximately 1455 cm−1. Although the mechanism of conversion from ACP and OCP to HAP is not fully understood yet, it has been reported that the crystalline phase is transformed in an aqueous solution, and this conversion is affected by the presence of coexisting substances such as proteins and minerals [30,32,33]. In particular, the mechanism by which the
conversion occurs during the protamine adsorption reaction is unknown; however, the XRD and FT-IR results suggested that ACP was converted to HAp.

As shown in Figure 5(b), the XRD pattern of OCP–Protamine exhibited diffraction peaks of OCP at $2\theta = 4.9^\circ$, 9.6$^\circ$, and 9.9$^\circ$. The FT-IR spectra, shown in Figure 6(b), indicated four P-O bands, characteristic of OCP, at approximately 1125, 1076, 1037, and 1025 cm$^{-1}$, and two sharp bands at 560–600 cm$^{-1}$ [22,26]. Thus, OCP likely maintained its crystalline structure after the protamine adsorption reaction in water.

Figure 5(c) shows the XRD pattern of HAp–Protamine. Diffraction peaks attributable to HAp could be observed at $2\theta = 31.9^\circ$, 32.9$^\circ$, and 39.9$^\circ$. In addition, the FT-IR spectra (Figure 6(c)) indicated bands of $v_3$ PO$_4^{3−}$ at approximately 1034 cm$^{-1}$, $v_1$ PO$_4^{3−}$ at approximately 962 cm$^{-1}$, and $v_4$ PO$_4^{3−}$ at approximately 604 and 565 cm$^{-1}$ [6,26]. It was considered that HAp maintained its crystalline structure after the protamine adsorption reaction in water.

The amounts of protamine adsorbed on the calcium phosphate powders are listed in Table 2. All the powders adsorbed more than 5.28 mg·g$^{-1}$ of protamine, and the powder derived from ACP corresponded to the highest amount of protamine adsorption. When the ACP was used as a starting powder, it converted to low crystalline carbonate-containing HAp during the protamine adsorption reaction, although more protamine could be adsorbed than that when HAp was used as the starting powder. Although the amount of protamine

---

**Figure 4.** Transmission electron microscopy (TEM) photographs (a, b1, b2, c) and selected area electron diffraction (SAED) patterns (a', b1', b2', c') of synthetic calcium phosphates: (a, a') ACP, (b1, b1') OCP (needlelike), (b2, b2') OCP (plate-like), and (c, c') HAp. Bars indicate 50 nm.
Adsorbed when OCP was used as the starting powder was lower than those when ACP or HAp was used, the crystal structure of OCP was maintained, which indicated that OCP could demonstrate its unique bone regeneration ability [9]. As described earlier, although multiple parameters are involved in the adsorption of protamine onto calcium phosphates, SSA was noted to be crucial for inducing the higher degree of protamine adsorption onto ACP and HAp than that onto OCP, considering the higher SSAs of ACP and HAp than that of OCP. Moreover, a characteristic feature of each starting powder was related to the protamine adsorption to a certain extent; in particular, ACP has an amorphous structure and high solubility [8,10], OCP has

**Figure 5.** XRD patterns of protamine-adsorbed calcium phosphates: (a) ACP-Protamine, (b) OCP-Protamine, and (c) HAp-Protamine.

**Figure 6.** FT-IR spectra of protamine-adsorbed calcium phosphates: (a) ACP-Protamine, (b) OCP-Protamine, and (c) HAp-Protamine.

**Table 2.** Amount of protamine adsorbed on synthetic calcium phosphates.

| Sample       | Charged solution (µg·cm⁻³) | Supernatant (µg·cm⁻³) | Adsorbed protamine (mg·g⁻¹)  |
|--------------|-----------------------------|------------------------|------------------------------|
| ACP-Protamine| 500 Trace                   |                        | 25.2 ± 0.15                  |
| OCP-Protamine| 500 382 ± 4.1               |                        | 5.28 ± 0.11                  |
| HAp-Protamine| 500 95.2 ± 6.0              |                        | 19.2 ± 0.13                  |

*Mean value ± standard deviation obtained from three independent experiments (n = 3).
needlelike and plate-like crystals and can incorporate organic compounds in the interlayer [34,35], and HAp has high crystallinity and stability [1,6]. Therefore, the adsorption of protamine onto inhouse-synthesized ACP, OCP, and HAp was confirmed in this study. Because the adsorption of protamine on commercially available DCPA and HAp has been confirmed [20,21], protamine can be adsorbed on various types of calcium phosphates.

3.3. Evaluation of antimicrobial property

Results of the examination of the antimicrobial property of various calcium phosphate powder against *S. aureus* are shown in Figure 7. The calcium phosphate powders (ACP, OCP, and HAp) did not exhibit any antibacterial property; however, the protamine-adsorbed calcium phosphate powders (ACP-Protamine, OCP-Protamine, and HAp-Protamine) exhibited antibacterial properties at concentrations of both 1 mg·cm⁻³ and 2 mg·cm⁻³. The vertical axis in Figure 7 shows the standard logarithm; a decrease in the value from 5 to 4 indicated that the bacterial count decreased from 100,000 to 10,000. These results indicated that all the protamine-adsorbed calcium phosphate powders exhibited sufficient antibacterial activity.

Figure 8 shows the concentration of protamine in the supernatant in the antibacterial property test. All the protamine-adsorbed calcium phosphate powders released protamine into the culture supernatant. The concentration of protamine in the culture supernatant was higher for ACP-Protamine (<9.7 µg·cm⁻³) and HAp-Protamine (<6.2 µg·cm⁻³) than that for OCP-Protamine (<1.3 µg·cm⁻³).

The release of protamine from protamine-adsorbed calcium phosphates is suspected to be complex and related to multiple parameters of protamine and the calcium phosphates, similar to the aforementioned protein binding property. The crystallinity of ACP-Protamine, whose ACP component was converted to HAp during protamine adsorption, was lower than that of HAp-Protamine; therefore, crystallinity was a factor responsible for the highest protamine release from...
ACP-Protamine among the investigated samples. Moreover, the incorporation of organic compounds by OCP in the interlayer contributed to protamine immobilization, leading to OCP-Protamine releasing the lowest amount of protamine among the examined specimens.

The antimicrobial property of the protamine-adsorbed calcium phosphate powders could be attributed to i) the action of protamine as a liquid factor released into the solution and ii) the interaction of the protamine adsorbed on the calcium phosphate with bacteria. Although the material form differs between bulk plate and particles, Honda et al. reported that the antimicrobial activity could be attributed to the released protamine in an experimental system based on HAp as a carrier [21]. In this study, the OCP-Protamine exhibited the same antibacterial property as those of ACP-Protamine and HAp-Protamine, even though the amount of protamine released was lower than those of the other two powders. This result could be explained by the fact that the antimicrobial property of OCP-Protamine was attributed to the protamine immobilized on the OCP particles (Route (iii)) rather than the released protamine (Route (ii)).

In general, OCP is an interlayer compound consisting of an HAp layer and a DCPD layer, and organic compounds can be incorporated in the interlayer [34,35]. The TEM results shown in Figure 4 indicated that only in the case of OCP were the two states of polycrystalline body and polycrystalline body growing into a single crystal were observed. In this scenario, the latter state has a more developed interlayer structure than the former state and is likely tightly bound by the intercalating part of the functional group of the protamine in the interlayer. Protamine is a protein with an isoelectric point of 12–13 and includes functional groups such as amino group on its surface. The TEM image shown in Figure 4 (b2) indicated that the OCPs prepared in this study had highly developed crystal planes. Such well-developed crystal planes are favorable for protein adsorption [36]. In other words, the antimicrobial property of OCP could be attributed to a different route than that of release owing to the strong immobilization of a portion of the protamine by OCP in a manner different from that of ACP and HAp.

Protamine-adsorbed calcium phosphates were successfully prepared and evaluated as biomaterials in this study. Because the prepared powders cannot be directly applied to the affected area in their original powder forms, they must be mixed with other raw materials and subsequently processed by compression or heating to be molded for application to affected areas. Protamine maintained its antimicrobial property even after these treatments (data not shown), and therefore, biomaterials molded using this powder are also expected to maintain their antimicrobial property. Although the required duration of protamine release and adsorption for clinical applications is not clear owing to insufficient data from in vivo antimicrobial studies, the antimicrobial properties of the prepared powders will be exhibited as long as protamine is present in affected area, because the antimicrobial properties could be considered as liquid and immobilized factors.

In summary, the prepared ACP-Protamine and OCP-Protamine exhibited sufficient antimicrobial properties and decreased the bacterial counts of S. aureus to more than one tenth. Although the antimicrobial properties of the prepared powders must be verified further, considering those of the protamine-adsorbed DCPA and HAp against S. mutans and Escherichia coli [20,21], the prepared powders are anticipated to exhibit antimicrobial behavior against a wide range of bacteria and act as promising antimicrobial biomaterials.

4. Conclusions

Protamine-adsorbed calcium phosphates were prepared from ACP and OCP and evaluated to develop antimicrobial biomaterials that could exhibit the biocompatibility of calcium phosphate and the antimicrobial property of protamine.

When ACP was used as the starting powder, it converted to HAp during the protamine adsorption reaction, although more protamine could be adsorbed than that when HAp was used as the starting powder. Moreover, although the amount of adsorbed protamine when OCP was used as the starting powder was lower than that when ACP or HAp was used, the crystal structure of OCP was maintained, and OCP-Protamine exhibited a similar antimicrobial property to that of ACP-Protamine and HAp-Protamine. Both ACP-Protamine and OCP-Protamine exhibited antimicrobial properties are can thus be considered promising candidates for antimicrobial biomaterials.

Disclosure statement

No potential conflict of interest was reported by the author(s).

References

[1] Jeong J, Kim JH, Shim JH, et al. Bioactive calcium phosphate materials and applications in bone regeneration. Biomater Res. 2019;23(1):1–11.
[2] Eliaz N, Metoki N. Calcium phosphate bioceramics: a review of their history, structure, properties, coating technologies and biomedical applications. Materials. 2017;10(4):334.
[3] Enax J, Eppe M. Synthetic hydroxyapatite as a biomimetic oral care agent. Oral Health Prev Dent. 2018;16(1):7–19.

[4] Boskey AL. Bone composition: relationship to bone fragility and antiosteoporotic drug effects. BoneKey Rep. 2013;2:447.

[5] Dorozhkin SV, Eppe M. Biological and medical significance of calcium phosphates. Angew Chem Int Ed. 2002;41(17):3130–3146.

[6] Koutosopoulos S. Synthesis and characterization of hydroxyapatite crystals: a review study on the analytical methods. J Biomed Mater Res. 2002;62(4):600–612.

[7] Vecstaudza J, Gasik M, Locs J. Amorphous calcium phosphate materials: formation, structure and thermal behaviour. J Eur Ceram Soc. 2019;39(4):1642–1649.

[8] Zhao J, Liu Y, Sun WB, et al. First detection, characterization, and application of amorphous calcium phosphate in dentistry. J Dent Sci. 2012;7(4):316–323.

[9] Suzuki O, Shiwaku Y, Hamai R. Octacalcium phosphate bone substitute materials: comparison between properties of biomaterials and other calcium phosphate materials. Dent Mater J. 2020;39(2):187–199.

[10] Posner AS, Betts F. Synthetic amorphous calcium phosphate and its relation to bone mineral structure. Acc Chem Res. 1975;8(8):273–281.

[11] Wekwejt J, Dzidzuszwcska M, Palubicka A. The problem of infections associated with implants – an overview. Eur J Med Technol. 2018;22(4):19–26.

[12] Francolini I, Vuotto C, Piozzi A, et al. Antifouling and antimicrobial biomaterials: an overview. Apmis. 2017;125(4):392–417.

[13] Gill TA, Singer DS, Thompson JW. Purification and analysis of protamine. Process Biochem. 2006;41(8):1875–1882.

[14] Islam NM, Itakura T, Motohiro T. Antibacterial spectra and minimum inhibition concentration of clupeine and salmine. Bull Jpn Soc Sci Fish. 1984;50(10):1705–1708.

[15] Suzuki K, Ando T. Studies on protamines: XVII. the complete amino acid sequence of clupeine YI. J Biochem. 1972;72(6):1433–1446.

[16] Miller BF, Abrams R, Dorfman A, et al. Antibacterial properties of protamine and histone. Science. 1942;96(2497):428–430.

[17] Kim YH, Kim SM, Lee SY. Antimicrobial activity of protamine against oral microorganisms. Biocontrol Sci. 2015;20(4):275–280.

[18] Zhou L, Matsumura H, Mezawa M, et al. Protamine stimulates bone sialoprotein gene expression. Gene. 2013;516(2):228–237.

[19] Marshall SH, Arenas G. Antimicrobial peptides: a natural alternative to chemical antibiotics and a potential for applied biotechnology. Electron J Biotechnol. 2003;6(3):271–284.

[20] Fujiki M, Abe K, Hayakawa T, et al. Antimicrobial activity of protamine-loaded calcium phosphates against oral bacteria. Materials. 2019;12(17):2816.

[21] Honda M, Matsumoto M, Aizawa M. Potential application of protamine for antimicrobial biomaterials in bone tissue engineering. Int J Mol Sci. 2020;21(12):4368.

[22] Shiwaku Y, Anada T, Yamazaki H, et al. Structural, morphological and surface characteristics of two types of octacalcium phosphate-derived fluoride-containing apatitic calcium phosphates. Acta Biomater. 2012;8(12):4417–4425.

[23] Cheng L, Weir MD, Hhk X, et al. Effect of amorphous calcium phosphate and silver nanocomposites on dental plaque microcosm biofilms. J Biomed Mater Res Part B. 2012;100B(5):1378–1386.

[24] Sugiuira Y, Obika H, Horie M, et al. Aesthetic silver-doped octacalcium phosphate powders exhibiting both contact antibacterial ability and low cytotoxicity. ACS Omega. 2020;5(38):24434–24444.

[25] Aizawa M. Processing of biocompatible apatite particles with well-controlled morphology and its application. Cosmetology. 2006;14:16–21. Japanese.

[26] Ban S, Hasegawa J, Maruno S. Synthesis of octacalcium phosphate and its transformation to apatite. Jsdmd. 1996;15(3):210–217.

[27] Yokota T, Nakano K, Nagaya M, et al. In vivo evaluation of porous hydroxyapatite ceramics including bone minerals using pig model. Mater Technol. 2018;33(10):689–697.

[28] Yokota T, Miki T, Honda M, et al. Fabrication and biological evaluation of hydroxyapatite ceramics including bone minerals. J Ceram Soc Jpn. 2018;126(2):99–108.

[29] Wang K, Zhou C, Hong Y, et al. A review of protein adsorption on bioceramics. Interface Focus. 2012;2(3):259–277.

[30] Pan H, Liu XY, Tang R, et al. Mystery of the transformation from amorphous calcium phosphate to hydroxyapatite. Chem Commun. 2010;46(39):7415–7417.

[31] Xin R, Leng Y, Wang N. In situ TEM examinations of octacalcium phosphate to hydroxyapatite transformation. J Cryst Growth. 2006;289(1):339–344.

[32] Kojima Y, Hayashi T, Yasue T, et al. Adsorption of bovine serum albumin on amorphous calcium phosphate in ringer solution. Phosphorus Res Bull. 1998;59:53–63.

[33] Petrakova NV, Teterina AY, Mikheeva PV, et al. In vitro study of octacalcium phosphate behavior in different model solutions. ACS Omega. 2021;6(11):7487–7498.

[34] Nomon H, Nishimura Y, Shiwaku J, et al. Characterization of layer-structured octacalcium phosphate/dwcarboxylate composite. Phosphorus Res Bull. 2005;18:127–134.

[35] Yokoi T, Goto T, Haru M, et al. Incorporation of tetra-carboxylic ions into octacalcium phosphate for the development of next-generation biofriendly materials. Commun Chem. 2021;4(1):1–9.

[36] Zhuang Z, Aizawa M. Protein adsorption on single-crystal hydroxyapatite particles with preferred orientation to α(b)- and c-axes. J Mater Sci Mater Med. 2013;24(5):1211–1216.