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Comparative analysis of bovine serum albumin adsorption onto octacalcium phosphate crystals prepared using different methods

Kaori TSUCHIYA, Ryo HAMAI, Susumu SAKAI and Osamu SUZUKI

Division of Craniofacial Function Engineering, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan

Corresponding author, Osamu SUZUKI; E-mail: suzuki-o@tohoku.ac.jp

This study compared bovine serum albumin (BSA) adsorption onto octacalcium phosphate (OCP) materials prepared from two wet preparations in the absence (w-OCP) and presence (c-OCP) of gelatin. Raman spectroscopy was used to analyze the BSA adsorption onto OCPs in a 150 mM Tris-HCl buffer containing 0.5 mM calcium and inorganic phosphate (Pi) ions at pH 7.4 and at 37°C. The degree of supersaturation of the supernatants after the adsorption was determined by measuring the ion composition. The results showed that BSA adsorption onto w-OCP was higher than that for c-OCP. The calcium ion concentration of the supernatant decreased for both w-OCP and c-OCP, whereas the Pi ion concentration increased, approaching OCP equilibria at different saturation levels. BSA adsorbed even onto c-OCP, which included a small amount of gelatin during c-OCP preparation. These results indicate that the biodegradability of w-OCP and c-OCP may be modulated through interactions with serum proteins.

Keywords: Octacalcium phosphate, Gelatin, Co-precipitation, Bovine serum albumin, Adsorption

INTRODUCTION

Octacalcium phosphate (OCP, Ca₈H₂(PO₄)₆·5H₂O) is an intermediate phase during the formation of hydroxyapatite (HA, Ca₁₀(PO₄)₆(OH)₂) from supersaturated calcium and phosphate solutions and has been considered a precursor of bone apatite. We reported that OCP is an osteoconductive and has been considered a precursor of bone apatite supersaturation (DS) in the calcifying solution. The synthesis conditions, particularly the degree of supersaturation of the supernatants after the adsorption was determined by measuring the ion composition. The extent of osteoconductivity of OCP-based materials depends on the material characteristics of OCP crystals, including their morphology, aspect ratio, and non-stoichiometric chemical composition, which affect bone formation capability and biodegradability. These material properties can be manipulated by changing the synthesis conditions, particularly the degree of supersaturation (DS) in the calcifying solution. The presence of additional components (beyond calcium and phosphate ions) in the calcifying solution, such as collagen and gelatin, also affects the properties of OCP crystals’ properties. The hypothesis that such modifications of OCP crystals affect their surface properties, which likely regulate the adsorption of serum proteins onto OCP in vitro, controlling their dissolution and biodegradability, is plausible. This hypothesis is based on our previous observations: 1) circulating serum proteins can be adsorbed onto OCP if implanted into calvaria, placed in the subcutaneous tissue in mice, or if immersed in rat serum in vitro; 2) while the dissolution and

hydrolysis of OCP into HA are enhanced in the presence of low concentrations of fluoride ions, its hydrolysis is slowed by the presence of bovine serum albumin, through its adsorption onto the OCP surface.

Previous studies showed that the presence of additional molecules in calcifying solutions does affect the morphology of calcium phosphate crystals, including OCP. Co-precipitation of OCP with collagen at room temperature produced wide, plate-like OCP crystals, whereas co-precipitation at high temperatures in the presence of alginate resulted in small, plate-like crystals. Furthermore, co-precipitation with gelatin at high temperatures yielded OCP crystals that were elongated toward the long axis. OCP co-precipitation with gelatin molecules, termed c-OCP, might have displayed highly soluble characteristics, because the composite that consisted of c-OCP and a spongy gelatin matrix material became highly biodegradable during regenerating rat calvaria bone defects. The composite was able to repair over 70% of the rat calvaria critical-size defects, accompanied by a decrease in the composite remnants. Composite materials have been shown to possess bone-healing properties and, therefore, could be used as bone substitute materials for autologous bone. Two OCP materials obtained from different supersaturated solutions had distinct crystal sizes and aspect ratios, and they exhibited distinct osteoblastic cell attachments in vitro, as well as distinct bone regeneration activity profiles upon implantation into critical-size defects in mouse calvaria. These results suggest that c-OCP may have a specific physicochemical property that underlies its unique biological response.

Another remarkable property of c-OCP is its high resorptive potential at the sites of bone formation. In fact, c-OCP tended to be replaced with newly formed
bone, owing to its biodegradation properties in rat calvaria defects\textsuperscript{15}. This degradation was more rapid than that seen for a biodegradable, highly porous β-tricalcium phosphate (β-TCP) material implanted in a rabbit tibia orthopedic long-bone defect model\textsuperscript{22}. These biodegradation behaviors are enhanced not only by dissolution arising from the thermodynamic metastability of these materials at physiological pH\textsuperscript{23-25} but also by direct resorption mediated by the activity of osteoclast-like cells at the implantation site\textsuperscript{11,18,22,26,27}.

Interactions between implanted OCP-based materials and serum proteins likely also affect the resorption of implanted materials. It has also been reported that small amounts of phosphoproteins adsorbed onto the substrate surfaces support HA crystal growth; however, this is modulated by the concentration of the surrounding solution\textsuperscript{28,29}. Moreover, some proteins may be adsorbed onto crystal growth sites for HA\textsuperscript{30} or specific planes of OCP crystals\textsuperscript{31,32}, suggesting that crystal growth sites may correspond to adsorption sites for calcium phosphate materials\textsuperscript{30-32}. Thus, protein adsorption could affect calcium phosphate dissolution\textsuperscript{16,31,32}. OCP can hydrolyze gradually into Ca-deficient HA via ion diffusion in the crystal lattice, namely, via topotaxial conversion as well as through the dissolution-precipitation process\textsuperscript{16}. OCP hydrolysis together with selective interactions between OCP and serum proteins such as serum albumin\textsuperscript{15,16} can progressively strengthen serum-crystal interactions\textsuperscript{16}. Furthermore, our previous study indicated that formation of new calcium phosphate crystals enhances serum protein adsorption onto the OCP surface \textit{in vitro}, in solutions that are supersaturated with OCP and HA\textsuperscript{33}. Based on these findings, we hypothesized that c-OCP may also interact with proteins at bone-forming sites, and such an interaction could affect its osteoconductivity. Adsorption of BSA onto OCP was previously shown to possess Langmuir-type properties\textsuperscript{16} and a high adsorption affinity\textsuperscript{16,34-36}. Here, we investigated the BSA adsorption onto c-OCP to examine how the presence of serum proteins, coupled with different synthesis conditions for OCP, affects c-OCP biodegradation, compared with OCP.

MATERIALS AND METHODS

\textit{Preparation of w-OCP and c-OCP}

OCP was synthesized using the wet synthesis method that was previously reported by Suzuki \textit{et al.}\textsuperscript{5}; the obtained material is hereafter denoted as w-OCP. Briefly, calcium and inorganic phosphate ion solutions were directly mixed under supersaturation with respect to OCP. Meanwhile, c-OCP was prepared according to the previously reported method\textsuperscript{12,18}, wherein calcium and phosphate ion solutions were mixed in the presence of 0.5% gelatin (wt:wt) to induce the co-precipitation of OCP with gelatin molecules. The w-OCP and c-OCP precipitates were separated from their reaction solutions and washed with deionized water, before drying for 24 h at 105°C. The dried precipitates were then passed through a 270 mesh testing sieve, to produce w-OCP and c-OCP granules with diameters <53 μm.

\textit{Adsorption test}

To prepare different BSA solutions saturated with respect to OCP (DS=1.0 regarding OCP phase), a 150 mM Tris-HCl buffer containing 0.5 mM Ca\textsuperscript{2+} and 0.5 mM inorganic phosphate (Pi) and BSA in the 0–0.75 mg/mL range were prepared by adding CaCl\textsubscript{2}·2H\textsubscript{2}O, KH\textsubscript{2}PO\textsubscript{4}, tris(hydroxymethyl)aminomethane (FUJIFILM Wako Pure Chemical, Osaka, Japan), and bovine serum albumin (BSA; molecular weight: 66 kDa, Sigma-Aldrich, St. Louis, MO, USA). The solution pH was adjusted to 7.4 with 1 M HCl at 37°C.

Granules of w-OCP and c-OCP (15 mg) in 3 mL-volume BSA solutions were incubated in 5.0 mL-volume Eppendorf tubes for 1 h at 37°C with rotation. The solutions were then centrifuged for 2 min at 3,900 rpm to obtain supernatants, and the BSA concentration was determined using the Bradford method with a CBB protein assay solution (Nacalai Tesque, Kyoto, Japan).

\textit{Characterization of w-OCP and c-OCP before and after incubation in the BSA solution}

The specific surface areas (SSAs) of the obtained w-OCP and c-OCP granules were measured using a Brunauer-Emmett-Teller (BET) surface area analyzer (Bel Sorb, Osaka, Japan). The Ca/P molar ratio for w-OCP and c-OCP, the Ca and P content in the 0.1 M HCl solution after dissolving the granules was measured using Calcium E and Phosphor C tests (FUJIFILM Wako Pure Chemical). Carbon, hydrogen, and nitrogen content for w-OCP and c-OCP were measured using a carbon hydrogen and nitrogen (CHN) elemental analyzer (vario EL cube, Elementar Analysensysteme, Langenselbold, Germany).

The zeta potentials of w-OCP, c-OCP, BSA, and gelatin were measured in a solution without BSA, using a zeta potential analyzer (ELS-Z2 MH, Otsuka Electronics, Osaka, Japan). The w-OCP and c-OCP solutions were sonicated prior to the measurements.

The w-OCP and c-OCP granules, separated from the BSA solutions, were washed with deionized water and subsequently lyophilized for 48 h in a vacuum freeze dryer (FDU-1200, Tokyo Rikakikai, Tokyo Japan). The w-OCP and c-OCP samples, before and after the incubations, were analyzed using power X-ray diffraction (XRD; MiniFlex 600, Rigaku Electrical, Tokyo, Japan). The XRD analysis was performed using Cu-Kα radiation at 40 kV and 15 mA with a scanning rate of 3°/s and 0.02° intervals from 2θ = 3° to 60°. The w-OCP and c-OCP crystal morphologies before and after the immersion into the 0.5 mg/mL BSA solution or for the control solution (without BSA) were observed using a field emission-type transmission electron microscope (FE-TEM; JEM-2100F, JEOL, Tokyo, Japan).

Granules of w-OCP and c-OCP before and after incubation were characterized using Raman spectroscopy (Model ApaRAMAN, LUCIR, Tsukuba, Japan); the characterization was performed for the 300–1,900 cm\textsuperscript{-1}}.
Table 1 Chemical composition, SSA, and elemental (carbon, hydrogen, and nitrogen) content for w-OCP and c-OCP

|        | Ca/P molar ratio | SSA (m²/g) | C (wt%) a) | H (wt%) a) | N (wt%) a) |
|--------|------------------|------------|------------|------------|------------|
| w-OCP | 1.29             | 24         | 0.28±0.09  | 1.25±0.01  | N.D.       |
| c-OCP | 1.24             | 25         | 2.64±0.16  | 1.56±0.03  | 0.76±0.05  |

a) Results are mean±standard deviation, over three replicates.

RESULTS

The Ca/P molar ratio for both w-OCP and c-OCP was lower than that for the stoichiometric composition of OCP (Table 1). The SSAs of w-OCP and c-OCP were 24 and 25 m²/g, respectively (Table 1). The carbon, hydrogen, and nitrogen contents in w-OCP and c-OCP were also measured using a CHN elemental analyzer before the incubation with BSA (Table 1). The carbon content for c-OCP was higher than that for w-OCP. Meanwhile, c-OCP contained 0.76±0.05 wt% nitrogen most probably derived from gelatin, whereas nitrogen was not detected in w-OCP.

In the XRD analysis, characteristic diffraction peaks corresponding to (100), (010), and (700) of the OCP crystal
structure were observed at 2θ=4.7°, 9.8°, and 33.6°, respectively, for w-OCP and c-OCP before and after the incubation with BSA (Fig. 1). The intensity of the (100) and (700) peaks for w-OCP before the incubation was higher than that for c-OCP. Upon the incubation with BSA, the intensity of these OCP-derived peaks decreased independently of the BSA concentration, whereas the peak intensity for c-OCP tended to be stable, even after the BSA incubation. No diffraction peaks, other than those for the OCP phase, were detected after the incubation of w-OCP and c-OCP with BSA.

TEM bright-field images of w-OCP and c-OCP before the incubation showed that both w-OCP and c-OCP crystals had plate-like structures (Fig. 2). The aspect ratio of the c-OCP crystals appeared to be higher compared with w-OCP. The plate-like structure of both w-OCP and c-OCP crystals was maintained, both after immersion into the control solution (without BSA) and after immersion into a 0.5 mg/mL BSA solution.

The amount of BSA adsorbed onto w-OCP and c-OCP rapidly increased with increasing BSA concentration up to 0.02 and 0.04 mg/mL, respectively (Fig. 3). The BSA concentration range for which this rapid increase was observed was lower for the w-OCP isotherm than for the c-OCP isotherm. Subsequently, the amount adsorbed onto adsorbents became saturated for both w-OCP and c-OCP, with w-OCP having a higher amount of adsorbed BSA than did c-OCP.

The calcium and Pi ion concentrations in the BSA solutions after the incubation with w-OCP and c-OCP were measured (Table 2). The Ca²⁺ concentration decreased, whereas the Pi ion concentration increased after the incubation with w-OCP and c-OCP. The Pi ion concentration in the solution incubated with c-OCP tended to be higher than that for w-OCP, regardless of the BSA concentration. The ion concentrations in the solution after the incubations were used to calculate the DS with respect to HA, OCP, and DCPD. All DS values were <1.0 with respect to DCPD in all solutions before and after the incubation with both adsorbents, indicating that the solutions were under the under-saturated condition with respect to DCPD. The DS values were 10⁻¹–10⁰ with respect to OCP after the incubation with c-OCP in the BSA solutions.
Table 2  Solution composition and DS after BSA adsorption onto w-OCP and c-OCP at pH 7.4, 37ºC, in a 150 mM Tris-HCl buffer for 1 h

| Material | BSA$^\circ$ mg/mL | Supernatant Ca$^\circ$ mM | Pi$^\circ$ mM | HA | OCP | DCPD |
|----------|-------------------|-------------------------|-------------|----|-----|------|
| Control solution | 0 | 0.50$^b$ | 0.50$^b$ | 9.97×10$^7$ | 1.09×10$^0$ | 8.29×10$^{-2}$ |
| 0 | 0.32 | 0.84 | 4.90×10$^7$ | 8.43×10$^{-1}$ | 8.87×10$^{-2}$ |
| 0.075 | 0.34 | 0.71 | 3.97×10$^7$ | 6.46×10$^{-1}$ | 7.97×10$^{-2}$ |
| 0.15 | 0.39 | 0.73 | 8.39×10$^7$ | 1.19×10$^{-1}$ | 9.34×10$^{-2}$ |
| 0.2 | 0.35 | 0.70 | 4.58×10$^7$ | 7.14×10$^{-1}$ | 8.13×10$^{-2}$ |
| 0.25 | 0.45 | 0.76 | 1.91×10$^8$ | 2.36×10$^{-1}$ | 1.12×10$^{-1}$ |
| 0.5 | 0.50 | 0.82 | 4.24×10$^8$ | 4.65×10$^{-1}$ | 1.35×10$^{-1}$ |
| 0.75 | 0.45 | 0.79 | 2.33×10$^8$ | 2.82×10$^{-1}$ | 1.18×10$^{-1}$ |
| w-OCP | 0 | 0.25 | 0.91 | 1.81×10$^7$ | 3.99×10$^{-1}$ | 7.51×10$^{-2}$ |
| 0.075 | 0.29 | 0.86 | 2.99×10$^7$ | 5.77×10$^{-1}$ | 8.12×10$^{-2}$ |
| 0.15 | 0.34 | 0.89 | 8.37×10$^7$ | 1.34×10$^{-1}$ | 1.01×10$^{-1}$ |
| 0.2 | 0.31 | 0.89 | 4.70×10$^7$ | 8.44×10$^{-1}$ | 9.01×10$^{-2}$ |
| 0.25 | 0.35 | 0.92 | 1.07×10$^8$ | 1.67×10$^{-1}$ | 1.08×10$^{-1}$ |
| 0.5 | 0.36 | 0.95 | 1.27×10$^8$ | 1.94×10$^{-1}$ | 1.22×10$^{-1}$ |
| 0.75 | 0.35 | 0.94 | 1.02×10$^8$ | 1.62×10$^{-1}$ | 1.07×10$^{-1}$ |
| c-OCP | 0 | 0.075 (mg/mL) | | | | |
| 0.075 | 0.15 (mg/mL) | | | | | |
| 0.25 | 0.25 (mg/mL) | | | | | |
| 0.5 | 0.25 (mg/mL) | | | | | |
| 0.75 | 0.25 (mg/mL) | | | | | |

$^a$ Objective adsorbate concentration.
$^b$ Objective calcium and phosphate ion concentrations.
$^c$ Averages over three replicates.

Fig. 4 Raman spectra of w-OCP before (a) and after the incubation in a 150 mM Tris-HCl buffer containing 0.075 (b), 0.15 (c), 0.20 (d), 0.25 (e), 0.50 (f), and 0.75 mg/mL (g) BSA with 0.5 mM Ca$^{2+}$ and 0.5 mM Pi ions.

Fig. 5 Raman spectra of c-OCP (a) before and (b) after the incubation in a 150 mM Tris-HCl buffer containing 0.075, (c) 0.15, (d) 0.20, (e) 0.25, (f) 0.50, and (g) 0.75 mg/mL BSA with 0.5 mM Ca$^{2+}$ and 0.5 mM Pi ions.

With both adsorbents, indicating saturation or slight under saturation with respect to OCP. The solutions tended to be closer to those of the saturated condition with respect to OCP after the incubation with c-OCP, compared with w-OCP. Meanwhile, the BSA solution after the incubation with both adsorbents maintained the supersaturated condition with respect to HA, based on the DS values >10$^7$ after the incubations.

To analyze the chemical structure of the adsorbents, w-OCP (Fig. 4) and c-OCP (Fig. 5) were analyzed using Raman spectrometry. Typical peaks assigned to $\nu_1$ PO$_4$, $\nu_1$ HPO$_4$ in the OCP structure were observed at 957 and 1,013 cm$^{-1}$, respectively, in the spectra of w-OCP and c-OCP, before and after the BSA incubation. After the incubation in solutions containing more than 0.20 mg/mL BSA, the peaks attributed to $\delta$(C-C) and amide I were observed at 1,447 and 1,656 cm$^{-1}$, respectively, in the spectra of w-OCP. Meanwhile, $\delta$(C-C) and amide I peaks were detected in the c-OCP spectra both before and after the incubations.
To analyze the amount of BSA adsorbed onto these adsorbents, curve fitting of the Raman spectra of w-OCP and c-OCP was performed before and after the BSA incubation. The separated peaks in the reference spectra of gelatin and BSA are shown in Figs. 6(a) and (b), respectively. In both reference spectra, the peaks attributed to amide III of β-sheet/random coils, α-helix, α-fibrous/γ(CH₃), and α-helix were detected at 1,230⁴⁴, 1,264⁴⁴, 1,312⁴⁵,⁴⁶, and 1,340 cm⁻¹⁴⁴, respectively. In addition, the peaks assigned to δ(C-C), amide II, and amino acids (tyrosine and phenylalanine) were observed at 1,450⁴⁴-⁴⁷ and 1,605 cm⁻¹⁴⁵,⁴⁶, respectively. In the region of amide I, the peaks attributed to α-helix and random coils were observed at 1,650 and 1,677 cm⁻¹⁴⁵,⁴⁶, respectively. These separated peaks were also present in the spectra of c-OCP before the incubation and for all adsorbents after the incubation in the 0.75 mg/mL BSA solutions, although the peaks were broader in the OCP.

![Raman spectra curve fitting](image)

**Fig. 6** Raman spectra curve fitting for (a) gelatin, (b) BSA, c-OCP (c) before and (d) after the incubation in a 150 mM Tris-HCl buffer containing 0.75 mg/mL BSA, and w-OCP (e) before and (f) after the incubation in the Tris-HCl buffer.

| Material                  | Peak height ratio  |
|---------------------------|--------------------|
|                           | 1,264 cm⁻¹/1,312 cm⁻¹ | 1,605 cm⁻¹/1,650 cm⁻¹ |
| Gelatin                   | 1.86               | 0.36               |
| BSA                       | 0.86               | 0.72               |
| c-OCP (Cont.)             | 1.42               | 0.51               |
| c-OCP (BSA 0.75)          | 0.94               | 0.74               |
| w-OCP (BSA 0.75)          | 0.77               | 0.90               |

**Table 3** Ratio of peak heights for 1,264 and 1,312 cm⁻¹ and for 1,605 and 1,650 cm⁻¹, estimated by curve fitting from the Raman spectra of gelatin, BSA, c-OCP, and w-OCP, before and after the incubation in a 150 mM Tris-HCl buffer containing 0.75 mg/mL BSA, 0.5 mM Ca²⁺, and 0.5 mM Pi ions.

| Material                  | Zeta potential (mV) ± standard deviation |
|---------------------------|----------------------------------------|
| w-OCP                     | -7.06±1.63                             |
| c-OCP                     | -1.59±0.45                             |
| Gelatin                   | 0.43±1.08                              |
| BSA                       | -10.44±0.85                            |

**Table 4** Zeta potentials of gelatin, BSA, w-OCP, and c-OCP in a 150 mM Tris-HCl buffer containing 0.5 mM Ca²⁺ ions and Pi ions at pH 7.4.

*Results are mean±standard deviation, over three replicates.
s spectra (Figs. 6(c–f)).

The peak height ratio was estimated from the curve fitting of the spectra of gelatin, BSA, c-OCP, and w-OCP (Table 3). The ratio of the peak height for the 1,264 to 1,312 cm$^{-1}$ peaks was higher for gelatin than for BSA. In contrast, the ratio for the 1,605 to 1,650 cm$^{-1}$ peaks was lower for gelatin than for BSA. Although the ratio of the peak heights for 1,605 and 1,650 cm$^{-1}$ slightly increased from 0.51 to 0.74, that for 1,264 to 1,312 cm$^{-1}$ decreased from 1.42 to 0.94 for c-OCP after the incubation in the 0.75 mg/mL BSA solution. The ratio for 1,264 to 1,312 cm$^{-1}$ and for 1,605 to 1,650 cm$^{-1}$ for w-OCP after the incubation with BSA was similar to the ratio observed for BSA.

Zeta potentials of w-OCP, c-OCP, and BSA, measured for the solutions containing 0.5 mM Ca$^{2+}$ and Pi ions, were negative (Table 4). In contrast, the potential of gelatin was slightly positive. The w-OCP zeta potential was lower than that for c-OCP in the presence of 0.5 mM Ca$^{2+}$ and Pi ions.

**DISCUSSION**

The present study assumed that the saturation can be attained sufficiently upon the introduction of OCP, with respect to both Ca$^{2+}$ and Pi ion concentrations and BSA adsorption$^{[16,48]}$. This saturation level of Ca$^{2+}$ and Pi ion concentrations has been used for expressing the solubility$^{[23]}$ and the tendency to dissolve of calcium phosphate materials, including OCP, under physiological pH and temperature conditions$^{[23,41]}$. The results obtained in the present study provided evidence that the synthesized OCP obtained in the presence of gelatin (c-OCP) had similar solubility as OCP obtained in the absence of such a protein (w-OCP), when the equilibrium was estimated under physiological conditions upon the BSA adsorption. However, the solubility equilibrium with respect to OCP in the case of c-OCP seems to be more consistent with the reported solubility of OCP$^{[41]}$ compared with w-OCP (obtained in the absence of gelatin), suggesting that the dissolutions for c-OCP and w-OCP vary to some extent with BSA adsorption. The present study, however, showed that the BSA adsorption can be estimated for c-OCP and w-OCP without changing their crystalline phases based on the XRD analytical results (Fig. 1).

Chemical analysis showed that both OCPs had similar chemical compositions and that calcium deficiency was associated with lower Ca/P molar ratios (Table 1). This non-stoichiometric tendency of the OCP composition was previously reported for synthetic w-OCP$^{[16,49]}$ and c-OCP synthesized in the presence of gelatin$^{[23]}$. The non-stoichiometric OCP was shown to have excess hydrogen on the surface$^{[16,50]}$, and the presence of a labile form of the non-stoichiometric surface acid phosphate (HPO$_4^{2-}$) in OCP was shown$^{[49]}$. The present adsorption analysis by BSA could consequently be compatible between c-OCP and w-OCP as adsorbents.

Our results indicate that BSA can be adsorbed onto both c-OCP and w-OCP, which is consistent with previous studies of BSA adsorption onto various calcium phosphate crystals, including OCP$^{[16,34-36,51-53]}$, and was characterized by an initial increase in the adsorption with the equilibrium concentration with BSA, followed by the adsorption saturation. The amount of BSA adsorbed onto both w-OCP and c-OCP surfaces first increased sharply with increasing BSA concentration and, thereafter, remained constant (Fig. 3). Interestingly, the amount of BSA adsorbed onto OCP was less than that adsorbed onto w-OCP, for all the examined BSA concentrations. The adsorbate solutions used here included 0.5 mM Ca$^{2+}$ and Pi ions, which were estimated as equilibria concentrations with respect to OCP$^{[16]}$. The dissolution of OCP and re-precipitation of calcium phosphate crystals appeared to be minimized, as confirmed by the stable OCP phases at all BSA concentrations, for both c-OCP and w-OCP (Fig. 1). The morphology of w-OCP and c-OCP crystals before and after the BSA incubation was preserved, and no de novo depositions on the original crystals were observed (Fig. 2). This finding also suggests that w-OCP and c-OCP dissolution could be suppressed in BSA solutions. Therefore, differences in the BSA adsorption strength could arise from the distinct surface properties of c-OCP and w-OCP, although serum albumin has been reported to be a strong inhibitor$^{[54]}$ or an accelerator$^{[33,35,41,55]}$ of calcium phosphate crystal growth, in vitro and in vivo.

The CHN elemental analysis demonstrated that small amounts of both nitrogen and carbon derived from gelatin were present in c-OCP before the BSA incubation, although nitrogen was not detected in w-OCP (Table 1). As expected from the results of the elemental analysis and previous studies of c-OCP$^{[32]}$, small numbers of gelatin molecules could remain in c-OCP crystals, even after thorough washing. Analysis of the Raman spectra after the BSA adsorption onto c-OCP and w-OCP supported this possibility (Figs. 4–6). The peak intensity ratios for 1,264$^{[44]}$ and 1,312 cm$^{-1}$$^{[45]}$ (α-helix and α-fibrous/α(CH$_3$) or for 1,605 and 1,650 cm$^{-1}$ (amino acid and α-helix)$^{[49]}$ (Table 3) indicated that BSA may have actually adsorbed onto both c-OCP and w-OCP. Although the BSA adsorption sites on c-OCP and w-OCP surfaces are unclear, the mechanism may involve some types of adsorption$^{[36-38]}$. In the present study, sites on the c-OCP crystals might have been occupied by gelatin molecules after the c-OCP preparation and, thus, could become unavailable for BSA adsorption. This decrease in the number of available binding sites may explain the reduced adsorption of BSA to c-OCP, compared with that for w-OCP. This theory has been previously used to explain the competitive adsorption of more than two adsorbate molecules$^{[58,59]}$.

To estimate the surface potentials of c-OCP and w-OCP, the zeta potentials of the two OCPs were analyzed (Table 4). In the analysis of the c-OCP and w-OCP zeta potentials, the negative potential decreased and approached zero in solutions with BSA only, w-OCP, and c-OCP, respectively. In the case of c-OCP, there was a negative potential, but the value was the closest to zero and may be associated with the presence of the hydrated
gelatin layer on c-OCP (Table 4)\(^{12}\). In terms of the zeta potential, one explanation for the difference between the BSA adsorptions for c-OCP and w-OCP could be the different surface potentials, wherein negatively charged BSA molecules are repelled electrostatically; hence, the protein cannot be adsorbed onto negatively charged surfaces (Fig. 3). Previous adsorption studies on calcium phosphate materials using basic, neutral, and acidic surfaces (Fig. 3) have shown that phosphate materials using basic, neutral, and acidic surfaces (Fig. 3) are adsorbed onto BSA molecules are repelled electrostatically; hence, the different surface potentials, wherein negatively charged protein cannot be adsorbed onto negatively charged surfaces (Fig. 3). Previous adsorption studies on calcium phosphate materials using basic, neutral, and acidic surfaces (Fig. 3) have shown that phosphate materials using basic, neutral, and acidic surfaces (Fig. 3) are adsorbed onto BSA molecules are repelled electrostatically; hence, the different surface potentials, wherein negatively charged protein cannot be adsorbed onto negatively charged surfaces (Fig. 3). Previous adsorption studies on calcium phosphate materials using basic, neutral, and acidic surfaces (Fig. 3) have shown that different surface charge of adsorbent proteins are the driving force behind the different adsorption strengths observed under the physiological condition\(^{34,60}\).

Results of this study regarding BSA as an OCP adsorbate provide new insights into interactions between serum proteins and OCP materials implanted in bone defects. In addition to the previous findings from the biochemical analyses of OCP\(^{14,15}\), the determination of whether serum proteins exhibit strong or weak adsorption onto c-OCP relative to w-OCP surfaces is of interest for maximizing bone repair properties. Indeed, the osteoconductivity and biodegradability of c-OCP together with gelatin in spongy matrix materials can allow the modification of OCP crystal characteristics\(^{18,22}\). The ability to reduce the adsorption of serum proteins, demonstrated in this study, could be a factor for improving the performance of c-OCP and tailoring the bioactivity of OCP. Further analysis of the interactions between OCP-based materials and serum proteins that occur during bone formation will better define the bioactive properties of these materials in the context of the effects of chemical variations in materials used to promote bone regeneration\(^7\).

CONCLUSION

The present study showed that c-OCP and w-OCP have different capacities for BSA adsorption, which affects their biodegradability. Further studies are underway, aiming to establish the linkage between the biodegradability and osteoconductivity of w-OCP and c-OCP.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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