Protein adsorption on needle-shaped hydroxyapatite prepared by hydrothermal treatment of mixture composed of CaHPO$_4 \cdot 2$H$_2$O and $\beta$-Ca$_3$(PO$_4$)$_2$

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The behavior of protein adsorption on needle-shaped hydroxyapatite (HAp) crystals was investigated and compared with that on irregularly shaped HAp crystals in phosphate buffer solutions. The proteins used were bovine serum albumin (BSA) and lysozyme chloride (LSZ). The effect of phosphate ions in the buffered solutions was evaluated at concentrations in the range 2–200 mol·m$^{-3}$. In 50 mol·m$^{-3}$ or lower buffer concentrations, the saturated amount of BSA adsorbed on needle-shaped HAp was greater than that on irregularly shaped HAp. The saturated amount of BSA adsorbed on needle-shaped HAp decreased with increasing phosphate buffer concentration. This resulted in little difference in BSA adsorption between the needle-shaped HAp and the irregularly shaped HAp at phosphate concentrations of 100 mol·m$^{-3}$ or higher. In contrast, the saturated amount of adsorbed LSZ increased with increasing phosphate buffer concentrations up to 10 mol·m$^{-3}$ and subsequently decreased with increasing phosphate concentrations between 10 and 200 mol·m$^{-3}$. There were no differences in the protein adsorption behaviors between the needle-shaped HAp and irregularly shaped HAp at a phosphate concentration of 200 mol·m$^{-3}$. A specific adsorption, useful for the separation of BSA and LSZ proteins was observed for the needle-shaped HAp at a low phosphate buffer concentration. This separation was caused by the large a-face surface area of the needle-shaped HAp by comparison with that of the irregularly shaped HAp.

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1. Introduction

When implanted in the body, hydroxyapatite (HAp; Ca$_{10}$(PO$_4$)$_6$(OH)$_2$) is known to have a high biological affinity, for example, good osteoconductivity.\(^1\),\(^2\) Therefore, it is widely used as an artificial substitute in the repair of bone tissues. Because of its protein adsorption characteristics, another popular application is in column packing for liquid chromatography.\(^3\),\(^4\)\(^)\) Previous reports concluded that the compositions and crystal structures significantly affect the adsorption of acidic and basic proteins such as bovine serum albumin (BSA) and lysozyme (LSZ), respectively. Zhu et al. reported differing behaviors of BSA and LSZ amongst powders of HAp, $\beta$-tricalcium phosphate ($\beta$-TCP; Ca$_3$(PO$_4$)$_2$) and biphasic composites of HAp and $\beta$-TCP.\(^5\) This phenomenon was ascribed to the electrostatic interaction between the proteins and the surfaces of the particles but the morphological effects of the particles were not described. Protein adsorption on HAp crystals is governed by the physicochemical properties of the crystals and is influenced by the size, morphology and pore structure of the HAp phase. HAp typically has two crystal faces: the a-face and the c-face. The a-face has positively charged sites, composed of two screw-axis calcium (Ca$^{2+}$) ions, while the c-face has negatively charged sites, connected to the oxygen ions belonging to the three phosphate groups.\(^6\),\(^7\) The unique characteristics of the different crystal faces enable the separation of proteins. Because of the dependence of these characteristics on the surfaces, it is worth investigating the synthesis procedures required to obtain needle-shaped crystals possessing high aspect ratios. Takagi et al. described the adsorption of BSA and LSZ on needle-shaped HAp, sintered at temperatures of 600, 1000 and 1200°C.\(^8\) In their reports, the adsorption behaviors of BSA and LSZ were shown to be affected by the sintering temperatures but details on morphological effects were not discussed in detail.

We recently developed micrometer-sized crystals of HAp with high aspect ratios, through hydrothermal processing of a mixture composed of dicalcium phosphate dihydrate (DCPD; CaHPO$_4 \cdot$2H$_2$O) and $\beta$-tricalcium phosphate ($\beta$-TCP; $\beta$-Ca$_3$(PO$_4$)$_2$).\(^9\) Because of its unique morphology, the protein adsorption behavior on needle-shaped HAp is expected to differ from that on irregularly shaped crystals so comparison between these two behaviors may clarify the effects of HAp morphology on protein adsorption. The purpose of this paper therefore, is to investigate the behavior of protein adsorption on needle-shaped HAp in comparison with protein adsorption on irregularly shaped HAp. Two types of proteins were used in this study: bovine serum albumin, as an acidic protein, and lysozyme, as a basic protein. The adsorption behavior of each protein on HAp crystals was evaluated by exposure of sample HAp powders to...
phosphate buffer solutions containing the proteins. The potential for separation of the proteins from solutions containing BSA and LSZ was also examined.

2. Experimental procedures

2.1 Materials

Needle-shaped crystals were prepared by hydrothermal processing, according to a previously reported method.\textsuperscript{9)} Pellets of the initial powders were prepared by mixing the reactants DCPD (Wako Pure Chemical Industries, Ltd.) and \(\beta\)-TCP (Taihei Chemical Industries Co., Ltd.) in a mass ratio of \(\beta\)-TCP/DCPD = 1/9. The mixed powder (0.2 g) was compacted by a uniaxial press (Shimadzu Co.) into cylindrical shapes under a pressure of \(1 \times 10^8\) Pa. The powdered pellets were hydrothermally treated in a designed vessel (Shikoku Rika Co., Japan). Specimens of the compacted powder were placed in an autoclave (100 cm\(^3\)) with 20 cm\(^3\) of an NH\(_4\)Cl/NH\(_3\) buffer solution with an NH\(_4\)Cl concentration of 2 mol dm\(^{-3}\) at pH 7.3. The solutions were hydrothermally exposed to the vapor phase at 160°C for 0.5 h, followed by immersion in the liquid phase at the same temperature. The solutions in the vessels were renewed after the samples had been immersed for 12 h and the samples were then soaked for a further 12 h. After liquid conditions in the vessel had been attained, the vessel was cooled to room temperature and the specimens were taken out. The recovered specimens were rinsed with ultrapure water and dried in air at 100°C. The resultant HAp had needle-like crystals, approximately 60 \(\mu\)m long and 1 \(\mu\)m thick, and had a molar ratio of Ca/P = 1.65, as determined by the method of Ioku et al.\textsuperscript{10)} The resultant powder is abbreviated N–HAp. On the other hand, HAp commercial powder, with a Ca/P molar ratio of 1.67, was supplied by Ube Material Industries, Ltd. This powder was heated in a muffle furnace (FO200, Yamato Scientific Co., Ltd.) at 900°C for 2 h in an ambient atmosphere. The heat-treated HAp powder is abbreviated I–HAp.

Samples of the specific surface areas of N–HAp and I–HAp were determined using the BET method with an instrument (NOVA 1000e, Yuasa Ionics Co., Ltd.). The specimens were then dried under vacuum conditions at 180°C for 2 h.

2.2 Protein adsorption

Phosphate buffers were prepared by dissolving an appropriate amount of sodium dihydrogen phosphate (NaH\(_2\)PO\(_4\), Nacalai Tesque Inc.) and disodium hydrogen phosphate (Na\(_2\)HPO\(_4\), Nacalai Tesque Inc.) in deionized water, resulting in 200 mol m\(^{-3}\) solutions at pH 7.4 at 37°C. The phosphate solutions were then diluted into a series of solutions with phosphate concentrations ranging from 2 to 100 mol m\(^{-3}\). BSA and LSZ were purchased from Wako Pure Chemical Industries, Ltd.). Each protein was dissolved in the series of phosphate buffers to give protein solutions with a concentration of 0.8 g dm\(^{-3}\). To estimate the selectivity of protein adsorption, buffer solutions containing both proteins were used at phosphate concentrations of 2 or 50 mol m\(^{-3}\).

Either N–HAp or I–HAp was soaked in the buffered protein solutions at a powder/solution ratio of 0.1 g cm\(^{-3}\). The solution was then kept in an incubator (FastGene\textsuperscript{TM}, Nippon Genetics Co., Ltd.) at 37°C. Because preliminary evaluations showed that protein adsorption reached a constant level after the samples were exposed for approximately 48 h, the HAp powders were maintained for 48 h in solutions containing a single protein, and for 7 d in solutions containing two proteins. After these periods, the supernatant fluid was collected from each solution using a centrifugal separator.

For the solutions containing a single protein, the protein concentration in the supernatant was determined by the Bradford assay, using a Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). The absorbance at 595 nm was measured against standard curves for the respective proteins, using an ultraviolet-visible (UV-vis) spectrophotometer (U–3410, Hitachi Ltd.). Mean values of the protein concentrations were determined from triplicate measurements for each solution. The accuracy of determination was estimated to be approximately \(\pm 5\%\) of the protein concentration and adsorptions of less than 0.05 mg m\(^{-2}\) were regarded as negligible (zero) values.

For the solutions containing two proteins, the presence of the proteins in the supernatant fluid was detected using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After soaking the N–HAp or I–HAp powders, the supernatant solution was obtained using a centrifugal separator. The protein solution (5 mm\(^3\)) was mixed with 5 mm\(^3\) of Laemmli sample buffer (62.5 mol m\(^{-3}\) Tris–HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, Bio-Rad Laboratories) containing 2-mercaptoethanol at 5 vol%, followed by heat treatment at 95°C for 5 min. The solution was then loaded onto a poly(acrylamide) gel (Bio-Rad Laboratories) for evaluation by electrophoresis at 200 V, using a running buffer (25 mol m\(^{-3}\) Tris, 192 mol m\(^{-3}\) glycine, 0.1% SDS, pH 8.3, Bio-Rad Laboratories) for 35 min. After electrophoresis, the gels were rinsed with ultrapure water, and stained with Coomassie brilliant blue G250 (BioSafe CBB G–250, Bio-Rad Laboratories) for 1 h, and subsequently rinsed with ultrapure water. The bands were evaluated against Bio-Rad’s protein prestained standards.

3. Results

Table 1 shows the characteristics of the N–HAp and I–HAp specimens examined. Figure 1 shows scanning electron micro-

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<th>Notation</th>
<th>Morphology</th>
<th>Ca/P atomic ratio</th>
<th>Specific surface area</th>
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<tbody>
<tr>
<td>N-Hap</td>
<td>Needle-shaped crystals</td>
<td>1.65</td>
<td>11 m(^2)g(^{-1})</td>
</tr>
<tr>
<td>I-Hap</td>
<td>Irregular-shaped crystals</td>
<td>1.67</td>
<td>19 m(^2)g(^{-1})</td>
</tr>
</tbody>
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Fig. 1. SEM images and powder X-ray diffraction patterns of N–HAp and I–HAp samples. All peaks on both diffraction patterns were assigned to hydroxyapatite, according to PDF#9–432.
scopic (SEM) images and powder X-ray diffraction (XRD) patterns. Needle-shaped crystals were observed for N–HAp, while irregularly shaped crystals were observed for I–HAp. The peaks on both diffraction patterns were assigned to HAp, according to PDF#9–432, and they had closely similar Ca/P molar ratios. The peak assignments are also given in Fig. 1. The relative intensity of peak 030 against peak 002 on the XRD patterns for N–HAp was larger than that for I–HAp. This means that N–HAp was elongated in the c-axis direction and had a larger area on the a-face than on the c-face. The specific surface area of N–HAp was smaller than that of I–HAp. A significant difference between the two was identified by the morphology of their crystals in the pellets.

Figure 2 shows changes in the adsorbed amounts of the protein on both N–HAp and I–HAp as a function of phosphate concentrations, at 48 h after exposure to the fluids containing BSA or LSZ. The adsorbed amounts were normalized by the surface area of each powder sample. (a) BSA and (b) LSZ. * indicates that adsorption was negligible, estimated as “0”.

Fig. 2. Changes in the amounts of proteins adsorbed on I–HAp after exposure to the fluid for 48 h, as a function of phosphate concentration. The adsorption amounts were normalized by the surface area of each powder sample. (a) BSA and (b) LSZ. * indicates that adsorption was negligible, estimated as “0”.

mg·m⁻² at a phosphate concentration of 2 mol·m⁻³ and when the phosphate concentration was increased to 10 mol·m⁻³, the adsorbed amount increased to 0.45 mg·m⁻². LSZ adsorption then decreased with increasing phosphate concentrations above 10 mol·m⁻³. In the case of I–HAp, the adsorbed amount of LSZ was about 0.16 mg·m⁻² up to a phosphate concentration of 20 mol·m⁻³ and it then decreased when the phosphate concentration was increased further. For I–HAp, LSZ adsorption was very low at a phosphate concentration of 100 mol·m⁻³ and almost no LSZ adsorption was observed at a phosphate concentration of 200 mol·m⁻³. At a phosphate concentration of 200 mol·m⁻³, the amount of adsorbed LSZ on N–HAp was almost same as that at 2 mol·m⁻³.

The adsorptions of BSA and LSZ on N–HAp in the buffer solutions containing both proteins were then compared with their adsorptions on I–HAp at phosphate concentrations of 2 and 50 mol·m⁻³. Figure 3 shows the results of SDS-PAGE images for the solutions containing both proteins before and after exposure to N–HAp or I–HAp for 7 d. After contact with N–HAp at a phosphate buffer concentration of 2 mol·m⁻³, the BSA band disappeared but the LSZ band was present. In contrast, both the BSA and the LSZ bands were virtually unchanged for the protein solution in the absence of N–HAp. A decreased intensity in both protein bands was observed after exposure to I–HAp at a phosphate buffer concentration of 2 mol·m⁻³, while no significant changes occurred at a phosphate concentration of 50 mol·m⁻³. These results indicate that N–HAp adsorbed BSA but not LSZ at phosphate buffer concentrations of 2 mol·m⁻³, while I–HAp adsorbed both the BSA and LSZ. At the higher phosphate concentration (50 mol·m⁻³), no protein adsorption occurred, irrespective of the HAp morphology.

4. Discussion

From the results described above, N–HAp, which has a needle shape with a higher surface area of the a-face than of the c-face, showed quite different protein adsorption behavior than I–HAp, which has an irregular-shaped morphology. Adsorption of BSA and LSZ was affected by the phosphate ion concentration in the buffer solution. For the adsorption of BSA and LSZ, the concentration of phosphate ions is more important than concentration of potassium ions co-existing in the solution, because previous reports show that phosphate ions have a high lyotropic effect on HAp crystals, while potassium ions have low chaotropic effects. Moreover, in HAp chromatography, the affinity of
phosphate for a protein is higher than that of the cation. Thus the dependence of protein adsorption behavior on the concentration of phosphate buffer is dominated by the existence of phosphate ions around the HAp crystals. For (low) phosphate concentrations of 2 mol·m$^{-3}$, it was shown that N–HAp (needle-shaped HAp) had a higher capacity for adsorbing BSA than I–HAp (irregularly shaped HAp). HAp has two types of crystal faces: positively charged $a$-face sites at around pH 7.4 and negatively charged $c$-face sites. BSA is an acidic protein, which is negatively charged at around pH 7.4, while LSZ is a basic, positively charged protein. The needle shape has a structure elongated along the $c$-axis of the hexagonal structure of HAp. The $a$-face has a calcium site for adsorption with negatively charged proteins and therefore the higher amounts of adsorbed BSA resulted from the higher $a$-face area of N–HAp. Calcium ions show a higher binding affinity for phosphate than for carboxyl groups.$^{13}$ If the phosphate concentrations are high, the negatively charged proteins adsorb on the $a$-face to reduce the degree of positive charge, resulting in the possibility of LSZ adsorption on HAp. This explanation accounts for the increased amounts of LSZ adsorbed on N–HAp in solutions that had phosphate concentrations ranging from 2 to 10 mol·m$^{-3}$. Under high phosphate concentrations, of more than 10 mol·m$^{-3}$, the interaction of phosphate ions with proteins in the solution, in addition to their adsorption on HAp, resulted in the prevention of the adsorption of proteins to HAp crystals.

Compared with the adsorption behavior of proteins on N–HAp, I–HAp did not show any particular differences between BSA and LSZ. To compare the selective adsorption of BSA and LSZ between N–HAp and I–HAp, the ratios of adsorbed proteins were calculated as LSZ/BSA and BSA/LSZ. Figure 4 shows the results for these ratios in phosphate concentrations ranging from 2 to 50 mol·m$^{-3}$. The higher concentration of phosphate was neglected because the adsorbed amounts of the proteins were too small to be estimated. For N–HAp, the ratio of BSA to LSZ adsorbed was approximately 5 when the phosphate concentration was 2 mol·m$^{-3}$. However, the ratio of BSA/LSZ for I–HAp was approximately 1, meaning that no selective adsorption occurred. At the higher phosphate concentration of 50 mol·m$^{-3}$, there was no selective adsorption of BSA against LSZ on either N–HAp or I–HAp. The high selectivity of N–HAp for adsorption of BSA over LSZ occurred specifically at lower phosphate concentrations. In contrast, when the phosphate concentration was about 20 mol·m$^{-3}$, the LSZ/BSA ratios showed the potential for I–HAp to selectively adsorb LSZ, by comparison with N–HAp. However, difficulty in separation was caused by the low capability of I–HAp to adsorb LSZ. This means that a well-designed morphology potentially allows control of the selectivity of proteins by HAp. Higher phosphate concentrations led to smaller differences in the adsorption of the proteins between the HAp samples, irrespective of morphology. These differences were observed even in the solution containing both BSA and LSZ proteins, although there may have been molecular interactions between the proteins having opposite net charges, in contrast to single protein systems.$^{5}$ The equal ratio between the two proteins may have allowed competitive adsorption of BSA against LSZ. At low phosphate concentrations (2 mol·m$^{-3}$), selective removal of the proteins was observed in the SDS-PAGE gels of the residual proteins in solution after N–HAp contact, whereas both proteins were removed from solution with I–HAp. Although the SDS-PAGE analysis was qualitative, we confirmed that the morphology of N–HAp produced highly selective adsorption, depending on the ion concentrations in the surrounding fluid.

5. Conclusions

The synthesized needle-shaped HAp (N–HAp) allowed for highly selective adsorption of bovine serum albumin when there was an appropriate concentration of phosphate in the solution. The selectivity was caused by the morphology of the N–HAp, which has a large $a$-face surface area. The designed morphology of HAp shows excellent potential for unique biological characteristics, triggered by the adsorption of proteins.

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References