PREPARATION AND CHARACTERIZATION OF MONODISPERSED METAL PHOSPHATE PARTICLES AND CHARACTERISTICS OF PROTEIN ADSORPTION ONTO VARIOUS APATITE PARTICLES

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Abstract: There exists a unique class of orthophosphates in which both the phosphorous and metal atoms are tetrahedrally coordinated by oxygen, therefore, to form metal phosphates. Various kinds of metal phosphate particles have many applications of uses in pigments, catalysis, adsorbents, and bioceramics. It is well known that calcium hydroxy apatite(Hap), a kind of calcium phosphate particles, also shows a high affinity for proteins and much attention has been focused on Hap and substituted Hap nanocrystals as sorbents. In this review, I will present (1) the preparation and characterization of monodispersed metal phosphate particles and (2) characteristics of protein adsorption onto various kinds of apatite particles based on our previous investigations.

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INTRODUCTION

Although carbon is often considered as the dominant element in terms of covalent bond formation, phosphorous can be undoubtedly be considered as a serious competitor for the position. The conventional notation for the electronic structure of the phosphorous atom is 1s$^2$ 2s$^2$ 2p$^6$ 3s$^2$ 3p$^3$, with three unpaired 3p electrons being available for bonding. Three, four and five essentially covalent linkages to other atoms are the most common, with the four-connected compounds being the most numerous of the three types. The classical Lewis octet structure for the orthophosphoric acid ions predicts the existence of three covalent bonds, and an electronic configuration of phosphorus completed by an outer shell of eight electrons as shown in Fig. 1.

![FIGURE 1 Electronic structure of phosphate ion.](image)

The salts of orthophosphoric acid possess at least an approximate tetrahedral distribution of four oxygen atoms around a central phosphorous atom. The covalent character of the phosphate ion is greater than that of the silicate ion but less than that of the sulfate ones. There exists a unique class of orthophosphates in which both the phosphorous and metal atoms are tetrahedrally coordinated by oxygen, therefore, to form metal phosphates. In this review, I will present (1) the preparation and characterization of monodispersed metal phosphate particles and (2) characteristics of protein adsorption onto various kinds of apatite particles, a series of metal phosphate, based on our previous investigations.

1. Preparation and characterization of monodispersed metal phosphate particles.

Various kinds of metal phosphate particles have many applications of uses in pigments, catalysis, adsorbents, and bioceramics. In order to develop the materials into high-quality products, the studies on the preparation and characterization of uniform spherical metal phosphate particles have been extensively done.

1.1 Cobalt phosphates

We have investigated the formation and characterization of the uniform spherical cobalt phosphate particles prepared by aging a solution containing CoSO$_4$ and Na$_2$HPO$_4$ at $80^\circ$C in the presence of urea and sodium dodecylsulfate (SDS)\textsuperscript{2,6}. Figure 2 is an example TEM picture of monodisperse cobalt phosphate particles precipitated. The XRD and gas adsorption measurements revealed that cobalt phosphate particles possess a thermally unstable slit-shaped layer structure (Fig. 3) and exhibit a high selective adsorption of H$_2$O by their molecular sieve effects (Fig. 4). By changing gas probe molecules with different diameters as displayed in Fig. 5, it can be determined that the layer distance is less than 0.353...
FIGURE 2 TEM picture of spherical cobalt phosphate particles.

FIGURE 3 Change in XRD patterns of uniform spherical cobalt phosphate particles treated at different temperatures. (a) 25, (b) 50, (c) 75, (d) 100, (e) 150, (f) 200, (g) 300, (h) 400, (i) 600, (j) 800°C.

It has been pointed out that anionic surfactant (sodium dodecyl sulfate: SDS) influenced the nucleation process and prevented particle agglomeration. Indeed, surfactant exhibited strong effect on the formation of colloidal particles as presented in Fig. 6, because it can be highly adsorbed on the nuclei of the precipitating particles. The diameter of the particles decreased steeply on anionic (SDS) and cationic surfactant (CTAC) systems, though nonionic one (NP-20) shows small effect on the particle size.

FIGURE 4 Monolayer capacity of water molecules (n_w) adsorbed on spherical cobalt phosphate particles. n_c in the inset represents the result for CO_2 molecule.

FIGURE 5 Monolayer capacity of probe gas molecules adsorbed on spherical cobalt phosphate particles.

FIGURE 6 Effects of surfactants on the particle diameter of spherical cobalt phosphate particles.

1.2 Aluminum phosphate

Aluminum phosphate is of interest in as
adsorbents, catalyst carriers, and possibly as catalysts. Some aluminum phosphate particles are known to show a high catalytic activity and a stronger surface acidity than silica-alumina cracking catalysts. On the other hand, Wilson et al. have reported that aluminum phosphate can be produced as a porous crystalline material as well as zeolite. This aluminum phosphate molecular sieve (AlPO-n) can be endowed with Brønsted acidity by isomorphous substitution of hetero atoms into their frameworks. Nowadays, AlPO-18 substituted with silicon and divalent metal (designated as SAPO-18 and MAPO-18, respectively) was synthesized and it was testified that these molecular sieves are excellent solid acid catalysts for methanol conversion to light alkanes. However, all these molecular sieve particles are not uniform both in shape and size. If we can prepare the particles with uniform shape and size, these characteristic properties would be more emphasized by the uniformity of the surface and inner structures of these particles, which permits us to design many high efficient products.

Spherical aluminum phosphate particles with a mean particle diameter of 477±16 nm can be produced from aging of a solution containing Al(NO$_3$)$_3$, Na$_2$HPO$_4$, and HNO$_3$ at 100°C for 19 h in 20-cm Teflon-lined screw-capped Pyrex test tube without agitation as shown in Fig. 7(A)). It was revealed from X-ray diffraction measurement and transmission electron microscope observation that amorphous particles are formed by agglomeration of small primary particles (Fig. 7(B)). The total weight loss from 25 to 1000°C (21.5 wt. %) and the Al/P molar ratio of the particles (1.04) offered that the chemical composition of the particles as AlPO$_4$.1.9H$_2$O, though the state of water in the material is uncertain due to its low crystallinity.

The particles exhibited a high selective adsorption of H$_2$O though they adsorbed small amount of N$_2$ and CO$_2$(See Fig. 8). This characteristic phenomenon was explained by rehydration of Al$^{3+}$ ions by H$_2$O molecules that were penetrated into the particles. Size of amorphous spherical aluminum phosphate particles was further controlled by using urea and isopropylamine (IPA) as is shown in Figs. 9 and 10. The mean particle diameter ($D_p$) of the particles decreased with increase in the concentration of urea by accelerating the precipitation reaction from decomposition of urea at elevated temperature. The $D_p$ also decreased by addition of IPA up to 6 mol% to Al ions under a constant urea concentration, while $D_p$ increased above the concentration because IPA produced electrolytes by reacting with HNO$_3$; the electrolytes reduce the range of Coulombic repulsion between charged primary particles. All these particles...
also adsorbed selectively H\textsubscript{2}O molecules. IPA does not act as a template but operates as an effective additive for controlling the particle size without altering the inner texture of spherical aluminum phosphate particles.

The size and pore structures of spherical aluminum phosphate particles are also controlled by adding various kinds of divalent and trivalent hetero metal ions\textsuperscript{17c). The addition of metal ions dramatically decreases the size of the spherical particles as shown in Fig. 11. Here the metal ions used were in the form of nitrates. The remarkable particle size reduction is observed on the systems doping trivalent Fe\textsuperscript{3+} ions rather than those doping divalent Co\textsuperscript{2+}, Zn\textsuperscript{2+} and Mn\textsuperscript{2+} ions. The incorporation of Fe\textsuperscript{3+} ions into the crystal lattice was observed on the systems doping trivalent Fe\textsuperscript{3+} ions to give solid solutions with a chemical composition of Al\textsubscript{1-x}Fe\textsubscript{x}PO\textsubscript{4}·nH\textsubscript{2}O, though no substitution occurred on divalent ions to attain particles with a chemical composition of AlPO\textsubscript{4}·nH\textsubscript{2}O. The divalent metal ions were adsorbed on primary fine particles to interfere with the particle growth. The divalent metal ions resided among the primary particles in secondary spherical aluminum phosphate particles were eliminated by rinsing and produced highly mesoporous materials without altering the particle morphology(Figs. 12). Similar mechanism was depicted on the particle formation in the presence of Fe\textsuperscript{3+} ions together with the incorporation into the crystal structure. The spherical aluminum phosphate particles produced with Co\textsuperscript{2+} ions exhibited electrically neutral surface in the aqueous medium, while those formed with Fe\textsuperscript{3+} ions manifested acidic surface nature due to phosphate ions exposed on particle surface.

1.3 Ferric phosphate

Ferric phosphate particles can be prepared by aging a solution dissolving Fe(ClO\textsubscript{4})\textsubscript{3} and H\textsubscript{3}PO\textsubscript{4} at 40–80°C for 16 h in a Teflon-lined screw-capped Pyrex test tube\textsuperscript{18}. The spherical or agglomerated fine particles are only precipitated with an extremely fast rate of reaction. TEM observation revealed that these particles grew in spherical structure by aggregation of primary small particles as same as nickel and aluminum phosphate ones. The size of spherical particles is decreased by increase in the solute concentration or raising the aging temperature; i.e., the formation of spherical particles was explained by a polynuclear layer mechanism proposed by Nielsen \textsuperscript{19}. The XRD patterns indicated that uniform spherical particles produced are amorphous, but they are crystallized to FePO\textsubscript{4} after calcining above 600°C. The voids between the primary particles within the secondary agglomerated particles constitute mesopores. The Fe/P molar ratio determined and weight loss in TG curves gave the chemical formulas of the particles as Fe(PO\textsubscript{4})\textsubscript{x}·(H\textsubscript{3}PO\textsubscript{4})\textsubscript{y}·nH\textsubscript{2}O (x: 0.93–1.00, y: 0–0.22, n: 2.4–2.7). The amorphous
spherical ferric phosphate particles also exhibit a high selective adsorption of H$_2$O by penetration of H$_2$O molecules into ultramicropores, produced after outgassing pretreatment, of that size is smaller than N$_2$ molecule. The more particles grew, the more adsorption selectivity of H$_2$O became remarkable.

1.4 Calcium phosphate particles
Calcium hydroxyapatite (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$, Hap) is utilized as an adsorbent material for liquid chromatography, because Hap is a major inorganic component of biological hard tissues as will be discussed in section 2. Although Hap is a metal phosphate, controlling the particle shape and size is very difficult because of its low solubility product. Since the hydrolysis of condensed phosphate ions, such as diphosphate(pyrophosphate, pp: P$_2$O$_7^{2-}$) and triphosphate (triphosphosphate, tpp: P$_3$O$_10^{5-}$) ions, is an endothermic process, the phosphate (orthophosphate: PO$_4^{3-}$) ions can be produced slowly by heating the solution, a so called forced hydrolysis reaction. Therefore, it can be expected that the forced hydrolysis reaction of the mixed solution of Ca$^{2+}$ and condensed phosphate ions can be applied for producing new calcium phosphate particles with unique shape and inner structure. According to these idea, we prepared calcium phosphate particles by aging a solution of CaCl$_2$[1.0x10$^{-3}$~1.0x10$^{-1}$ mol/dm$^3$] and Na$tpp$(sodium tripolyphosphate, Na$tpp$: Na$_5$P$_3$O$_10$:1.0x10$^{-3}$~1.0x10$^{-1}$ mol/dm$^3$) sodium triphosphate at 100~150°C for 16 h in a Teflon-lined screw-capped Pyrex test tube. The rod-like and spherical particles were precipitated at an extremely fast rate of reaction under 100°C as shown in Fig. 13.

The spherical particles were only produced at 100°C by aggregation of primary small particles. The rod-like particles are CaNa$_2$P$_2$O$_7$·4H$_2$O but the uniform spherical ones are amorphous and the latter ones were crystallized to β-Ca$_2$P$_2$O$_7$ after being calcined above 600°C. The hydrolysis of tpp ions has been expressed as the following two reactions (1) and (2).

$$\begin{align*}
P_3O_{10}^{5-} + 2H_2O & \rightarrow 3PO_4^{3-} + 4H^+ \quad (1) \\
2P_2O_{10}^{5-} + H_2O & \rightarrow 3P_2O_7^{4-} + 2H^+ \quad (2)
\end{align*}$$

The reaction (1) represents that tpp ions are completely hydrolyzed to orthophosphate ions. In the case of the reaction (2), tpp ions are not completely hydrolyzed and still remain pp ions. In our experiment, the reaction (2) would be occurred. The aging temperature drastically varied the particle shape under conditions for producing uniform spherical particles; solid spherical particles were produced up to the aging temperature of 115°C but balloon-like hollow spheres were precipitated at 120°C as is displayed the TEM pictures in Fig. 14.

However, large and irregular agglomerates made up of extremely small rod-like particles were precipitated after aging the solution above 130°C. TEM observation and ICP-AES measurements revealed that the balloon-like hollow spheres were produced by dissolving the interior of solid spherical particles after reinforced their shell by the adsorption of unhydrolyzed tpp. The spherical and balloon-like calcium phosphate particles with amorphous crystal form showed high selective adsorption of H$_2$O by rehydration of Ca$^{2+}$ ions with penetrating H$_2$O molecules into the particles as well as aluminum and ferric phosphate ones.
2. Characteristics of protein adsorption onto various kinds of apatite particles.

It is well known that a synthetic Hap, a kind of calcium phosphate particles, shows a high affinity for proteins and much attention has been focused on Hap and substituted Hap nanocrystals as sorbents to remove pathogenic proteins from blood in blood purification therapy \(^{27,28}\) or to act as a carrier for protein delivery \(^{29,30}\). Hap is in the space group P6\(_3\)/m; its unit cell parameters are \(a=b=0.943\) nm and \(c=0.688\) nm, and it possesses two different binding sites (C and P sites) on the particle surface as is depicted the crystal structure of Hap in Fig. 15. Thus, it contains a multiple-site binding character for proteins \(^{31-33}\). After dispersing Hap particles in aqueous media, calcium atoms (Ca(II) atoms in Fig.15a) are exposed on the Hap surface by dissolution of OH ions at the particle surface to produce rich in calcium ions or positively charged sites to bind to acidic groups of proteins, so called as C sites. These C sites are arranged on \(ac\) or \(bc\) particle face in a rectangular manner with the interdistances of 0.943 nm and 0.344 nm (c/2) for the \(a\) (or \(b\)) and \(c\) directions, respectively (Fig. 15a). The P sites, negatively charged adsorbing sites, each formed by six oxygen atoms belonging to three crystal phosphate ions, are arranged hexagonally on the \(ab\) particle face with a minimal interdistance in both \(a\) and \(b\) directions equal to \(|a|=|b|\) = 0.943 nm (Fig. 15b). In addition, Hap is the most stable calcium phosphate under physiological conditions. Hence, Hap is widely applied for separating various proteins using as a column for a high-performance liquid chromatograph (HPLC) apparatus and many essential studies have been reported \(^{30-32}\). In the past decade, the authors’ group has been conducted fundamental studies on the adsorption of acidic bovine serum albumin (BSA), neutral myoglobin (MGB), and basic lysozyme (LSZ) onto various kinds of synthetic Hap particles. \(^{34,35}\) In these studies, we investigated by changing many factors of Hap particles, such as calcium-to-phosphorus (Ca/P) atomic ratio, kinds of divalent cations (Ca, Sr and Ca-Sr solid solution), mean particle length, contents of carbonate ions incorporated and heat treatment temperature. The synthesis methods of various kinds of apatite particles as employed in the following sections were briefly described. The original Hap particles were synthesized in a 20 dm\(^3\) Teflon screw-capped vessel under nitrogen atmosphere by mixing 20 dm\(^3\) of a 20 mM Ca(OH)\(_2\) solution with 0.167 dm\(^3\) of a 10 wt. % H\(_3\)PO\(_4\) solution, both of which were prepared using CO\(_2\)-free distilled water. The resulting white suspension was further aged at 100°C for 48 h. The Ca-Sr solid solution (CaSrHap) particles were synthesized by aging the precipitates (at 100°C for 48 h) formed from the reaction of a mixed solution of Ca(OH)\(_2\) and Sr(OH)\(_2\) with H\(_3\)PO\(_4\) in Teflon vessel under CO\(_2\)-free conditions as well as a preparation for Hap ones. The carbonate calcium hydroxyapatite (Cap) containing various amounts of carbonate from 0 to 10.1 wt. % were prepared at a pH of 8.4±0.2 at 100°C by feeding 0.25 dm\(^3\) of 0.54 M Ca(CH\(_3\)COO)\(_2\) solution into 9 dm\(^3\) of mechanically stirred 0.036 M NaH\(_2\)PO\(_4\) solution containing 0 – 0.1 M NaHCO\(_3\) under nitrogen atmosphere in a Teflon vessel. The properties and TEM micrographs of typical apatite particles are shown in TABLE 1 and Fig. 6, respectively.

The properties of proteins used in these studies were listed in Table 2.

2.1 BSA adsorption

The adsorption isotherms of BSA on Hap, strontium hydroxyapatite(SrHap) and calcium-strontium
TABLE 1. Properties of typical apatite particles.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sr/(Ca+Sr) atomic ratio</th>
<th>(Ca+Sr)/P atomic ratio</th>
<th>NaHCO₃ [mol dm⁻³]</th>
<th>CO₃²⁻ ion [wt. %]</th>
<th>specific surface area [m²/g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hap</td>
<td>0.000</td>
<td>1.57</td>
<td>0.00</td>
<td>0.0</td>
<td>124</td>
</tr>
<tr>
<td>CaSrHap-1</td>
<td>0.004</td>
<td>1.49</td>
<td>0.00</td>
<td>0.0</td>
<td>116</td>
</tr>
<tr>
<td>CaSrHap-2</td>
<td>0.120</td>
<td>1.50</td>
<td>0.00</td>
<td>0.0</td>
<td>131</td>
</tr>
<tr>
<td>CaSrHap-3</td>
<td>0.260</td>
<td>1.54</td>
<td>0.00</td>
<td>0.0</td>
<td>132</td>
</tr>
<tr>
<td>CaSrHap-4</td>
<td>0.564</td>
<td>1.63</td>
<td>0.00</td>
<td>0.0</td>
<td>122</td>
</tr>
<tr>
<td>CaSrHap-5</td>
<td>0.751</td>
<td>1.69</td>
<td>0.00</td>
<td>0.0</td>
<td>67</td>
</tr>
<tr>
<td>CaSrHap-6</td>
<td>0.876</td>
<td>1.70</td>
<td>0.00</td>
<td>0.0</td>
<td>42</td>
</tr>
<tr>
<td>SrHap</td>
<td>1.000</td>
<td>1.51</td>
<td>0.00</td>
<td>0.0</td>
<td>53</td>
</tr>
<tr>
<td>Cap-1</td>
<td>0.000</td>
<td>1.62</td>
<td>0.01</td>
<td>1.5</td>
<td>85</td>
</tr>
<tr>
<td>Cap-2</td>
<td>0.000</td>
<td>1.57</td>
<td>0.05</td>
<td>4.7</td>
<td>98</td>
</tr>
<tr>
<td>Cap-3</td>
<td>0.000</td>
<td>1.60</td>
<td>0.10</td>
<td>10.1</td>
<td>102</td>
</tr>
</tbody>
</table>

FIGURE 16 TEM micrographs of typical apatite particles.

TABLE 2. Properties of proteins.

<table>
<thead>
<tr>
<th>protein</th>
<th>isoelectric point</th>
<th>molecular weight (Da)</th>
<th>size (nm)</th>
<th>numbers of functional groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>4.7</td>
<td>62100</td>
<td>4.14</td>
<td>680</td>
</tr>
<tr>
<td>LSZ</td>
<td>11.1</td>
<td>14600</td>
<td>3.35</td>
<td>155</td>
</tr>
<tr>
<td>MGB</td>
<td>7.0</td>
<td>17800</td>
<td>3.45</td>
<td>34</td>
</tr>
</tbody>
</table>

hydroxyapatite(CaSrHap: solid-solution), in aqueous solution of 10⁻⁴ KCl at 15°C, are pseudo-Langmuir type. The saturated amounts of adsorbed BSA (nBSA) are the plateau values of the adsorption isotherms increased with Ca/P, Sr/P and (Ca+Sr)/P ratios as shown in Fig.17(a)34. This result suggests that the electrostatic attractive force between BSA and various kinds of apatite particles is a determining factor of nBSA. The large acicular particles of SrHap with flat surface are advantageous for the adsorption of negatively charged BSA by the large number of positively charged adsorption sites on the exposed ac or bc crystal planes. It was suggested therefore that the texture of the particle is significant in the adsorption of BSA.

The adsorption isotherms of BSA on carbonate hydroxyapatite(CaSrHap: solid-solution), in aqueous solution of 10⁻⁴ KCl at 15°C, are pseudo-Langmuir type. The saturated amounts of adsorbed BSA (nBSA), which are the plateau values of the adsorption isotherms increased with Ca/P, Sr/P and (Ca+Sr)/P ratios as shown in Fig.17(a)34. This result suggests that the electrostatic attractive force between BSA and various kinds of apatite particles is a determining factor of nBSA. The large acicular particles of SrHap with flat surface are advantageous for the adsorption of negatively charged BSA by the large number of positively charged adsorption sites on the exposed ac or bc crystal planes. It was suggested therefore that the texture of the particle is significant in the adsorption of BSA.

FIGURE 17 Relationship between nₕ of BSA, LSZ and MGB and the cation/P molar ratio of various kinds of synthetic Hap particles.

(a) BSA: (○) Hap, (□) SrHap, (◊) CaSrHap
(b) MGB: (○) Hap, (□) SrHap, (◊) CaSrHap

FIGURE 18 Plots of nₕ of MGB, BSA and LSZ as a function of particle length of (a) Hap and (b) Cap particles.

calcium hydroxyapatites (Cap) containing various amounts of carbonate ions from 0 to 10.1 wt.% are also pseudo-Langmuir type35. The nₕ values are decreased with increase in CO₃²⁻ ion content in Caps. This fact could be explained by the difference in the particle textures of Caps, the large rod-like particles with flat surface are advantageous for the adsorption of negatively charged BSA by the large number of positively charged adsorption sites on the exposed ac or bc crystal planes. This result indicates again that the particle texture is thought to be significant in the adsorption of BSA as well as Hap, SrHap and CaSrHap. The nₕ values for the synthesized Hap particles are strongly dependent upon their mean
particle length ($L_p$) as shown in Fig. 18, while that of LSZ ($n_5^{LSZ}$) onto the Hap particles as will be described in a next section exhibited a minor dependence on the $L_p$. This result provides definitive evidence that the C sites exposed on ac or bc crystal faces are the adsorption sites for BSA.

2.2 LSZ adsorption

The adsorption of LSZ onto various kinds of synthetic Hap as employed in BSA adsorption experiments has been investigated at the same condition of BSA. The adsorption isotherms of LSZ onto these HAPs exhibit the pseudo-Langmuir type as well as those of BSA. The surface charge of the particles is reversed to positive one by adsorption of positively charged LSZ molecules. The saturated pseudoULangmuir type and the surface charge of the particles are appreciably smaller than those of BSA. Specifically adsorb onto phosphate ions on oxygen atoms of three phosphate ions on faces and/or negatively charged sites formed by six amino groups, though the low probability of the latter mode was insinuated.

2.3. MGB adsorption

The adsorption of MGB onto various kinds of colloidal synthetic Hap utilized above has been also compared. The adsorption rate of MGB onto Hap was comparable to that of LSZ and was faster than that of BSA. This distinction was explained by the difference in the molecular mass of these proteins (MGB: 17,800 Da, LSZ: 14,600 Da, BSA: 67,200 Da); the diffusion rates of the smaller MGB and LSZ molecules to the Hap surface are faster than that of the larger BSA. The adsorption isotherms of MGB onto these synthetic Haps exhibit the pseudo-Langmuir type and the surface charge of the MGB-covered Haps showed almost constant negative value and is independent of the MGB concentration for all the Haps. The values of the saturated amounts of adsorbed MGB($n_5^{MGB}$) for Hap are independent on the Ca/P atomic ratio of the materials as is shown in Fig.17(b), supporting less importance of an electrostatic interaction between MGB and Haps because MGB molecules are electrostatically neutral. It can presumed that MGB molecules are adsorbed onto phosphate ions exposed on ac and bc crystal faces by the van der Waals attractive force without taking advantage of the electrostatic interaction. As we pointed out before that $n_5^{BSA}$ depends strongly on the particle length by the specific electrostatic interaction between negatively charged carboxylic acid groups of BSA and positively charged C sites on the exposed ac or bc crystal faces, implying the importance of C sites for the adsorption of acidic proteins. However, $n_5^{MGB}$ slightly increases with an increase in particle length as same as LSZ(Fig. 18), suggesting that the positively charged C sites do not play a key role on the adsorption of neutral protein MGB. Similar minor importance of C sites for BSA, though LSZ slightly desorbed as is shown in

![FIGURE 19 Adsorption-desorption isotherms of BSA on three different Haps. Open and filled symbols represent adsorption and desorption isotherms, respectively.](image-url)
pre-adsorption of BSA, where LSZ is adsorbed onto BSA-covered Hap as illustrated in Fig. 21. On the other hand, the amount of adsorbed BSA is not changed by the pre-adsorption of LSZ (Fig. 22). The enhancement of adsorption of positively charged LSZ is induced by an electrostatic attractive force through pre-adsorption of negatively charged BSA molecules with a high coverage. Hence the double protein adsorbed layers are formed. However, since the coverage of LSZ onto Hap is considerably low, no stimulation of BSA adsorption occurs on the LSZ-covered surface.

2.5. Effects of surface hydrophobicity of Hap
Actually, adsorbent surface properties such as hydrophobicity and electric charge density as well as environmental conditions like pH, temperature and ionic strength are often taken as experimental variables in protein adsorption studies. The effects of surface hydrophobicity of Hap on the protein adsorption behavior have been examined by grafting oleylphosphate (OP) molecules on Hap surface. The $n_s^{BSA}$ value strongly depends on number of oleyl groups on the particle surface ($n_0$) as shown in Fig. 23. The $n_s^{BSA}$ values of BSA exhibit a maximum around $n_0=0.8$, though $n_s^{LSZ}$ ones continuously.
increase up to $n_o=2.0$ and are saturated. The $n_s^{LSZ}$ values on OP-grafted Hap particles increase with increase in $n_o$. This difference in the dependence of $n_s^{BSA}$ and $n_s^{LSZ}$ values on $n_o$ observed could be interpreted as follows. In the case of BSA, the hydrophobic interaction between hydrophobic parts of proteins and oleyl groups on the Hap may enhance the accumulation of proteins on the particle surface to increase the $n_s^{BSA}$. However, since the negative charge of OPU-grafted Hap particles was increased with increase in $n_o$, repulsion forces between negatively charged BSA molecules and OP-grafted Hap particles would be increased with increase in $n_o$. Therefore, $n_s^{BSA}$ decrease at $n_o \geq 1.3$. On the contrary, LSZ molecules are positively charged. Therefore, besides the increasing the hydrophobic interaction an enhancement of electrostatic attractive force between LSZ and OP-grafted Hap particles with increase in $n_o$ appears to be exert for raising the $n_s^{LSZ}$.

2.6. Competitive and Cooperative Adsorptions

The adsorption rate of LSZ is faster than that of BSA due to its larger diffusion coefficient, though the saturated adsorption amount of LSZ is less than that of BSA\textsuperscript{40}. The rates of BSA adsorption onto Hap do not change by the addition of LSZ, while those of LSZ are considerably reduced by addition of BSA with forming (BSA$\cdot$LSZ$\cdot$) agglomerates in the solution. The cooperative adsorption behavior of LSZ has been observed in the presence of lower amounts of BSA due to the preferential adsorption of the larger (BSA$\cdot$LSZ$\cdot$) agglomerates as can be seen in Fig. 24. However, in the case of higher BSA content, the adsorption of LSZ was inhibited by capturing the LSZ molecules in the (BSA$\cdot$LSZ$\cdot$) agglomerates. The similar cooperative adsorption behavior of BSA was also observed on all systems examined in the presence of various amounts of LSZ.

2.7. Effects of solvent (acetonitrile:AN)\textsuperscript{41}

The CD spectra revealed that the fraction of $\alpha$-helical content of BSA is remarkably decreased at AN concentration above 30 vol. % while $\beta$-sheet content is increased. On the other hand, the percentages of random coil and turn contents are only slightly decreased. In addition to this secondary structural change of BSA, the UV spectra suggested that the tertiary structure of protein molecules is also changed by the addition of large amounts of AN; BSA molecules associate to form molecular aggregates at [AN]$>40$ vol. %. The contraction of BSA molecule at low AN concentration (10–20 vol. %) gives its small cross-sectional area, providing a large amount of adsorption ($n_s^{BSA}$) as is shown in Fig. 25A, though $n_s^{BSA}$ is decreased above 30 vol. % AN by enlargement of BSA molecules with solvation and unfolding some $\alpha$-helix domains. Also, the $n_s^{LSZ}$

![FIGURE 24 Adsorption isotherms of LSZ on Hap from BSA-LSZ mixtures in the presence of various amounts of BSA(A) and their em(B). The changes of the amounts of adsorbed BSA are displayed in (C). Concentration of BSA: (●) 0, (□) 2.5, (▽)5.0, (△)7.5 and (○) 10.0 mg/cm$^3$.](image-url)

2.8. Immunogamma globulin adsorption

The adsorption rate of IgG is similar to that of BSA and is slower than that of MGB and LSZ\textsuperscript{42}. This fact has been interpreted by the difference in the structural stability and molecular weight (146,000Da for IgG) of these proteins. The adsorption isotherms of IgG onto the Hap particles exhibited the pseudo-Langmuir type. The saturated amount of adsorbed IgG values ($n_s^{IgG}$) for the particles with the mean particle length less than 70 nm(open circle symbol) was decreased with increasing the Ca/P ratio. The adsorption behavior of IgG molecules was very similar to that of basic LSZ though IgG has zero net-charge as is
FIGURE 25 Adsorption isotherms of BSA(A) on Hap and their em(B).

[Acetonitrile]: (■) 0, (○) 10, (▲) 20, (▽) 30, (◄) 50.

FIGURE 26 (a) Plots of $n_s^{IgG}$ as a function of Ca/p ratio and (b) plots of $n_s^{IgG}$, $n_s^{BSA}$, $n_s^{MGB}$ and $n_s^{LSZ}$ as a function of particle length of Haps (● >70 nm, ○<70 nm).

shown in Fig. 26(a). The $n_s^{IgG}$ value is increased with increase in the mean particle length of Haps, though the relationship was less significant than BSA but similar to MGB and LSZ(Fig. 26(b)). The similar adsorption behavior of IgG and LSZ suggested that the $F_{ab}$ parts of IgG molecules preferentially adsorb onto Hap to provide the reversed Y-shaped conformation of IgG as displayed in Fig. 27. The change of the adsorption mode of IgG molecules from the reversed Y-shaped conformation to side-on by “spreading” the $F_c$ part of IgG molecules onto particle surface within longer adsorption time has been suggested. The $n_s^{IgG}$ value is increased with increasing the electrolyte concentration by screening the intra- and intermolecular electrostatic interactions of proteins.

FIGURE 27 Theoretical $n_s^{IgG}$ values for different adsorption configuration.

2.9. Study on adsorption mechanism of proteins through ionic concentration measurements

The kinetic studies of dissolution and ion-exchange properties of synthetic Hap particles in the absence and presence of proteins have been examined. In the absence of proteins, Hap particles slightly dissolve to give low amounts of calcium ([Ca$^{2+}$]=0.09–0.14 µmol m$^{-2}$) and phosphate ([PO$_4^{3-}$]=0.01–0.08 µmol m$^{-2}$) ions in KCl, CaCl$_2$, BaCl$_2$ and AlCl$_3$ solutions. The [Ca$^{2+}$] increases with increase in the Ca/P ratio of Hap, while the [PO$_4^{3-}$] decreases. The [Ca$^{2+}$] and [PO$_4^{3-}$] are independent of the ionic strength. Ba$^{2+}$ and Al$^{3+}$ ions are completely ion-exchanged with calcium ions in Hap lattice within 2 hr. In the presence of BSA, the Hap particles dissolve slightly faster than the systems without protein. This fact was explained by a complexation of dissolved ions to functional groups of BSA. The adsorption of BSA induced a reduction of [Ca$^{2+}$] and [PO$_4^{3-}$] in the aqueous medium and minima appeared on [Ca$^{2+}$] and [PO$_4^{3-}$] profiles before the BSA adsorption reached a saturation as shown in Fig. 28. This result suggests that the adsorption of BSA onto Hap is governed by Ca$^{2+}$ ions complexing to BSA molecules (called as binding effect) together with the operation of C sites. The addition of BaCl$_2$ and AlCl$_3$ steeply increases the $n_s^{BSA}$ values at the initial adsorption step by the strong binding effect of these di- and tri-valent cations between BSA and Hap.
However, after eliminating these cations from Hap surface by the ion-exchange reaction, the binding effects disappear and $n_{BSA}$ decrease as shown in Fig. 28. Since the number of functional groups is small, the binding effect of the counter ions is only slightly detected for the systems with divalent and tri-valent ions on the adsorption systems of LSZ.

2.10. Microcalorimetric Study

To clarify the adsorption mechanism of proteins onto Hap, the adsorption ($\Delta H_{\text{ads}}$) and desorption ($\Delta H_{\text{des}}$) enthalpies of BSA, MGB and LSZ onto Hap have been measured by using a flow microcalorimeter (FMC). The amounts of protein adsorbed ($\Delta n_{\text{ads}}$) and desorbed ($\Delta n_{\text{des}}$) were also measured simultaneously by monitoring the protein concentration of downstream from the FMC with a UV detector. In this experiment, Hap was prepared by hydrolyzing CaHPO$_4$·2H$_2$O (DCPD) with different concentrations of NaOH. The crystallinity of Hap was improved by increase in the NaOH concentration. The $\Delta n_{\text{ads}}$ values were also measured statically by a batch method in each system. The $\Delta n_{\text{ads}}$ values measured by the FMC and static measurements fairly agreed with each other. The $\Delta H_{\text{BSA}}^{\text{ads}}$ is decreased with the improvement of Hap’s crystallinity, in other words by increase in NaOH concentration, suggesting that the BSA adsorption readily proceeded onto Hap as shown in Fig. 29. This fact indicates a high affinity of Hap to protein. This affinity was further recognized by $\Delta H_{\text{BSA}}^{\text{des}}$ because its positive value is increased by the improvement of Hap’s crystallinity. These opposite tendencies on $\Delta H_{\text{BSA}}^{\text{ads}}$ and $\Delta H_{\text{BSA}}^{\text{des}}$ reveal that Hap possesses a high adsorption affinity to BSA (i.e., enthalpy facilitated protein adsorption but hindered its desorption). The fraction of BSA desorption is also decreased with increase in the improvement of Hap’s crystallinity, confirming the high affinity of Hap to protein.

FIGURE 28 Changes in amounts of adsorbed BSA, concentrations of cations and PO$_4^{3-}$ ions, pH and em of Hap particles as a function of incubation time in various electrolytes. Concentration of electrolytes was fixed at 1x10$^{-4}$M(0.05 µmol/m$^2$). Full and open (italic) symbols represent the data obtained from the systems with and without BSA.
Similar results were observed on the LSZ system, though the enthalpy values were smaller than those of BSA. In the case of neutral MGB, $\Delta H_{\text{ads}}^{\text{MGB}}$ also exhibited the results similar to those of BSA and LSZ systems. However, due to its weak adsorption by the van der Waals force, $\Delta H_{\text{des}}^{\text{MGB}}$ was small and almost zero. Hence, the fraction of MGB desorption was less dependent on the crystallinity of Hap.

2.11. Effect of surface modification with pyrophosphoric acids (PP$_a$)

The PP$_a$ modified Hap particles (abbreviated as PP-Hap) possessed anchored polyphosphate (PP: P-{O-PO(OH)}$_n$-OH) branches on their surfaces$^{45}$. The saturated amounts of adsorbed BSA ($n_{\text{BSA}}^{\text{ads}}$) were increased three-fold by the surface modification with PP$_a$ though they were independent of the [PP$_a$](Fig. 30)$^{45}$. Furthermore, the fraction of BSA desorption was independent of the [PP$_a$]. This enhancement of BSA adsorption onto the PP-Hap is due to the hydrogen bonding between oxygen and OH groups of the PP-branches and functional groups of BSA molecules. In the case of LSZ, a more higher adsorption enhancement was observed; the saturated amount of adsorbed LSZ ($n_{\text{LSZ}}^{\text{ads}}$) for Hap modified at [PP$_a$]=6 mmol/dm$^3$ was nine-fold than that for Hap unmodified. This remarkable adsorption enhancement was explained by a three-dimensional binding mechanism; LSZ molecules were trapped inside of the PP-branches. Hence, a fraction of LSZ desorption was decreased with an increase in the [PP$_a$]; as more PP-branches are presented on the surface the higher retardation of LSZ desorption was induced. It was expected from their small size that MGB absorb between the PP-branches as well as LSZ. However, the amounts of adsorbed MGB ($n_{\text{MGB}}^{\text{ads}}$) did not vary and were independent of the [PP$_a$] due to the small numbers of functional groups of MGB. In addition, no dependence of the fraction of MGB desorption on the [PP$_a$] was observed. The schematic diagram of proteins adsorbed on PP-Hap is displayed in Fig. 31. The anchored structure of the PP-branches developed on the Hap surface to provide three-dimensional protein adsorption spaces was proved by a comparative experiment that was elucidating the effect of pyrophosphate ions for BSA adsorption onto Hap$^{45}$.

![FIGURE 29 Plots of $\Delta H_{\text{ads}}$, $\Delta H_{\text{des}}$, $\Delta n_{\text{ads}}$, $\Delta n_{\text{des}}$ and fraction of desorption for three kinds of proteins as function of [NaOH].](image)

$\Delta H_{\text{ads}}$, $\Delta H_{\text{des}}$, $\Delta n_{\text{ads}}$, $\Delta n_{\text{des}}$ and fraction of desorption for three kinds of proteins as function of [NaOH]. (○, △, □) adsorption, (●, ▲ , ■) desorption.
FIGURE 30 Adsorption (a) and desorption (a’) isotherms of proteins onto PP-Hap particles and their zeta potential (b) at each adsorption process. [PP] mmol/dm³, (○) unmodified, (▽) 2, (Δ) 4, and (□) 6.

[Graphs showing adsorption isotherms]

FIGURE 31 Schematic diagram of proteins adsorbed on PP-Hap particles. (No structural rearrangement of protein molecules was drawn.)

FIGURE 32 Adsorption isotherms of BSA onto Hap nanocrystals produced at various β−alanine/Ca ratios. β/Ca ratio: (○) 0, (□) 0.4, (●) 1.0, (▲) 2.0, (X) 4.0, (P) 10.0

2.12. Adsorption behavior of proteins onto positively charged Hap

Positively charged Hap nanocrystals have been prepared by using β-alanine and clarified the adsorption affinity of these surface amide functionalized Hap nanocrystals to proteins⁴⁶. The zeta potential (zp) of β-alanine-Hap nanocrystals prepared at β-alanine/Ca ~0.4 and 1.0 of those incorporating β-alanine exhibited positive charge at pH≤5.9. The saturated amounts of adsorbed BSA for the positively charged β-alanine-Hap nanocrystals
FIGURE 33 XPS spectra of Hap particles after treated at various temperatures. (a) Ca$_{2p}$, (b) O$_{1s}$ and (c) P$_{2p}$.

were increased 2.3–2.4-fold by their electrostatic attraction force between positively charged β-alanine-Hap nanocrystals and negatively charged BSA molecules (Fig. 32).

2.13. Effects of heat treatment of Hap particles

The effects of heat treatment of Hap particles on the protein adsorption have been investigated. The Hap single phase is continued up to the heat treatment temperature of 600°C. However, after treated above 800°C in air, β-Ca$_3$(PO$_4$)$_2$ (β-TCP) phase slightly appeared. Also, the FTIR and XPS measurements indicated that a partially dehydrated oxyhydroxyapatite (pd-OHap) is formed after treated at high temperature as shown the XPS spectra in Fig. 33. The saturated amounts of adsorbed BSA ($n^B_{B}A$) did not vary on the Hap particles after heat treated at 200 and 400°C. However, $n^B_{B}A$ values were increased by raising the heat treatment temperature above 600°C as shown in Fig. 34. The adsorption of BSA was increased up to ca. 1.4. This adsorption coverage of BSA ($\theta_{B}SA$) over the unity suggests that the BSA molecules densely adsorbed and a part of BSA molecules adsorbed as end-on type on the Hap particle surface or BSA molecules became contracted. Similar adsorption behavior has been observed on the
particles is observed on the adsorption of MGB (Fig. 36). The increases of $n_{\text{B}}^{\text{BSA}}$ and $n_{\text{L}}^{\text{LSZ}}$ have been explained by the increase of calcium and phosphate ions in the solutions dissolved from β-TCP formed after heat treatment of Hap, especially treated at high temperature. The dissolved Ca$^{2+}$ and PO$_4^{3-}$ ions may act as binders between proteins and Hap surfaces; the adsorption of Ca$^{2+}$ ions on the Hap surface offers an adsorption site for BSA owing to its positive charge. In the case of adsorption of positively charged LSZ molecules, PO$_4^{3-}$ ions act as a binder in an opposite way. Since the MGB molecules are neutral, therefore, no binding effect of both ions was observed.

In closing, I hope this review will contribute to the researchers in the fields of biomaterials, biomineralization and biosensor.

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