FULL PAPER

Immobilization and collection of enzymes by hydroxyapatite/maghemite composite particles with magnetism

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Apatite nuclei (ApN) were precipitated by raising the pH of simulated body fluid (SBF) with ion concentrations nearly equal to those of human blood plasma. The maghemite (γ-Fe2O3) particles were attached to the ApN, and the particles were subsequently soaked in SBF adjusted at pH = 7.60, 36.5 °C for one day. By this treatment, the ApN induced hydroxyapatite (HA) formation and the γ-Fe2O3 particles were encapsulated with HA particles with approximately 1–2 μm in diameter. The specific surface area of thus-obtained HA/Fe2O3 particles was almost 27 times as large as that of the commercially obtained HA particles. Urease or superoxide dismutase (SOD) was immobilized on the surface of the HA/Fe2O3 particles in ultrapure water, and the particles were collected by using a neodymium magnet. It was indicated that more than 90 % of urease or SOD was collected by using the HA/Fe2O3 particles. By using the urease immobilized on the HA/Fe2O3 particles, furthermore, urea dispersed in buffered solution almost completely decomposed. As the immobilization efficiency of urease increased, the urea decomposition was promoted.

Key-words : Hydroxyapatite, Maghemite, Composite particles, Magnetism, Enzyme collection, Enzyme activity

1. Introduction

Hydroxyapatite [HA: Ca10(PO4)6(OH)2], which is a main inorganic component of living bone, has attracted much attention as a biomaterial with high affinity to living bone and cells. Also, HA is well known to have a sufficient adsorption ability of biopolymers such as enzymes, proteins, and biomolecules. From these properties, HA has been well studied as one of the important biomaterials in not only orthopedic and dental fields but also drug and gene delivery techniques.

When the pH of simulated body fluid (SBF), whose ion concentrations and pH value are similar to those of human blood plasma, is increased, homogeneous nucleation of calcium phosphate occurs because of an increase of supersaturation respect to calcium phosphate. As a result, fine particles of calcium phosphate precipitates in the solution. In a recent decade, we found that the fine particles were highly active to induce HA formation in physiological SBF and referred to the particles as ‘apatite nuclei’ (ApN). By using the ApN, we encapsulated various kinds of particles with HA in our previous study. First, we attached the ApN on the surface of the core particles with 1–5 μm in particle size. Next, we immersed the ApN-adsorbed core particles in SBF. By this treatment, the ApN induced HA formation on the surface of the core particles, and the HA coated the surface of the core particles. This preparation method of core–shell particles can be applied to various kinds of bioinert metals, ceramics, and polymers as core particles.

Maghemite (γ-Fe2O3) has ferrimagnetism and is stable because Fe in γ-Fe2O3 is not to be oxidized anymore in comparison with that in magnetite (Fe3O4). By applying γ-Fe2O3 to the above preparation process as core particles, HA/Fe2O3 particles with both affinities to biomolecules of HA and magnetism of γ-Fe2O3 will be obtained. Because the HA/Fe2O3 particles possess both bioaffinity and magnetism, they are thought to be suitable carriers for enzyme immobilization, which can adsorb enzymes on the surface of HA and can be collected by the magnetic field.

Urease [molecular weight (MW) = 480 kDa, isoelectric point (pI) = 5.1] is one of the metalloenzymes which possesses Ni at its active centers and promotes urea decomposition by hydrolysis. In the previous study, we investigated the amount of collected urease and the presence or absence of urea decomposition ability using a constant amount of urease. However, it is essential to investigate the effect of the amount of HA/Fe2O3 particles on that of collected urease and the effect of the amount of immobilized urease on the speed of urea decomposition. Also, the pI of enzymes affected the amount of immobilized

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‡ Preface for this article: DOI http://doi.org/10.2109/jcersj2.128.P11-1

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enzymes. However, it is important to investigate the collected amount of enzymes with similar pI and different MW, too.

In this study, we fabricated the HA/Fe$_2$O$_3$ particles based on our previous method using ApN and γ-Fe$_2$O$_3$ particles. Then, we examined the urease immobilization property of the HA/Fe$_2$O$_3$ particles by using the various amount of HA/Fe$_2$O$_3$ particles and the various initial amount of urease. We examined the effect of the amount of immobilized urease on the specific activity by comparison of the decomposition speed of urea.

To investigate the immobilization amount of other kinds of metalloenzymes, also, superoxide dismutase (SOD) (MW = 16 kDa, pI = 4.75), which is an enzyme for the decomposition of radical oxygen, was immobilized on the HA/Fe$_2$O$_3$ particles. The effect of the amount of HA/Fe$_2$O$_3$ particles on the amount of immobilized SOD was investigated.

2. Materials and methods

2.1 Preparation of SBF

SBF was prepared by the method certified as ISO 23317 using NaCl$_2$ (Fujifilm Wako Pure Chemical, Osaka, Japan), NaHCO$_3$ (Hayashi Pure Chemical, Osaka, Japan), KCl (Hayashi Pure Chemical), K$_2$HPO$_4$·3H$_2$O (Nacalai Tesque, Kyoto, Japan), MgCl$_2$·6H$_2$O (Hayashi Pure Chemical), CaCl$_2$·6H$_2$O (Hayashi Pure Chemical), and Na$_2$SO$_4$ (Hayashi Pure Chemical). To accelerate HA formation, the SBF was adjusted at pH = 7.60, 36.5 °C, which is higher than the physiological condition (pH = 7.40, 36.5 °C), by dissolving tris(hydroxymethyl)aminomethane (Hayashi Pure Chemical) and 1 M HCl (Hayashi Pure Chemical) in this study. The solution is denoted as ‘solution for accelerated HA formation (SAHAF)’ hereafter.

2.2 Preparation of ApN

The pH of the SAHAF was raised to 8.60 by dissolving tris(hydroxymethyl)aminomethane at 25.0 °C. By this treatment, ApN were precipitated in the SAHAF. The ApN were collected by suction filtration using a membrane filter with 0.05 μm for average pore size (Merck Millipore, Burlington, MA), washed with ultrapure water, and dried at 36.5 °C.

2.3 Preparation of HA/Fe$_2$O$_3$ particles

Commercially obtained γ-Fe$_2$O$_3$ particles (Particle diameter <50 nm, Sigma-Aldrich, St. Louis, MO) were used in this study. The 10 mg of γ-Fe$_2$O$_3$ particles and 30 mg of ApN prepared by the above procedure were mixed in 1 L of ultrapure water using an ultrasonic cleaner for 10 min. Subsequently, the particles were collected by suction filtration using a membrane filter with 0.3 μm for average pore size (Merck Millipore). Thus-treated γ-Fe$_2$O$_3$ particles were dispersed in the SAHAF and kept for one day. Thus-obtained HA/Fe$_2$O$_3$ particles were collected by suction filtration, washed with ultrapure water, and dried at 36.5 °C. For the reference, γ-Fe2O3 particles were soaked in SAHAF for seven days.

2.4 Analyses of HA/Fe$_2$O$_3$ particles

The surface of the obtained HA/Fe$_2$O$_3$ particles was measured by X-ray diffraction instrumentation (XRD: RINT 2500, Rigaku, Tokyo, Japan) with Cu-Kα radiation at 50 kV, 300 mA, field emission scanning electron microscope (SEM: SU6600, Hitachi High-Technologies, Tokyo, Japan) and energy dispersive X-ray analyzer (EDX: XFlash® 5010, Bruker AXS, Fitchburg, WI). We measured specific surface area of the HA/Fe$_2$O$_3$ particles by Brunauer–Emmett–Teller (BET) specific surface area distribution measurement instrumentation (BELSORP-mini II, MicrotrackBel., Osaka, Japan). For the reference, that of commercially obtained HA particles (Fujifilm Wako Pure Chemical) was also measured by BET.

2.5 Estimation of collection efficiency of SOD

SOD solution was prepared by dispersing 4.2 kunits of Cu-Zn type SOD (Fujifilm Wako Pure Chemical) in 15 mL of ultrapure water with ultrasonication for 5 min. The pH value of the SOD solution was 5.7. The 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, or 100 mg of the HA/Fe$_2$O$_3$ particles were dispersed in 5 mL of ultrapure water with ultrasonication for 5 min. Subsequently, both solutions were mixed well. The dispersed HA/Fe$_2$O$_3$ particles were collected using a neodymium magnet and removed from the SOD solution. Subsequently, water in the remained solution was evaporated by heating, and then 20 mL of ultrapure water was added. To estimate the collected amount of SOD, the amount of Zn, which exists at an active center of the SOD, was measured by inductively coupled plasma atomic emission spectrometer (ICP-AES; ICPS-7510, Shimadzu, Kyoto, Japan). For the reference, 4.2 kunits of the SOD were added in 20 mL of ultrapure water, and the amount of Zn was measured by the same procedure.

2.6 Estimation of collection efficiency of urease

Urease solution was prepared by dispersing 25, 50, or 100 mg of urease (Fujifilm Wako Pure Chemical) in 15 mL of ultrapure water with ultrasonication for 5 min. The pH values of the urease solution were 6.45 for 25 mg of urease, 6.71 for 50 mg of urease, and 6.88 for 100 mg of urease, respectively. The 25, 50, 100, 200, or 300 mg of the HA/Fe$_2$O$_3$ particles were dispersed in 15 mL ultrapure water with ultrasonication for 5 min. Subsequently, both solutions were mixed well and then applied ultrasonic vibration for 15 min. The dispersed HA/Fe$_2$O$_3$ particles were collected using a neodymium magnet and removed them from the urease solution. The water in the remained urease solution was evaporated by heating. Then, the remained urease was decomposed by adding both 60% HNO$_3$ (Fujifilm Wako Pure Chemical) and 60% HClO$_4$ (Fujifilm Wako Pure Chemical), and then 20 mL of ultrapure water was added. The amount of Ni, which exists at an active center of urease, was measured by ICP-AES. For the reference, 25, 50, or 100 mg of urease was decomposed by the same process, 30 mL ultrapure water was added, and then the amount of Ni contained in urease was measured by ICP-AES.
2.7 Evaluation of enzyme activity of urease immobilized on HA/Fe₂O₃ particles

The enzyme activity of urease was evaluated by the urease-glutamate dehydrogenase (GLDH) method. The urease-GLDH method is one of the methodologies to measure urea nitrogen based on hydrolyzation by urease reaction [Eq. (1)] and the GLDH reaction by generated NH₃ [Eq. (2)].

\[
\begin{align*}
\text{NH}_3 + \text{HOOC(CH}_2)_2\text{COCOOH} + \text{NADPH} &\rightarrow \text{HOOC(CH}_2)_2\text{CH(NH}_2)\text{COOH} + \text{H}_2\text{O} + \text{NADP}^+ \\
\end{align*}
\]

This measurement is carried out by measuring changes in an absorption maximum of nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) at 340 nm. The value of the enzyme activity of urease was calculated by using Eq. (3), which is based on Lambert–Beer’s law.

\[
A = \frac{(E_1 - E_2) \times 1}{6.22} \times \frac{1}{1} \times \frac{1}{2} \times 3.0 \\
\times \frac{1}{0.1 \times 0.0005} \times \frac{10}{S}
\]

A: Enzyme activity [units·min⁻¹]
E₁: Changes in absorbance in the test [min⁻¹]
E₂: Changes in absorbance in the blank test [min⁻¹]
6.22: Absorbance index of NADPH [mM⁻¹·cm⁻¹]
3.0: Total volume of the solution [mL]
0.0005: Concentration of urease [mg·mL⁻¹]
S: Accurate amount of added urease [mg]

First, the following Solution A–G was prepared by the following procedure.

2.7.1. Solution A (0.01 M of phosphate buffer): 1.74 g of K₂HPO₄·3H₂O and 7.44 g of EDTA-2Na (Fujifilm Wako Pure Chemical) were dissolved in 800 mL of distilled water, and subsequently, this solution was adjusted to pH = 6.9–7.1 with 1 M NaOH (Fujifilm Wako Pure Chemical). Then, the total volume of this solution was adjusted to 1 L with distilled water to prepare 0.01 M of phosphate buffer.

2.7.2. Solution B (0.05 M Tris-HCl buffer): 1.2 g of tris(hydroxymethyl)aminomethane was dissolved in 150 mL of distilled water, and this solution was adjusted to pH = 7.9–8.1 with 1 M HCl. Then, the total volume of this solution was adjusted to 200 mL with distilled water to prepare a 0.05 M Tris-HCl buffer.

2.7.3. Solution C: 3.65 g of 2-oxoglutaric acid (Fujifilm Wako Pure Chemical) was dissolved in 75 mL of distilled water, and this solution was adjusted to pH = 4.9–5.1 with 1 M NaOH (Fujifilm Wako Pure Chemical). Then, the total volume of this solution was adjusted to 100 mL with distilled water.

2.7.4. Solution D: 68 mg of β-NADPH (Fujifilm Wako Pure Chemical) was dissolved in distilled water and adjusted total volume to 5 mL.

2.7.5. Solution E: 0.192 mL of 10,400 units·mL⁻¹ L-GLDH (Toyobo, Osaka, Japan) was dissolved in distilled water, and the total volume was adjusted to 2 mL to prepare 1,000 units·mL⁻¹ GLDH solution.

2.7.6. Solution F: 69 mL of Solution B, 0.3 mL of Solution C, 1.8 mL of Solution D, and 0.9 mL of distilled water were mixed. Then, 24 mL of the above-mixed solution, 0.5 mL of Solution E, and 3.5 mL of distilled water were mixed to prepare a Tris-HCl buffer containing 2-oxoglutaric acid, β-NADPH, and GLDH.

2.7.7. Solution G (urea solution): 3.6 g of urea (Fujifilm Wako Pure Chemical) was dissolved in distilled water to prepare 10 mL of urea solution.

In order to immobilize urease with ca. 200 or 400 mg g⁻¹ of immobilization efficiency per a unit amount of the HA/Fe₂O₃ particles, urease was immobilized on the surface of the HA/Fe₂O₃ particles by the method described in Section II-6 by using 25 mg of urease and 50 mg of the HA/Fe₂O₃ particles or using 200 mg of urease and 300 mg of the HA/Fe₂O₃ particles. As a result, the HA/Fe₂O₃ particles where urease was immobilized at 200 or 433 mg g⁻¹ of immobilization efficiency per a unit amount of the HA/Fe₂O₃ particles were obtained.

The collected HA/Fe₂O₃ particles and urease were dispersed in Solution A, and the total volume was adjusted to 100 mL. Then, 1 mL of this solution was taken, and the total volume was adjusted to 200 mL using ice-chilled Solution A. For the reference, 10 mg of urease was added to Solution A, and a urease solution adjusted to 200 mL by the same method was prepared.

The 0.1 mL of the urease solution and 2.8 mL of the Solution F were placed in an absorption cell (10 mm length) and heated in an incubator at 36.5 °C for 5 min. Then, 0.1 mL of Solution G was added to this solution and measured the absorption of β-NADPH at 340 nm using an ultraviolet–visible spectrophotometer (UV–VIS) (V-550, JASCO, Tokyo, Japan). By this treatment, urea degradability of immobilized urease was examined. As a blank test, a sample containing 0.1 mL of Solution A instead of the urease solution was also measured.

3. Results and discussion

Figure 1 shows the SEM image and the EDX profile of the γ-Fe₂O₃ particles soaked in SAHAF for seven days. Only the γ-Fe₂O₃ particles were observed in the SEM image, and peaks of Ca and P, which are the main constituents of HA, were not found. This result means that HA formation was not induced on the surface of γ-Fe₂O₃ particles by only soaking the γ-Fe₂O₃ particles in SAHAF.

Figure 2 shows XRD patterns of the untreated γ-Fe₂O₃ particles and the HA/Fe₂O₃ particles prepared by mixing γ-Fe₂O₃ particles and ApN, filtering, and then keeping the γ-Fe₂O₃ particles in SAHAF for one day. For the untreated γ-Fe₂O₃ particles, diffraction peaks of γ-Fe₂O₃ were
observed around 2θ = 30, 36, and 43°. For the HA/Fe₂O₃ particles, on the other hand, the intensity of the peaks attributed to γ-Fe₂O₃ was significantly decreased, and broad diffraction peaks of HA were newly observed around 2θ = 26, 27.5, and 32°. This result revealed that the γ-Fe₂O₃ particles were encapsulated with HA matrix. In the SEM observation, it can be seen that the particle size of the obtained HA/Fe₂O₃ particles was approximately 1–2 μm in diameter. Because the average particle size of the untreated γ-Fe₂O₃ particles was less than 50 nm as described in ‘Materials and Methods,’ it is considered that several γ-Fe₂O₃ particles included in the HA matrix. From the viewpoint of the size of the γ-Fe₂O₃ particles, it is considered that γ-Fe₂O₃ nanoparticles were attached to the ApN, and the ApN grew to HA spherical particles in SAHAF. In the EDX spectrum of HA/Fe₂O₃ particles, a peak of Mg was detected, too. Such existence of minute Mg is also characteristic of HA formed in SBF. On the other hand, a peak of Mg was not detected on the commercially obtained HA. It is considered that this Mg might be contained in a part of the HA crystal structure, such as the Ca site.

Figure 4 shows (a) adsorption isotherm of nitrogen gas and (b) specific surface area of the commercially obtained HA particles and the obtained HA/Fe₂O₃ particles obtained by the BET measurement.
specific area than the commercially obtained HA particles. This is because the HA/Fe₂O₃ particles consisted of many fine crystallites consisted of HA, as shown in Fig. 3(a).

Figure 5 shows the estimated collection efficiency of SOD estimated by the amount of Zn obtained by ICP-AES. (b) is an enlarged figure of (a) in the range of less than 10 mg of the added HA/Fe₂O₃ particles. Each dotted straight line shows a 100 % collection efficiency of SOD.

Figure 6 shows the relationship between the estimated collection amount of urease or that per 1 g of HA/Fe₂O₃ particles and the amount of the added HA/Fe₂O₃ particles. Each dotted line shows ideal values when assuming that all the urease in the solution was collected.

that a large amount of urease tended to be collected when much amount of the HA/Fe₂O₃ particles were added. In the case of the 25 mg urease solution, the amount of the collected urease was kept around 23 mg when more than 200 mg of the HA/Fe₂O₃ particles were added. The amount of collected urease per 1 g of HA/Fe₂O₃ particles was decreased along the ideal inverse proportion curve when more than 200 mg of HA/Fe₂O₃ particles. In the case of the 50 mg urease solution, the amount of the collected urease was kept around 49–50 mg when more than 200 mg of the HA/Fe₂O₃ particles were added. In the case of the 100 mg urease solution, the amount of the collected urease was reached to approximately 92 mg when 300 mg of the HA/Fe₂O₃ particles were added. Its collection efficiency corresponded to the ideal inverse proportion curve only at 300 mg of the HA/Fe₂O₃ particles. These results indicate that approximately 90 % of dispersed urease could be collected by using the HA/Fe₂O₃ particles and applying the magnetic field generated by the neodymium magnet also in this case. Because both SOD and urease were successfully collected, it was suggested that the HA/Fe₂O₃ particles were applicable for both acidic enzymes.

On the other hand, it can be seen that the immobilization behavior of urease and SOD was different. Although the pl of urease and SOD is almost a similar level, the molecular weight of urease is nearly 30 times as much as SOD. Hence, it is speculated that the difference in the molecular weight of enzymes might affect their immobilization behavior.

Figure 7 shows the relationship between the estimated amount of collected urease or that per 1 g of Fe₂O₃ particles and the amount of urease initially dispersed in the solution. Fig. 7. Relationship between (a) estimated amount of collected urease or (b) that per 1 g of Fe₂O₃ particles and the amount of urease initially dispersed in the solution. Each dotted straight line shows ideal values when assuming that all the urease in the solution was collected.
Fe₂O₃ particles were continuously increased along the ideal straight line as the initial amount of dispersed urease was increased. In the case of 100 or 200 mg of the HA/Fe₂O₃ particles, both amount of collected urease and that per 1 g of added Fe₂O₃ particles was clearly lower than the ideal value in the case of 100 mg of the initial amount of urease, although these values were increased along the ideal line in the case of less than 50 mg of the initial amount of urease. In the case of 25 or 50 mg of the HA/Fe₂O₃ particles, both the amount of collected urease and that per 1 g of added Fe₂O₃ particles were significantly lower than the ideal values in the entire range of the initial amount of urease.

The above phenomena can be summarized as follows. When the amount of HA/Fe₂O₃ increased, the amount of collected urease per 1 g of HA/Fe₂O₃ particles decreased. In this case, however, even when the amount of initial urease increased, the amount of collected urease per 1 g of HA/Fe₂O₃ particles was near the ideal collection amount. On the other hand, when the amount of HA/Fe₂O₃ decreases, the amount of collected urease per 1 g of HA/Fe₂O₃ particles increases. In this case, however, when the initial amount of urease increased, the amount of collected urease per 1 g of HA/Fe₂O₃ particles was considerably lower than the ideal collection amount. From this result, it is considered that limitation of the amount of collected urease was related to the amount of the added HA/Fe₂O₃ particles, and more urease could be collected when more HA/Fe₂O₃ particles were dispersed in the urea solution.

Figure 8 shows changes in absorbance of β-NADPH at 340 nm when adding 0 mg of urease, 10 mg of urease, 10 mg of immobilized urease (immobilization efficiency of urease: 200 mg g⁻¹ in immobilization efficiency), and 22 mg of immobilized urease (433 mg g⁻¹ in immobilization efficiency) in urea solution. (b) is an enlarged figure of (a) during approximately 10 min of the reaction time.

Figure 9(a) shows a comparison of specific enzyme activity per unit amount of urease between 10 mg of not-immobilized urease, 10 mg of immobilized urease (200 mg g⁻¹ in immobilization efficiency), and 22 mg of immobilized urease (433 mg g⁻¹ in immobilization efficiency) per a unit amount of (a) urease and (b) HA/Fe₂O₃ particles.
unit amount of the HA/Fe₂O₃ particles among 10 mg of immobilized urease and 22 mg one. The specific enzyme activity of the 10 mg of immobilized urease was 6.1 kunits·g⁻¹. In contrast, that of the 22 mg of immobilized urease was 27.2 kunits·g⁻¹, which is about 4.5 times of the 10 mg of immobilized urease. It is suggested that the specific enzyme activity of immobilized urease became larger by increasing enzyme immobilization efficiency.

We recognize several limitations in this study. The first is about the collection amount of enzymes. To evaluate the collection amount of enzymes, we applied the ICP-AES method to measure the metallic component in enzymes. Hence, the effect of free metal ions was not considered strictly. In order to solve this problem, the colorimetric method to measure the metallic component in enzymes. Hence, the efficiency of enzymes on the HA/Fe₂O₃ particles also showed urea decomposition ability in an aqueous solution in high efficiency by the immobilization of urease. The decomposition rate is related to the immobilization efficiency of urease on the HA/Fe₂O₃ particles. This study showed that the HA/Fe₂O₃ particles possessed a potential as promising carriers for applications of enzyme immobilization technique.

Acknowledgment This work was partly supported by Kansai Research Foundation for Technology Promotion.

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