Biomimetic Method for Production of Magnetic Hydroxyapatite Microcapsules for Enzyme Immobilization

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We fabricated hydroxyapatite microcapsules encapsulating γ -Fe₂O₃ particles by biomimetic method using Apatite Nucleus and SBF. In general, hydroxyapatite adsorbs many types of biomolecules like enzyme on its surface. In the present study, to examine enzyme immobilization property of the microcapsules, we immobilized seven types of enzymes with different isoelectric point in phosphate buffered saline (pH 7.40). From results of material analyses, we successfully fabricated hydroxyapatite microcapsules encapsulating γ -Fe₂O₃ with lower crystallinity than that of stoichiometric hydroxyapatite. In comparison with monoclinic hydroxyapatite, the microcapsules adsorbed more enzymes due to the larger specific surface area. As enzyme in the buffer was moderately charged, immobilization efficiency showed the highest value. It is suggested that immobilization efficiency was affected by pI of enzyme and that enzyme immobilization occurred by not only electrical double layer interaction but also ion interaction and other interactions.

Key words: Hydroxyapatite, Magnetic property, Enzyme immobilization, Isoelectric point, Biomimetic method

1. INTRODUCTION

Hydroxyapatite constitutes approximately 69% of human living bones. The crystalline of hydroxyapatite shows needle-like or plate-like and it has a-plane and c-plane. The a-plane of hydroxyapatite is positively charged due to calcium ion and the c-plane is negatively charged due to phosphate groups and hydroxide ion [1, 2]. Hydroxyapatite has an ability to adsorb biomolecules like protein and enzyme. Protein molecules are locally charged negatively and positively by the charged amino acid residues such as aspartic acid, glutamic acid, lysine, arginine and histidine [3]. Therefore, it is suggested that adsorption occurs by electrostatical interaction between protein and hydroxyapatite [4-6].

When the pH or temperature of simulated body fluid (SBF) [7-9] is raised, fine particles of amorphous calcium phosphate are precipitated. In our recent studies, the fine particle highly induces hydroxyapatite formation in SBF and named it Apatite Nucleus (AN) [10].

In our previous studies, we fabricated some kinds of hydroxyapatite microcapsules covering core particles through hydroxyapatite formation induced by AN. The core particles we used were Ag microspheres [11], silicagel microspheres [12], magnetite microspheres [13] and maghemite (γ -Fe₂O₃) nanoparticles [14]. γ -Fe₂O₃ has ferrimagnetism and is chemically stable. The hydroxyapatite microcapsules encapsulating γ -Fe₂O₃ particles can immobilize urease on their surfaces and we can collect them by applying magnetic field. Furthermore, the urease immobilized on the microcapsules can hydrolyze urea [15].

Enzyme immobilization as we immobilized urease enhances pH and thermal stability, and allows us to

reuse enzyme by separation of immobilized enzyme and products. Compared to inorganic catalyst, enzyme is environmentally friendly and thus the enzyme immobilization microcapsules we fabricated are promising environmental materials. In this study, to examine the enzyme immobilization property of the microcapsules, we immobilized seven types of enzymes with different isoelectric point (pI) on the surfaces of the microcapsules.

2. MATERIALS AND METHODS

2.1 Preparation of SBF and Apatite Nucleus

We prepared SBF by Kokubo's method [7-9]. We dissolved reagent-grade NaCl (Wako Pure Chemical Industries, Ltd., Japan), NaHCO₃, KCl (Hayashi Pure Chemical Ind., Ltd., Japan), K₂HPO₄·3H₂O (nacalai tesque, Japan), MgCl₂·6H₂O, CaCl₂ and Na₂SO₄ (Hayashi Pure Chemical Ind., Ltd., Japan) in distilled water and buffered it at pH 7.40 with tris(hydroxymethyl)aminomethane (Hayashi Pure Chemical Ind., Ltd., Japan) and 1 M HCl at 36.5 °C.

The pH value of SBF was raised to 8.40 by dissolution of tris(hydroxymethyl)aminomethane at 25.0 °C. Then, microwave at 700 W was irradiated to the SBF for 9 min and ANs were precipitated in the SBF. It was collected by percolation using a nitrocellulose membrane filter with 50 nm for average pore size (Merck Millipore, USA), rinsed with distilled water, and dried at 36.5 °C.

2.2 Preparation of phosphate buffered saline

We prepared phosphate buffered saline (PBS) by dissolving reagent-grade NaCl (137 mM), KCl (2.68 mM), Na₂HPO₄·12H₂O (10.0 mM, Wako Pure

Chemical Industries, Ltd., Japan) and KH_2PO_4 (1.76 mM, Wako Pure Chemical Industries, Ltd., Japan) and buffering at pH 7.40 with 1 M HCl at 36.5 °C.

2.3 Fabrication and analyses of magnetic hydroxyapatite microcapsules

Ten mg of $\gamma\text{-Fe}_2\text{O}_3$ particles (Aldrich, USA, particle diameter < 50 nm) and 30 mg of ANs were added in 1000 ml of distilled water and dispersed with ultrasonic vibration for 10 min. By this treatment, ANs were attached on the surfaces of $\gamma\text{-Fe}_2\text{O}_3$ particles and immersed them in SBF at pH 7.60 at 36.5 °C for 24 hours. Hydroxyapatite was formed by ANs and we obtained magnetic hydroxyapatite microcapsules covering whole surfaces of $\gamma\text{-Fe}_2\text{O}_3$ particles. They were collected by percolation using a nitrocellulose membrane filter with 300 nm for average pore size (Merck Millipore, USA), rinsed with distilled water, and dried at 36.5 °C. The surface structure and size of the microcapsules were observed by field emission scanning electron microscopy (FE-SEM; SU6600, Hitachi High-Technologies, Japan). The X-ray diffraction (XRD) patterns of samples were obtained by Ultima IV (Rigaku, Japan) with Cu-K α radiation. The elemental composition of the microcapsules were analyzed by energy dispersive X-ray analysis (EDX; XFlash® 5010, Bruker, Germany) and fourier transform infrared spectroscopy (FT-IR; FT-720, HORIBA, Japan).

2.4 Enzyme immobilization on the magnetic hydroxyapatite microcapsules

The suspension of the microcapsules (4 mg/ml in PBS) was treated by ultrasonic vibration for 5 min. The same volume of enzyme solution (2 mg/ml in PBS) was added to the suspension of the microcapsules and mixed well for 24 hours. The enzymes used in this study were pepsin (Aldrich, USA, pI=2.6), invertase (Aldrich, USA, pI=3.8), urease (Wako Pure Chemical Industries, Ltd., Japan, pI=5.0), peroxidase (Wako Pure Chemical Industries, Ltd., Japan, pI=7.2), α -chymotrypsin (Aldrich, USA, pI=8.75), trypsin (Aldrich, USA, pI=10.1) and lysozyme (Wako Pure Chemical Industries, Ltd., Japan, pI=11.0). The microcapsules after mixing were collected by centrifugation and the concentration of enzyme in the supernatant was determined by Bradford's method [16] with ultraviolet and visible spectrophotometer (UV-VIS; U-5100, Hitachi High-Technologies, Japan). Bovine serum albumin (nacalai tesque, Japan) was used as a standard. The immobilization efficiency was calculated by following equation,

$$\text{Immobilization efficiency [\%]} = \frac{C_0 - C_1}{C_0} \times 100$$

where C_0 is the initial concentration of enzyme (mg/ml) and C_1 is the enzyme concentration after immobilization (mg/ml). The above-mentioned experiment using commercially available monoclinic hydroxyapatite powder (Wako Pure Chemical Industries, Ltd., Japan, 4 mg/ml in PBS) instead of the microcapsules was conducted for comparison. All measurements were conducted in triplicate and standard errors were calculated.

3. RESULTS AND DISCUSSION

3.1 Analyses of the magnetic hydroxyapatite microcapsules

Fig.1 shows the SEM image of the hydroxyapatite microcapsules fabricated by using ANs and SBF. It can be seen that the whole surface of the microcapsule was coated by needle-like crystallites and the diameter of the microcapsule was 1-2 μm similar to our previous studies [14,15].

As shown in EDX profile (Fig.2), peaks of calcium and phosphorous derived from a component of calcium phosphate, and peaks of Fe derived from a component of $\gamma\text{-Fe}_2\text{O}_3$, were observed. The FT-IR spectra of $\gamma\text{-Fe}_2\text{O}_3$ particles, the microcapsules and monoclinic hydroxyapatite are shown in Fig.3. In the FT-IR spectrum of monoclinic hydroxyapatite (Fig.3C), characteristic bands assigned to the phosphate groups were observed at 1095, 1060, 1024 and 960 cm^{-1} due to the stretching mode of the P-O bond. The FT-IR spectrum of the microcapsules (Fig.3B), the bands assigned to the phosphate groups were newly observed at 1105, 1051, 1027 and 958 cm^{-1} in comparison with that of the untreated $\gamma\text{-Fe}_2\text{O}_3$ particles (Fig.3A). From the EDX profile and the FT-IR spectra data, it is indicate that calcium phosphate was formed on the surface of $\gamma\text{-Fe}_2\text{O}_3$ particles in SBF and the $\gamma\text{-Fe}_2\text{O}_3$ particles were encapsulated with the calcium phosphate.

The XRD patterns of (A) the microcapsules and (B) the untreated $\gamma\text{-Fe}_2\text{O}_3$ particles are presented in Fig.4. In the diffraction pattern of the microcapsules, new diffraction peaks of hydroxyapatite were appeared at 22, 28, 32, 35, 40, 46 and 49 ° in addition to that of the untreated $\gamma\text{-Fe}_2\text{O}_3$ particles. The peaks of hydroxyapatite exhibited broad shape, implying that hydroxyapatite with lower crystallinity than that of stoichiometric hydroxyapatite covered $\gamma\text{-Fe}_2\text{O}_3$ particles while soaking in SBF.

Fig.1 SEM image of the hydroxyapatite microcapsules fabricated by using ANs and SBF at pH 7.60.

Fig.2 EDX profile of the hydroxyapatite microcapsules fabricated by using ANs and SBF at pH 7.60.

Fig.3 FT-IR spectra of (A) γ -Fe₂O₃ particles, (B) the hydroxyapatite microcapsules and (C) monoclinic hydroxyapatite.

Fig.4 XRD patterns of (A) the microcapsules and (B) the untreated γ -Fe₂O₃ particles.

3.2 Enzyme immobilization of the magnetic hydroxyapatite microcapsules in two types of buffers

The seven types of enzymes which have various isoelectric point (pI=2.6, 3.8, 5.0, 7.2, 8.75, 10.1 and 11.0) were immobilized on the microcapsules and monoclinic hydroxyapatite in PBS and carbonate/bicarbonate buffer. Fig.5 shows enzyme immobilization efficiency of (A) monoclinic hydroxyapatite and (B) the microcapsules in PBS as a function of pI, respectively. In both figures, α -chymotrypsin was immobilized the most and it can be seen that there is no relationship between immobilization efficiency and pI of enzymes. In addition, it is indicated that the amount of enzyme on the microcapsules was more than that of monoclinic hydroxyapatite due to the larger specific surface area of the microcapsules [15]. Then we introduced the absolute value of the difference between pH of the enzyme solution and pI of enzyme ($|pH-pI|$) instead of pI as the x-axis (Fig.6 (A), (B)). When the value of $|pH-pI|$ is closed to zero, free enzyme in the solution is electrostatically neutral and as the value of $|pH-pI|$ becomes larger, enzyme is strongly charged. As shown in Fig.6 (A), (B), immobilization efficiency did not increase or decrease linearly but had the maximum value. In other words, when the enzyme was moderately charged ($|pH-pI|=1\sim 2$), it was immobilized the most on the microcapsules and monoclinic hydroxyapatite. It is suggested that enzyme immobilization occurred by not only electrical double layer interaction but also other interaction such as van der Waals force and locally ion interaction.

The conformational change on immobilized enzymes is an important factor in enzyme immobilization. They occur enzyme denaturation and inactivation. It is reported that the secondary structure of enzyme immobilized on needle-like hydroxyapatite was well maintained [5]. In this study, we immobilized enzymes on the microcapsules which were covered by needle-like hydroxyapatite crystallites. Therefore, we considered that the conformational changes of the enzymes immobilized on the microcapsules did not occur.

Fig.5 Enzyme immobilization efficiency of (A) monoclinic hydroxyapatite and (B) the microcapsules in PBS as a function of pI.

Fig.6 Enzyme immobilization efficiency of (A) monoclinic hydroxyapatite and (B) the microcapsules in PBS as a function of $|\text{pH}-\text{pI}|$. (a) pepsin, (b) invertase, (c) urease, (d) peroxidase, (e) α -chymotrypsin, (f) trypsin and (g) lysozyme.

4. CONCLUSION

We fabricated hydroxyapatite microcapsules encapsulating $\gamma\text{-Fe}_2\text{O}_3$ and immobilized seven types of enzymes with different pI in PBS. Immobilization efficiency of each enzyme on the microcapsules was higher than that of each enzyme on monoclinic

hydroxyapatite because of the larger specific surface area of the microcapsules. There is no relationship between immobilization efficiency and pI of enzymes and then we introduced the absolute value of the difference between pH of the enzyme solution and pI of enzyme ($|\text{pH}-\text{pI}|$) instead of pI. When the enzyme was moderately charged, it was immobilized the most. It is suggested that enzyme immobilization occurred by not only electrical double layer interaction but also other interactions.

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