One-step preparation of organic-inorganic hybrid capsules based on simultaneous gelation and silicification

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Enzyme encapsulation within alginate capsules usually suffers from low encapsulation efficiency and leakage of enzyme. In this study, alginate-aminosilane hybrid capsules with high encapsulation efficiency, good retention and high storage stability of enzyme have been prepared by a novel method, in which viscous solution containing enzyme, calcium chloride as a crosslinker, and 3-(2-aminoethylamino)propyltrimethoxysilane as an aminosilane was dripped into alginate solution, facilitating the formation of alginate-aminosilane hybrid shell at alginate/droplet interface. Results showed that the hybrid capsules could encapsulate formate dehydrogenase with molecular weight of 74 kDa with encapsulation efficiency of 98% and have the ability to protect the enclosed enzyme from denaturation by chaotropic effect of calcium ion. Furthermore, the capsules were reused efficiently for 20 cycles without loss of the enzyme activity. The approach developed in this study could evolve as a generic platform of enzyme immobilization for various biotechnological applications.

KEYWORDS
formate dehydrogenase, organic-inorganic hybrid microcapsule, silane coupling agent, sodium alginate, sol-gel

1 | INTRODUCTION

Over the last decade, a significant challenge in the research of biotechnology is to construct bioreactor systems with single or multiple enzymes for various applications including chemical, pharmaceutical, food industries, and biofuel cells.1-3 To construct bioreactors, immobilization of enzymes into or onto insoluble support materials is usually prerequisite to ensure reusability of enzymes and permit easy biocatalyst-product separation.

Alginate biopolymer has been recognized as a promising candidate for a support of enzyme and cell immobilization because of its biocompatibility and gelation ability with divalent cations or cationic polymers at ambient conditions.4-6 Numerous papers have shown alginate capsules can encapsulate enzymes with variety of molecular weights. On the other hand, however, due to high porosities of alginate gel matrix, leakages of enzymes with molecular weights of less than about 300 kDa are usually observed,7 which needs to engineer its structure with other components such as polymers, inorganics, and/or crosslinkers.8-10

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Recently, biomimetic modification of alginate-based spheres (beads and microcapsules) with various inorganics such as silicon alkoxides, silane coupling agents, sodium silicate, hydroxyapatite, calcium phosphate, calcium carbonate, and titania has gained interest to overcome the aforementioned limitation because of their superior properties such as lower leakage of enzyme, less swelling, and higher stability compared to original alginate gel spheres. Most approaches, however, still remain some issues to be solved such as complicated multisteps procedure, significant loss of enzyme during capsule preparation, and gradual leakage of enzyme from hybrid spheres during reusing. Therefore, a simple, effective, and versatile procedure of microcapsule preparation that encapsulate enzyme with high efficiency should be pursued to provide robust catalyst for biocatalysis.

Herein, we report a novel one-step method of alginate-aminosilane hybrid microcapsules with high encapsulation efficiency and high stability of enzyme. As shown in Figure 1, an aqueous solution containing polyvinylpyrrolidone (PVP) as a thickener, calcium chloride (CaCl₂) as a crosslinker, and 3-(2-aminoethylamino)propyltrimethoxysilane (AAPTS) as an inorganic source was prepared, and dripped into sodium alginate solution though a needle, yielding microcapsules with AAPTS-alginate hybrid shell (AEA-capsule).

As an example, formate dehydrogenase (FDH), an enzyme that is widely investigated as a catalyst for regeneration of NADH in enzymatic syntheses of chiral compound with dehydrogenases but difficult to encapsulate by conventional alginate-based microcapsules due to its low molecular weight (74 kDa), was chosen as the model enzyme for the present study.

The purpose of this study is to prepare aminosilane-alginate hybrid capsules by using the new one-step preparation method to solve the low encapsulation efficiency of the conventional alginate microcapsules without impairing its prominent properties. The effect of preparation conditions on encapsulation efficiency of enzyme, long-term storage stability, and reusability were investigated in this paper.

2 EXPERIMENTAL

2.1 Materials

Sodium alginate and CaCl₂ were purchased from Kanto Chemical Co, Inc. The PVP (a molecular weight of 130 kDa) was purchased from Alfa Aesar. The FDH from *Candida boidinii* (EC.1.2.1.2) was purchased from Roshe. The NAD⁰ and NADH were available from Oriental yeast Co, Inc. The AAPTS and N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid were purchased from Tokyo Kasei Kogyo Co, Inc. All other chemicals used in the experiments were reagent grade and were used without further purification.

2.2 Preparation of AAPTS-alginate hybrid microcapsules

The preparation of AEA-capsules was conducted with a homemade coaxial air-stripping extrusion device. In brief, 25 mM N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer solution containing 7.5% (v/v) PVP, CaCl₂, AAPTS, and 2 U/ml FDH with an appropriate pH value was prepared as a core solution. The core solution was dripped into a
vigorously stirred 1% (w/v) sodium alginate solution (5 ml) through the inner nozzle using a microsyringe pump at a volumetric flow rate of 0.2 ml/min. Nitrogen gas was passed through the outer nozzle in order to control the droplet size. After 2 minutes of dripping, the mixture was kept undisturbed for 4 minutes, and then diluted threefold by adding distilled water. After another 30 seconds, the capsules were collected by filtration. Alginate microcapsules (Alg-capsule) were also prepared in the same manner except with no addition of AAPTS.

The surface morphology of AEA-capsules was evaluated from microscopic images captured by a digital camera (Canon A570IS powershot) connected to an optical microscope (Kenis, Japan, Model-LB). For the imaging by scanning electron microscopy, capsules were cut in half and washed with water for several times to eliminate PVP core solution and then freeze dried. Then, the samples were sputter coated with platinum and observed using field-emission scanning electron microscope (FE-SEM S-4500, Hitachi).

2.3 Activity measurement of FDH encapsulated in AEA-capsules and Alg-capsules

For activity measurement of FDH-encapsulated AEA-capsules and Alg-capsules, 5.8 mL of 0.05 M Tris-HCl buffer solution (pH 7.5) containing 0.02 M CaCl₂, NAD⁺, and the capsules (0.2-0.3 g) was prepared, and was incubated at 30°C for 30 minutes with gentle stirring (150 rpm). After that, the FDH-catalyzed reaction was started at 30°C by adding 0.2 ml of sodium formate solution into the reaction mixture and continued for 60 minutes. The concentrations of NAD⁺ and sodium formate at the beginning of the reaction were 1 mM and 30 mM, respectively. Samples (0.8 mL) were taken out at various intervals and the concentration of NADH produced in the solution was determined by measuring the absorbance at 340 nm using spectrophotometer (V-530, Jasco), and then the samples were returned to the reaction solution.

The encapsulation efficiency (%) was defined as the percentage of the activity of FDH in the core solution taken from AEA-capsules with respect to that in the original core solution before encapsulation as follows 19-21:

\[
\text{Encapsulation efficiency (\%)} = \frac{E_{n_{\text{encapsulated}}}}{E_{n_{\text{initial volume}}}} \times 100. \tag{1}
\]

\(E_{n_{\text{encapsulated}}}\) is the activity of FDH in the core solution (μmol-min⁻¹·g-core⁻¹) obtained by crushing AEA-capsules. \(E_{n_{\text{initial volume}}}\) is the activity of the core solution (μmol-min⁻¹·g-core⁻¹) before encapsulation.

The activity yield was determined by Equation (2)

\[
\text{Activity yield (\%)} = \frac{A_{c_{\text{capsule}}}}{A_{c_{\text{free}}}} \times 100. \tag{2}
\]

\(A_{c_{\text{capsule}}}\) is the activity of the encapsulated FDH (μmol-min⁻¹·g-capsule⁻¹). \(A_{c_{\text{free}}}\) is the activity of FDH in the core solution (μmol-min⁻¹·g-core⁻¹) obtained by crushing AEA-capsules. The core solution in the capsules was taken out as follows: the adsorbed water on the surface of the capsules was removed by filter paper, and then the capsules in a microtube were crushed and squeezed using a pestle. The obtained core solution (0.05 mL) was used for activity measurement to obtain \(E_{n_{\text{encapsulated}}}\) and \(A_{c_{\text{free}}}\).

2.4 Reusability assay

The reusability test of the encapsulated FDH was performed by conducting the activity measurement of encapsulated FDH. Experiments were performed using the same AEA-capsules in the reactor for 20 cycles. At the end of each batch, the AEA-capsules were separated by mesh and washed with water, followed by reusing the AEA-capsules in a new reaction cycle without any treatment. The recycling efficiency (%) was defined as the percentage of the activity of AEA-capsules at certain cycles with respect to that at first cycle as follows:

\[
\text{Recycling efficiency (\%)} = \frac{\text{activity in the } n^{\text{th}} \text{ order}}{\text{activity in the } 1^{\text{st}} \text{ order}} \times 100. \tag{3}
\]
3 RESULTS AND DISCUSSION

3.1 Observation of AEA-capsules

Alginate microcapsules with alginate shells and aqueous core are generally prepared by dripping viscous solution containing CaCl₂ as a crosslinker into sodium alginate solution (Alg-capsules). In the present method, the organic-inorganic hybrid capsules with alginate-AAPTShybrid shell and aqueous core (AEA-capsules) were produced in the same manner, except with addition of AAPTTS in a core solution. While some thickeners such as carboxymethylcellulose, xanthan gum, and starch have been used for preparing Alg-capsules, PVP, a water-soluble and low toxic to living tissues, is selected as thickener in this study because it has no carboxyl groups in its structure, and preliminary test showed that AAPTTS remained stable in soluble form in aqueous PVP solution.

As can be seen from Figure 2A and 2B, the AEA-capsule was spherical in shape with uniform size, and the shell membrane was much thinner (below 10 μm) compared to that of Alg-capsule (about 200 μm). The SEM images showed that the surface of AEA-capsule (Figure 2C) was smoother than that of Alg-capsule (Figure 2D). Furthermore, it can be seen from Figure 2E that AEA-capsule have a homogeneous structure with the thickness of ~10 μm. EDX analysis confirmed the existence of Si (AAPTTS) and calcium in the shell of AEA-capsule (Figure 2F). Our previous studies, describing two-step preparation method of alginate-aminosilane hybrid capsules, have shown that soaking of Alg-capsules in an aqueous solution containing aminopropyltriethoxysilane (APTES) induced gradual shrinkage of alginate shell along the thickness direction with time course and finally formed thin shell membrane with homogenous structure. The results of the morphological and EDX analyses were consistent with those obtained in the previous study. Therefore, these results suggested that AAPTTS was successfully incorporated into alginate shell via an electrostatic interaction between positively charged amino groups of AAPTTS and negatively charged carboxyl groups of alginate along with polycondensation of AAPTTS.

FIGURE 2 Optical images of (A) an AEA-capsule and (B) alginate microcapsules (Alg-capsule). The FE-SEM images of the surface of (C) AEA-capsule and (D) Alg-capsule. E, Cross-section of an AEA-capsule shell; F, The result of EDX analysis of the shell of an AEA-capsule
3.2 Encapsulation of FDH in AEA-capsules

Encapsulation of FDH in AEA-capsules was successfully demonstrated, and AEA-capsules were found to facilitate the reduction of NAD\(^+\) to NADH coupled with formate oxidation. Then, effects of encapsulation conditions such as AAPTS concentration, CaCl\(_2\) concentration and pH of core solution on enzyme encapsulation were investigated with different core solutions prepared by changing one of the three factors.

It can be seen from Figure 3A that encapsulation efficiency of FDH in AEA-capsules was increased with an increase of AAPTS concentration at 0.05 M CaCl\(_2\) in the core solution. On the other hand, the increase of CaCl\(_2\) concentration at 0.1 M AAPTS resulted in the decrease of the encapsulation efficiency (Figure 3B). In the preparation process, the electrostatic interaction of alginate with AAPTS could be competitive with calcium ion. Thus, the increase of AAPTS or decrease of CaCl\(_2\) in a core droplet might result in the increase of AAPTS molecules at droplet/alginate interface, leading to the rapid electrostatic interaction of alginate by AAPTS. On contrary, the increase of calcium ion at droplet/alginate interface might inhibit the electrostatic interaction of alginate with AAPTS. Furthermore, the increase in pH of core solution led to the increase of encapsulation efficiency, and 98% of the encapsulation efficiency was achieved at below pH 7 (Figure 3C). In the case of the two-step preparation, based on the fabrication of Alg-capsules and subsequent electrostatic interaction of APTES with alginate,\(^{24}\) the encapsulation efficiency was below 40%, due to the leakage of FDH during the fabrication process of Alg-capsules before treatment with APTES. In the present method, the electrostatic binding of AAPTS to alginate and gelation of alginate by CaCl\(_2\) at droplet/alginate interface would occur simultaneously. Therefore, such a high encapsulation efficiency would be due to a rapid formation of the hybrid shells before FDH leaked from the core droplet in alginate solution.

3.3 Effects of capsule size

Generally, enzymatic reactions inside microcapsules are limited by the diffusion of substrates. Thus, the size of microcapsules may be one of the most important parameters of enzyme immobilization, as reducing the size of microcapsules will reduce the diffusion distance of the substrate to enzyme inside the microcapsules. In this study, AEA-capsules with different sizes were prepared by changing the size of droplets containing FDH, AAPTS, and CaCl\(_2\) solution under
different nitrogen flow. Figure 4 shows the results of activity yield ($\eta$), encapsulation yield (En), and $\eta$En with microcapsules of different diameters. $\eta$En indicates the efficiency of the enzyme activity based on the initial amount of the enzyme applied to the encapsulation. As expected, the activity yield increased with decreasing capsule diameter. In contrast, encapsulation efficiency (En) remained almost constant with diameters ranging from 1.6 to 3.0 mm, but decreased with smaller diameter of 1.3 mm. A decrease of microcapsule size corresponds to an increase in a surface area of a microcapsule. Since the leakage of FDH from a droplet per unit surface area would be constant, the decrease of En at smaller diameter would be due to the increased surface area of the droplet. The relationship between $\eta$En and the diameter of the AEA-capsule revealed that $\eta$En had the maximum value when the size of the AEA-capsules was around 1.6 mm diameter under the experimental condition.

### 3.4 Storage stability of encapsulated FDH

The long-term storage stability of immobilized enzymes is required for wide range of industrial applications. We therefore evaluated the activity of FDH during storage at 4°C over 50-day period for (1) AEA-capsules in a buffer solution (pH 7.5, 0.05 M Tris-HCl buffer) containing 1 mM NAD$^+$ and 0.1 M calcium chloride (CaCl$_2$), (2) free FDH in the same buffer solution, and (3) free FDH in the buffer solution without the addition of CaCl$_2$. The free FDH in the buffer solution with or without CaCl$_2$ were found to retain 14 and 82% of its initial activity, respectively, during 50 days (Figure 5). The decrease of the enzyme activity in the presence of CaCl$_2$ was likely due to denaturation of FDH by chaotropic effect of calcium ion.$^{25,26}$ In contrast, the AEA-capsules retained their full activity during 50 days in the presence of CaCl$_2$. These results indicate that AEA-capsules have the excellent ability to protect FDH from the negative effect of CaCl$_2$ and improve the storage stability of FDH.

### 3.5 Reusability of FDH-encapsulated AEA-capsules

The reusability of AEA-capsules, demonstrated by examining loss in activity of AEA-capsules during reusing cycles (Figure 6), showed that no loss in catalytic activity was found for AEA-capsules after 20 repeated batches. In contrast, the rapid decrease in the catalytic activity was observed in the case of Alg-capsules. It is well known that FDH have a molar weight of 76 kDa and is difficult to retain in alginate gels due to their large pores. Encapsulation of FDH within alginate-based hybrid/composite spheres was studied by some researchers. Lu et al.$^{14}$ confirmed gradual leakage of FDH.
from alginate-silica hybrid beads, obtained by using tetramethoxysilane and alginate. Zhang et al in 2009 showed that FDH encapsulated in alginate/chitosan-hydroxyapatite composite capsules retained more than 60% initial activity after seven repeated cycles due to prevention of FDH leakage from the composite capsules. Furthermore, many effective step-by-step preparation methods for the modification of alginate-based microspheres with inorganic materials such as silicate, hydroxyapatite, calcium phosphate, calcium carbonate, and titania have been applied for encapsulation of enzyme with molecular weight ranging 14.1 to 27.5 kDa. However, gradual leakage of enzymes was still found.14-17,27-36 Considering that, in enzyme encapsulation, it is a common principle to physically prevent the permeation of enzyme through capsule membrane, the result suggests that AEA-capsules have smaller pores compared to those obtained by the other methods. The high reuse stability of AEA-capsules might be attributed to the dense and homogenous hybrid structure formed by three-dimensional organization of AAPTS in the framework of calcium alginate matrix. Therefore, the results demonstrated that AEA-capsules have a superior performance over other step-by-step preparation method with alginate-based microcapsules.

4 CONCLUSIONS

We have demonstrated the successful preparation of enzyme-encapsulated organic-inorganic hybrid microcapsules without impairing the handling capacity of alginate. The fabrication process simply encapsulates the enzyme inside the microcapsule with high encapsulation efficiency of 98%. The study on the effect of capsule size on the encapsulation and activity yields of FDH showed that the optimal size of the capsule for efficient encapsulation of FDH was 1.6 mm in diameter. Compared to conventional alginate-based microcapsules, the AEA-capsules demonstrated unprecedented storage and reuse stabilities. The approach developed in this study could evolve as a generic platform of enzyme immobilization for various biotechnological applications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

AUTHOR CONTRIBUTIONS

Fumio Kurayama, Conceptualization-Lead, Data curation-Lead, Formal analysis-Lead, Funding acquisition-Supporting, Investigation-Lead, Methodology-Lead, Project administration-Lead, Validation-Lead, Visualization-Lead, Writing-original draft-Lead, Writing-review & editing-Lead; Newaz Bahadur, Writing-review & editing-Supporting; Masahide Sato, Investigation-Supporting; Takeshi Furusawa, Writing-review & editing-Supporting; Noboru Suzuki, Funding acquisition-Lead, Resources-Lead, Supervision-Furusawa, Equal, Writing-review & editing-Supporting.
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