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Encapsulation of catalase into nanochannels of an inorganic composite membrane

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ABSTRACT

Enzymes, especially those known as membrane proteins existing in plasma membranes, direct important and complicated reactions in living bodies. Thus, attempts have been made to extract such enzymes from living bodies, and immobilize and accumulate them on supports to effectively use their functions for catalysis [M. Hartmann, Chem. Mater. 17 (2005) 4577–4593]. However, enzymes extracted from living bodies tend to aggregate in the absence of detergents or at high concentrations, resulting in a loss of their activities [Y. Urabe, T. Shiomi, T. Itoh, A. Kawai, T. Tsunoda, F. Mizukami, K. Sakaguchi, ChemBioChem 8 (2007) 668–674]. We have, however, succeeded in assembling a highly durable membrane capable of high-density accumulation and providing a regular array of catalase by encapsulating it in mesoporous silica synthesized in the pores of an alumina membrane. The artificial biomembrane showed not only activity similar to that of the native catalase for the decomposition of H_2O_2 but also much higher stability; the catalase immobilized in the membrane still retained its original activity even after being employed 160 times in decomposing H_2O_2, whereas the native lost its activity after 40 cycles.

Enzymes have been neglected as catalysts in organic synthesis due to high enzyme cost, contamination of products by residual protein and low stability. However, the encapsulation of proteins into a mesoporous silicate results in increased resistance to organic solvents, or surroundings, in comparison with the native [3–9]. It is well known that membrane proteins direct an array of proteins and complicated reactions in living bodies. These facts strongly suggest that if enzymes were immobilized in the pores of an inorganic membrane, the inorganic membrane with the enzyme could be a very useful reactor for organic synthesis. Recently, the synthesis of nanoporous silicas in anodic alumina pores has been tried elsewhere [10–14], and the resultant composite membranes have shown high potential as artificial membrane supports due to the high number of interconnected pores. However, for the artificial biomembrane to act effectively, the direction of mesoporous silica channels prepared in the pores of a porous alumina membrane obtained by anodic oxidation should be the same as the alumina columnar pore direction, i.e., perpendicular to the face of the base alumina membrane (Fig. 1a and b) [10,15]. As can be seen from the SEM images (Fig. 1c and d) and TEM image (Fig. 1e) of the surface of the silica–alumina composite membrane slightly etched with a 10% phosphoric acid solution, each mesoporous silica tube in its alumina pore is perpendicular to the alumina membrane face, having a diameter of about 200 nm, corresponding to that of the alumina columnar pore. The nanochannels of the silica tube run predominantly parallel to the wall of the columnar alumina pore (Fig. 1e). When single component helium was fed into the membranes, the helium permeances for the base and composite were approximately 8000 and 500 nmol m^{-2} s^{-1} p^{-1}, respectively, indicating that the pores of the base membrane narrowed because of the mesoporous silica preparation there. For binary mixtures of helium and water at 40 °C, the membranes showed the helium permeances depicted in Fig. 2 as a function of the partial pressure of water, i.e., the permeance of the base membrane was constant to approximately 8000 nmol m^{-2} s^{-1} p^{-1} for the mixtures containing water vapor, but that of the composite membrane decreased to 5.6 nmol m^{-2} s^{-1} p^{-1} at a relative water pressure of 0.63, indicating that as the water vapor condenses in the silica pores, it inhibits the permeation of helium molecules. Converting the relative vapor pressure to the effective pore diameter for permeation by a Kelvin

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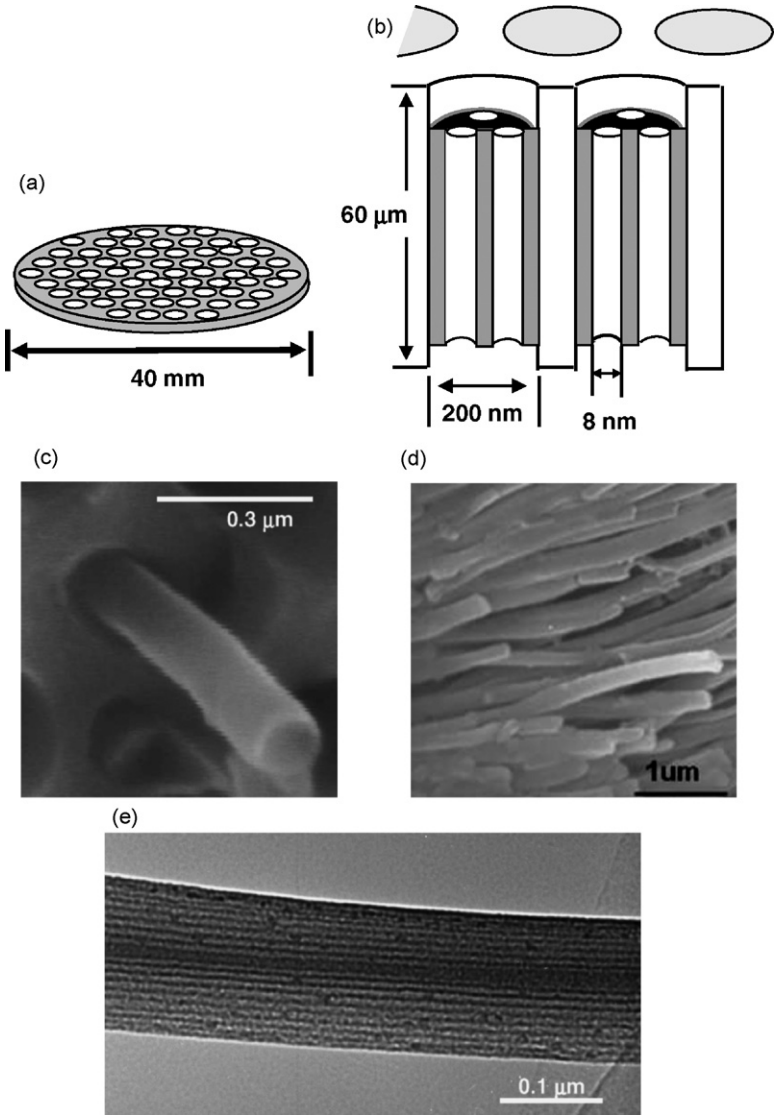


Fig. 1. Schematic illustration of: (a) the anodic alumina membrane used in this work; (b) the assembly of silica-surfactant nanochannels (channel diameter = 8 nm) formed inside the columnar alumina pore (pore diameter = 200 nm). Typical SEM images: (c) cross-sectional view of the alumina membrane; (d) columnar structures formed in the alumina pore after treatment with the precursor solution. Typical TEM image of columnar mesoporous silica: (e) side-view of columnar structures formed in the alumina pore. The columnar structures were obtained as a white precipitate by complete etching of the alumina membrane and collected by filtration.

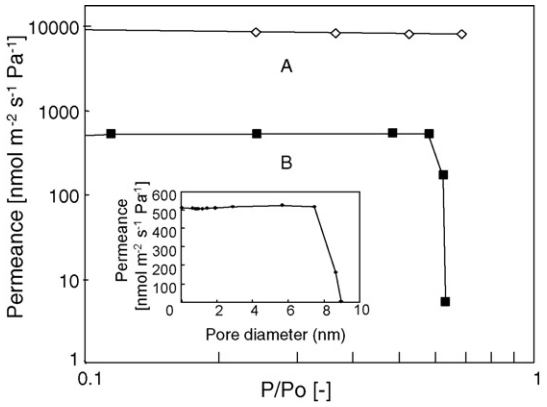


Fig. 2. The permeation properties of the anodic alumina membrane (curve A) and NAM (curve B) as a function of the partial pressure of water vapor in the feed stream for binary mixtures of helium and water vapor at 40 °C.

equation [16], the average pore diameter obtained was ca. 8 nm (see inset in Fig. 2), which is comparable to the diameter observed by TEM [15]. Thus, the alumina columnar pores of the composite membrane are filled with silica tubes consisting of nanochannels, which run parallel to the columnar pore and perpendicular to the base membrane face, with a channel diameter of about 8 nm, and the composite membrane is a silica nanochannel-incorporated alumina membrane (NAM).

Recently, confinement of enzymes into inorganic nanospaces has been a subject of investigation [3–9], anticipating that the enzymes will become stabilized or more tolerant of environmental factors such as temperature, pressure, media, etc. We ourselves have reported that the encapsulation of enzymes in mesoporous silicates not only increases the stability of enzymes but also results in new capabilities [2,8,9]. Naturally, it should then be reasonable to expect the assembly of a high-performance, artificial membrane accumulating enzymes confined in inorganic nanospaces, but no such reports have been published to date. The silica–alumina composite membrane mentioned above at last enables the assembly of

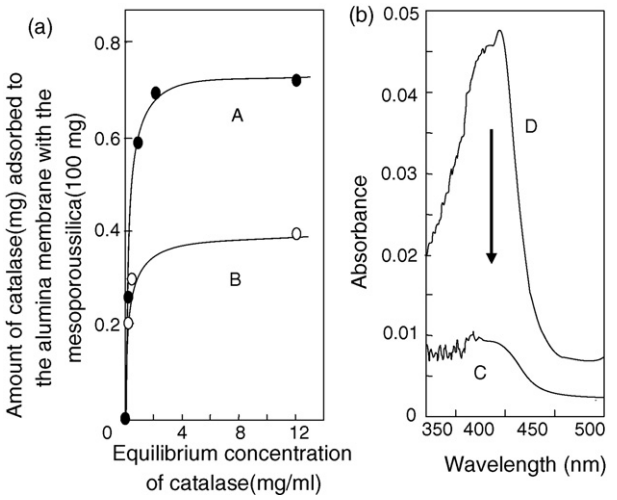


Fig. 3. (a) The adsorption of catalase onto NAM (curve A) and the anodic alumina membrane (curve B) in water, measured spectrophotometrically with respect to the equilibrium concentrations of catalase and catalase-membrane. (b) Curve C: absorption of supernatant solution obtained after the composite membrane containing catalase (0.7 mg/100 mg) was completely etched with 10 ml of phosphoric acid, i.e., the alumina part was completely dissolved. Curve D: absorption of the phosphoric acid containing the catalase (0.7 mg/10 ml) amount corresponding to the amount of catalase adsorbed into the composite membrane.

an artificial biomembrane capable of accumulating enzymes and arranging them regularly.

For the introduction of an enzyme into the silica nanochannels, a catalase, which consists of four subunits containing heme with a molecular weight of 250,000 (size: ca. 7 nm × 8 nm × 10 nm; PDB, 1TGU) that completely loses its activity when the subunits dissociate to the individuals, was chosen [17,18]. Fig. 3a shows the adsorption of catalase onto the base and composite membranes by immersing them in an aqueous solution containing the catalase. From the comparison of curves A and B in Fig. 3a, it is found that the composite membrane can effectively immobilize catalase, but the immobilizing ability of the base or anodic alumina membrane is much lower. On the other hand, the BET surface areas of NAM decreased with an increase in the amount of catalase adsorbed into the pores of NAM (Table 1). This suggests, consistent with the reported data [2,8,9], that the catalase was encapsulated into the silica nanochannels. First, we attempted to dissolve only the alumina part of the composite membrane containing catalase. When the composite membranes containing catalase were completely etched with phosphoric acid, the UV–vis absorption of the resultant solutions showed the existence of catalase in the concentration corresponding to the amount of catalase adsorbed onto the membrane. The quantity of catalase detected in the supernatant solution after centrifugation, however, was below 20% (Fig. 3b, curve C) of the amount of catalase adsorbed onto the composite membrane (Fig. 3b, curve D), suggesting that over 80% ($D_{at\ 395\ nm} - C_{at\ 395\ nm}$) of catalase introduced into the composite membrane still existed in nanochannels of the silica part not etched with phosphoric acid

Table 1
Physicochemical properties of the catalase-NAM

Compound	Catalase adsorbed (mg/100 mg NAM)	Specific surface area ($m^2\ g^{-1}$)
Catalase-NAM	0	25.3
	0.16	21.0
	0.36	17.2
	0.71	9.8
Alumina membrane	–	6.4

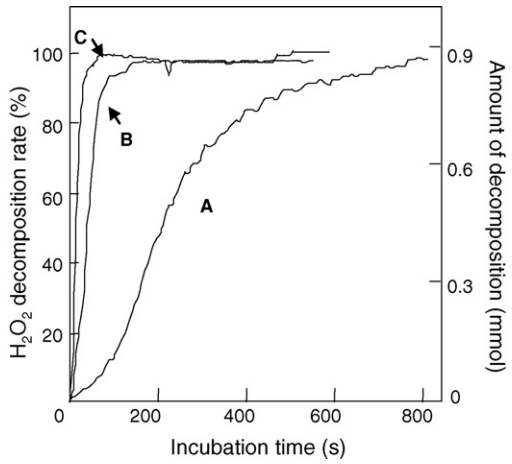


Fig. 4. H_2O_2 decomposition of catalase in water for catalase-NAM (A), catalase-FSM-16 (B) and native catalase (C).

and remained in the precipitation after centrifugation. Accordingly, we conclude that most of the catalase is adsorbed onto the silica nanochannels in the composite membrane. However, the catalase adsorbed into the membrane (Fig. 4, curve A) shows a low activity rate compared to catalase-FSM-16 (Fig. 4, curve B) or the native (Fig. 4, curve C). This is probably attributable to the slow diffusion of H_2O_2, delaying its coming into contact with the catalase in the silica channels in the alumina pores. We went on to incubate catalase and the catalase-encapsulated composite membrane with 0–0.2 mg/ml trypsin, a proteolytic enzyme that can completely break up catalase, and tested their respective activities for the decomposition of hydrogen peroxide. As Fig. 5 shows, in the native, the addition of 0.0025 mg/ml trypsin resulted in a significant reduction in activity (to ca. 2%). On the other hand, the catalase-encapsulated composite membrane exhibited little reduction of activity, even following the addition of 0.1 and 0.2 mg/ml trypsin solutions (100%). Although trypsin is small enough to enter the silica channels of the composite membrane, it seems to become mostly trapped on the alumina pore walls and at the silica channel entrances of the composite membrane before contacting the catalase in the silica nanochannels. These results further indicate that the catalase is encapsulated in the silica channels in the alumina pores of the composite membrane, consistent with the finding mentioned in the preceding paragraph.

The composite membranes, both with and without catalase, were applied to the decomposition of H_2O_2. The former showed very high activity, similar to that of the native catalase just extracted

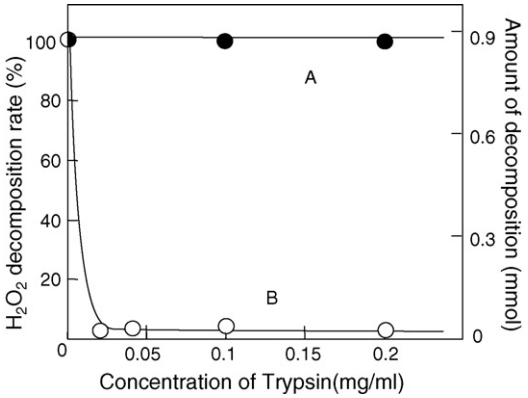


Fig. 5. Plots of activity retained with increasing trypsin (0–0.2 mg/ml). Curve A: catalase (0.7 mg) in NAM. Curve B: free catalase (0.7 mg).

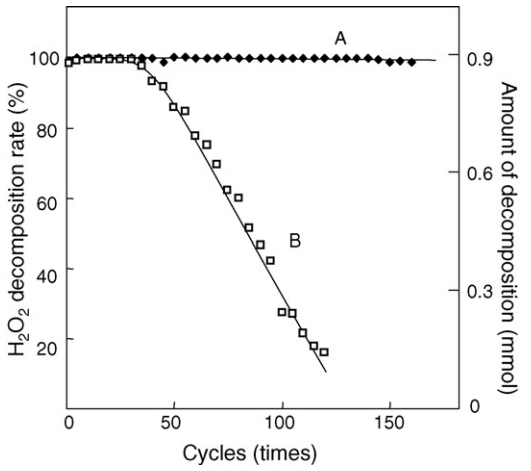


Fig. 6. Cycling-life test of catalase-NAM after incubation of 36 days at 4 °C. Curve A: catalase in NAM membrane (100 mg: 0.7 mg as catalase). Curve B: free catalase (0.7 mg).

from a living body, but, naturally, the latter was entirely ineffective for decomposition. Interestingly, it is well known that enzymes extracted from living bodies progressively lose their activity [2,18]; catalase encapsulated in the silica nanochannels, however, does not lose its activity easily. Fig. 6 shows the H_2O_2 decomposition profile of the catalase-encapsulated composite membrane after the membrane had been incubated for 36 days at 4 °C, together with the profile of the native catalase incubated under the same conditions. The catalase-encapsulated composite membrane could still completely decompose H_2O_2, even after 160 cycles. Moreover, during the reaction, no release of catalase from the membrane was observed. However, the native begins to deactivate after 40 cycles and decomposes only 16% of the H_2O_2 fed to it after 120 cycles. It is thus found that the encapsulation of catalase in the silica nanochannels increases its stability, prolonging its life time.

So far, it has commonly been recognized that the immobilization of enzymes in supports lowers their activity [1]. This is attributed to the fact that most reactants find it difficult to approach enzymes enclosed within the supports, and, furthermore, enzymes sometimes change, to a greater or lesser degree, their conformation or structure when immobilized. However, in this study, such lowering in the catalase activity was not observed, and the catalase-encapsulated composite membrane, i.e., the artificial biomembrane, continued to show a very high capacity for H_2O_2 decomposition. This seems to be due to the fact that catalase hardly changes its original structure with encapsulation [2,9], and also that the silica nanochannels still possess some margins for small molecules or H_2O_2 to pass freely, even after encapsulation.

In conclusion, an artificial biomembrane was able to be produced by introducing an enzyme, catalase, into silica nanochannels prepared in the columnar pores of an anodic alumina membrane. The encapsulation of the enzyme in the silica nanochannels enables high-density accumulation of the enzyme without aggregation, arranging it regularly, increasing its stability, and thus prolonging its lifetime.

1. Experimental

1.1. Characterization

SEM images were measured with a field-emission SEM (Hitachi S-800). The sample membrane was fixed on a SEM stage using carbon tapes and the SEM measurements performed after deposition of a thin Pt/Pd layer using an ion-sputter (Vacuum Device Inc., Model MSP-10, Japan). TEM images were measured on a JEM-200EX (JEOL) equipped with a field-emission gun and operated at 200 kV. DTA–TG curves were collected on a Thermo-plus TG8120 thermal analyzer (Rigaku Co., Ltd., Japan) at a heating rate of 10 °C min^{-1}. Nitrogen adsorption and desorption measurements at 77 K were carried out on a Belsorp-Max (Japan BEL Co., Ltd., Japan).

1.2. Preparation of composite and catalase-encapsulated membranes

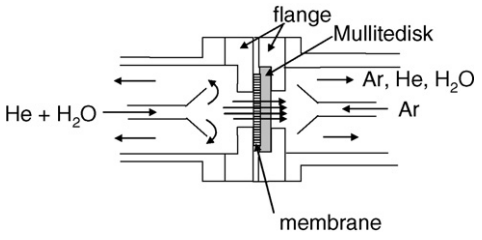
The precursor solutions containing a triblock copolymer surfactant (P123) were prepared according to the literature, with some modifications [10,15]. A typical procedure for synthesis was as follows. The P123 (1.0 g) was dissolved in a mixture of ethanol (15 g), HCl (0.10 g of 37 wt.% aqueous solution), and water (2.0 g). This mixture was stirred for 1 h at 60 °C in a flask with a reflux condenser. TEOS (2.13 g) was then added to the mixture, and the final mixture was stirred for a further 17 h at 60 °C. This mixture was immediately used as the precursor solution for the preparation of the silica–alumina composite membrane mentioned below.

An anodic alumina membrane was set in an ordinary membrane filtration apparatus and the precursor solution (4 ml) was dripped onto the alumina membrane. Moderate aspiration was applied by using a diaphragm vacuum pump (Model UN820, KNF Neuberger, Inc.), so that the precursor solution penetrated the columnar alumina pores. After the dripped precursor solution completely penetrated the alumina pores, the alumina membrane, including the precursor solution, was dried under aspiration for 10 min. The resulting sample was then calcined at 500 °C for 6 h in air to obtain the silica–alumina composite membrane.

A catalase-encapsulated membrane was prepared as follows. To 10.0 ml of catalase (0–120 mg) dissolved in water was added the above composite membrane. The membrane was then kept standing for 12 h at 4 °C to establish an adsorption equilibrium. The amount of catalase adsorbed into the pores of the composite membrane was determined by measuring the absorbance of the supernatant at 406 nm, which is characteristic of the absorption band of the catalase, and additionally verified by the TG decline on the TG-DTA curves.

1.3. Gas permeation tests

Permeation properties through the membrane were determined for binary mixtures of helium and steam at 40 °C using the setup shown in Scheme 1 [19]. The membrane was connected to stainless-steel flanges using a resin (Varian, Torr Seal). To reinforce the membrane, a porous mullite disk (Nikkato Corp., *F*, diameter = 25 mm, thickness = 2 mm, porosity = 22%) was placed between the permeate side of the membrane and the stainless-steel flange. The membrane was fixed to a permeation cell using Teflon sealing rings. Water vapor was fed to the membrane by bubbling with helium, and the composition was adjusted by mixing with helium.



Scheme 1. Schematic of gas permeation test.

Most of the feed mixture did not permeate the membrane, but escaped from the cell. Argon was purged to the permeate side of the membrane as a sweep gas, and argon and the permeated helium and water vapor evacuated from the cell. The compositions of the feed and permeated mixtures were analyzed by a gas chromatograph (GC-TCD, Agilent Technologies, A3000) equipped with PLOT-Q as a separation column. The flow rate of the mixture was determined by a flow meter.

1.4. Pulverized biomembrane activity measurement

The composite membrane containing catalase was first pulverized to obtain powder composites for the comparison of activity of native with catalase-FSM-16. The catalase activity of the catalase-encapsulated composite membrane was measured using hydrogen peroxide as the substrate. The pulverized biomembrane (2 mg: 0.014 mg as catalase), catalase-FSM-16 (2 mg: 0.014 mg as catalase), or catalase (0.014 mg) was suspended in 3 ml of water at 2 °C containing hydrogen peroxide (1%; 0.297 mol/l). The catalase activity of pulverized membrane in water was spectrophotometrically determined by measuring the absorbance decrease at 270 nm.

1.5. Stability against proteolytic enzyme, trypsin

The enzymatic stability of the catalase-encapsulated composite membrane was measured by treatment with the proteolytic enzyme, trypsin, followed by the evaluation of the catalase activity using hydrogen peroxide. The membrane (100 mg: 0.7 mg as catalase) was set in an ordinary membrane filtration apparatus after incubation for 3 h at 37 °C in the absence of trypsin (0–0.2 mg/ml) and 3 ml of 1% hydrogen peroxide (0.297 mol/l) was dropped onto the membrane. The amount of dismutation of hydrogen peroxide was spectrophotometrically determined by measuring the absorbance at 270 nm. For comparison, free catalase (0.7 mg) incubated for 3 h at 37 °C in the absence of trypsin (0–0.2 mg/ml), was adsorbed on to mesoporous silica and set on an ordinary membrane filtration apparatus, after which the 1% hydrogen peroxide (0.297 mol/l) was dropped onto the catalase.

1.6. Cycling-life test of catalase

The catalase activity of the catalase-encapsulated composite membrane was measured using hydrogen peroxide as the substrate. The membrane (100 mg: 0.7 mg as catalase) was set in an ordinary membrane filtration apparatus after incubation for 36 days at 4 °C and 1% hydrogen peroxide (0.297 mol/l) was dripped onto the membrane. The amount of dismutation of hydrogen peroxide was spectrophotometrically determined by measuring the absorbance at 270 nm. Comparison with native catalase was performed by fixing the catalase on mesoporous silica whose pores cannot accommodate it. Specifically, the free catalase (0.7 mg), incubated for 36 days at 4 °C, was adsorbed on mesoporous silica (pore diameter: 2.7 nm) set on an ordinary membrane filtration apparatus, and 1% hydrogen peroxide (0.297 mol/l) was dripped onto the catalase.

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