Enzyme catalytic membrane based on a hybrid mesoporous membrane†

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Immobilization of glucose oxidase (GOD) within a hybrid mesoporous membrane with 12 nm pore diameter was successfully achieved, resulting in catalytically high efficiency during flow of a glucose solution across the membrane.

Enzyme encapsulation within a porous inorganic host has provoked significant interest because it can render enzymes more mechanically robust and thermally stable as well as more easily separable from the reaction media.[1–3] Mesoporous silica materials have been considered as a suitable host due to their inherent characteristics of high surface area and pore volume, tunable pore size accommodating dimensions of enzymes, and mechanical stability.[4,5] Until now, mesoporous silica particles have been usually used as the host[6–8] and high activities of enzymes within the host have been reported.[9] However, as the enzyme is encapsulated within small pores, its kinetics for a complete catalytic reaction process is often reduced compared to that of the enzyme in a solution because of the rate limitations imposed by slow diffusion within the small pores.[10,11] Hence, shortening of the generally prolonged reaction time is demanded for rapid enzyme catalytic reaction. In addition, the use of mesoporous silica particles requires their separation from a reaction medium by centrifugation or filtration.[12] Although the separation of a mesoporous silica host is easier than that of enzyme, reduction of the separation process is favored for a simple and rapid scheme. Herein, we propose a method to solve those problems by applying a hybrid mesoporous membrane composed of mesoporous silica within a porous anodic alumina membrane (Fig. 1).

Recently, we[13,14] and other research groups[15–17] reported a method to fabricate hybrid mesoporous membranes with an ordered pore structure ranging from 3.4 to 12 nm. Since mesoporous silica can be formed within all alumina pores in the anodic alumina membrane (AAM) of 2 to 4 cm in diameter, the resulting hybrid mesoporous membrane allows transport of molecules through silica mesopores by embedding the membrane between two solution phases.[18,19] In the present study, glucose oxidase (GOD) was covalently immobilized within the hybrid mesoporous membrane and the resulting GOD-immobilized membrane (GOD-M) was applied for conversion of glucose to gluconic acid and hydrogen peroxide. As shown schematically in Fig. 1, the GOD-M can be used for conversion of glucose by using a conventional filtration apparatus. Herein, we demonstrated rapid glucose conversion by passing a glucose solution through the GOD-M under moderate aspiration (Fig. 1). The proposed scheme allows conversion of glucose without separation of the mesoporous silica host from the reaction media. In addition, our experimental results showed that the GOD-M has an efficient catalytic activity for glucose, indicating that an enzyme catalytic membrane based on a hybrid mesoporous membrane is useful for the study of enzyme chemistry as well as for practical applications in biosensors and catalysts.

The hybrid mesoporous membrane was prepared by using Pluronic F127 ($(PEO)_{106}(PPO)_{70}(PEO)_{106}$) as a structure-directing agent.‡ As shown in Fig. 2(a), columnar mesoporous silica was formed inside the AAM pores and had a length of *ca.* 50 ± 2 μm. Some authors reported that mesoporous silica could be formed within an alumina membrane by soaking the alumina membrane in a precursor solution.[20,21] In contrast to this method, we have used an aspiration-induced infiltration approach that prevents any formation of mesoporous silica on the membrane surface. This is a significant advantage for the application of the hybrid mesoporous membrane to the glucose conversion because the unfavorable formation of mesoporous silica on the membrane surface may prevent smooth solution flow across the membrane.

The mesostructure of the columnar mesoporous silica was observed by transmission electron microscopy (TEM) after calcination of the hybrid mesoporous membrane at 600 °C. The mesostructure shown in TEM images (Fig. 2(b) and (c), (d)) was similar to that found for mesoporous silica formed inside alumina pores by using Pluronic P123 as the structure-directing agent and could be ascribed to the 3D concentric spherically layered structure.[14] As typically shown in Fig. 2(c)

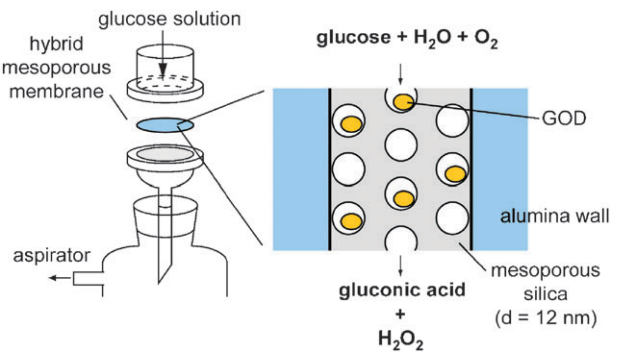

Fig. 1 Schemes for the glucose conversion by using a GOD-immobilized hybrid mesoporous membrane under moderate aspiration.

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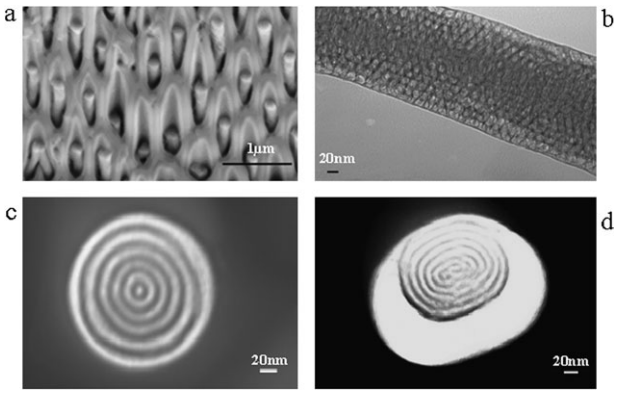

Fig. 2 SEM and TEM images of mesoporous silica. (a) Columnar mesoporous silica inside the alumina pores. (b) TEM side-view of columnar mesoporous silica. (c), (d) TEM top views of columnar mesoporous silica with concentric stacked-doughnuts structure.

and (d), shrinkage of mesoporous silica was sometimes observed inside the alumina pores.[22] This shrinkage might be induced by the decomposition of Pluronic F127 and the condensation of the silica walls during the calcination process. The pore diameter was estimated by the measurements of nitrogen adsorption/desorption isotherms and the value was 12 nm (ESI, Fig. S1†).

The GOD encapsulation was done by covalent attachment on the inner wall of the silica mesopores, because if GOD is noncovalently encapsulated, it may be eluted from the silica mesopores during flow of the glucose solution. After removal of Pluronic F127 inside the silica mesopores by calcination, 3-aminopropyltrimethoxysilane (APTMS) was immobilized on the inner pore surface. Then, GOD was covalently attached *via* a linker of glutaraldehyde (GA) and the GOD-M was obtained. The weight of GOD encapsulated was estimated by thermal gravimetric and differential thermal analysis (TG-DTA) and the estimated value was *ca.* 15 mg per 1 g of the GOD-M (ESI, Fig. S2†).

The BET surface area of the hybrid mesoporous membrane was estimated as 18 m^2 g^{-1} from the nitrogen adsorption/desorption isotherm measurements (ESI, Fig. S1†). Here, we assumed an occupied area of a single-molecule thick layer of GOD on the silica surface as 6 × 6 nm because unit cell dimensions of GOD are 6.0 nm × 5.2 nm × 7.7 nm.[23] From this assumption, the occupied area for 15 mg of GOD was calculated as 2 m^2 and the surface coverage ratio of GOD in the GOD-M was *ca.* 0.11.

When a solution passed through the GOD-M under aspiration, no detectable leakage of GOD was recognized. In contrast, the leakage of GOD was observed when GOD was electrostatically encapsulated in the mesoporous silica modified by APTMS only. Accordingly, we concluded that GOD was covalently attached *via* the GA linker in the GOD-M.

The GOD-M was set in an ordinary filtration apparatus and 1 ml of glucose solution was poured on the membrane surface. By applying moderate aspiration, the glucose solution passed through the GOD-M at a flow speed of 1 ml per 50 min. The sample solution that passed through the GOD-M was collected in a glass container and the concentration of hydrogen peroxide in it was measured by a traditional colorimetric

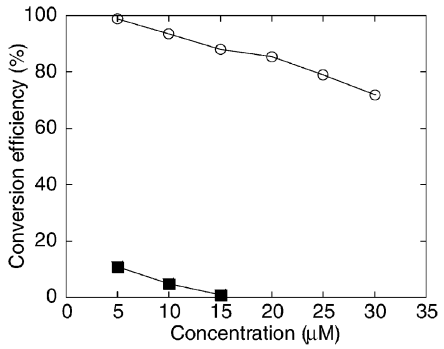

Fig. 3 Conversion efficiency with varying concentration of glucose solution on different supports. ○: GOD-M; ■: GOD immobilization within AAM.

method using peroxidase and 3,3′,5,5′-tetramethylbenzidine. For 5 μM glucose solution, the conversion efficiency was 99%. The effect of the concentration of glucose on the conversion efficiency is shown in Fig. 3. The conversion efficiency decreased as the concentration of glucose increased. For the GOD-M prepared in this study, the surface coverage of GOD was low (0.11) and this low surface coverage would be ascribed to the decrease in the conversion efficiency with increasing the glucose concentration. Hence, an improvement of the surface coverage of GOD would be important to create an effective GOD catalytic membrane.

We further examined the conversion efficiency of a biocatalyst membrane in which GOD was immobilized within AAM pores which lacked mesoporous silica. As a result, it was confirmed that the conversion efficiency for the GOD-immobilized AAM (GOD-AAM) was much lower than that for the GOD-M. This low efficiency for the GOD-AAM could be ascribed to the large pore diameter (*ca.* 200 nm) of AAM compared to that for the hybrid mesoporous membrane (12 nm), since the encounter frequency of glucose with GOD immobilized on inner pore walls would increase as the pore diameter decreased. Thus, the GOD-M synthesized in this study has a higher conversion efficiency than nanoporous membrane systems (*e.g.* AAM) with large pore diameters.

We also investigated the effect of the flow speed on the conversion efficiency. As shown in Fig. 4, the conversion efficiency dropped as the flow speed increased. Because of the very low amount of immobilized GOD (surface coverage

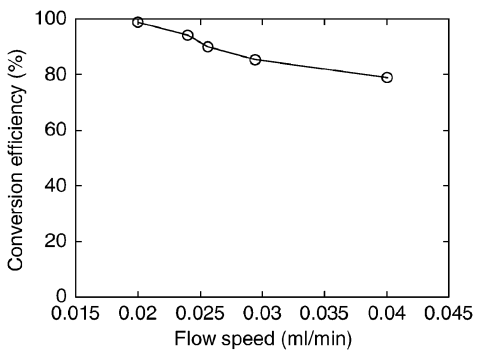

Fig. 4 Conversion efficiency with varying flow speed. Concentration of glucose was 5 μM with 0.01 M sodium phosphate buffer (pH = 7).

ratio of GOD = 0.11), the glucose could not be catalyzed entirely when it passed through the membrane at high speed. Furthermore, when the surfactant template was removed by calcination, shrinkage of the mesoporous silica occurred and the passage of glucose through the membrane could occur without it being catalyzed.

The reusability of the immobilized enzyme is an important factor in the biocatalyst membrane. The conversion efficiency was examined after prolonged storage of the GOD-M in a 0.01 M sodium phosphate buffer solution at 4 °C. After storage for 10 days, the retained conversion efficiency was about 57% of the original efficiency. The decay in the conversion efficiency could be attributed to denaturation of GOD in the GOD-M. In addition, hydrolysis of silica matrix should be taken into account for the decay. Probably, hydrolysis of the silica matrix in the phosphate buffer solution was the cause of the decay in the conversion efficiency after 10 days.

In summary, a hybrid mesoporous membrane with 12 nm pore diameter was synthesized by using Pluronic F127 as a structure-directing agent, and GOD was covalently immobilized within the hybrid mesoporous membrane which was then applied for oxidation of glucose to gluconic acid and hydrogen peroxide. The hybrid mesoporous membrane with GOD showed high conversion efficiency of glucose and the conversion efficiency was 8 to 9 times higher than that observed for the AAM with GOD. This study showed that the hybrid mesoporous membrane could be an excellent substrate for immobilization of enzymes. This kind of enzyme catalytic membrane could potentially be used in the development of applications in various fields such as enzymatic sensors, biocatalysts, and bio-fuel cells.

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Notes and references

‡ *Synthesis*

Mesoporous silica was synthesized by using Pluronic F127 as a structure-directing agent. The precursor solution was prepared following the literature procedure.[24] 0.92 g of Pluronic F127 were dissolved in a solution containing 17.66 g of ethanol, 0.2 g of 1 M HCl, and 1.38 g of water. The solution was refluxed at 30 °C for 1 h with stirring and a homogeneous solution was obtained. Then, 2.0 g of TEOS was added to the homogeneous solution with slow stirring for 5 h to form a silica–surfactant sol. Porous anodic alumina membranes (pore diameter = *ca.* 100 nm, thickness = *ca.* 60 μm, diameter of membrane = 25 mm) were obtained from Whatman (Anodisk). An alumina membrane was set in an ordinary membrane filtration apparatus, and the precursor solution was dropped onto the alumina membrane. Moderate aspiration was applied so that the precursor solution penetrated into the columnar alumina pores, then the membrane was dried in air at room temperature. The resulting membrane was calcined by increasing temperature slowly from room temperature to 600 °C for 8 h.

Immobilization of enzyme onto the surface of inorganic supports

Grafting aminopropyl functional groups covalently on the silica surface of the hybrid mesoporous membrane was carried out by using the reaction between silanol groups and 3-aminopropyltrimethoxysilane (APTMS) in dry methanol. Typically, one piece of membrane was immersed into a solution containing 9 ml of dry methanol and 1 ml of APTMS, and this was refluxed for 4 h at 60 °C. After cooling slowly, the membrane was washed with methanol and dried under reduced pressure for 2 h. The APTMS-grafted membrane was set in an ordinary membrane filtration apparatus, and 1ml glutaraldehyde (GA) (2.5 wt%) solution was dropped onto the alumina membrane. Moderate aspiration was applied so that the GA solution flowed through the membrane at room temperature. After that, the membrane was thoroughly rinsed using a 0.01 M sodium phosphate buffer solution in order to remove trace amounts of glutaraldehyde and to avoid cross-linking after addition of enzyme. One piece of the membrane was immersed into 1ml of an enzyme solution (2.5 mg ml^{-1}) and shaken at room temperature for 20 h at 80 rpm. The membrane was washed using a 0.01 M sodium phosphate buffer solution several times, and then kept in the same buffer solution at 4 °C.

1 C. Lei, Y. Shin, J. Liu and E. J. Ackerman, *J. Am. Chem. Soc.*, 2002, **124**, 11242.
2 U. T. Bornscheuer, *Angew. Chem., Int. Ed.*, 2003, **42**, 3336.
3 Y. Han, S. S. Lee and J. Y. Ying, *Chem. Mater.*, 2006, **18**, 643.
4 J. Fan, C. Yu, F. Gao, J. Lei, B. Tian, L. Wang, Q. Luo, B. Tu, W. Zhou and D. Zhao, *Angew. Chem., Int. Ed.*, 2003, **42**, 3146.
5 J. L. Blin, C. Gerardin, C. Carteret, L. Rodehuser, C. Selve and M. J. Stebe, *Chem. Mater.*, 2005, **17**, 1479.
6 J. F. Diaz and K. J. J. Balkus, *J. Mol. Catal. B: Enzym.*, 1996, **2**, 115.
7 I. Gill and A. Ballesteros, *J. Am. Chem. Soc.*, 1998, **120**, 8587.
8 Y. Wang and F. Caruso, *Chem. Mater.*, 2005, **17**, 953.
9 H. R. Luckarift, J. C. Spain, R. R. Naik and M. O. Stone, *Nat. Biotechnol.*, 2004, **22**, 211.
10 A. Corma, *Chem. Rev.*, 1997, **97**, 2373.
11 F. Goettmann and C. Sanchez, *J. Mater. Chem.*, 2007, **17**, 24.
12 X. Wang, J. C. C. Chan, Y. Tseng and S. Cheng, *Microporous Mesoporous Mater.*, 2006, **95**, 57.
13 A. Yamaguchi, F. Uejo, T. Yoda, T. Uchida, Y. Tanamura, T. Yamashita and N. Teramae, *Nat. Mater.*, 2004, **3**, 337.
14 A. Yamaguchi, H. Kaneda, W. Fu and N. Teramae, *Adv. Mater.*, DOI: 10.1002/adma.200701368.
15 Y. Wu, G. Cheng, K. Katsov, S. W. Sides, J. Wang, J. Tang, G. H. Fredrickson, M. Moskovits and G. D. Stucky, *Nat. Mater.*, 2004, **3**, 816.
16 Q. Lu, F. Gao, S. Komarneni and T. E. Mallouk, *J. Am. Chem. Soc.*, 2004, **126**, 8650.
17 Z. Yang, Z. Niu, X. Cao, Z. Yang, Y. Lu, Z. Hu and C. C. Han, *Angew. Chem., Int. Ed.*, 2003, **42**, 4201.
18 A. Yamaguchi, T. Yoda, S. Suzuki, K. Morita and N. Teramae, *Anal. Sci.*, 2006, **22**, 1501.
19 T. Yamashita, S. Kodama, M. Ohto, E. Nakayama, S. Hasegawa, N. Takayanagi, T. Kemmei, A. Yamaguchi, N. Teramae and Yukio Saito, *Anal. Sci.*, 2006, **22**, 1495.
20 B. Platschek, N. Petkov and T. Bein, *Angew. Chem., Int. Ed.*, 2006, **45**, 1134.
21 B. Yao, D. Fleming, M. A. Morris and S. E. Lawrence, *Chem. Mater.*, 2004, **16**, 4851.
22 A. Y. Ku, S. T. Taylor and S. M. Loureiro, *J. Am. Chem. Soc.*, 2005, **127**, 6934.
23 H. J. Hecht, H. M. Kalisz, J. Hendle, R. D. Schmidt and D. Schomburg, *J. Mol. Biol.*, 1993, **229**, 153.
24 L. A. Villaescusa, A. Mihi, I. Rodriguez, A. E. Garcia-Bennett and H. Miguez, *J. Phys. Chem. B*, 2005, **109**, 19643.