One-pot Synthesis and Characterization of Laccase-entrapped Magnetic Nanobeads

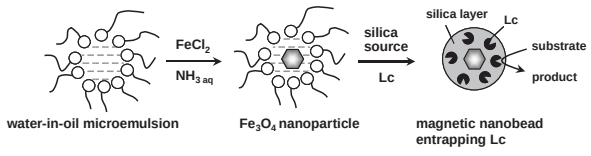
Hideki Matsune, Hitoshi Jogasaki, Masato Date, Sakae Takenaka, and Masahiro Kishida*
Department of Chemical Engineering, Graduate School of Engineering, Kyushu University, 744 Moto-oka, Nishi-ku, Fukuoka 819-0395

(Received August 21, 2006; CL-060955; E-mail: kishida@chem-eng.kyushu-u.ac.jp)

We report the simple one-pot synthesis of magnetic nanoparticles coated with silica-gel layers that entrap laccase (Lc) by the water-in-oil microemulsion method. The amorphous porous structure of the silica layer enables the entrapped Lc to react with substrates in solution. The enzymatic activity is studied using Michaelis–Menten kinetics. The reusability of the magnetic nanoparticles is examined.

Magnetic particles that immobilize enzymes have attracted increasing attention as magnetically recoverable biocatalysts.[1,2] The use of nanometric materials as enzyme supports offers some advantages due to the high specific surface area of the nanoparticle and the low mass-transfer resistance of substrates compared to those of conventional submicron particles.[3] Enzymes have been immobilized on supports by many methods such as impregnation, ion exchange, covalent linking,[1] and entrapment in porous matrices.[2] The covalent chemical linkage is often used for the fabrication of enzyme-immobilized magnetic nanoparticles with high enzymatic activity comparable to that of the free enzyme. However, this method is a surface modification process that requires several steps, which generally include anchor coating, linker binding, and enzyme immobilization, followed by purification in each step.[1] A more simple technique is preferable for the easy immobilization of desired enzymes on magnetic nanoparticles. The sol–gel entrapment of enzymes in silica particles is a promising technique due to ease of preparation without covalent modification. The entrapped enzymes react with the substrates migrating through the pore network of silica.[2–4]

In this letter, we report the simple one-pot synthesis of magnetic nanoparticles coated with silica-gel layers that entrap laccase (denoted by Lc; EC 1.10.3.2) by using the water-in-oil microemulsion method. Lc is a well-defined multicopper oxidase and has been widely used for the polymerization of phenolic compounds.[5] The Lc-entrapped silica layer was produced by the entrapment of Lc during the formation of this layer by the hydrolysis and copolymerization of silicon alkoxide (Scheme 1). The following is a description of the typical synthesis process. The synthesis procedure described in a previous study[7] for the preparation of Fe_3O_4/SiO_2 core/shell nanoparticles (referred to as magnetic nanobeads) was modified for the entrapment of enzyme molecules in the silica layers. Nonionic surfactant polyoxyethylene (15) cetyl ether was dissolved in cyclohexane at 50 °C (0.5 M, 100 mL). After the addition of 1.1 mL of 1.4 M Fe_2Cl_2 (aq) and 1 mL of 28 wt % NH_3 (aq) in the solution with vigorous mechanical stirring, the color of the solution gradually changed from bright red-brown to dark brown, which is a characteristic of Fe_3O_4 formation. Excess Fe^{2+} ions were oxidized by oxygen dissolved in the solution during stirring; this resulted in the formation of particles that consisted of the mixed-valence complex of $Fe^{II}Fe^{III}{}_2O_4$. After stirring for 3 min, 1.1 mL of 1.4 M Fe_2Cl_2 (aq) was added again to initiate the growth of Fe_3O_4 particles. Lc-entrapped silica-gel layers around the Fe_3O_4 core particles were prepared by the addition of 6 g of a mixture of 3-aminopropyltriethoxysilane (APTS) and tetraethoxysilane (TEOS) (APTS/TEOS molar ratio = 0.25), followed by the addition of phosphate buffer saline (PBS) solution (pH = 5.3) containing Lc. APTS was used for the formation of a hydrophilic silica layer. Finally, the magnetic nanobeads were washed twice with acetonitrile and deionized water by using a magnet or by centrifugation and then stored at 4 °C. The adsorption method was also examined for reference. Lc-adsorbed magnetic nanobeads were prepared by immersing magnetic nanobeads with amino moiety in Lc/PBS solution for 1 night, followed by repeated washing with PBS solution until the supernatant after magnetic recovery showed no enzymatic activity.



Scheme 1. Preparation of a magnetic nanobead that entraps Lc enzyme molecules within its silica layer.

The powder X-ray diffraction pattern of the nanobeads showed peaks due to the Fe_3O_4 (magnetite) phase and a broad halo due to amorphous silica (See Supporting Information 1).[8] The spherical core/shell nanostructures were observed in the TEM image of the nanobeads (Figure 1). Most of the nanobeads were composed of the Fe_3O_4 core nanoparticles with diameters of ca. 6 nm located nearly at the center of spherical silica particles with an average diameter of 30.6 nm (standard deviation = ±3.3 nm). The Lc molecules in the silica layers were not observed in the TEM image because of their low contrasts. The total amount of Lc entrapped within the nanobeads was estimated from the amount of copper ions of Lc (extracted by

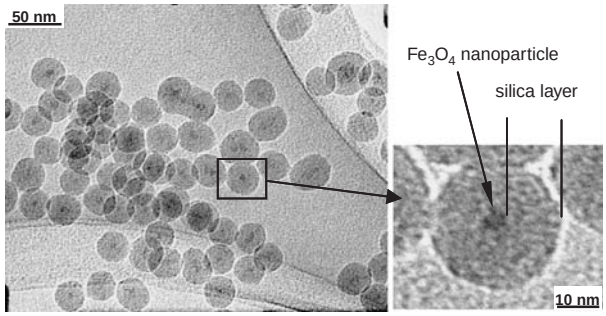


Figure 1. TEM image of laccase-entrapped magnetic nanobeads.

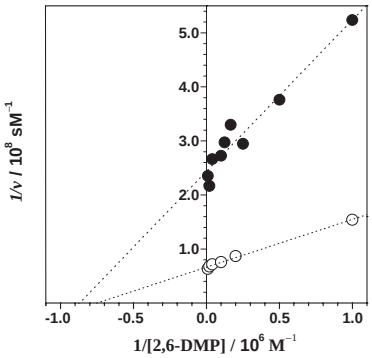


Figure 2. Lineweaver–Burk ($1/v$ vs $1/[2,6\text{-DMP}]$) plots for oxidative reaction of 2,6-DMP with entrapped (●) and free (○) Lc (at pH = 5.3, 38 °C, and [Lc] = 0.03 μM).

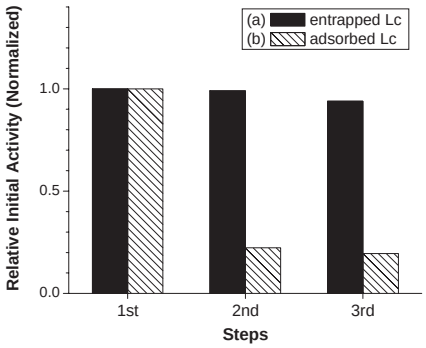


Figure 3. Initial reaction rates of Lc (a) entrapped within magnetic nanobeads and (b) adsorbed on magnetic nanobeads after magnetic recovery.

HCl (aq) from the silica layer that was burned, then dissolved with KOH (aq)) by inductively coupled plasma-mass spectrometry. An Lc loading of 2.6 μg per mg of the nanoparticles was observed, corresponding to 77% of Lc entrapped in the nanobeads during the synthesis.

The enzymatic activity of Lc was examined from the oxidative reaction of 2,6-dimethoxyphenol (2,6-DMP) in the PBS solution.[5] The initial reaction rate (v) was determined by monitoring the absorbance of polymerized DMP by UV–vis spectrophotometry ($\varepsilon = 49.6\,\mathrm{mM^{-1}\,cm^{-1}}$ at 469 nm).[6] Figure 2 shows the Lineweaver–Burk plots ($1/v$ vs $1/[2,6\text{-DMP}]$) of the entrapped and free Lc enzyme molecules ([Lc] = 0.03 μM). The plots gave straight lines, which demonstrate that the reaction of 2,6-DMP with Lc follows the Michaelis–Menten model. The Michaelis constant (K_m) of the entrapped Lc was similar to that of free Lc (12 and 13 μM, respectively). However, the different intercept values on the y axis demonstrate that the apparent V_{max} value of the entrapped Lc is 27% of the V_{max} value of free Lc. In general, enzymes entrapped in the porous matrix tend to exhibit the greater K_m value and the less V_{max} value than the free enzymes.[3,4] This was considered to be due to the conformational distortion of the entrapped enzyme or the diffusion limit of the substrates and products in the pores of the gel structure. On the other hand, in our case, the K_m value of the entrapped Lc was comparable to that of free Lc, thereby suggesting that the enzymatic property of Lc in the nanobeads was similar to that of free Lc, and that the internal diffusion effect of the substrates on K_m was negligible. This implies that the reaction rate was primarily determined by the Lc entrapped in the pores in the surface region of the nanobeads. The reduction in V_{max} indicates that a fraction of the trapped Lc showed no enzymatic reaction due to the caging deep inside the nanobeads or deactivation by the chemicals at the synthesis.

The reusability of Lc-entrapped magnetic nanobeads was examined and compared with that of Lc-adsorbed nanobeads. For this purpose, the following steps were repeated: recovery of the nanobeads with a permanent magnet, redispersion of these beads in a new reaction solution, and measurement of the enzymatic activity. Figure 3 shows the initial reaction rates of the adsorbed and entrapped Lc after recovery. While the recovery ratio of the magnetic nanobeads was constant in each step (See Supporting Information 2),[8] the activity of the Lc-adsorbed nanobeads drastically decreased when the steps were repeated; this was probably due to the desorption of Lc molecules from the nanobeads during the reaction. On the other hand, the Lc molecules entrapped within the nanobeads retained their activities at least after 3 steps. The Lc-entrapped nanobeads could be quantitatively recovered without loss of the activity. This indicates that the Lc molecules showed no desorption due to the entrapment within the silica layer of the nanobeads and the porous structure of this layer enabled the entrapped Lc to react with the 2,6-DMP molecules in solution. The one-pot synthesis method described above is a relatively simple and efficient technique for the preparation of enzyme-immobilized magnetic nanobeads.

References and Notes

1 X. Gao, K. M. K. Yu, K. Y. Tam, S. C. Tsang, *Chem. Commun.* **2003**, 2998; H. M. R. Gardimalla, D. Mandal, P. D. Stevens, M. Yen, Y. Gao, *Chem. Commun.* **2005**, 4432; L. M. Rossi, A. D. Quach, Z. Rosenzweig, *Anal. Bioanal. Chem.* **2004**, *380*, 606.
2 H.-H. Yang, S.-Q. Zhang, X.-L. Chen, Z.-X. Zhuang, J.-G. Xu, X.-R. Wang, *Anal. Chem.* **2004**, *76*, 1316.
3 I. Hinberg, R. Korus, K. F. O'Driscoll, *Biotechnol. Bioeng.* **1974**, *16*, 943.
4 S. Braun, S. Rappoport, R. Zusman, D. Avnir, M. Ottolenghi, *Mater. Lett.* **1990**, *10*, 1; T. K. Jain, I. Roy, T. K. De, A. Maitra, *J. Am. Chem. Soc.* **1998**, *120*, 11092; D. Avnir, S. Braun, O. Lev, M. Ottolenghi, *Chem. Mater.* **1994**, *6*, 1605; S. A. Yamanaka, F. Nishida, L. M. Ellerby, C. R. Nishida, B. Dunn, J. S. Valentine, J. I. Zink, *Chem. Mater.* **1992**, *4*, 495.
5 J. Tominaga, J. Michizoe, N. Kamiya, H. Ichinose, T. Maruyama, M. Goto, *J. Biosci. Bioeng.* **2004**, *98*, 14.
6 H. Wariishi, K. Valli, M. H. Gold, *J. Biol. Chem.* **1992**, *267*, 23688.
7 T. Tago, T. Hatsuta, K. Miyajima, M. Kishida, S. Tashiro, K. Wakabayashi, *J. Am. Ceram. Soc.* **2002**, *85*, 2188.
8 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

Published on the web (Advance View) October 28, 2006; doi:10.1246/cl.2006.1356