Immobilization of *Trametes versicolor* Laccase on Magnetically Separable Mesoporous Silica Spheres

Yufang Zhu,*,† Stefan Kaskel,*,† Jianlin Shi,‡ Tobias Wage,§ and Karl-Heinz van Pée§

Institut für Anorganische Chemie, Technische Universität Dresden, Mommsenstr. 6, Dresden 01069, Germany; State Key Laboratory of High Performance Ceramics and Superfine Microstructures, Shanghai Institute of Ceramics, Chinese Academy of Sciences, 1295 Ding-xi Road, Shanghai 200050, P. R. China; and Professur für Allgemeine Biochemie, Technische Universität Dresden, Bergstraße 66, Dresden 01069, Germany

Received May 10, 2007. Revised Manuscript Received July 21, 2007

Magnetic mesoporous silica spheres (MSS) with large pore size were prepared by reducing Fe^{3+}-containing mesoporous silica spheres. Laccase from *Trametes versicolor* was immobilized on magnetic mesoporous silica spheres through physical adsorption and covalent attachment methods. Laccase oxidizes 2,2′-azinobis(3-ethylbenzthiazolin-6-sulfonate) (ABTS) to the green cation radical ($ABTS^{\bullet+}$) as a model system; the immobilized laccase retained the activity and exhibited higher resistance to pH changes and thermal stability. The immobilized laccase obtained through covalent attachment almost has no leaching and can retain above 70% of activity after 10 consecutive operations. More interesting, the immobilized laccase can be separated quickly using an external magnetic field. Therefore, the magnetite-containing mesoporous silica spheres are a promising support for enzyme immobilization.

Introduction

Laccase (benzenediol:oxygen oxidoreductase; E.C. 1.10.3.2) is a copper-containing oxidase, which is able to catalyze a one-electron oxidation of various phenols, substituted polyphenols, aromatic substrates, benzenethiols, and a series of other oxidizable compounds with the simultaneous reduction of dioxygen to two molecules water.[1–5] The substrate specificity of laccase has suggested its potential to remove pollutants from the environment without creating the harsh side effects associated with many other methods.[6] However, despite laccase having intrinsic appreciable stability, the enzyme is often easily inactivated in practical application due to a wide variety of environmental conditions. In addition, it is also difficult to be separated from the reaction system for reuse, which limits the further industrial application of laccase.[7]

It is well-known that the immobilization of enzymes on insoluble supports provides an effective way to perform enzyme reuse and to improve its stability. Laccase has been successfully immobilized on many different types of supports, such as activated carbon,[6] porous glass,[8] kaolinite,[9] polymer beads and membranes,[10,11] magnetic chitosan,[12] and polystyrene microspheres.[13] To the best of our knowledge, the studies of laccase immobilized on magnetically separable mesoporous silica spheres have not been reported. Mesoporous silica materials have attracted much attention as promising supports for the immobilization of enzymes because of their large surface areas, tunable pore sizes and volumes, and well-defined surface properties for modification. There have been a variety of reports describing the use of mesoporous silica materials for the immobilization of enzymes, and the enzymatic activities can be retained and the stabilities also can be improved to some extent.[14–22] On

* Corresponding authors. Yufang Zhu: phone +49-351-46334710, fax +49-351-46337287, e-mail yufang.zhu@chemie.tu-dresden.de. Stefan Kaskel: phone +49-351-46334885, fax +49-351-46337287, e-mail stefan.kaskel@chemie.tu-dresden.de.

† Institut für Anorganische Chemie, Technische Universität Dresden.

‡ Chinese Academy of Sciences.

§ Professur für Allgemeine Biochemie, Technische Universität Dresden.

(1) Thurston, C. F. *Microbiology* **1994**, *140*, 19.
(2) Felici, M.; Artemi, F.; Luna, M.; Speranza, M. *Chromatography* **1985**, *320*, 435.
(3) Xu, F. *Biochemistry* **1996**, *35*, 7608.
(4) Cai, W.; Martin, R.; Lemaure, B.; Leuba, J. L.; Petiard, V. *Plant Phydiol. Biochem.* **1993**, *31*, 441.
(5) Dodor, D. E.; Hwang, H.-M.; Ekunwe, S. I. N. *Enzyme Microbial Technol.* **2004**, *35*, 210.
(6) Davis, S.; Burns, R. G. *Appl. Microbiol. Biotechnol.* **1992**, *37*, 474.
(7) Fagain, C. O. *Enzyme Microbiol. Technol.* **2001**, *33*, 137.
(8) Rogalski, J.; Dawidowicz, A.; Jozwik, E. *J. Mol. Catal. B: Enzym.* **1999**, *6*, 29.
(9) Hu, X.; Zhao, X.; Hwang, H.-M. *Chemosphere* **2007**, *66*, 1618.
(10) Annibale, A. D.; Stazi, S. R.; Vinciguerra, V. *J. Biotechnol.* **2000**, *77*, 265.
(11) Jolivalt, C.; Brenon, S.; Caminade, E. *J. Membr. Sci.* **2000**, *180*, 103.
(12) Jiang, D.-S.; Long, S.-Y.; Xiao, H.-Y.; Zhou, J.-Y. *Biochem. Eng. J.* **2005**, *25*, 15.
(13) Pich, A.; Bhattacharya, S.; Adler, H.-J. P.; Wage, T.; Taubenberger, A.; Li, Z.; van Pée, K.-H.; Böhmer, U.; Bley, T. *Macromol. Biosci.* **2006**, *6*, 301.
(14) Diaz, J. F.; Balkus, K. J. *J. Mol. Catal. B: Enzym.* **1996**, *2*, 115.
(15) Yiu, H. P.; Wright, P. A.; Botting, N. P. *J. Mol. Catal. B: Enzym.* **2001**, *15*, 81.
(16) Takahashi, H.; Li, B.; Sasaki, T.; Miyazaki, C.; Kajino, T.; Inagaki, S. *Chem. Mater.* **2000**, *12*, 3301.
(17) He, J.; Li, X.; Evans, D. G.; Duan, X.; Li, C. *J. Mol. Catal. B: Enzym.* **2000**, *11*, 45.
(18) Pandya, P. H.; Jasra, R. V.; Newalker, B. L.; Bhatt, P. N. *Microporous Mesoporous Mater.* **2004**, *77*, 67.
(19) Zhu, Y.; Shen, W.; Dong, X.; Shi, J. *J. Mater. Res.* **2005**, *20*, 2682.
(20) Zhang, X.; Guan, R.-F.; Wu, D.-Q.; Chan, K.-Y. *J. Mol. Catal. B: Enzym.* **2005**, *33*, 43.
(21) Petri, A.; Gambicorti, T.; Salvadori, P. *J. Mol. Catal. B: Enzym.* **2004**, *27*, 103.
(22) Galarneau, A.; Mureseanu, M.; Atger, S.; Renard, G.; Fajula, F. *New J. Chem.* **2006**, *30*, 562.

10.1021/cm071265g CCC: $37.00
Published on Web 12/04/2007

the other hand, magnetic support technology is a promising strategy for the preparation of the immobilized enzymes because it can be easily recovered using an external magnetic field and recycled for iterative uses. Furthermore, the use of magnetic supports can reduce the capital and operational costs.[23–28] For example, Schüth et al. reported a kind of magnetically separable hydrogenation catalyst by using Co-containing mesoporous carbon.[23,24] Ulman et al. demonstrated a successful method to immobilize proteins on pure γ-Fe_2O_3 magnetic nanoparticles for biological use.[25] Kim et al. reported a magnetic and stable enzyme system by the combined use of two different kinds of nanostructured materials: magnetic nanoparticles and mesoprous silica.[26]

In this paper, we reported the development of a magnetically separable immobilized laccase system based on magnetite-containing mesoporous silica spheres (Fe_3O_4@MSS) as supports. Laccase was immobilized on Fe_3O_4@MSS support using physical adsorption and covalent attachment methods. To evaluate the activity and stability of the immobilized laccase, we use laccase-catalyzed oxidization of 2,2′-azinobis(3-ethylbenzthiazolin-6-sulfonate) (ABTS) to the green cation radical ($ABTS^{\bullet+}$) as a model system for test. The activity of the immobilized laccase was retained to a large extent, and its stability was apparently improved.

Experimental Section

Preparation of Mesoporous Silica Spheres (MSS). 4.0 g of P123 ($EO_{20}PO_{70}EO_{20}$, Aldrich) and 5.0 g of KCl were dissolved in 120 g of H_2O and 23.6 g of HCl (37 wt %) at room temperature until the solution became transparent, and then 3.0 g of mesitylene (Fluka) was added. After stirring for 2 h, 8.5 g of tetraethyl orthosilicate (TEOS, Fluka) was added and stirred for 10 min. Then the mixture was kept under static conditions at 35 °C for 24 h, followed by another 24 h at 100 °C. The resultant precipitates were filtered, washed, and dried at 100 °C. The products were then calcined in air at 510 °C for 8 h to remove the templates. The sample was named MSS.

Preparation of Magnetite-Containing Mesoporous Silica Spheres (Fe_3O_4@MSS). Fe_3O_4@MSS was synthesized following a previously reported method.[29,30] A typical procedure was performed as follows: 0.5 g of $Fe(NO_3)_3 \cdot 9H_2O$ (Aldrich, 98%) was dissolved in 20 mL of ethanol, followed by addition of 1 g of MSS. After stirring at room temperature until all the solution had been evaporated, a dry powder was obtained. After drying at 100 °C in air, the sample was reduced by heating at 400 °C (heating rate of 3 °C/min) for 4 h under a 7% H_2−93% Ar atmosphere. The sample was named Fe_3O_4@MSS.

Modification of Fe_3O_4@MSS with Amino Groups. After activation at room temperature in vacuum, 0.5 g of Fe_3O_4@MSS was added to 50 mL of toluene solution containing 1.0 mL of $(C_2H_5O)_3SiCH_2CH_2CH_2NH_2$ (Fluka). After being stirred at 60 °C for 20 h, the mixture was extensively washed with acetone and air-dried. The sample was named Fe_3O_4@MSS-NH_2.

Immobilization of Laccase on Mesoporous Supports. In the first procedure (physical adsorption), 100 mg of Fe_3O_4@MSS support was suspended in 15 mL of phosphate buffer (pH 7.0) containing a certain amount of laccase (3−21 mg). The mixture of the supports and laccase solution was slowly stirred at room temperature for 12 h. Subsequently, the laccase immobilized on Fe_3O_4@MMS particles was separated by a magnet. Then the particles were washed with 10 mL of buffer solution by shaking for 5 min and separated quickly using a magnet. The washing procedure was repeated eight times. Finally, the immobilized particles were used directly for the activity measurement. The sample was named E-Fe_3O_4@MSS.

In the second procedure (covalent attachment), 100 mg of Fe_3O_4@MSS-NH_2 support was first stirred at room temperature for 1 h with glutaraldehyde solution (0.5 mL of 25 wt % glutaraldehyde solution in 10 mL of phosphate buffer (pH 7.0)). After separation and extensive washing with H_2O, the resulting supports were used to carry out the immobilization of laccase following the same conditions as the first procedure. The sample was named E-Fe_3O_4@MSS-NH_2.

Determination of Laccase Activity. The activity of the free and immobilized laccase was determined spectrophotometrically in a reaction medium containing 0.13% (v/v) ABTS (Sigma) as substrate in phosphate buffer (pH 3.0−7.0) at room temperature in the absorbance at 420 nm. A suitable amount of laccase (0.05−0.4 mg) was added to the substrate solution and stirred immediately. Five minutes later, the absorbance of the supernatant was determined using a UV–vis spectrophotometer. The molar extinction coefficient for the oxidation of ABTS at 420 nm is 36×10^{-3} M^{-1} cm^{-1}. One unit of activity is defined as the amount of enzyme required to oxidize 1 μmol of substrate per minute.

To determine the resistance to pH changes, the activities of the free and immobilized laccase were determined by measuring the activities after they were put in different buffer solutions (pH 3.0–7.0) for 6 h. The temperature–activity profiles of the free and immobilized laccase were determined in the buffer solution of 10–70 °C at pH 3.6. Here the activity was expressed in relative units [%] where the maximal activity value at a certain pH value and temperature was set at 100%.

Characterization. The small-angle X-ray diffraction (SAXRD) patterns were measured on a Bruker AXS Nanostar using Cu $K\alpha_1$ radiation (1.5405 Å) and 105 cm sample-to-detector distance. The wide-angle X-ray diffraction (WAXRD) pattern was obtained on a Stoe Stadi P powder diffractometer equipped with a curved germanium (111) monochromator and linear PSD using Cu $K\alpha_1$ radiation (1.5405 Å) in transmission geometry. Scanning electron microscopy (SEM) was carried out on a Zeiss DMS 982 Gemini field emission scanning electron microscope at 4.0 kV. Transmission electron microscopy (TEM) was performed using a JEOL 2100F electron microscope operated at 200 kV. The UV/vis absorption spectra were measured using a Shimadzu UV-1650PC spectrophotometer. N_2 adsorption–desorption isotherms were obtained on a Nova 2000 pore analyzer at 77 K under continuous adsorption condition. BET and BJH analyses were used to determine the surface area, pore size distribution, and pore volume. Magnetic measurement was carried out on a vibrating sample magnetometer (VSM) at room temperature.

(23) Lu, A.-H.; Schmidt, W.; Matoussevitch, N.; Bönnemann, H.; Spliethoff, B.; Tesche, B.; Bill, E.; Kiefer, W.; Schüth, F. *Angew. Chem., Int. Ed.* **2004**, *43*, 4303–4306.
(24) Lu, A.-H.; Li, W.-C.; Kiefer, A.; Schmidt, W.; Bill, E.; Fink, G.; Schüth, F. *J. Am. Chem. Soc.* **2004**, *126*, 8616.
(25) Dyal, A.; Loos, K.; Noto, M.; Chang, S. W.; Spagnoli, C.; Shafi, K. V. P. M.; Ulman, A.; Cowman, M.; Gross, R. A. *J. Am. Chem. Soc.* **2003**, *125*, 1684.
(26) Kim, J.; Lee, J.; Na, H.; Kim, B.; Youn, J.; Kwak, J.; Moon, K.; Lee, E.; Kim, J.; Park, J.; Dohnalkova, A.; Park, H.; Gu, M.; Chang H.; Grate, J. W.; Hyeon, T. *Small* **2005**, *1*, 1203.
(27) Reetz, M. T.; Zonta, A.; Vijayakrishnan, V.; Schimossek, K. *J. Mol. Catal. A: Chem.* **1998**, *134*, 251.
(28) Yang, H.; Zhang, S.; Chen, X.; Zhuang, Z.; Xu, J.; Wang, X. *Anal. Chem.* **2004**, *76*, 1316.
(29) Arruebo, M.; Galan, M.; Navascues, N.; Tellez, C.; Marquina, C.; Ibarra, M. R.; Santamarýa, J. *Chem. Mater.* **2006**, *18*, 1911.
(30) Jiao, F.; Jumas, J.-C.; Womes, M.; Chadwick, A. V.; Harrison, A.; Bruce, P. G. *J. Am. Chem. Soc.* **2006**, *128*, 12905.

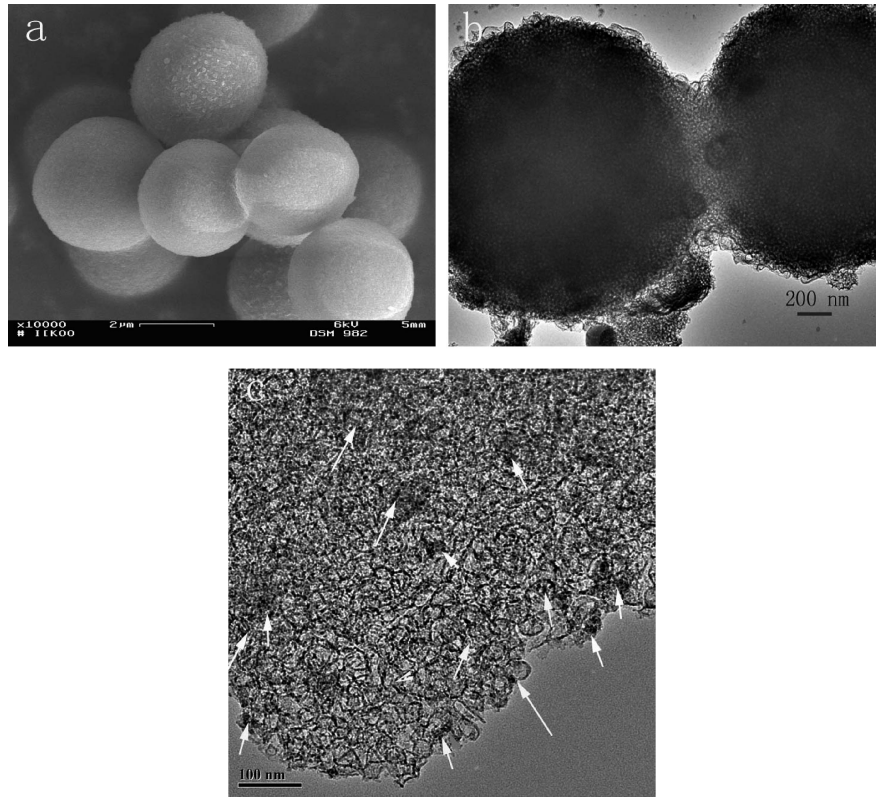

Figure 1. SEM (a) and TEM (b, c) images of Fe_3O_4@MSS.

Results and Discussion

Preparation of Fe_3O_4@MSS Support. Figure 1a shows a typical SEM image of Fe_3O_4@MSS materials. It can be observed clearly that the morphology of Fe_3O_4@MSS is dominated by spheres, and the average size of the spheres is around 2 μm. The TEM images of Fe_3O_4@MSS spheres are shown in Figure 1b,c. These Fe_3O_4@MSS spheres possess a disordered mesoporous structure consistent with the structural features of mesostructured cellular foams (MCFs) reported previously.[31] It also can be found that the magnetite nanoparticles are distributed inside MSS spheres (these magnetite nanoparticles are indicated with arrows) (Figure 1c).

The SAXRD patterns of Fe_3O_4@MSS spheres before and after modification are shown in Figure 2. Both patterns show two broad reflection peaks, which indicates the presence of a mesporous structure without the long-range ordering.[32,33] After being modified with amino groups, the SAXRD pattern has negligible changes, which suggests the mesopore network is intact. The WAXRD pattern of Fe_3O_4@MSS (inset of Figure 2) can be easily indexed to Fe_3O_4 according to the reflection peak positions and relative intensities,[30] confirming the presence of magnetite nanoparticles inside MSS spheres. This is consistent with the result obtained from the TEM observation of the same sample.

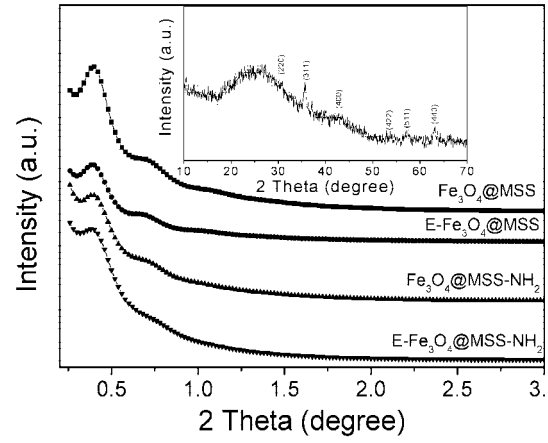

Figure 2. Small-angle XRD patterns of the supports before and after laccase immobilization. The inset is the wide-angle XRD pattern of Fe_3O_4@MSS.

The porosity of Fe_3O_4@MSS spheres before and after modification has been investigated by nitrogen sorption analyses. The isotherms are of type IV and show steep hysteresis of type H1 at high relative pressures (Figure 3), which is typical for mesoporous materials that exhibit capillary condensation and evaporation and possess large pore sizes (inset of Figure 3). It suggests that these spheres would be suitable for the support because of the easier diffusion of the substrates and products. For Fe_3O_4@MSS spheres modified with amino groups, the amount of nitrogen adsorption decreases apparently due to amino groups grafted on the pore surface.

Figure 4a shows the magnetization curve measured at room temperature for Fe_3O_4@MSS. The curve presents a very small hysteresis loop. The M_s (magnetization saturation)

(31) Winkel, P. S.; Lukens, W. W.; Yang, P.; Margolese, D. I.; Lettow, J. S.; Ying, J. Y.; Stucky, G. D. *Chem. Mater.* **2000**, *12*, 686.
(32) Deere, J.; Magner, E.; Wall, J. G.; Hodnett, B. K. *Chem. Commun.* **2001**, 465.
(33) Yokoi, T.; Yoshitake, H.; Tatsumi, T. *J. Mater. Chem.* **2004**, *14*, 951.

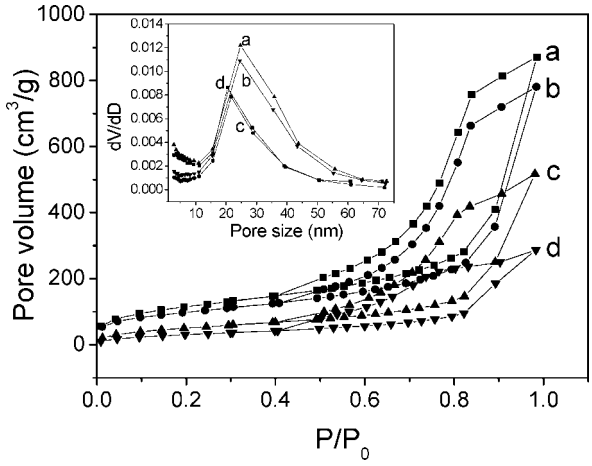

Figure 3. Nitrogen adsorption–desorption isotherms of the supports before and after laccase immobilization and the corresponding pore size distributions calculated from the adsorption branches (a, Fe_3O_4@MSS; b, E-Fe_3O_4@MSS; c, Fe_3O_4@MSS-NH_2; d, E-Fe_3O_4@MSS-NH_2).

value is about 6.05 emu/g. The remnant M_r is about 0.49 emu/g (defined as the magnetization at $H = 0$), and the coercivity H_c is about 44 Oe (defined as the field magnitude necessary to obtain $M = 0$). Therefore, this indicates that Fe_3O_4@MSS supports showed paramagnetic behavior, and their separation in liquid media can be controlled by magnetic fields. The separation properties of these supports were not affected by the modification of the supports and the immobilization of laccase on the supports. Even after laccase immobilized on Fe_3O_4@MSS and Fe_3O_4@MSS-NH_2 oxidized ABTS to $ABTS^{\bullet+}$, the immobilized laccase also can be separated quickly by applying an external magnetic field (Figure 4b).

Immobilization of Laccase. From N_2 adsorption–desorption isotherms and the structure parameters of Fe_3O_4@MSS and Fe_3O_4@MSS-NH_2 before and after the immobilization of laccase (Figure 3 and Table 1), the surface area, pore volume, and pore size are apparently decreased after the supports immersed in laccase solutions for 12 h. This indicates laccase has been immobilized on Fe_3O_4@MSS and Fe_3O_4@MSS-NH_2. When laccase was immobilized on the supports, the SAXRD patterns still show two reflection peaks and have no apparent change (Figure 2), which suggests that the immobilization of laccase did not destroy the mesoporous structure of the supports.

To determine the immobilized amount of laccase on Fe_3O_4@MSS and Fe_3O_4@MSS-NH_2, equilibrium adsorption

Table 1. Structural Parameters of the Supports before and after Laccase Immobilization

samples	S_{BET} (m^2/g)	V_p (cm^3/g)[a]	D_p (nm)[b]
Fe_3O_4@MSS	421	0.628	24.7
E-Fe_3O_4@MSS	354	0.548	24.4
Fe_3O_4@MSS-NH_2	193	0.385	21.6
E-Fe_3O_4@MSS-NH_2	119	0.286	20.5

[a] The single point adsorption total volume at $P/P_0 = 0.892$. [b] D_p is the peak pore size in pore size distribution from the adsorption branch.

experiments were used. When the equilibrium mixtures of laccase and support suspensions are separated, the free laccase concentrations in the supernatant can be measured by means of UV–vis spectrophotoscopic measurements, and then the immobilized amount of laccase can be calculated. Figure 5a shows the immobilized amounts of laccase on the supports as a function of laccase concentration. It can be observed that the immobilized amount of laccase increases with the increase of the laccase concentration. Up to a certain laccase concentration, no more laccase can be immobilized. Therefore, it can be estimated that the immobilized amounts of laccase on Fe_3O_4@MSS and Fe_3O_4@MSS-NH_2 can reach about 82 and 59 mg/g, respectively. Here the immobilized amount of laccase on Fe_3O_4@MSS-NH_2 is lower than that on Fe_3O_4@MSS, which may be related to the relatively low surface area of Fe_3O_4@MSS-NH_2. In addition, two $-$COH groups of glutaraldehyde molecule were possibly covalently attached with the amino groups on the surface of the channels before laccase immobilization, which decreases the amount of functional groups to react with laccase molecules. Figure 5b shows the immobilized amounts of laccase on the supports as a function of time when the laccase concentration is 1.0 mg/mL. The immobilization of laccase on Fe_3O_4@MSS is fast, and equilibrium adsorption can be achieved in 6 h. However, it takes more than 12 h for Fe_3O_4@MSS-NH_2 to reach equilibrium. Such slow immobilized behavior of Fe_3O_4@MSS-NH_2 may be due to the decreased pore size causing hindered diffusion effects.

Activity of the Immobilized Laccase. It is essential for the application of the immobilized laccase that the immobilized laccase should retain a high activity. To investigate the activity of the immobilized laccase, we employed the oxidation of ABTS by laccase to test laccase activity. The product of this reaction is the green cation radical ($ABTS^{\bullet+}$) with an absorption maximum at 420 nm, providing a convenient spectrophotometer test method. The specific activities of the free and immobilized laccase in phosphate

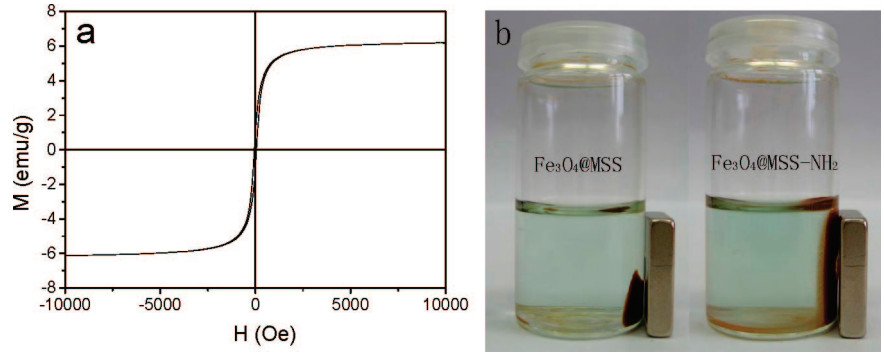

Figure 4. (a) Magnetization curve measured at room temperature for Fe_3O_4@MSS. (b) Photograph of the immobilized laccases in the reaction media under an external magnetic field.

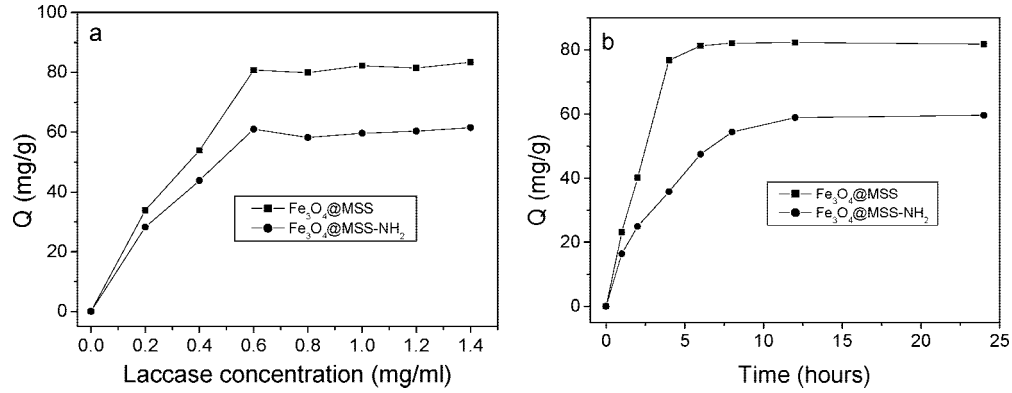

Figure 5. (a) Immobilized amounts of laccase on the supports as a function of laccase concentration. (b) Immobilized amounts of laccase on the supports as a function of time.

Table 2. Imobilization Capacity and Activity of the Free and Immobilized Laccase

samples	immobilization capacity (mg/g)	specific activity of laccase (U/mg)	recovery yield of laccase (%)
free laccase		0.476	100
E-Fe_3O_4@MSS	82	0.378	79.4
E-Fe_3O_4@MSS-NH_2	59	0.271	56.9

buffer of pH 4.5 at 20 °C are given in Table 2. It can be found that the activity of laccase was retained to a large extent, 79.4% and 56.9%, respectively, when laccase was immobilized on Fe_3O_4@MSS and Fe_3O_4@MSS-NH_2. This demonstrates that the immobilized laccase is not denatured; ABTS can easily diffuse into the mesoporous channels of supports and be oxidized by the immobilized laccase. Here much lower specific activity was observed for the Fe_3O_4@MSS-NH_2 than Fe_3O_4@MSS, which can be explained that the more active sites of laccase are not available for taking part in the reactions when laccase is immobilized on supports by covalent attachment, and the method of covalent attachment itself is adverse to the activity of the immobilized laccase.[17]

The activity of the free and immobilized laccase at different pH values is shown in Figure 6a. The free and immobilized laccases exhibit maximal activity at pH 3.6. The immobilized laccase shows higher resistance to changes in pH value of the medium, and much higher activity values were obtained for the immobilized laccase in a medium from pH 5.0 to pH 7.0. Figure 6b shows the effect of temperature on the activity of the free and immobilized laccase. The maximal activity is observed in the temperature range 10–30 °C and followed by a stepwise decrease of activity with the increase of temperature. However, laccase immobilized on Fe_3O_4@MSS and Fe_3O_4@MSS-NH_2 supports retains a higher activity at the same temperature range of 40–70 °C compared with the free laccase.

To investigate the time-dependent thermal stability of the immobilized laccase, the free and immobilized laccases were treated at 60 °C in buffer solution of pH 3.6 for variable incubation periods. Samples were withdrawn every 30 min, and then their activities were measured immediately. Figure 7 shows the activity of the free and immobilized laccase treated at 60 °C as a function of time. The activity of the immobilized laccase decreased more slowly than that of free laccase. Four hours later, the residual activities of free laccase and laccase immobilized on Fe_3O_4@MSS and Fe_3O_4@MSS-NH_2 were 16.4%, 61.2%, and 72.7%, respectively.

Apparently, laccase immobilized on Fe_3O_4@MSS and Fe_3O_4@MSS-NH_2 shows higher pH and thermal stabilities than free laccase, since mesoporous channels of supports can keep laccase from injuring due to direct exposure environmental change. However, when comparing the pH and thermal stabilities of the two immobilized laccases, it can be found that the Fe_3O_4@MSS-NH_2 supports provide better results than the Fe_3O_4@MSS supports. Because covalent attachment provides much stronger binding interaction between laccase and support than physical adsorption, laccase immobilized on Fe_3O_4@MSS-NH_2 reduces the occurrence of drastic conformational changes than laccase immobilized on Fe_3O_4@MSS, thus resulting in an increased pH resistance and thermal stability.

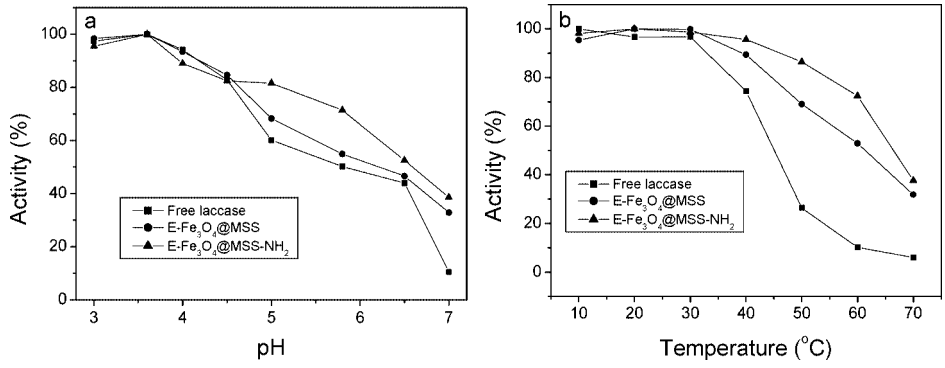

Figure 6. (a) Activity of the free and immobilized laccase at different pH values. (b) Effect of temperature on the activity of the free and immobilized laccase.

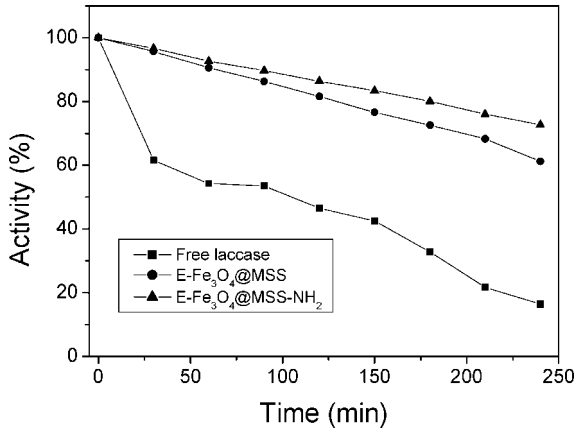

Figure 7. Activity of the free and immobilized laccase as a function of time at 60 °C.

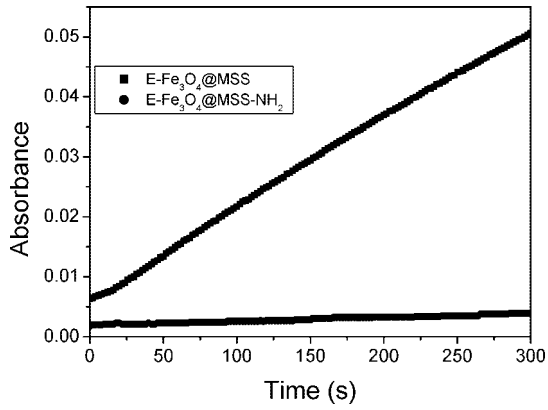

Figure 8. UV absorbancen of ABTS oxidized by the leaching solutions of two immobilized laccases at the wavelength of 420 nm.

The leaching of the immobilized laccase is disadvantageous for practical applications. Here, we also investigated the leaching behavior of the two immobilized laccases. For leaching experiments, the two immobilized laccases were stirred in phosphate buffer of pH 3.6 for 1 h, and then the supernatants were separated from the solutions. Figure 8 shows the UV absorbance of ABTS oxidized by leached solutions of the two immobilized laccases at the wavelength of 420 nm. In the case of laccase immobilized on Fe_3O_4@MSS-NH_2, the UV absorbance of $ABTS^{\bullet+}$ almost has no increase. However, the UV absorbance of $ABTS^{\bullet+}$ increases with the increase of time for laccase immobilized on Fe_3O_4@MSS. This suggests that laccase immobilized on Fe_3O_4@MSS-NH_2 has very little leaching, but for laccase immobilized on Fe_3O_4@MSS, leaching is significant.

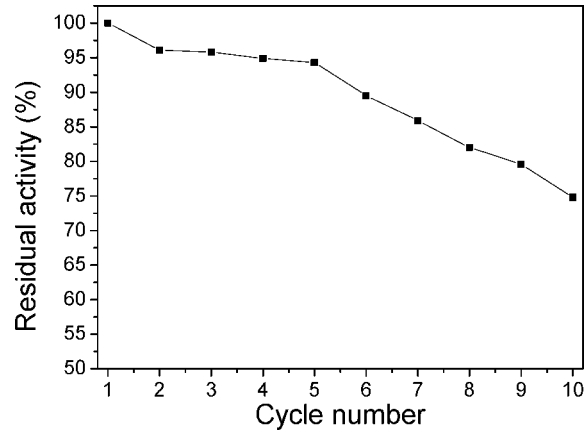

Figure 9. Reusability of laccase immobilized on Fe_3O_4@MSS-NH_2.

The reusability of laccase immobilized on Fe_3O_4@MSS-NH_2 is shown in Figure 9. The immobilized laccase retained above 70% residual activity after 10 consecutive operations, which indicates that laccase immobilized on Fe_3O_4@MSS-NH_2 performed a good reusability.

Conclusions

A magnetically separable mesoporous silica sphere has been prepared by reducing Fe^{3+}-containing mesoporous sphere. Laccase from *Trametes versicolor* could be immobilized on these magnetically separable mesoprous silica spheres through physical adsorption and covalent attachment methods, and the activities of both immobilized laccases were retained to some extent. The thermal and pH stabilities of both immobilized laccases were improved apparently as compared to the free laccase. More importantly, the immobilized laccase offered the advantage of quick separation in a magnetic field. However, the immobilized laccase obtained through physical adsorption exhibited the laccase leaching during the reaction, while the immobilized laccase obtained though covalent attachment almost has no leaching. Furthermore, the immobilized laccase obtained though covalent attachment showed good reusability, and it can retain above 70% of activity after 10 consecutive operations.

Acknowledgment. The authors gratefully acknowledge the support provided by the Alexander von Humboldt Foundation.

CM071265G