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Surface Modification of Halloysite Nanotubes with Dopamine for Enzyme Immobilization

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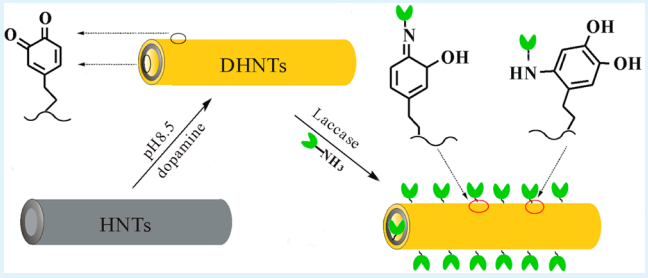
Supporting Information

ABSTRACT: Halloysite nanotubes (HNTs) have been proposed as a potential support to immobilize enzymes. Improving enzyme loading on HNTs is critical to their practical applications. Herein, we reported a simple method on the preparation of high-enzyme-loading support by modification with dopamine on the surface of HNTs. The modified HNTs were characterized by transmission electron microscopy, Fourier transform infrared spectroscopy, and X-ray photoelectron spectroscopy analyses. The results showed that dopamine could self-polymerize to adhere to the surface of HNTs and form a thin active coating. While the prepared hybrid nanotubes were used to immobilize enzyme of laccase, they exhibited high loading ability of 168.8 mg/g support, which was greatly higher than that on the pristine HNTs (11.6 mg/g support). The immobilized laccase could retain more than 90% initial activity after 30 days of storage and the free laccase only 32%. The immobilized laccase could also maintain more than 90% initial activity after five repeated uses. In addition, the immobilized laccase exhibited a rapid degradation rate and high degradation efficiency for removal of phenol compounds. These advantages indicated that the new hybrid material can be used as a low-cost and effective support to immobilize enzymes.



KEYWORDS: *halloysite nanotubes, dopamine, enzyme immobilization, modification, laccase*

INTRODUCTION

As a kind of effective and selective biocatalyst, enzymes have attracted significant attention in many fields such as industrial synthesis, pharmacy industry, and environmental protection. However, their use is currently limited because of significant drawbacks such as their low thermal stability, instability, relatively high cost, and difficulties associated with recovery and reuse. The previous studies have revealed that immobilizing enzymes on the supports could effectively solve these problems.[1−4] So, various supports, including polymeric organic and inorganic materials, have been explored to immobilize enzymes.[5,6] The inorganic materials as supports outperform the organic ones in many aspects, such as high stability or antiswelling ability, high resistance to microbial corrosion, and better reusability.[7] Some inorganic supports like clays, layered double hydroxides, silica gels, amorphous aluminum phosphate, alumina, and zeolites have been utilized as supports for enzyme encapsulation and immobilization.[7−9] These traditional supports with pore sizes of 3−10 Å (roughly the same size as small molecules) have found wide application in enzyme immobilization, but their relatively small pore sizes deny access to large-molecule and/or bulky reactants and thus limit their further applications. Recently, some porous nanomaterials, such as carbon nanotubes, mesoporous silica, and nonsilica materials (MCM-41), have been explored to solve this problem; however, the high cost and difficulty in preparation of these mesoporous materials limited their practical applications on a large scale.[10]

Halloysite is a type of natural kaolinite mineral. Compared with kaolinite [$Al_2Si_2O_5(OH)_4$], halloysite [$Al_2Si_2O_5(OH)_4 \cdot 2H_2O$] contains additional water molecules between the alumina/silica layers. The hydration of −Al−OH and −Si−OH groups reduces bonding interactions between the neighboring alumina/silica layers and thus drives the layers to curve and form a particularly hollow tubular structure.[11−13] In such a way, halloysite nanotubes (HNTs) with inner diameters of 5−20 nm are more suitable for entrappping macromolecules than the intercalated kaolinite and montmorillonite with small pore sizes of 0.3−2 nm (roughly the same size as small molecules).[11,14] Furthermore, the exterior and interior surfaces of HNTs are composed respectively of siloxane (Si−O−Si) groups and a gibbsite-like array of aluminol (Al−OH) groups, yielding a negatively charged outer surface and a positively charged inner lumen within a range of pH values.[12] The

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halloysite versatile surface features derived from its special structure provide opportunities for advanced applications in fields such as electronics, catalysis, separation, and functional materials.[15−19] The halloysite has also been recognized as a fine lumen or support to immobilize a broad variety of biologically active substances including drugs, enzymes, and DNA.[20] However, the intrinsic inorganic property and the absence of abundant functional groups of halloysite result in low loading and weak bonding between biomolecules and nanotubes. Surface modification of HNTs has been demonstrated to be an effective method to improve biomolecule loading on HNTs. For instance, the maximum enzyme loading of halloysite modified with chitosan could reach as large as 21.5 mg/g, higher than 3.1 mg/g of pristine halloysite.[21] Halloysite functionalized with selective protein could be used as target-specific coatings to capture special biomolecules.[22] To the best our literature knowledge, there are few studies on the modification of HNTs with a biomimetic molecule, dopamine, to load biomolecules.

Because Messersmith and co-workers pioneered the single-step formation of polymer-film-based dopamine on various substrates, surface modification with polydopamine has already become an efficient and feasible method to endow biological functionality on inorganic materials.[23,24] Meanwhile, this method is not involved in complex linkers and is free of organic solvents, making it suitable for biomaterial applications. Furthermore, the abundant functional groups (i.e., catechol and amine) existing on the modified surface could enhance the binding ability for various biomolecules.[24,25] On the basis of this idea, we designed a new hybrid support by modification with dopamine on the surface of HNTs in the present work. We selected laccase (EC 1.10.3.2) as a model enzyme to elucidate the enzyme-immobilizing ability of dopamine-modified halloysite nanotubes (DHNTs). The results showed that DHNTs had an excellent capacity for laccase immobilization. Compared to the free laccase, the immobilized laccase displayed more excellent properties of thermal stability, storage stability, and reusability, which indicates that new hybrid nanotubes can be used as low-cost and relatively effective supports to immobilize enzymes.

EXPERIMENTAL SECTION

Materials. The HNT mineral was obtained from clay minerals in Henan, China. The source material was crushed and sieved to eliminate aggregates prior to use. Dopamine was supplied by Yuancheng Technology Development Co., Ltd. (Wuhan, China). Tris(hydroxymethyl)aminomethane, laccase (EC 1.10.3.2, 13.6 U/mg) from *Trametes versicolor*, and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) were obtained from Sigma-Aldrich (USA). Coomassie brilliant Blue G-250 and bovine serum albumin were purchased from Solarbio Company (Beijing, China). All other chemicals were used without further purification as analytical grade.

Characterization. The microstructures of the nanotubes were observed using transmission electron microscopy (TEM; FEITEC-NAIG2). The Fourier transform infrared (FTIR) spectra of the samples were measured by a FTIR spectrometer (IR300, Nocolet). X-ray photoelectron spectroscopy (XPS) spectra were recorded on a Thermo VG ESCALAB250 X-ray photoelectron spectrometer. A Shimadzu UV−vis spectrophotometer (UV-2450, Shimadzu) was used to analyze the concentration and activity of the enzyme.

Preparation of Modified HNTs. DHNTs were prepared in the following manner. A sample of HNTs was dispersed in deionized water to obtain a HNT suspension (10 mg/mL, 50 mL). After 0.5 h of ultrasound treatment, the pH value of the suspension was adjusted to alkalescence (pH 8.5) by tris(hydroxymethyl)aminomethane. Then, 0.2 mg of dopamine powder was added into the HNT suspension being stirred at 30 °C. The final mixture was stirred for 360 min to obtain HNTs coated with black insoluble polymers. The resulting black product was separated by centrifugation and washed with distilled water repeatedly until the black filtrate became colorless and transparent.

Determination of Enzyme Loading. Laccase was selected as a target enzyme to be immobilized on DHNTs. The immobilization of laccase was carried out as follows: DHNTs was added into a centrifuge tube charged with 10 mL of a laccase solution of citric acid/Na_2HPO_4 buffer (pH 5.0). The mixture was maintained in a shaking bed at 25 °C for 600 min, and the immobilized laccase was separated by centrifugation and washed with 40 mL of a buffer solution three times to remove the free laccase. Then the wet immobilized enzyme was stored at 4 °C for subsequent enzyme assays.

The Bradford method was used to determine enzyme loading on DHNTs by measuring of the initial and final concentrations of protein within the immobilization medium solutions.[26] A calibration curve was plotted using Coomassie Brilliant Blue G-250 solutions as standards (0−24 mg/L). The enzyme concentration in the solution can be determined with UV−vis spectrophotometry by measuring the absorbance at 595 nm. The amount of enzyme immobilized onto DHNTs was calculated by mass balance with the following equation:

$$\text{enzyme loading (mg/g)} = \frac{(C_0 - C_1)V}{W_s} \times 100\%$$

where C_0 is the initial enzyme concentration (mg/mL), C_1 is the final enzyme concentration (mg/mL), V is the enzyme volume (mL), and W_s is the support dose added (g).[13]

Assay of the Laccase Activity. The laccase activity was measured as the oxidation of ABTS according to the method in the literature.[27] The activities of free and immobilized laccase were obtained spectrophotometrically by generation of the ABTS radical. The relative enzymatic activity was related to a percentage of this highest activity (100% represents the highest enzymatic activity). The activity recovery was calculated from the value of the activity of the initial laccase solution divided by the activity value of immobilized laccase obtained immediately after the immobilization procedure.

Removal of 2,4-Dichlorophen. The degradation assay of 2,4-dichlorophen was performed at 30 °C with a reaction mixture of immobilized laccase and a 2,4-dichlorophen solution. The mixture was incubated for 600 min under continuous vibration. The percentage of 2,4-dichlorophen degradation was determined by measuring the changes of the supernatant absorption using a double-beam UV−vis spectrophotometer. The colorimetric assay solution was composed of a diluted sample (50.0 mL), a buffer solution (5.0 mL, 100 mM aqueous ammonia), a potassium ferricyanide reagent [1.0 mL, 80 mg/mL of $K_3Fe(CN)_6$ in water], and a 4-aminoantipyrene (4-AAP) solution (1.0 mL, 2% 4-AAP in water). The resulting compound was a quinone-type dye with a characteristic absorption peaking at 510 nm.[28] The absorbance was converted to the concentrations of 2,4-dichlorophen according to a calibration curve. The removal efficiency was calculated with the following formula:

$$\text{removal efficiency} = \frac{C_0 - C_1}{C_0} \times 100\%$$

where C_0 and C_1 are the initial and final 2,4-dichlorophen concentrations (mg/mL) in the solution, respectively.

RESULTS AND DISCUSSION

Strategy for Immobilizing Laccase on DHNTs. The method of polydopamine surface modification and laccase immobilization is illustrated in Figure 1. First, HNTs were immersed in an alkaline aqueous solution of dopamine for several hours to form an adherent polydopamine coating on nanotubes (DHNTs). Second, DHNTs were used as supports to immobilize laccase. The residual quinone functional groups presented in the polydopamine coating are reactive toward

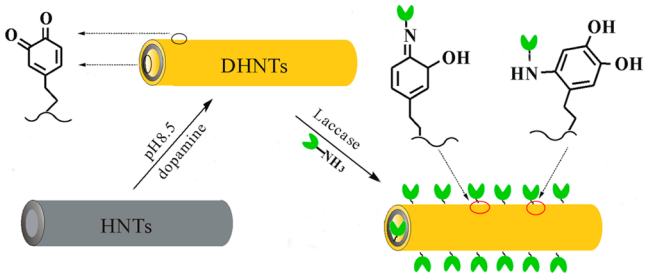

Figure 1. Schematic description of the polydopamine coating and subsequent laccase immobilization on DHNTs.

nucleophilic groups, and laccase can couple covalently with polydopamine through Michael addition and/or Schiff base formation.[29−31] The active quinone groups at the surface of DHNTs may react with the amino groups of laccase, which results in laccase immobilization on the surfaces of DHNTs.[30]

Characterization of HNTs and DHNTs. Figure 2 shows the TEM images of the pristine HNTs and DHNTs prepared in

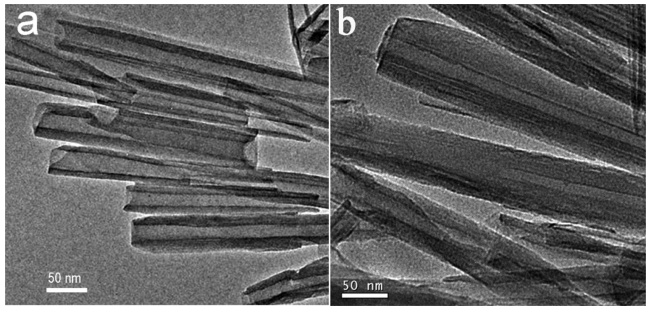

Figure 2. TEM micrographs of HNTs (a) and DHNTs (b).

this work. The images of HNTs (Figure 2a) show that HNTs possess a hollow tubular structure and both ends are open. The lengths of the HNTs range from 500 to 1000 nm, and the inner diameters range from 15 to 35 nm. The tube wall thicknesses of HNTs vary in the range of 10−15 nm. The tube wall of DHNTs becomes thicker than that of HNTs (Figure 2b), indicating that a dopamine polymer layer had formed on the surface of the HNTs. To elucidate morphology changes of HNTs and DHNTs, the inner and outer diameters of nanotubes before and after modification were measured from TEM images. The morphological parameters of the HNT and DHNT samples are summarized in Table S1 in the Supporting Information (SI). It is noted that the accuracy of these dimensions is limited by the number of nanotubes observed in the TEM images. The results suggested that the average outer diameter of DHNTs increased about 9 nm (from 7.5 to 10 nm), while the inner diameter decreased about 6 nm (from 5.5 to 7 nm) compared with the pristine HNTs, indicating that the dopamine polymer layers existed on both the inner and outer surfaces of HNTs.

Thermogravimetric analysis (TGA) of the HNT and DHNT samples was further carried out to test the amount of dopamine modified on the surface of HNTs (Figure S1 in the SI). The TGA curve of pristine HNTs reveals a weight loss of 14.23 wt % from 105 to 775 °C, corresponding to the removal of surface hydroxyl groups (−OH). As for DHNTs, a weight loss of 17.66 wt % within the same temperature range is displayed, 3.43 wt % more than HNTs, which can be attributed to the additional thermal loss of loaded polydopamine. One may conclude that the amount of polydopamine is approximately 3.43 wt %.

Figure 3 shows a FTIR spectral comparison of HNTs and DHNTs. In the FTIR spectrum of HNTs, absorption bands at

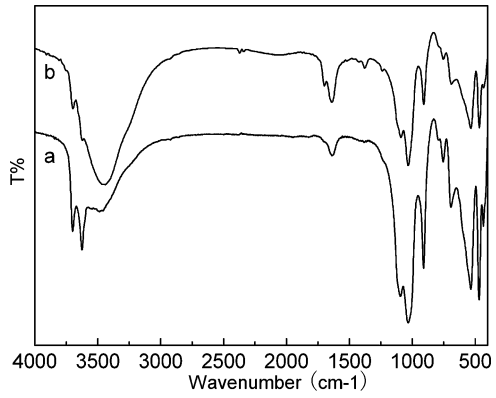

Figure 3. FTIR spectra of (a) HNTs and (b) DHNTs.

3701 and 3628 cm^{-1} are ascribed to −OH groups. The band at 910 cm^{-1} is assigned to the bending vibration of Al−OH. Others bands at 1000−1100 and 450−550 cm^{-1} are due to the Si−O stretching and Si−O bending vibrations, respectively. After modification by dopamine, new peaks appear at 3440 and 1698 cm^{-1}. The vibration band at 3440 cm^{-1} is attributed to symmetric and asymmetric NH_2 stretching vibrations. The absorption band at 1698 cm^{-1} is associated with the stretching vibration of the carbonyl group on polydopamine. The above results may indicate that polydopamine, the sole source for C−H and N−H, has been successfully formed on HNT surfaces.

The presence of polydopamine films on DHNTs was confirmed by XPS measurement in Figure 4. The XPS survey spectra of DHNTs show the presence of O, C, N, Si, and Al (Figure 4a). The complicated nature of polydopamine on DHNTs was revealed by the different chemical states of elemental C, O, and N in deconvoluted core-level spectra (Figure 4b−d). The main peak in the C 1s spectra (Figure 4b) at 286.5, 285.8, 284.5, and 282.9 eV, respectively, corresponding to C═O bonds (C−N and C−OH), C−H, and aromatic C in the dopamine polymer. In the O 1s peak (Figure 4c), two peaks were observed at 531.7 and 532.5 eV, which were due to the O atoms of polydopamine in the form of quinone and catechol. The high-resolution spectra of the N 1s peak are shown in Figure 4d. The main peak at 399.9 eV indicated the presence of R_2NH and RNH_2, while the small shoulder at 401.8 eV was attributed to R_3N. This result indicated that the adhesive polydopamine film formed on the surface of HNTs through self-polymerization.

Immobilization of Laccase. The immobilization capacity of DHNTs was evaluated by investigating the laccase loading. As shown in Figure 5, the amount of laccase immobilized on DHNTs increased with increasing laccase concentration in the medium. When the laccase concentration was 4.0 mg/mL, the laccase loading increased to 168.8 mg/g. However, when the laccase concentration was more than 3 mg/mL, a decline in the activity recovery of the immobilized laccase was observed. The loading reaches a maximum at a high enzyme concentration (~5 mg/mL), and there is a slightly continuous decrease in the enzyme activity when the enzyme concentration exceeds 4 mg/mL. A similar phenomenon was also observed on other supports.[5] It may be explained that excessive laccase loading

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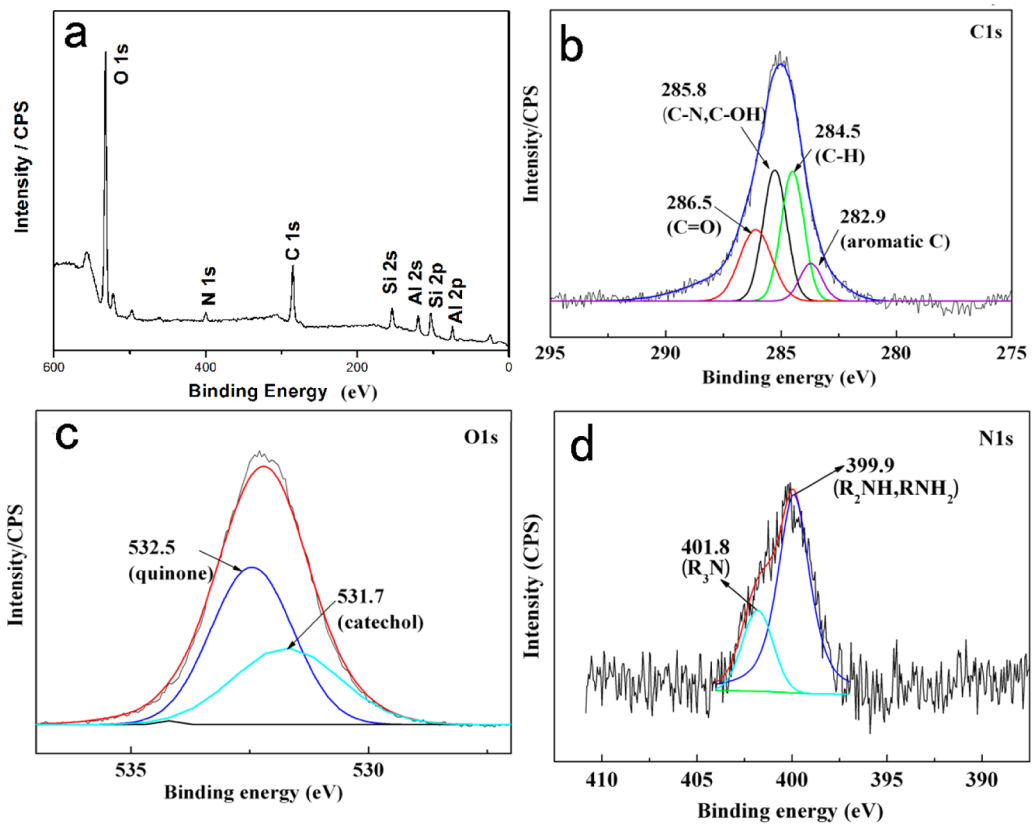

Figure 4. XPS analyses of DHNTs: (a) survey scan and (b) C 1s, (c) O 1s, and (d) N 1s core levels.

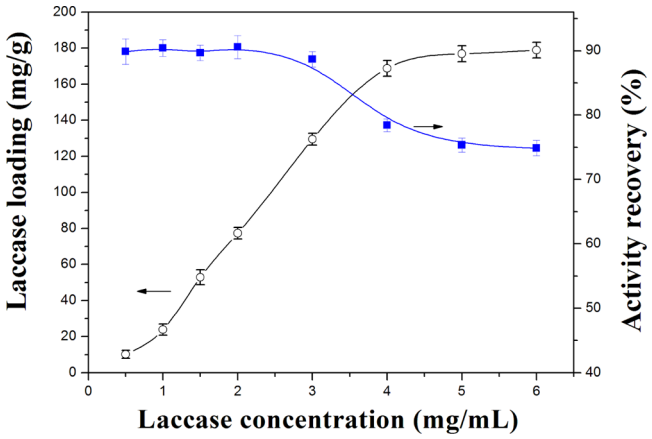

Figure 5. Effect of the initial laccase concentration on the laccase loading and activity recovery.

would easily lead to the crowding or agglomeration of enzyme molecules onto the surface of supports. Consequently, the resulting steric constraints could restrain dispersion and transmission of the substrate and product, exhibited as a reduction of the activity.[5] Therefore, the optimum laccase concentration was chosen as 4.0 mg/mL. In this case, the laccase loading is efficient (activity recovery higher than 92%) without sacrificing the unnecessary use of excess enzyme.

Meanwhile, compared with the enzyme loading of 21.5 mg/g on pristine HNTs modified with chitosan at an initial laccase concentration of 4.0 mg/mL,[21] the enzyme loading on DHNTs can reach as high as 168.8 mg/g, nearly 7 times as high as that on HNTs. Such a high loading capacity can be attributable to modification of polydopamine on the outer surface and/or inner lumen of the nanotubes, which introduces abundant functional groups to effectively bind laccase. The electrostatic interactions associated with the surface charges could affect enzyme immobilization in some cases.[17] However, the covalent linking may play a more dominant role in the interactions between the enzyme and DHNTs in this case.

According to the inner and outer diameter changes of the nanotubes in Table S1 in the SI, we can calculate that the ratio of increased volume inside and outside the nanotubes is 1:3.9, indicating that the ratio of polydopamine modified on the inner and outer nanotubes is approximately 1:3.9. Considering that the functional groups of polydopamine could enhance significantly the binding ability for laccase molecules, it is deduced that the loading percentage outside the nanotubes is much higher than that inside the nanotubes, although it is difficult to measure the accurate amount of laccase loaded outside or inside the nanotubes.

Stability of Immobilized Laccase. Enzyme immobilization is favorable for its applications. The effect of the temperature on the stability of laccase is shown in Figure 6a. The traces indicate that the immobilized laccase attained a smaller rate of thermal inactivation relative to the free laccase. At 60 °C, the free enzyme only retained 34% activity after a 280 min test, while the immobilized one showed a significant resistance to thermal inactivation (retaining 85% activity). These results suggest that the thermal stability of immobilized laccase is superior at the higher temperature. The reason could be attributed to the preservation of the structure and active site of the enzyme by immobilization on the support.[32] The improved thermal stability would extend the application of the immobilized enzymes.

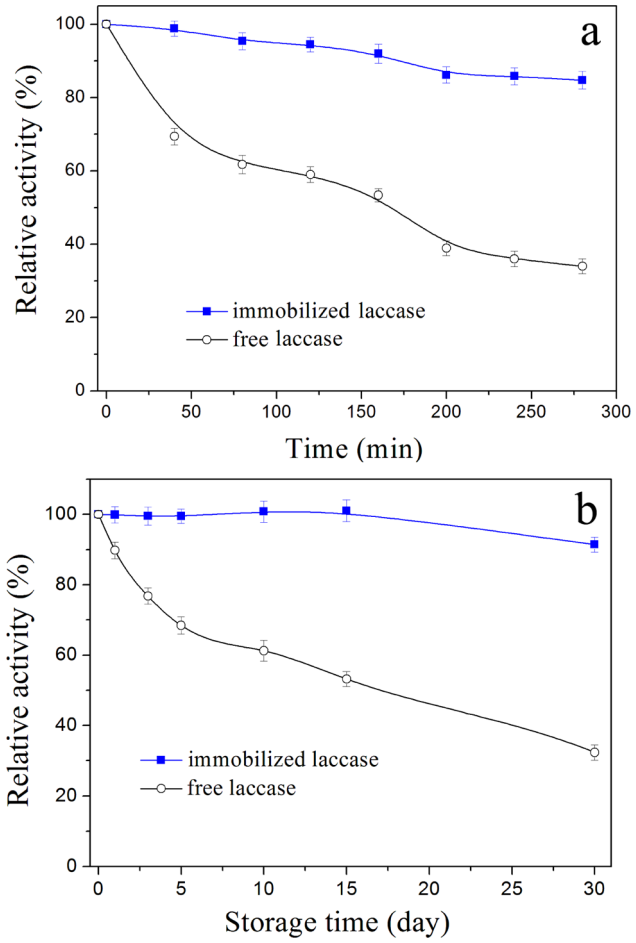

Figure 6. Thermal stability at 60 °C (a) and storage stability at 4 °C (b) of the free and immobilized laccase (4.0 mg/mL).

The storage stability of immobilized laccase in a phosphate buffer (100 mM, pH 5.0) was much better than that of the free laccase at 4 °C. From Figure 6b, it could be observed that the free laccase retained 32.31% of the initial activity after 30 days, while the immobilized laccase still held more than 90% of the initial activity after the same period. The improved storage stability was due to increasing stabilization of its active conformation by multipoint bond formation between the support and laccase molecule.[33−35] The interactions between the enzymes and supports with different geometries could affect the activities of the enzyme. It is generally believed that highly curved surfaces could reduce the possibility of enzyme denaturation and suppress lateral interactions between adjacent enzymes, thus allowing for adsorbed enzyme to maintain its structural integrity and activity.[36,37] The laccase immobilized on the curving surface of DHNTs could retain higher activity compared with flat surfaces. Furthermore, compared with the enzymes immobilized outside the nanotubes, the enzymes encapsulated inside the nanotubes could be protected by the shell of nanotubes, and they could retain higher activity in some external environments.

Besides the high thermal and storage stabilities, the practical application of immobilized enzymes in the industry also requires excellent reusability. The reusability stability of immobilized laccase was determined at optimum activity conditions using immobilized laccase on DHNTs. As shown in Figure 7, an activity loss of 8% was observed within the initial

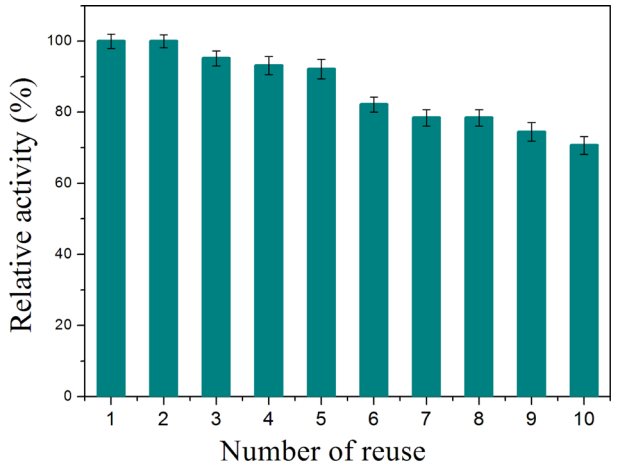

Figure 7. Reusability of immobilized laccase.

five cycles. Since the sixth cycle, the activity remained in a state of slowdown for another five cycles. After 10 repeated uses, the immobilized enzyme retained more than 70% of its initial activity. The activity loss may be due to enzyme leakage during washing and enzyme deactivation during repeated uses.[38,39]

Removal of 2,4-Dichlorophen. Some enzymes have been used as biocatalysts to remove phenolic compounds, among which laccase can react with aqueous phenolic compounds and form insoluble products. They could be easily removed from the aqueous solution. Phenolic compounds of 2,4-dichlorophen were selected as target organic contaminants to evaluate the removal efficiency of immobilized laccase in the present work. As described in Figure 8, a high removal rate was observed in

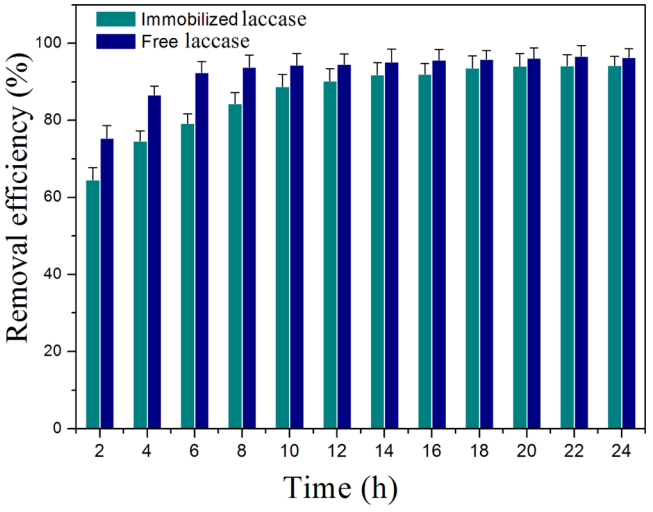

Figure 8. Removal efficiency of 2,4-dichlorophen by the free and immobilized laccase with time.

the initial 2 h, and about 65% of the total 2,4-dichlorophen (50 mg/L) was degraded. After a period of 2 h, the reaction rate decreased, possibly because of the simultaneous decrease in the concentration of the reacting species.[39,40] When the reaction of 2,4-dichlorophen degradation lasted for 10 and 24 h, the removal efficiencies of 2,4-dichlorophen (50 mg/L) reached 90.2% and 94.6%, respectively. Compared to the free laccase (Figure 8), the immobilized laccase displayed lower removal efficiency, especially in the initial stage. When the reaction time extended over 20 h, the removal efficiency by immobilized

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laccase was close to that by free laccase. For instance, the removal efficiency by free laccase reached 96.22% at a time of 20 h, only 2.1% higher than that by immobilized laccase (94.12%). Considering excellent reusability, thermal stability, and other properties of immobilized enzymes, the immobilized laccase is more applicable to practical applications.

CONCLUSIONS

A DHNT hybrid was successfully prepared by the self-polymerization of dopamine on a natural HNT surface. The modified HNTs showed an excellent loading capacity for laccase compared with the natural HNTs. When they were used as enzyme supports, they exhibited a high capacity of 168.79 mg/g for laccase immobilization. Both the thermal and storage stabilities of laccase were greatly elevated after immobilization. Reusability was also improved, and the activity of immobilized laccase could maintain 70% of the initial activity after 10 cycles. Finally, immobilized laccase was utilized to remove 2,4-dichlorophen from wastewater, which achieved a removal efficiency as high as 94%. On the basis of these results, it was concluded that the DHNT hybrid could be used as an effective support for biomacromolecule immobilization.

ASSOCIATED CONTENT

Supporting Information

TGA and structural data for HNTs and DHNTs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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