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Tuning Laccase Catalytic Activity with Phosphate Functionalized Carbon Dots by Visible Light

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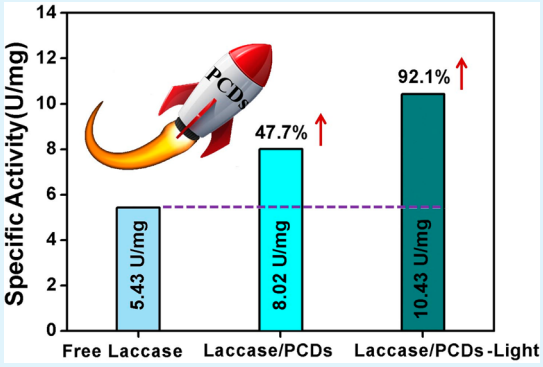
Supporting Information

ABSTRACT: The phosphate functionalized carbon dots (PCDs) with high biocompatibility and low toxicity can be used as efficient additives for the construction of laccase/PCDs hybrids catalyst. A series of experiments indicated that the activity of laccase/PCDs was higher than that of free laccase (increased by 47.7%). When laccase/PCDs hybrids catalyst was irradiated with visible light (laccase/PCDs-Light), its activity was higher than that of laccase/PCDs hybrids without light irradiation (increased by 92.1%). In the present system, the T1 Cu in laccase was combined with the phosphate group on PCDs, which can increase binding capacity of laccase/PCDs hybrids and substrate. Further, the visible light irradiation increased the donating and accepting electronic capability of the laccase/PCDs hybrids, improving their catalytic activity.



KEYWORDS: *carbon dots, phosphate modification, laccase, activity tuning, visible light*

1. INTRODUCTION

Enzyme based catalysts have received considerable attention because of their fascinating catalytic properties, such as high activity, selectivity, and specificity, and their widespread applications (e.g., catalysis, chemical/biological sensing, and medical diagnosis).[1−3] Laccase, as a typical multicopper oxidase, containing four copper ions (such as T1 Cu, T2 Cu, and a pair of T3 Cu) distributed into three locations (T1, T2, and T3 sites) can oxidize a variety of substrates (phenols, polyphenols, and anilines) and was widely applied in industrial oxidation reactions.[4,5] In general, changing pH, temperature, and substrate concentration can regulate and control the activity of laccase, However, most of these regulative methods perturb the enzyme or substrate reaction environment. Recently, some new techniques may enhance the enzyme activity, involving chemistry of proteins, microbiology, and protein engineering. Also, various inorganic materials (metal ions such as Ca^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+}; gold nanoparticles; graphene oxide; reduced graphene oxide; carbon nanostructues; Cu salts; etc.)[6,7] have been tested in combination with laccase to construct the enzyme based hybrid catalyst. In these systems, the inorganic species could change the enzyme activity; however, the mechanism of change was still unclear, and also their activity cannot be regulated efficiently.

Carbon dots (CDs) with low toxicity have shown unique photoinduced electron transfer and photoluminescence (PL) properties.[7−16] In our previous work, CDs were used to modulate the porcine pancreatic lipase catalytic activity by visible light.[17] It should be noted that the surface functionalization of CDs could further modify their physical and photochemical properties. At the same time, it also provides prerequisite active sites for the photocatalyst design.[17] Based on these advantages of CDs, here, we fabricated the laccase/phosphate modification carbon dots (PCDs) hybrids and investigated the effect that PCDs and visible light have on the catalytic behavior of laccase. The catalytic activity of laccase/PCDs was higher than the activity of laccase (increase about 47.7%). When the laccase/PCDs were irradiated by visible light (laccase/PCDs-Light), their catalytic activity increased about 92.1% vs laccase/PCDs hybrids. In the present system, the specific activity of laccase/PCDs hybrids was gradually increased with increasing light intensity, which was linearly dependent within a low light intensity (<80 mW/cm^2) input, while with the light intensity over 80 mW/cm^2, the specific activity of laccase/PCDs hybrids reached the maximum value and plateaued. Further, the functional group also plays a very important role in the PCDs effect on the activity of laccase via the interaction between the phosphate group and T1 Cu in laccase.

2. EXPERIMENTAL SECTION

Test Instruments and Experiment Chemicals. High-resolution TEM (HRTEM) and transmission electron microscopy (TEM) images were obtained by a FEI-Tecnai F20 (200 kV). The UV−vis spectra were characterized by a LAMBDA 750 photometer, while the photoluminescence (PL) analysis used the Horiba JobinYvon (Fluoro Max 4) Luminescence Spectrometer. The Fourier transform infrared

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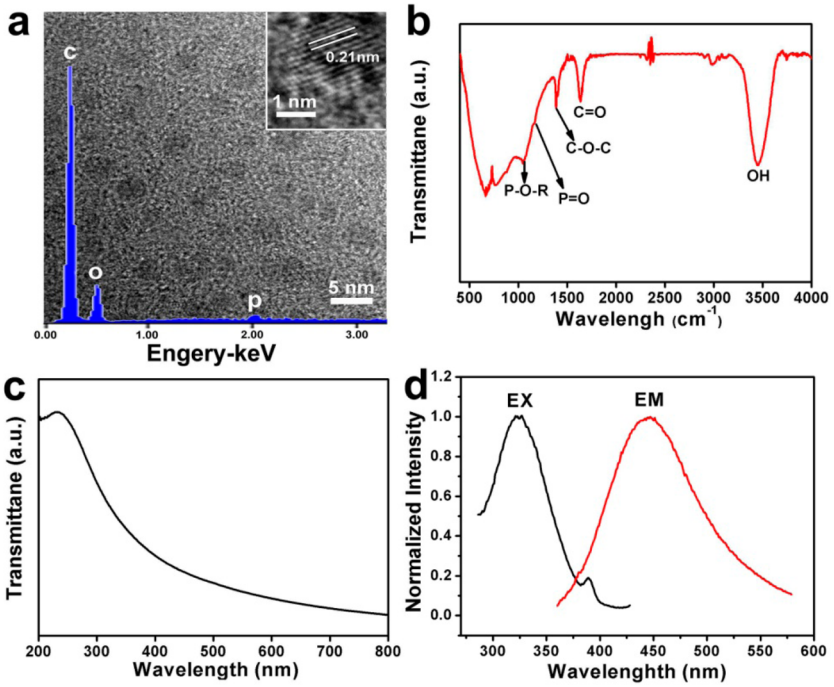

Figure 1. (a) TEM, HRTEM, and EDX images of PCDs. (b) FTIR spectra of PCDs. (c) UV−vis absorption of PCDs. (d) PL excitation (black) and emission (red) spectrum of the PCDs in aqueous solution.

spectra (FTIR) of carbon dots were acquired with a Varian Spectrum GX spectrometer. The circular dichroism spectroscopy (CD) was measured with a Model 410 circular dichroism spectrometer. In the entire experiment, we used a xenon lamp (300 W) as the light source. The laccase (≥5.43 U/g) and the ABTS (2,2-azinobis (3-ethylbenzothiazolin-6-sulfinic acid) diammonium salt) were purchased from USA Sigma-Aldrich and Adamas-bate, respectively. All other chemicals were analytical grade. Except as noted, in the whole experiment these two kinds of solution were used throughout: PB buffer (pH = 6.0, 0.01 M) and ultrapure water (Millipore, ≥18 MΩ cm).

Preparation of PCDs, CDs, and CDs-NH_2. All chemicals were not purified in the experiment. The two graphite rods were placed into the phosphoric acid, respectively. Their distance is 7.5 cm. The direct current power of 20 V was applied to the graphite electrodes. After 120 h, the anode graphite rod was corroded. At the same time, we obtained the dark-yellow solution that was filtered by filter paper. In order to remove the graphite particles, the filtrate was centrifuged at 22 000 rpm for 30 min. After that, the raw solution was dialyzed by the semipermeable membrane (MWCO 14 000) to dispose the phosphoric acid. After filter treatment, the obtained solution was water-soluble PCDs (0.005 mg/mL).

The CDs and CDs-NH_2 were synthesized according to our previous paper.[17]

Preparation of PCDs-Cu, PCDs-Cu1, and PCDs-Cu2. In the first, the 500 μL CuOAc solution (0.2 μM, 0.4 μM, and 0.6 μM) was added dropwise in 10 mL PCDs solution (0.005 mg/mL), stirring at 277 K for 30 min. Afterward, the mixture was placed in a Teflon-lined stainless steel autoclave (20 mL), and the stainless steel autoclave was heated to 353 K for 12 h. After that, the solution was dialyzed to remove the excess Cu^{2+}, and then the PCDs-Cu (0.2 μM CuOAc as raw material), PCDs-Cu1 (0.4 μM CuOAc as raw material), and PCDs-Cu2 (0.6 μM CuOAc as raw material) were obtained, respectively.

Synthesis of Laccase/PCDs. In the first, 1 mL PCDs solution (0.005 mg/mL) was dissolved in 1 mL PB solution (pH = 6.0), and the mixture was stirred for 30 min. Thereafter, 10 mg laccase powder was dissolved in 20 mL PB solution (pH = 6.0) and the mixture was stirred at 277 K for 24 h. After that, the solution was centrifuged at 2000 rpm to dispose of the insoluble part. Finally, a series of different volumes PCDs (0.0025 mg/mL) (250, 300, 350, 400, 450, 500, and 550 μL, respectively) were dropwise added in the 10 mL laccase solution and the mixture was stirred at 277 K for 24 h.

Synthesis of Laccase/PCDs-Cu. The synthesis method of Laccase/PCDs-Cu was similar to that of Laccase/PCDs.

Activity Assays of Enzyme. The entire experiment must be run three times in order to ensure the accuracy of the test data. Using ABTS as a substrate, the activity of laccase, laccase/PCDs, and laccase/PCDs-Light can be evaluated spectrophotometrically.[18] Specifically, the laccase solution (0.5 mg/mL) and substrate solution (0.5 mM) were mixed quickly, and the absorbance of the oxidized product was recorded at 421 nm. The catalytic temperature was 35 °C.

Native-Polyacrylamide Gel Electrophoresis (Native-PAGE). Native-PAGE was performed according to the method of Laemmli.[19] Protein solutions were mixed at a 1:1 (v/v) ratio with the Native-PAGE sample buffer (1 mL Tris-HCl (0.1 M, pH = 6.0), 0.1 mg bromophenol blue, 7 mL ultrapure water, and 1 mL glycerol (87%)). Mixtures of laccase/PCDs complexes and the free laccase were loaded on the Native-PAGE gels, respectively. The gels subjected to electrophoresis at a constant current of 15 mA using a JY 600 Cell apparatus (Atto Co., Beijing, China). After electrophoresis, the Native-PAGE gels were stained with Coomassie Brilliant Blue R-250 (concentration: 0.25%).

Circular Dichroism Spectroscopy (CD). CD spectra of free laccase and laccase/PCDs were tested with the Model 410 circular dichroism spectrometer (scan speed: 50 nm/min; path length of quartz cuvette: 1 mm). All the CD spectra were corrected by deducting the blank spectra (PB solution) and at least tested six times.[18]

Detection of Michaelis−Menten Equation for Laccase. The Michaelis−Menten equation is a classical model of enzyme kinetics. It can reflect the relationship between the substrate concentration [S] and the initial velocity (V).

$$V = \frac{[S]V_{max}}{[S] + K_m} \quad (1)$$

V_{max} and K_m represent the maximum velocity of the enzymatic reaction and Michaelis constant, respectively. The curve defined the relationship between 1/[S] and 1/V. In a word, the Lineweaver−Burk plot can be received by inversing the equation to this form:

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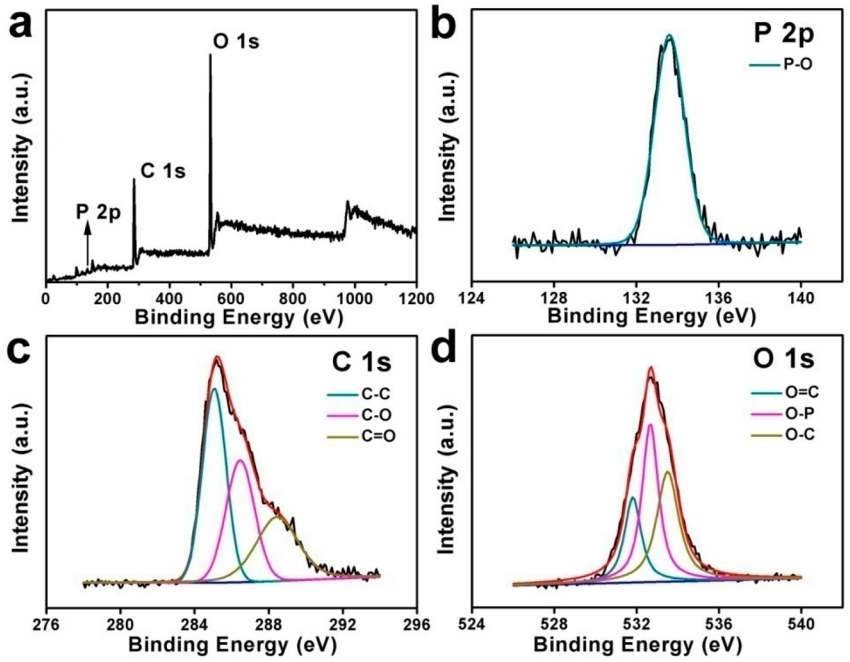

Figure 2. (a) XPS spectrum of the PCDs. (b) P 2p spectrum of the PCDs. (c) C 1s spectrum of the PCDs. (d) O 1s spectrum of the PCDs.

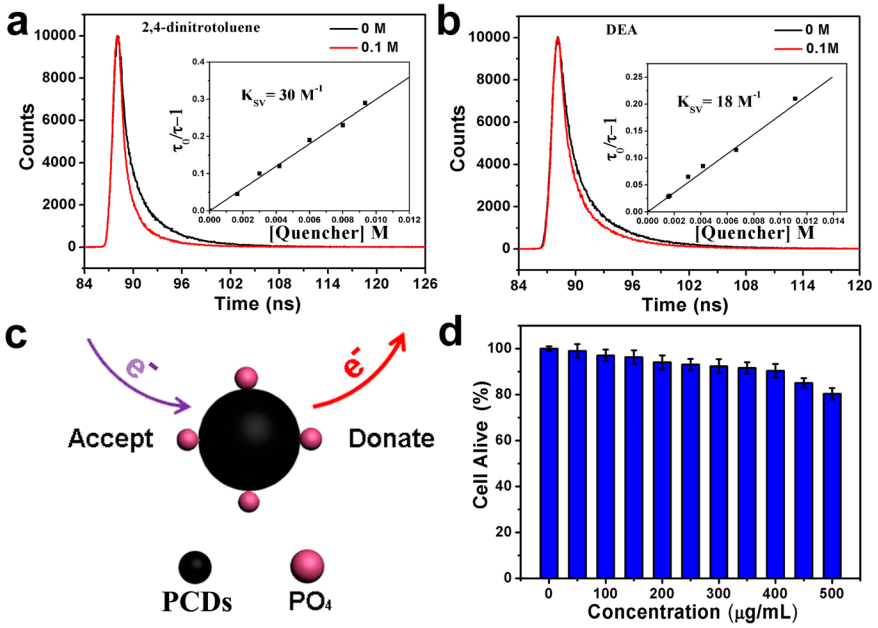

Figure 3. Photoluminescence decays of the PCDs with (a) 2,4-dinitrotoluene and (b) DEA, respectively (λ_{ex} = 485 nm, using the 550 nm narrow band-pass filter as monitor). Insets: Stern−Volmer plots of the PCDs (λ_{ex} = 485 nm). (c) PCDs electron donating and accepting schematic diagram. (d) Absorbance based viability assay to HeLa cells exposed to different concentrations of PCDs.

$$\frac{1}{V} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \quad (2)$$

By plotting $1/V$ with $1/[S]$, we can obtain a straight line. The ordinate intercept is the value of $1/V_{max}$, and the abscissa intercept is the value of $-1/K_m$. Thus, the values of K_m and V_{max} can be obtained by calculating.

Cytotoxicity Analysis. The HeLa cell line was purchased from the Cell Bank of Chinese Academy of Science. The cell of culture conditions was at 37 °C in 5% CO_2 (medium type: standard medium). Cells were incubated in a 96-well plate for 24 h before PCDs treatment. Different concentrations of PCDs were added into cells. After 48 h, the relative viabilities of the cell were determined by MTT (colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). Cells were lysed by SDS (acidulated sodium dodecyl sulfate). Absorbance was recorded at 570 nm using microplate reader (Bio-Rad 680, U.S.A.). In order to gain the accurate data, at least three independent experiments were carried out.

3. RESULTS AND DISCUSSION

Figure 1a shows the TEM image of PCDs, demonstrating that PCDs are monodisperse and uniform, and then the diameters of the PCDs are about 3.0 ± 0.5 nm. At the same time, the DLS of PCDs also reveals that the diameter of PCDs is about 3.0 ± 0.5 nm. (shown in Figure S1). The PCDs possesses good water

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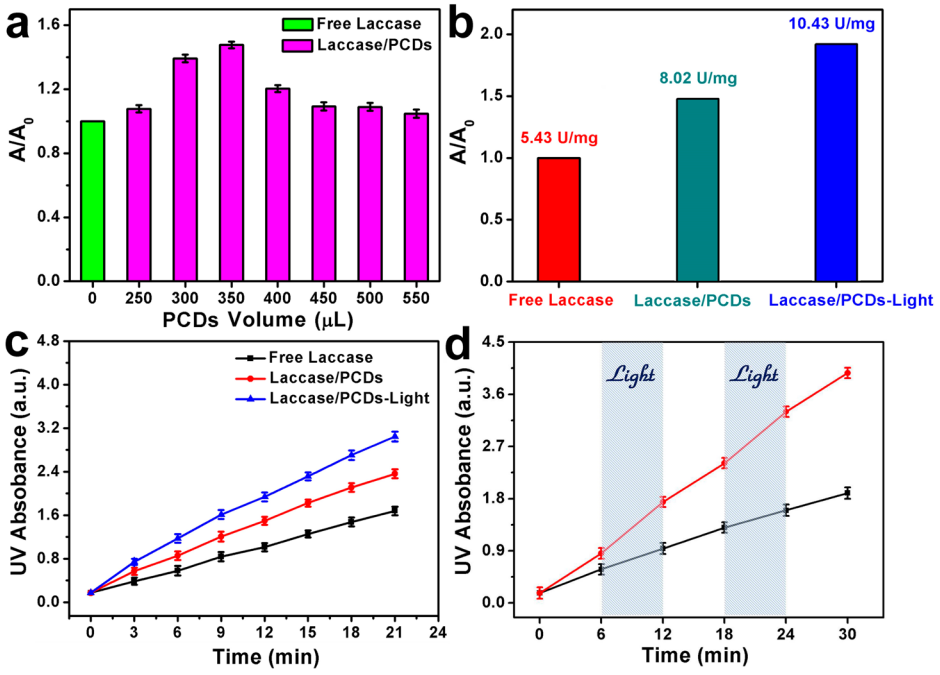

Figure 4. (a) Histogram of enzyme specific activity vs the different volume of PCDs (0.0025 mg/mL) (free laccase: green, laccase/PCDs: pink). (b) Specific activity of the free laccase, laccase/PCDs, and laccase/PCDs-Light (A: activity of hybrids, A_0: activity of free laccase). (c) Substrate degradation of UV absorbance for free laccase, laccase/PCDs, and laccase/PCDs-Light. (d) Catalytic ability of laccase/PCDs (red line) and free laccase (black line) under visible light and without visible light (recorded every 6 min).

solubility. Figure 1a (inset image) shown the HRTEM image of PCDs, revealing the lattice spacing of PCDs is about 0.21 nm. At the same time, we also tested the EDX spectrum of PCDs. The experimental data reveal that the PCDs contain C, P and O elemental. The FTIR spectroscopy of PCDs can prove that the surfaces of PCDs have the functional groups (Figure 1b, red line). The peaks at 1080, 1640, and 3400 cm^{-1} correspond to the vibrations of C—O, C═O, and C—OH bond, respectively.[20,21]The broad peak between 2900 and 2200 cm^{-1} is typical for phosphate. The peaks at 1164 and 1049 cm^{-1} are related to the vibration of P═O and P−O−R (R=alkyl), respectively.[22,23] The good water solubility of PCDs was due to these hydrophilic functional groups. That greatly extended their application in water system. Figure 1c showed the UV−vis absorption spectrum of PCDs (black line), revealing a typical absorption peak is about 235 nm. This is attributed to the $\pi-\pi^*$ transition corresponding to carbon core C═C units. The experimental results are consistent with absorption peak of polycyclic aromatic hydrocarbons.[16,24,25]

Figure 1d shows that the maximum excitation of PCDs was 320 nm and the maximum emission wavelength was 450 nm. The PL spectra of PCDs with different excitation wavelengths were shown in Figure S2. When the excitation wavelength was 320 nm, the PL spectra of PCDs had the strongest emission peak located at 450 nm. With changing excitation wavelengths (300−370 nm), the emission spectra of PCDs show a gradual red-shift phenomenon, which is consistent with the results reported before.[26,27]

To further confirm successful phosphorus group functionalization, X-ray photoelectron spectroscopy (XPS) of PCDs was investigated, and then the XPS spectrum of PCDs in Figure 2a shows the P 2p peak at 134.1 eV, the C 1s peak at 284.6 eV, and the O 1s peak at 532.1 eV.[28] The high-resolution P 2p peak in Figure 2b reveals the presence of P−O bonding (133.7 eV) in PCDs,[28−31] confirming the successful modification of phosphate on the surface of CDs. The C 1s peak in Figure 2c can be divided into three different components located at 284.7, 286.6, and 288.5 eV, which are attributed to C—C, C—O, and C═O bonding, respectively.[32−35] The O 1s peak in Figure 2d can also be divided into three component peaks: 531.8, 532.7, and 533.5 eV. The peaks at 531.8 and 533.5 eV can be assigned to O═C and O—C bonding, respectively, whereas the peak at 532.7 eV indicates O—P bonding in agreement with the P 2p peak results.[36]

Figure 3a and b shows that the luminescence decay of PCDs that were quenched by the well-known electron acceptor 2,4-dinitrotoluene and electron donor *N*,*N*-diethyl aniline (DEA). (λ_{ex} = 485 nm, with the 550 nm narrow band-pass filter as monitor.) As shown in Figure 3a and b insets, the Stern−Volmer quenching constants ($K_{SV} = \tau F^{\circ} kq$) are 30 and 18 M^{-1}, respectively. As shown in Figure 3c, this result indicates that the PCDs possess electron donating and accepting properties.

The HeLa cell line was used in the determination of the in vitro cytotoxicity of PCDs. As shown in Figure 3d, it showed metabolic activity of HeLa cells coincubated with PCDs. These cells were incubated in 96 well plates containing different amounts of PCDs. After 48 h, the cell viability was not significantly reduced even at ultrahigh PCDs concentrations (up to 500 μg/mL), demonstrating that the PCDs have good biocompatibility for the organism. The experimental data indicate that PCDs can be applied in biological fields due to the small size, water solubility, low toxicity, electron donating property, and electron accepting property.

In the following experiments, the laccase/PCDs were obtained by a wet chemical method. We investigated how adding different volumes of PCDs solution (0.005 mg/mL) influenced the catalytic activity of laccase in the laccase/PCDs. Figure 4a reveals the effect of different volumes of PCDs (from 250 to 550 μL) on the activity of laccase, that their activity was volume-dependent. In contrast, laccase showed the highest

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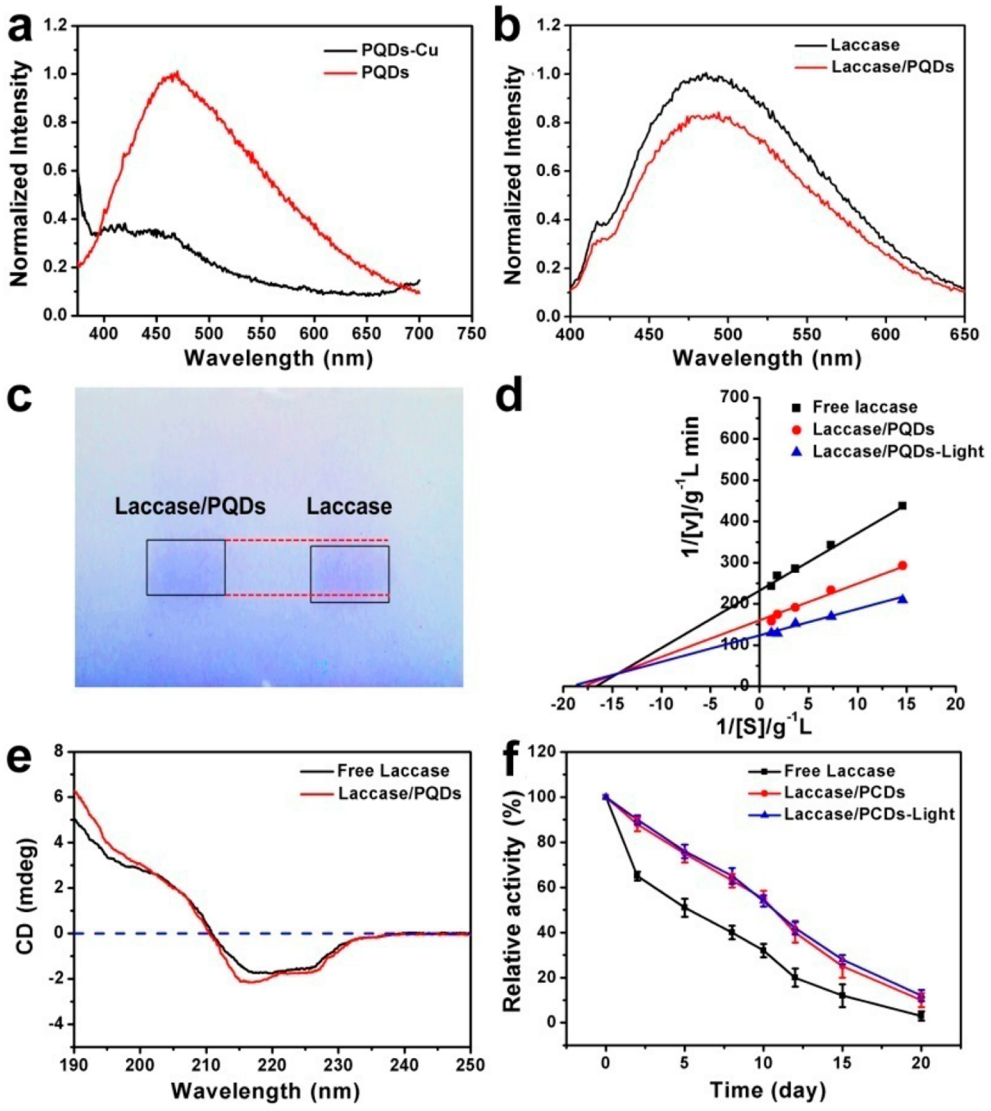

Figure 5. (a) PL absorption of PCDs and PCDs-Cu (λ_{ex} = 360 nm). (b) PL absorption of PCDs and laccase/PCDs (λ_{ex} = 360 nm). (c) Native-PAGE of the free laccase and laccase/PCDs hybrids (12% gels) under visible light. (d) Lineweaver−Burk plot of laccase (black line), laccase/PCDs (red line), and laccase/PCDs-Light (blue line). (The concentrations of ABTS were 0.125, 0.25, 0.5, 1.0, and 1.5 mM, PB (pH = 6.0, 0.01 M).) (e) CD spectra of free laccase (black line) and laccase/PCDs (red line). (f) Relative lifetime of free laccase, laccase/PCDs, and laccase/PCDs-Light in PB (pH = 6.0) at 308 K.

activity when the volume of PCDs was 350 μL, which is 47.7% higher than that of free laccase. However, when the added volume of PCDs is more (or less) than 350 μL, the specific activity of laccase/PCDs hybrids are also higher than that of free laccase (increasing about 20.4%, 9.3%, 9%, 4.8%, 7.8%, and 39.3% vs free laccase). These results revealed that the PCDs can affect the activity of laccase. At the same time, we also found that when the laccase/PCDs hybrids (PCDs: 350 μL, 0.0025 mg/mL) were irradiated by visible light, the activity of laccase/PCDs was 92.1% higher than that of laccase as show in Figure 4b.

In order to research the photochemical properties, the photoswitched catalytic ability of laccase/PCDs hybrids (PCDs: 350 μL, 0.0025 mg/mL) was defined by a series of ABTS oxidation reactions. Figure 4c showed that the oxidation ratio of the laccase/PCDs-Light hybrids increased faster than those of laccase/PCDs hybrids and free laccase in the same time. After 21 min, their oxidation ratio (Abs/min) was 0.072, 0.104, and 0.137 for laccase (black line), laccase/PCDs (red line), and laccase/PCDs-Light (blue line), respectively. PCDs without visible light increased the catalytic activity of laccase by about 44.4% (oxidation ratios were 0.072. to 0.104); when the laccase/PCDs were irradiated by the visible light the PCDs can increase the catalytic activity of laccase by about 90.3% (oxidation ratio of ABTS from 0.072 to 0.137). As shown in Figure S4 the results of the control experiment revealed that the ABTS cannot be oxidized by the PCDs under the visible light or not. These results confirm that PCDs can control the activity of laccase using visible light. So, the laccase/PCDs should possess switching catalytic activity properties under visible light.

In order to study the role of visible light in the catalytic reaction, we tested the catalysis ratios (using the ABTS oxidation reaction with laccase and laccase/PCDs as the catalysts) vs the same reaction time under the visible light or not. Figure 4d showed that the oxidation ratio of ABTS was about 0.148 (6−12 min and 18−24 min), under irradiating light,. but it was about 0.113 without irradiation (0−6 min and 12−18 min) for the red line. These data reveal that the visible

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light can affect the catalytic reactions. On the other hand, as shown in Figure 4d, when using laccase as the catalyst, there was little change in the oxidation ratio of ABTS under the visible light or not, but the oxidation ratio of ABTS decreases by 15%. The above experimental results demonstrate that laccase/PCDs can act as a photoswitched catalyst.

In order to prove that PCDs can conbine with laccase, a large number of experiments were carried out. As shown in Figure 5a, the fluorescence intensity of pure PCDs was higher than that of PCDs-Cu and the emission peak position did not change significantly. This result reveals that Cu^{2+} had coordinated with some PCDs form PCDs-Cu. Figure 5b showed that the PL spectrum of the laccase (black line) was the similar to the PL spectrum of the laccase/PCDs (red line), but their fluorescence intensity was different. The result revealed that PCDs should be connected to the laccase by noncovalent bonds. In order to further probe the interaction between PCDs and laccase in present laccase/PCDs hybrids, the PCDs and laccase were mixed in PB solution (pH = 6.0) and the laccase/PCDs hybrids formed were separated by Native-PAGE. As shown in Figure 5c, the laccase and laccase/PCDs stain the Native-PAGE gels by Coomassie Brilliant Blue R-250 under visible light. The results revealed that laccase is much faster than the laccase/PCDs hybrids. The polyacrylamide gel electrophoresis results prove that the PCDs and laccase formed stable laccase/PCDs hybrids and they are stable in the electric field. The results in Figure 5b and c demonstrate that the laccase/PCDs are formed by noncovalent bonds.[17]

In the following experiment, to define how PCDs influence the activity of laccase when in the laccase/PCDs, we tested and calculated Michaelis−Menten kinetics of the laccase and laccase/PCDs. The K_m can reflect the binding capacity of the substrate with enzyme. The value of K_m was determined by Lineweaver−Burk plot analysis for most of the enzymes.[37−39] The values of K_m and V_{max} can be obtained from the slope and intercept values. The Lineweaver−Burk plot of laccase, laccase/PCDs, and laccase/PCDs-Light are shown in Figure 5d, so, the K_m value of the free laccase, the laccase/PCDs, and laccase/PCDs-Light was determined to be about 59.97 mg/L, 56.99 mg/L, and 53.84 mg/L, respectively. The K_m value can be approximated by expressed enzyme and substrate affinity. The K_m value of the laccase/PCDs and laccase/PCDs-Light was determined to be 56.99 mg/L and 53.84 mg/L, respectively, which are all lower than that of free laccase. In other words, the substrate affinity of laccase/PCDs and laccase/PCDs-Light is higher than that of free laccase. The V_{max} values of laccase, laccase/PCDs, and laccase/PCDs-Light were determined to be 4.28 mg/L·min, 6.32 mg/L·min, and 8.22 mg/L·min, respectively. From this, we inferred that PCDs can increase the activity of laccase under the visible light or not.

The determination of the protein structure was carried out by CD spectroscopy. In order to probe whether PCDs will affect the structure of laccase, we measured the CD spectra of laccase to evaluate the structural change of laccase induced by PCDs. Figure 5e is the CD spectra of free laccase (black line) and laccase/PCDs (red line), which indicates that there are some significant changes in the conformation of laccase (especially, the secondary structure of laccase). The negative peak at 216 nm (β-sheet) and 227 nm (β-turn) obviously decreased, which indicated the increase of β-sheet and β-turn content of laccase/PCDs hybrids. The experiment data reveal that the structure of laccase/PCDs has significant changes compard to the free laccase. Further, to investigate the lifetime of free laccase and laccase/PCDs, the recycled catalyst experiments were carried out with ABTS solution (0.05 M) in 20 days. As shown in Figure 5f, after 5 days the catalytic activity of free laccase, laccase/PCDs, and laccase/PCDs-Light was reduced by about 49%, 25%, and 24%, respectively. After about 20 days, the catalytic activity of free laccase was 0.163 U/mg, which was reduced by 97%, and then the activity of laccase/PCDs and laccase/PCDs-Light was reduced by 90% (0.543 U/mg) and 88% (0.652 U/mg). Therefore, we deduced that the relative lifetimes of free laccase and laccase/PCDs are about 20 days. Then the experimental data reveal that PCDs can protect the laccase from damage.

To research how the light intensity affected the activity of laccase/PCDs, specific activity experiments with the different light intensity were investigated. Figure 6 shows the dependence of light intensity on specific activity of laccase/PCDs hybrids. It suggests that the specific activity of laccase/PCDs hybrids would increase with the increase of light intensity. When the light intensity reaches 80 mW/cm^2, the specific activity of laccase/PCDs hybrids remains relatively unchanged, which suggests that 80 mW/cm^2 is the equilibrium light intensity in these specific activity experiments, and the value of specific activity (based on the free laccase activity) is calculated to be about 10.43 U/mg for laccase/PCDs hybrids.

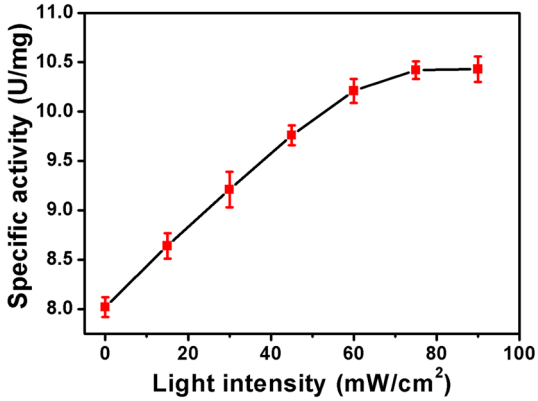

Figure 6. Dependence of current specific activity on light intensity.

The followed experiments further confirm that the PCDs were more likely to combine with laccase (T1 Cu) and then increase activity. Based on the research on toxicity, the experimental data reveal that PCDs-Cu, PCDs-Cu1, and PCDs-Cu2 have good biocompatibility for the organism, indicating that they are of use in biological fields (as shown in Figure S3). At the same time, we surveyed how the addition of different volumes of PCDs-Cu (PCDs-Cu concentration is 0.0025 mg/mL) affect the specific activity of laccase in the laccase/PCDs-Cu hybrids system. Figure 7a reveals the effect of different volumes of PCDs-Cu (from 250 to 550 μL) on the activity of laccase, and its activity is dependent on the volume of PCDs-Cu. The experimental results indicate that when the volume of PCDs-Cu is 300 μL the specific activity of laccase/PCDs-Cu reaches 7.23 U/mg, which is 33.1% higher than that of free laccase. When more (or less) than 300 μL PCDs-Cu is added, the activity of laccase/PCDs-Cu hybrids decreases but is still higher than that of free laccase (increasing about 27.8%, 3.35%, 4.53%, 1.08%, 0.41%, and 2.21% vs free laccase). Further, the activity of laccase/PCDs-Cu hybrids was about 7.58% lower than that of laccase/PCDs hybrids for the same

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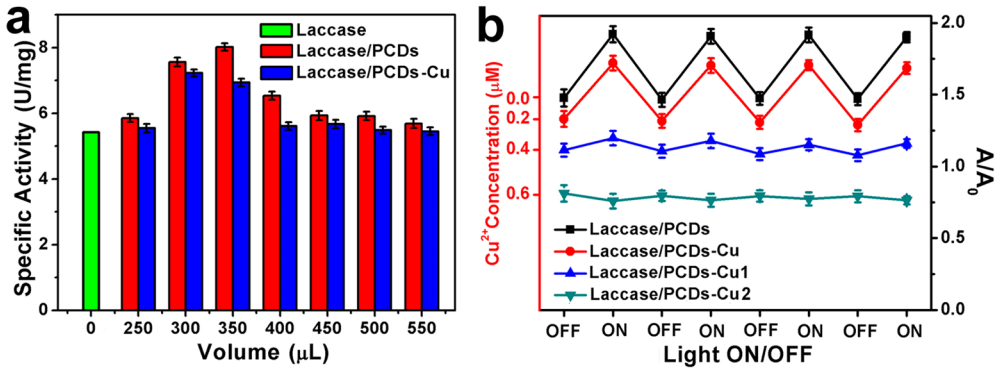

Figure 7. (a) Histogram of laccase/PCDs-Cu (PCDs-Cu: 0.0025 mg/mL) and laccase/PCDs (PCDs: 0.0025 mg/mL) specific activity in different volumes of PCDs and PCDs-Cu solution compared with that of free laccase, respectively (free laccase: green, laccase/PCDs: red, laccase/PCDs-Cu: blue). (b) A/A_0 of laccase/PCDs (black trace: PCDs, 350 μL), laccase/PCDs-Cu (red trace: PCDs-Cu, 350 μL), laccase/PCDs-Cu1 (blue trace: PCDs-Cu1, 350 μL), and laccase/PCDs-Cu2 (green trace: PCDs-Cu2, 350 μL) under the visible light or not (A: activity of hybrids, A_0: activity of free laccase).

volume of PCDs solution. In order to study the role of PCDs in the reaction, we test the value of A/A_0 (A: the activity of hybrids, A_0: the activity of free laccase) under the visible light or not. As shown in Figure 7b (black line), A/A_0 was 1.92 (increased about 30.1%) with light irradiation (6 min), but it was 1.46 (decreased about 23.8%) without irradiation (6 min). These data showed that the light irradiation can affect the catalytic activity. On the other hand, as shown in Figure 7b, when using the laccase/PCDs-Cu, laccase/PCDs-Cu1, and laccase/PCDs-Cu2 as the catalyst, there were obvious changes in the A/A_0 value under the visible light or not. For the laccase/PCDs-Cu and laccase/PCDs-Cu1, the A/A_0 values were 1.72 and 1.20 (increased about 29.3% and 8.1%) with light irradiation (6 min), but they were 1.32 and 1.11 (decreased about 23.3% and 8.3%) without irradiation (6 min). For laccase/PCDs-Cu2 hybrids, the A/A_0 value was 0.81 and 0.76 (less than 1), that reveals the activity of laccase/PCDs-Cu hybrids is lower than that of free laccase under the visible light or not. Here, it should be noted that laccase is about 6.7 nm × 5.8 nm × 5.0 nm (as shown in Figure S9a) and the diameter of PCDs is 3.0 ± 0.5 nm. For laccase, the specific four-electron reduction of oxygen relies on electron transfer between the T1 copper center and the T2/T3 copper centers which react with oxygen.[40−42] In this system, the T1 copper center is located close to the external surface of the protein shell (as shown in Figure S9b). Thus, the above experiments collectively show that, in laccase/PCDs catalyst, the PCDs should combine with the T1 Cu in free laccase.

In order to acquire the photocontrolled mechanism of laccase/PCDs, a large number of tests were run. The results revealed that the surface groups of PCDs can affect the activity of laccase/PCDs, which is a main factor involved in their visible-light-controlling property. Figure 8a shows that there was little change in the activity of the laccase (dark 5.43 U/mg, light 4.62 U/mg) under the visible light or not. In other words, the enzyme activity was decreased 0.9% under the visible light. Next, various carbon dots with different kinds of surface functional groups were selected and used as laccase based catalysts, and we also studied the catalytic activity of these laccase/carbon dot based catalysts. For laccase/CDs (the diameter of CDs was 4.0 ± 0.5 nm) system, carbon dots (CDs) with quenching constants (KSV = $\tau F^{\circ} kq$) of about K_1 = 31 M^{-1} and K_2 = 21 M^{-1} are shown in Table S1 and Figure S5.

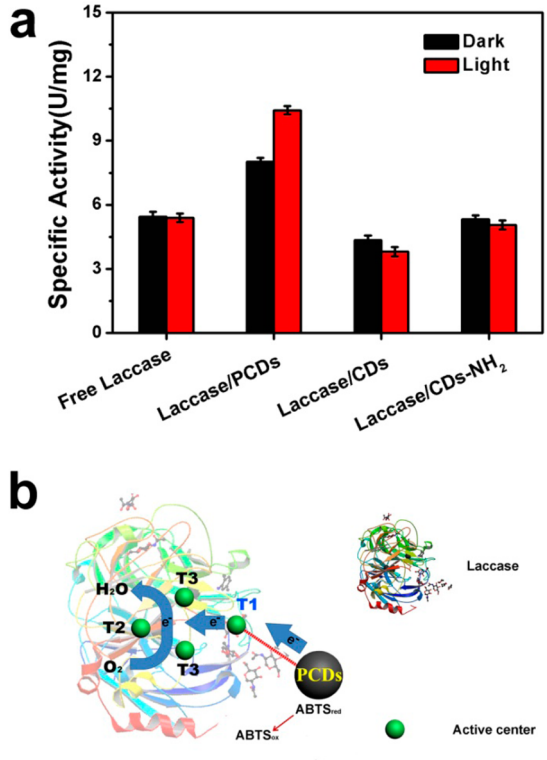

Figure 8. (a) Specific catalytic activity of free laccase, laccase/PCDs, and laccase/CDs, laccase/CDs-NH_2 under the visible light or not. (b) Illustration of electron transfer pathways for laccase/PCDs hybrids.

The main surface groups of CDs are −OH and −COOH shown in Figure S6.

The system was irradiated by light. As shown, the activity of laccase/CDs was lower than that of no visible light irradiation (decrease 14.2%). For the laccase/CDs-NH_2 (the diameter of CDs-NH_2 was 4.0 ± 0.5 nm) system, CDs-NH_2 with quenching constants ($K_{SV} = \tau F^{\circ} kq$) of about K_1 = 21.6 M^{-1} and K_2 = 18.8 M^{-1} are shown in Table S1 and Figure S7. The main surface groups of CDs-NH_2 are −OH, −COOH, and −NH_2 shown in Figure S8. The laccase/CDs-NH_2 system was irradiated by light. As shown, their activity was lower than that of no visible light irradiation (decrease 50.1%). The data revealed that the

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surface functional group of carbon dots is another important factor to change the activity of laccase.

Based on the results above, we propose a mechanism for visible-light-controlling catalytic behavior of laccase/PCDs hybrids. As shown in Figure 8b, laccase has multiple catalytic centers (T1−T3). The catalytic mechanism of laccase involves oxidation of the substrate at the T1 Cu site with concomitant reduction of O_2 to H_2O in trinuclear copper cluster (T2/T3 Cu cluster) placed 12−13 Å away, without releasing H_2O_2.[5] And then, it is well-known that the inclusion of the T1 Cu is important for effective reduction of oxygen.[43] Because the surfaces of PCDs have many kinds of groups (−OH, −COOH, and $-PO_3$) and contain an important functional group, which is $-PO_3$ groups, the PCDs are more likely to combine with laccase (T1 Cu). PCDs have electron donating and accepting properties, and the surfaces of PCDs contain many kinds of functional groups (such as −OH, −COOH, and $-PO_3$) which may help to draw the ABTS. At the same time, the PCDs (on the surface of laccase) are good for increasing the substrate affinity capacity. So the oxidized ABTS ($ABTS_{ox}$) can transfer electrons (e^-) to PCDs and then PCDs passed e^- to the T1 Cu of laccase. Finally, e^- is intramolecularly delivered to the T2 Cu and T3 Cu.[44] Then, the activity of laccase/PCDs hybrids (dark: 8.02 U/mg, light: 10.43 U/mg) is higher than that of free laccase (5.43 U/mg) under the visible light or not.

4. CONCLUSION

In summary, we investigated the effect that PCDs and visible light have on the catalytic behavior of laccase. We proved that the activity of laccase can be controlled by PCDs in the dark or under visible light, and the activity of laccase/PCDs was higher than that of laccase (increase by 47.7%). That is because the laccase (T1 Cu) can combine with PCDs whose surface has many kinds of groups (−OH, −COOH, and $-PO_3$) and can increase the laccase/PCDs hybrids and substrate binding ability. When laccase/PCDs were irradiated by the visible light (laccase/PCDs-Light), the activity of laccase/PCDs-Light was higher than that of laccase/PCDs hybrids (increased by 92.1%). The addition of visible light irradiation initiates the increased donating and accepting electronic capability of the laccase in laccase/PCDs, which enhances their activity. The dates of the Michaelis−Menten equation can also prove this opinion. With the combined functionalities of the protein and inorganic material, the laccase/PCDs hybrid system is likely to have important applications in biofuel cells, bioanalytical devices, biosensors, and industrial biocatalysis. This method could be applied in the enzyme field and could be hopefully used to develop hypercatalytic enzymes.

■ ASSOCIATED CONTENT

Supporting Information

TEM images; UV−vis spectra; PL spectra; FTIR spectra. 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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