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Simultaneous Enhancement of Bioactivity and Stability of Laccase by Cu^{2+}/PAA/PPEGA Matrix for Efficient Biosensing and Recyclable Decontamination of Pyrocatechol

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ABSTRACT: Simultaneously enhancing the catalytic bioactivity and stability of enzyme is still an intractable issue in the enzymatic study. Herein, a facile and effective approach was designed to immobilize and modify laccase on a Cu^{2+}-adsorbed pyrene-terminated block copolymer [poly (acrylic acid)/poly (poly (ethylene glycol) acrylate)] (PAA/PPEGA), which was prepared via well-controlled reversible addition-fragmentation chain transfer polymerization. PAA provided the supporting matrix for firm immobilization of Cu^{2+}, an enzyme bioactivity inducer, onto the microstructure of laccase, while avoiding any contamination of the heavy metal Cu^{2+} into the following application system. The water-soluble, biocompatible and nontoxic PPEGA was used as an ideal modifier to improve the laccase stability. Accordingly, the modified laccase exhibited enhanced catalytic bioactivity and stability simultaneously to 447% and 237%, respectively. The modified laccase was immobilized on the highly oriented pyrolytic graphite surface and large-area graphene papers through π–π stacking interactions between the pyrene moiety of PAA/PPEGA and the π-conjugated graphene-like surface. The as-prepared portable solid-state electrochemical laccase biosensor showed lowest detection limit of 50 nM (S/N ≥ 3) and long-term stability for pyrocatechol detection. Besides, the laccase immobilization on graphene paper provided efficient pyrocatechol decontamination platform with convenience and recyclability, which could retain the laccase bioactivity of 176% after 8 consecutive operations.

Laccase is a kind of promising biocatalyst with many possible applications, including bioremediation,[1] chemical synthesis,[2] wine stabilization[3] and biosensing.[4] Despite the wide applications of laccase, the potential use is severely hindered by its low stability under different storage/working environments.[5] Therefore, enhancing the stability of laccase is the first issue to be solved. Current methods to stabilize laccase rely on immobilizations,[6] encapsulation,[7] or complexation with hydrogel,[8] fire-fighting foams or polyelectrolytes.[9] However, bioactivities of laccase after the above stabilization were all decreased obviously. For example, Soares et al have found that the polyethylene glycol (PEG)–enzyme in vitro assays showed a higher stability in human serum sample but the enzyme bioactivity only retained 30% of the original bioactivity.[10] Thus, increasing the bioactivity of laccase was also a difficult challenge to face up. Till now, only several ways have been found to increase the laccase bioactivity, such as the addition of Cu^{2+},[11] photocatalyst[12] or microorganisms[13] to the laccase solution. Therein, the addition of Cu^{2+}, an enzyme bioactivity inducer, into the laccase solution was an effective way to improve the enzyme bioactivity but the new pollutant was introduced to the following enzymatic application system simultaneously since Cu^{2+} belongs to one heavy metal.[11] Fixing Cu^{2+} on a solid-state supporting matrix should be one way to solve this problem, but no related work has been presented. In addition, laccase stabilities were not taken into consideration among these methods.[11-13] Therefore, it is still a great challenge but make great sense to design an effective methodology which can increase the bioactivity and stability of laccase simultaneously while avoiding secondary pollution for their further utilization.

Pyrocatechol, a kind of phenolic compound, is of great environmental concern, since it reacts with different biomolecules, like DNA, protein, membranes and leads to unrepairable damage.[14] Hence, rapid, sensitive and precise detection and decontamination of pyrocatechol and its derivatives are attracting increasing attention in environmental protection. At present, many analytical methods based on chemical,[15] spectrophotometry[16,17] and gas chromatographic techniques[18] have been developed for the detection of pyrocatechol. However, these methods are often accompanied with disadvantages such as needing derivatization or pre-concentration steps, expensive and/or not portable test equipments.[19] In the last few years, laccase biosensors have been applied for pyrocatechol determination because of their unique merits such as fast, specific, accurate and highly sensitive.[20] However, laccase biosensors were usually short-lived and in exorbitant price due to the low stability of laccase.[21] Besides, for the decontamination of large amount of pyrocatechol pollutant, laccase biosensor is limited because of its small area. Therefore, the immobilization of laccase on insoluble and portable supports with large area could provide an effective and recyclable way to detect and

decontaminate pyrocatechol.[22] Till now, laccase has been successfully fixed on various types of supports, such as magnetically silica spheres,[23] polystyrene microspheres[24] and silica beads.[25] But the separation after decontamination was complicated or needing additional magnetic field. Graphene paper is with extensible large surface areas, well-defined π-conjugated surface properties for modification, simple segregation operation and environmental friendliness,[26] which is expected to be as an ideal promising supports for the biomolecular immobilization, nevertheless, the immobilization of enzyme such as laccase on graphene papers has not been reported yet.

It is well known that poly (acrylic acid) (PAA) is a negative charged polyelectrolyte which can provide abundant carboxylic groups as chelators to bind heavy metal ions.[27] Previous studies have shown that PAA could form stable complexes with metal ions (e.g. Cu^{2+}) via co-ordination interactions because of its high density of carboxyl[28] for heavy metal removal from waste water. Meanwhile, laccase can also be incorporated on PAA through the esterification reaction between the surface amino groups on laccase and the carboxylic acid group of PAA.[28] Thus, PAA is anticipated to be artfully as an ideal polymer to immobilize Cu^{2+} onto the microstructure of laccase. Moreover, poly (ethylene glycol) acrylate (PEGA), a water soluble, biocompatible and nontoxic polyether, is an ideal polyether to modify enzyme in order to improve its solubility and stability because PEGylation could impart molecules many pharmacological advantages such as biochemical and pharmacological characteristics.[29-31] Therefore, PAA/PPEGA copolymer combining the advantages of these two respective polymers should have synergic effects on enhancing both the bioactivity and stability of laccase. However, as far as we all know, relevant researches have never been reported till now. Reversible addition-fragmentation chain transfer (RAFT) polymerization[32] belongs to a well-controlled free radical polymerization, which provides a versatile platform for molecular engineering of biomolecules in mild reaction conditions.[33] Moreover, different kinds of functionalized copolymers can be easily synthesized via the accurate selection of monomers with required functional groups such as carboxyl, hydroxy and sulfydryl.[34,35]

In this study, pyrene terminated PAA/PPEGA block copolymer was prepared via RAFT polymerization and used to immobilize laccase and Cu^{2+} coinstantaneously. The obtained modified laccase (Cu^{2+}/PAA/PPEGA-laccase) exhibited improved bioactivity (447%) and enhanced stability (237%), respectively, while avoiding the the secondary pollution of Cu^{2+} in the following applications. Moreover, a laccase biosensor for pyrocatechol determination was prepared through the modification of highly oriented pyrolytic graphite (HOPG) with pyrene-functionalized Cu^{2+}/PAA/PPEGA-laccase via π−π stacking interactions. Impressively, the as-prepared biosensor shows satisfied sensitivity and stability for detection of pyrocatechol. The use of large-area graphene paper was tested for the immobilization of enzymes to decontaminate pyrocatechol effectively in a recyclable way. The synthesized copolymers, modified enzyme, as-prepared laccase sensors were characterized by ^{1}H NMR, dynamic light scattering, Fourier transform infrared, sodium dodecyl sulfate polyacrylamide gel electrophoresis, energy dispersive analysis, cyclic voltammetry assays, transmission electron microscope, etc. accordingly to confirm their characteristics and unique properties, respectively.

EXPERIMENTAL SECTION

Materials 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS, AR) was bought from TCI Co. Ltd (Shanghai, China). 2,2'-Azobis (isobutyronitrile) (AIBN, 98%) was purchased from Sigma-Aldrich (Steinheim, Germany). Fungal laccase (E.C. 1.10.3.2, 0.5 U/mg solid)) from the white-rot basidiomycete fungus *Trametes* (*Coriolus, Polyporus*) *versicolor* and dialysis membrane (MWCO, 500, 1000, 50000) were bought from Shanghai Yuanye Bio-Technology Co. Ltd. (Shanghai, China). The RAFT agent, 4-cyano-4-ethyl-trithiopentanoic acid (CETP) was prepared using a well-defined procedure by Liu et al.[36] The stock solution of laccase (1 mg/mL) was freshly prepared in 0.1 M acetic acid buffer solution (ABS, pH 5), which was prepared with acetic acid and sodium acetate. Working solution of pyrocatechol (0.1 M) was freshly prepared in 0.1 M ABS (pH 5.0) prior to usage. Other chemicals used herein are shown in the Supporting Information.

Apparatus Infrared spectroscopy was measured on a Perkin-Elmer Spectrum One Fourier transform infrared (FTIR) spectra. The diameter and size distributions of native laccase and modified laccase were tested using dynamic light scattering (DLS, Malvern Instruments). Ultraviolet spectrophotometer (UV) spectra were recorded on a UV/V-16/18 UV spectrophotometer (Shanghai, Mapada). ^{1}H NMR spectra was obtained on a JNM-ECP 600 (600 MHz) spectrometer. The Mn of laccase and modified laccase were monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Energy dispersive analysis (EDS) was obtained from an EDS detector attached to the SEM microscope.

Enzymatic Bioactivity and Stability Assay Details of synthesis process for pyrene-functionalized Cu^{2+}/PAA/PPEGA-laccase are presented in the Supporting Information. Bioactivity of laccase was determined at 25 °C, using ABTS as the substrate.[37] The assay mixture contained 0.5 mM ABTS, 0.1 M ABS (pH 5.0), and 1 mg/mL laccase solution. Oxidation of ABTS was determined by monitoring the absorption at 420 nm. One unit (U) of laccase bioactivity was defined as the amount of enzyme oxidizing 1 μmol of ABTS per min at 25 °C. To analyze the stability of laccase, the solutions of both laccase (1 mg/mL) and modified laccase (1 mg/mL) were incubated at 70 °C. The enzymatic bioactivity was measured at different time periods with the aforementioned method.[38] All of the tests were repeated for three times, and the average result was reported. All data are reported as the mean ± standard deviation. Data were considered to be statistically significant at $P < 0.05$.

Fabrication of the Solid-state Pyrocatechol Biosensor The fabrication process of the solid-state pyrocatechol biosensor was described as follows: the HOPG was incubated in Cu^{2+}/PAA/PPEGA-laccase solution (1 mg/mL) for 12 h at 4 °C, then washed with ultrapure water to clean the modified laccase unstably absorbed and finally dried at 4 °C for 3 h to afford Cu^{2+}/PAA/PPEGA-laccase modified HOPG. All the relevant tests of the pyrocatechol biosensor were performed in aerobic condition during the experiment.

The Immobilization of Cu^{2+}/PAA/PPEGA-laccase on Graphene Paper In order to further study the effect of PAA/PPEGA chains on the bioactivity and stability of laccase after being immobilized on graphene paper, pyrene functionalized laccase (pyrene-laccase) was prepared as illustrated in the Supporting Information. The immobilization process of py-

rene-laccase and Cu^{2+}/PAA/PPEGA-laccase on graphene paper was described as follows: two graphene papers were immersed in pyrene-laccase (1 mg/mL) and Cu^{2+}/PAA/PPEGA-laccase solution (1 mg/mL) for 24 h at 4 °C, respectively, then washed with ultrapure water to get rid of the unstable modified laccase.

RESULTS AND DISCUSSION

The Possible Microstructure of the Cu^{2+}/PAA/PPEGA-laccase and Its Mechanism of High Bioactivity and Stability As illustrated in Figure 1A, Cu^{2+}/PAA/PPEGA-laccase with higher bioactivity and stability was successfully prepared and its possible microstructure is shown in Figure 1B. Laccase obtains four copper ions that distribute into three sites (T_1, T_2, and T_3 sites, defined according to spectroscopic properties) in which electrons from the reducing substrate (ABTS) are extracted by the T_1 site and then transfer to the T_2/T_3 center (where molecular oxygen is reduced and $ABTS^+$ is generated).[39] Combining Cu^{2+} with laccase has positive effect on the electron transfers between T_1, T_2, and T_3 sites,[40] therefore, the bioactivities of Cu^{2+}/PAA/PPEGA-laccase are significantly improved compared to those of native laccase. Moreover, it can be speculated that the presence of PAA/PPEGA chains could hinder the conformational transitions of laccase in the environment of high temperature since the polymer chains provide moderate microenvironment to facilitate the maintenance of the laccase's conformation. Therefore, the thermal stability of PAA/PPEGA-laccase can be maintained through resisting the unfolding transition at the laccase transition temperature, comparing to the native one in buffer solution.[38,41] Besides, it was also reported that the aggregation of the enzyme could be easily induced by temperature variance, mainly because of the destruction of the enzyme's tertiary structure.[42] In consequence, the introduction of PAA/PPEGA polymer chains could minimize the thermal inactivation of laccase as the result of the aggregation effect.[43] The scientificity and reasonability of the imagined microstructure of Cu^{2+}/PAA/PPEGA-laccase will be proved by our following bioactivity and stability tests.

The Synthesis and Characterization of Pyrene functionalized CETP (PCETP), Laccase, PAA/PPEGA and PAA/PPEGA-laccase As illustrated in Scheme S1, CETP was first modified with pyrene functional groups to obtain the PCETP for the synthesis of pyrene-terminated PAA/PPEGA via RAFT polymerization. As demonstrated in Figure S1A, the peak signals at 8.30-7.90 ppm should correspond to the pyrene aromatic protons in 1-pyrenemethanol. The peaks at 1.80, 2.65 and 2.90 ppm represent the three methylene groups from the RAFT agent. The peak at 1.25 ppm shows the existence of terminal methyl group. The PAA/PPEGA-laccase was successfully confirmed by ^1H NMR as indicated in Figure S1D. The peak signals at 5.25 (l_1), 3.00 (l_2), 2.70 (l_3) should correspond to the main characteristic peak signals of laccase as shown in Figure S1B. The peaks at 3.60 (t, 2H, l), 3.30 (d, 2H, g), 2.30 (d, 2H, i), 1.90(t, 1H, h), 1.60 (t, 1H, j), 1.50 (s, 3H, m) should correspond to the main characteristic peak signals of PAA/PPEGA as shown in Figure S1C. All of these results confirmed the successful synthesis of pyrene-functionalized PAA/PPEGA-laccase.

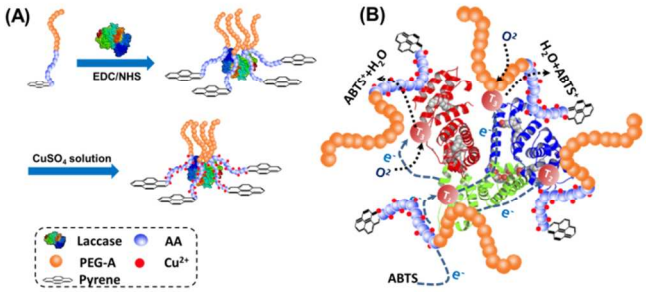

Figure 1. (A) Schematic illustration of synthesis of Cu^{2+}/PAA/PPEGA-laccase (B) Possible microstructure of the Cu^{2+}/PAA/PPEGA-laccase and proposed schematic illustration for the decomposition of ABTS.

In order to study the relationship between the bioactivity, stability and the ratio of [PAA]/[PPEGA], the PAA/PPEGA copolymers with different composition were prepared as shown in Table S1. Comparisons among Entries 1-9 revealed that the increasing amount of PAA would bring significantly positive effect on the bioactivity of Cu^{2+}/PAA/PPEGA-laccase. However, the half-life of Cu^{2+}/PAA/PPEGA-laccase was reduced with the decreasing amount of PPEGA, which indicated the increasing amount of PPEGA would bring positive effect on the stability of laccase. Therefore, PAA/PPEGA (Mn = 54200, Entry 5 in Table S1) was performed using a reduced ratio of [PAA]/[PPEGA](60:40) in order to afford the higher bioactivity and stability.

The laccase from *Trametes Versicolor* has eight lysine residues, about four lysine residues are on or near their surface,[44] which could contribute to the reaction with PAA/PPEGA. After extensive purification to remove any unconjugated PAA/PPEGA by dialysis, the Mn of PAA/PPEGA-laccase was subject to SDS-PAGE analysis. It was found that the Mn of laccase was increased from 120 kDa to 260−320 kDa after conjugation (Figure 2A). Some residual of native laccase still existed after reaction with PAA/PPEGA (60 kDa). Based on the SDS-PAGE analysis, it was found that there were more than four PAA/PPEGA chains attached on each laccase.

TEM and DLS were used to characterize the size distribution of the PAA/PPEGA-laccase. TEM indicated the size of PAA/PPEGA-laccase was about 100-200 nm as shown in Figure 2B. As shown in Figure 2C, the DLS results revealed that the hydrodynamic diameter of native laccase was ranging from 80 to 100 nm, which was consistent with the values previously reported.[45] What's more, it also showed a small size distribution at about 10 nm, which may be resulted from the laccase residual. The DLS data for the PAA/PPEGA indicated a diameter of about 50 nm and PAA/PPEGA-laccase showed a primary population with the size of about 150-250 nm. These results are similar to those from the TEM analysis.

The UV−vis spectrum showed that there was a slight blue-shift of PAA/PPEGA-laccase compared to the native one, which indicated that the microenvironment polarity around the lysine residues of laccase had changed to be a less polar environment (Figure 2D).[38,46] This could be due to the amidation reaction between the carboxy groups of the PAA and the amino groups of laccase. As shown in Figure 2D,

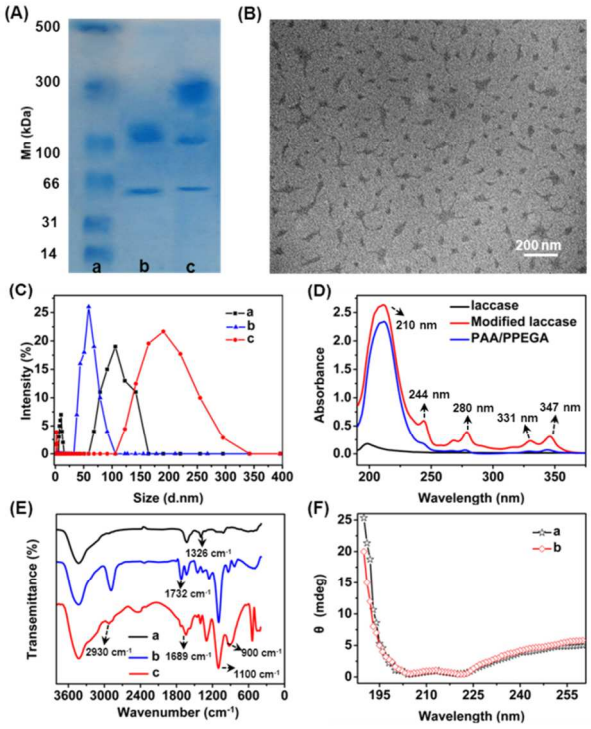

Figure 2. (A) SDS-PAGE of (a) Mn markers, (b) native laccase, (c) PAA/PPEGA-laccase. (B) TEM images of PAA/PPEGA-laccase (C) DLS of (a) laccase, (b) PAA/PPEGA and (c) PAA/PPEGA-laccase. (D) UV–vis absorbance of the solutions of laccase, PAA/PPEGA and PAA/PPEGA-laccase. (E) FT-IR spectra of (a) laccase, (b) PAA/PPEGA and (c) PAA/PPEGA-laccase. (F) Circular dichroism (CD) spectra of (a) laccase and (b) PAA/PPEGA-laccase.

the peaks at 280 nm, 331 nm and 347 nm should be attributed to the pyrene groups of the copolymer and the peaks at 210 nm and 244 nm can be ascribed to the carboxyl groups and trithiocarbonate groups of PAA/PPEGA respectively.[47] These evidences indicated that the pyrene-terminated PAA/PPEGA chains have been successfully attached onto laccase. Further analysis of the laccase, PAA/PPEGA and PAA/PPEGA-laccase were performed using FTIR (Figure 2E). Characteristic peaks in FTIR spectrum of PAA/PPEGA at 1100, 1732 cm^{-1} were observed, which can be attributed to C-O and C=O vibrational stretching from PPEGA and the -COOH groups from PAA respectively. The unconjugated laccase showed major peaks at 1100, 1400 and 1689 cm^{-1}, while PAA/PPEGA-laccase showed major peaks at 900, 1100, 1326, 1400 and 1689 cm^{-1}, respectively. Comparing the FTIR spectrum of PAA/PPEGA-laccase and native one, the new peaks at 900 cm^{-1} should be attributed to the out-of-plane bending vibration of benzyl groups. The band at 1326 cm^{-1} can be assigned to the stretching and bending vibrations of the C-N bond of the amide III band. The increase of the intensity at 1689 cm^{-1}, which should be attributed to the carbonyl stretching vibration of amide carbonyl functionality, was clearly observed.[38] All of the results above indicated the successful conjugation between laccase and PAA/PPEGA.

The catalytic bioactivity of enzyme is regulated by the structure of the protein. CD spectra were measured to estimate the secondary structural change of laccase caused by PAA/PPEGA. As shown in Figure 2F, the PAA/PPEGA and laccase formed stable complexes. Both the native laccase and PAA/PPEGA-laccase exhibited two obvious minima of α-helix content at 203 and 222 nm, respectively, which indicated that the attachment of the PAA/PPEGA chains on the surface of laccase would not affect the secondary conformation of laccase.[48]

Enzymatic Bioactivity Test In order to investigate whether the laccase is still active after conjugation with PAA/PPEGA or Cu^{2+}/PAA/PPEGA, the bioactivity of laccase, PAA/PPEGA-laccase and Cu^{2+}/PAA/PPEGA-laccase were tested in different working conditions. ABTS could be oxidized by laccase and then produce the stable dark green cation radical ($ABTS^{+}$).[49] The absorbance values for the product were recorded by an UV-visible spectrophotometer at 420 nm as shown in Figure 3A, and a calibration curve (absorbance vs. reaction time) was plotted to calculate enzyme bioactivity compared with that of native laccase, which indicated the bioactivity of Cu^{2+}/PAA/PPEGA-laccase was much higher than that of native one.[50] The images of the color of ABTS solution was recorded every 20 s as indicated in Figure 3B. It can be observed that the color of right cuvette (Cu^{2+}/PAA/PPEGA-laccase) was much darker than the left one (laccase) and barely changed after 120 s while the left one reached a balance till 480 s. All of these results demonstrated that the bioactivity of Cu^{2+}/PAA/PPEGA-laccase was much higher than that of native laccase. The exact bioactivity values were calculated using following equation:

$$\text{Bioactivity} = D \times \Delta A_{420} \times V_t \times (e \times V_s \times d)^{-1}$$

D: Dilution ratio

ΔA_{420}: The change in absorbance values of $ABTS^{+}$ at 420 nm

V_t: The volume of reaction solution

V_s: The volume of laccase solution

e: Mole absorbance value, 6.22×10^{-3} mol·cm^2/L

D: Light path, 1cm

The bioactivity of the PAA/PPEGA-laccase was also evaluated at 25 °C. In order to compare easily with the native laccase, the bioactivity of native laccase was set as the standard (100%). As shown in Figure 3C, the enzymatic bioactivity of PAA/PPEGA-laccase and Cu^{2+}/PAA/PPEGA-laccase were shown to be 27% lower and 347% higher than that of native one, respectively. Moreover, when the native laccase was incubated in $CuSO_4$ solution and then dialyzed for 24 h, no obvious change of the bioactivity could be observed as shown in Figure S3. This observation clearly suggests the special effect of Cu^{2+} on the bioactivity laccase and Cu^{2+} was undoubtedly immobilized on the copolymers. The laccase and modified laccase displayed Michaelis−Menten kinetics (Table S2), with k_{cat} values of the Cu^{2+}/PAA/PPEGA-laccase being about 3 times larger than that of the unmodified one calculated from Lineweaver-Burk plot (Figure S4),[20,51] which could also imply that the Cu^{2+}/PAA/PPEGA-laccase displayed a much higher catalyzing rate.[52]

To explore the optimum laccase working conditions, the bioactivities at different temperature and pH conditions were studied. Analysis of the available data showed that the apparent optimum temperature and pH of laccase were about 30 °C and 5.5, respectively (Figure 3D and 3E). In the temperature range below 30 °C, the bioactivity of laccase remained at a high level while decreased apparently above 30 °C. In addition, laccase lost almost all of its bioactivity when temperature

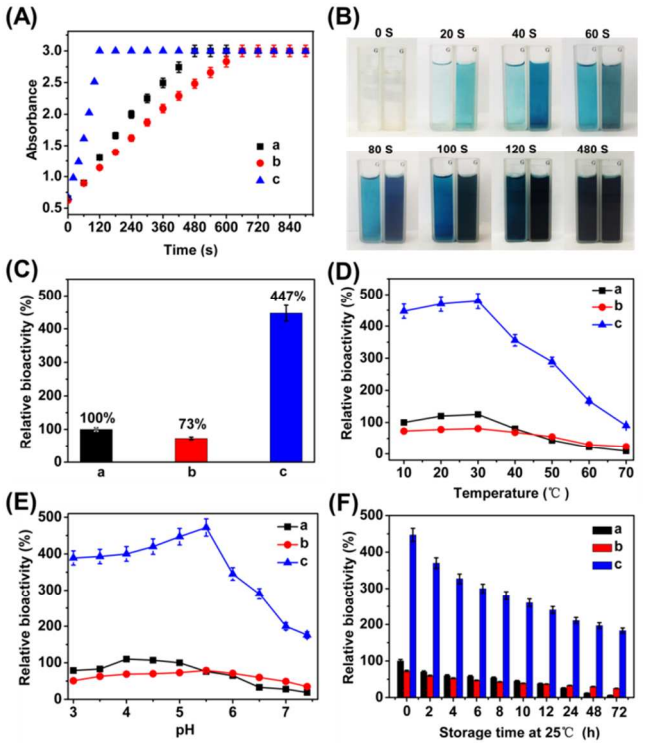

Figure 3. (A) Laccase bioactivity was tested using ABTS as the substrate. (0.5 mM ABTS, 0.1 M ABS (pH 5.0) and 1 mg/mL laccase solution). Oxidation of ABTS was monitored as absorption increased at 420 nm. (a) native laccase, (b) PAA/PPEGA-laccase and (c) Cu^{2+}/PAA/PPEGA-laccase (B) Images of color change of ABTS solution decomposed by native laccase (left cuvette) and Cu^{2+}/PAA/PPEGA-laccase (right cuvette). (C) Relative bioactivities of (a) laccase, (b) PAA/PPEGA-laccase and (c) Cu^{2+}/PAA/PPEGA-laccase at 25 °C (D) at different temperature from 10 to 70 °C (E) at different pH value from 3 to 7.5, (F) after incubation in ABS solution at 25 °C for three days.

reached 70 °C. It can be seen that laccase prefers an acidic condition as shown in Figure 3E. Moreover, the bioactivities of modified and native laccase were monitored for 72 hours during the incubation in ABS solution at 25 °C. As shown in Figure 3F, the laccase lost almost all of its original bioactivity after 3 days, while about half of original bioactivity of modified laccase still remained. It suggests that the stability of modified laccase has been significantly increased compared to the native laccase.

Stability Test of the Laccase To further study the thermal stability, the bioactivities of laccase, PAA/PPEGA-laccase and Cu^{2+}/PAA/PPEGA-laccase were tested in different working conditions. It was found that Cu^{2+} has no obvious effect on the stability of laccase. The stability of Cu^{2+}/PAA/PPEGA-laccase was similar to that of PAA/PPEGA-laccase, therefore, PAA/PPEGA-laccase was chosen as the representative to study their stability. Firstly, 4 °C is the proper storing temperature of enzyme. The bioactivity of the conjugated laccase remained almost unchanged within the first 72 hours storage at 4 °C as shown in Figure 4A. Vacuum freeze-drying technique, an effective method of water removal to obtain the final products with high quality, was used to dry the PAA/PPEGA-laccase to enhance the long-term stability and simultaneously facilitate the manipulation and transportion.[38] The laccase bioactivity of the freeze-dried powder was tested periodically. Figure 4B showed that the native laccase suffered obvious bioactivity drops after freeze-drying, about 40% of bioactivity was lost compared with its original bioactivity in the first month and the bioactivity slowly dropped within 3 months storage in a dry powder status at 4 °C. However, the freeze-dried PAA/PPEGA-laccase bioactivity retained more than 70% of its original bioactivity after 90 days. When the modified laccase and the native one were incubated at 70 °C in ABS solution, respectively, the bioactivity was tested every 10 minutes. It was observed that laccase nearly lost all of its bioactivity after 30-min incubation, while the PAA/PPEGA-laccase still exhibited approx. 31% of its original bioactivity (Figure 4C). Besides, in the first 10-min incubation, an obvious decrease in PAA/PPEGA-laccase bioactivity was obtained, and subsequently the bioactivity decreased slowly through the whole assay. After 90-min incubation, about 15% of bioactivity still remained. As indicated in Figure 4D, the PAA/PPEGA-laccase showed about 80% bioactivity after three freeze−thaw cycles,[52] while the native laccase only retained 60% bioactivity after the first cycle and almost all of its bioactivity was lost after the fourth one.

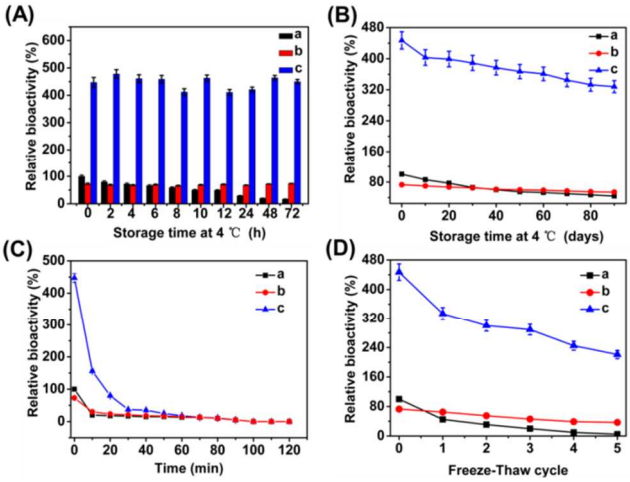

Figure 4. (A) Relative bioactivities of (a) native laccase, (b) PAA/PPEGA-laccase and (c) Cu^{2+}/PAA/PPEGA-laccase incubated in ABS solution at 4 °C for three days. (B) in 3 months. (C) at 70 °C, (D) after five freeze−thaw cycles.

Half-life Test of the Laccase and Modified Laccase The obvious increase of the thermal stability of conjugated laccase usually indicates that the PAA/PPEGA can prolong the half-life of laccase. The assay of half-life for laccase and modified laccase under different incubation conditions are shown in Table S3. An obvious increase of the half-life for the conjugated laccase can be easily observed from the table at different working conditions.

Fabrication and Characterizations of the Pyrene-terminated Cu^{2+}/PAA/PPEGA-laccase Modified Biosensor The fabrication of the solid-sate biosensor based on the pyrene-terminated Cu^{2+}/PAA/PPEGA-laccase is illustrated in Figure 5A. The HOPG was first incubated in ABS solution of pyrene-terminated Cu^{2+}/PAA/PPEGA-laccase (pH=5) for 12 h at 4 °C to afford the Cu^{2+}/PAA/PPEGA-laccase modified HOPG in one step. Based on the stable non-covalent π-π stacking between the pyrene moiety and highly HOPG with graphene basal plane, an efficient and stable solid-state biosensor can be successfully fabricated. In order to confirm the π-π stacking between the pyrene moiety and graphene, the fluorescence spectroscopy of Cu^{2+}/PAA/PPEGA-laccase and

graphene/Cu^{2+}/PAA/PPEGA-laccase composite were tested. As shown in Figure S5 the emission fluorescence of pyrene groups of Cu^{2+}/PAA/PPEGA-laccase was mostly quenched after being attached onto graphene, which confirmed the successful attachment of pyrene groups on graphene.[47] The EDS of the bare HOPG surface (Figure 5B) evidenced the existence of carbon. After incubation of the HOPG in Cu^{2+}/PAA/PPEGA-laccase solution, the EDS of the modified HOPG indicated the presence of Cu^{2+} (Figure 5C). As can be seen from the curve a of Figure 5D, no

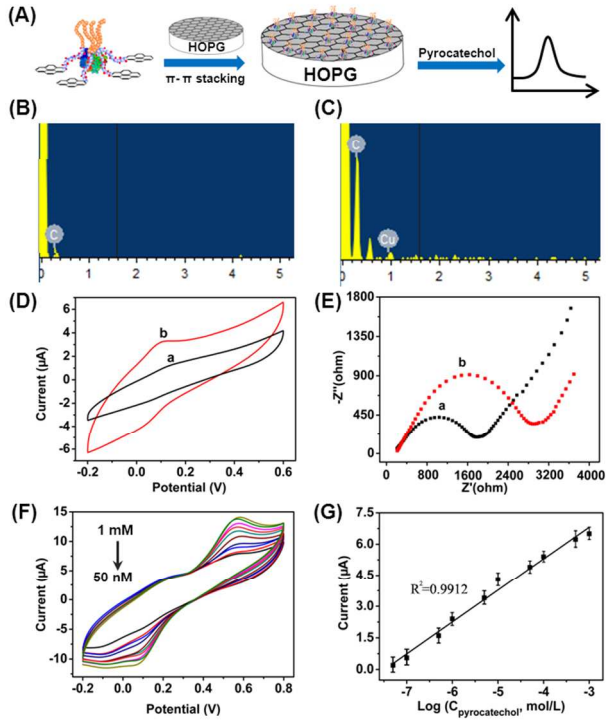

Figure 5. (A) Schematic illustration for facile fabrication of a solid-sate biosensor based on the pyrene-terminated Cu^{2+}/PAA/PPEGA-laccase, which showed obvious cyclic voltammogram (CV) signals. EDS of (B) bare HOPG surface and (C) Cu^{2+}/PAA/PPEGA-laccase modified HOPG surface. (D) CVs of the (a) bare HOPG and (b) as-prepared biosensor at the scan rate of 100 mV s^{-1} in 0.1 M ABS (pH 5.0). (E) (a) Bare HOPG and (b) as-prepared biosensor in 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) containing 50 mM KCl, respectively. (F) The CVs of Cu^{2+}/PAA/PPEGA-laccase biosensor in 0.1 M ABS (pH 5) against different concentrations of pyrocatechol. (G) The relationship between the anodic peak currents and the logarithm of different pyrocatechol concentrations.

obvious peak was observed for the bare electrode. Nevertheless, a pair of redox peaks could be clearly seen on the Cu^{2+}/PAA/PPEGA-laccase modified electrode (curve b). The anodic and cathodic peak potentials caused by the copper ions in laccase were observed at 0.105 V and 0.052 V at the scan rate of 100 mV s^{-1}, which were close to the values previously observed.[53] The separation of peak potential was 53 mV, which was close to the separation of peak potential (51 mV) for electrochemical reaction with one electron transfer process.[54] As shown in Figure 5E, the electron transfer resistance (R_{et}) for the bare HOPG electrode (curve a), calculated from the semicircle of impedance spectra, was 1800 Ω.[55] However, the R_{et} was increased to 3000 Ω for the Cu^{2+}/PAA/PPEGA-laccase modified one (curve b). The significant increase of the R_{et} was in agreement with the results of CV, which indicated that the HOPG electrode was successfully modified with Cu^{2+}/PAA/PPEGA-laccase. All of these results confirmed the successful modification of the HOPG electrode with the Cu^{2+}/PAA/PPEGA-laccase.

Subsequently, the CVs of the biosensor at different scan rates in 0.1 M ABS solution were recorded in Figure S7A, from which a large redox wave with oxidation peak at near 0.15 V was observed, which was consistent with the characteristic redox wave of laccase. Both the cathodic and anodic currents were proportional to the scan rate from 50 to 200 mV/s (R^2=0.9947 and R^2=0.9910, respectively) as shown in Figure S7B, which indicated the electrochemical reaction of the PAA/PPEGA-laccase was a surface-bound electrochemical process.[56,57] To further evaluate the biological sensing properties of the as-prepared biosensor for pyrocatechol, CV of pyrocatechol were carried out. As shown in Figure 5F, the CV performances of the as-prepared biosensor in the different concentration of pyrocatechol (50 nM to 1 mM) were measured. Figure 5G showed a good linear correlation in the pyrocatechol concentration between 50 nM and 1 mM (R^2 = 0.9912). The detection limit of the biosensor was 50 nM (S/N $\geqslant$ 3).

Stability and Reusability of Cu^{2+}/PAA/PPEGA-laccase Biosensor The as-prepared biosensor was stored in refrigerator for 30 days at 4 °C, the peak current of the oxidation of pyrocatechol was just decreased by 4.23% as shown in Figure S6, illustrating that Cu^{2+}/PAA/PPEGA-laccase immobilized on electrode possessed excellent stability. The relative standard deviation of the peak currents for the oxidation of pyrocatechol of 3 different electrodes was just 4.79%, which indicated an excellent fabrication reproducibility of the biosensor.

The Recyclable Use of Cu^{2+}/PAA/PPEGA Laccase on Graphene Paper Surface The use of pyrene-laccase and Cu^{2+}/PAA/PPEGA-laccase as an effective decontamination agent were investigated by immobilizing them on the graphene paper surface (Figure 6A). Briefly, two graphene papers were immersed in pyrene-laccase (1 mg/mL) and Cu^{2+}/PAA/PPEGA-laccase solution (1 mg/mL) for 24 h at 4 °C respectively, followed by rinsing with ultrapure water successively to remove the unstably bound laccase. Then, these two graphene papers were immersed in the solution of 10 mM pyrocatechol solution and the decontamination efficiency was analyzed via the color change of the solution. After 360 min of decontamination test as shown in Figure 6B, the Cu^{2+}/PAA/PPEGA-laccase (left) showed higher decontamination efficiency compared to the pyrene-laccase (right) via the color change of pyrocatechol solution, which indicated the Cu^{2+}/PAA/PPEGA-laccase retained a higher bioactivity. The high decontamination efficiency clearly suggested a great potential for Cu^{2+}/PAA/PPEGA-laccase immobilized graphene paper as an effective, recyclable and economical decontaminant formulation. The bioactivity and reusability of pyrene-laccase, PAA/PPEGA-laccase and Cu^{2+}/PAA/PPEGA-laccase are shown in Figure 6C and D. It can be found that the bioactivity of Cu^{2+}/PAA/PPEGA-laccase after the immobilization was enhanced to 317% and 347% at 25 and 30 °C, respectively. Moreover, the immobilized Cu^{2+}/PAA/PPEGA-laccase retained above 176% residual bioactivity after eight consecutive operations as shown in Figure 6D, which indicated that Cu^{2+}/PAA/PPEGA-laccase immobilized on graphene paper performed a good reusability.

CONCLUSIONS

In summary, we have successfully designed a Cu^{2+} adsorbed PAA/PPEGA copolymer matrix for laccase immobilization and modification, which made the laccase exhibit significantly improved bioactivity (447%) and enhanced stability (237%). The as-prepared Cu^{2+}/PAA/PPEGA-laccase could be generalized to the development of other laccase-based decontaminant systems for environmental protection. For example, the pyrene-functionalized Cu^{2+}/PAA/PPEGA-laccase has been successfully immobilized on the HOPG surface and large-area graphene papers through non-covalent π–π stacking interactions. The as-prepared electrochemical biosensor exhibited varied advantages such as simplicity, highly sensitivity (lowest detection limit of 50 nM), long-term stability (~95% of its preliminary electrochemical signals can be retained after intermittent use over 4-week period) and cost-effectiveness.

Besides, the bioactivities of Cu^{2+}/PAA/PPEGA-laccase immobilized on the graphene papers were retained at a high bioactivity level (317%). Furthermore, the immobilized Cu^{2+}/PAA/PPEGA-laccase offered the advantage of quick separation and good reusability. Above 176% of bioactivity still retained after 8 consecutive operations of decontaminating pyrocatechol solution. The developed approach will make great sense to the improvement of enzyme properties, biosensors, target-specific design of copolymers, and environmental protection, etc.

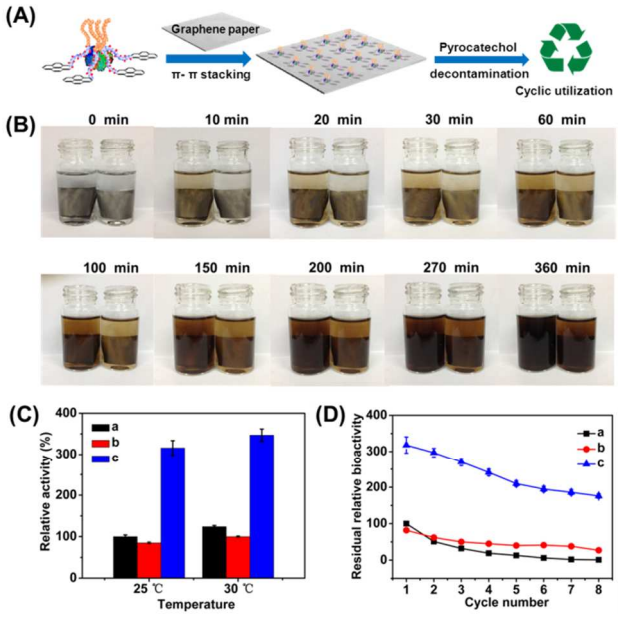

Figure 6. (A) Schematic illustration for the preparation of recyclable Cu^{2+}/PAA/PPEGA-laccase modified graphene paper. (B) Colorimetric monitoring of the pyrocatechol solution decomposed by pyrene-laccase (right) and Cu^{2+}/PAA/PPEGA-laccase (left) immobilized on the graphene paper surface. (C) Relative bioactivities of pyrene-laccase (a) PAA/PPEGA-laccase (b) and Cu^{2+}/PAA/PPEGA-laccase (c) immobilized on the graphene paper surface at 25 and 30 °C, respectively. (D) Reusability assays of pyrene-laccase (a), PAA/PPEGA-laccase (b) and Cu^{2+}/PAA/PPEGA-laccase (c) immobilized on the graphene paper surface.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / ‡[1]Tao Chen and Yuanhong Xu contributed equally to this work.

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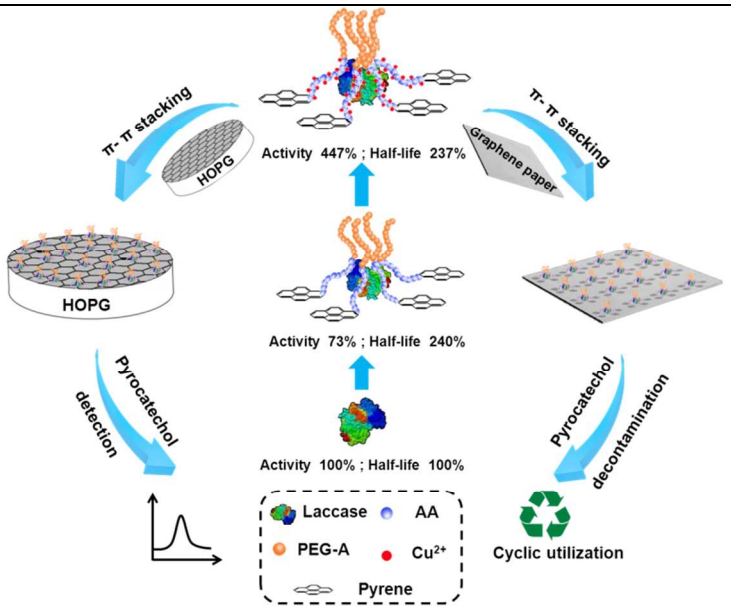

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