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PAPER

In situ encapsulation of laccase in nanofibers by electrospinning for development of enzyme biosensors for chlorophenol monitoring

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A biosensor based on *Trametes versicolor* laccase (Lac) was developed for the determination of phenolic compounds. The biosensor was prepared by *in situ* electrospinning of a mixture of polyvinyl alcohol (PVA), Lac, PEO–PPO–PEO (F108) and gold nanoparticles (Au NPs), where F108 was used as an enzyme stabilizing additive and Au NPs was used to enhance the conductivity of the biosensor. Laser confocal scanning microscopy and electrochemical impedance spectroscopy proved that the enzyme was successfully encapsulated into the electrospun nanofibers. Under the optimal conditions, the lowest detection limit was found to be 0.04 μM (S/N = 3) for 2,4-DCP and the highest detection limit was found to be 12.10 μM for 4-CP. The sensitivity of the biosensor obtained in the linear range for chlorophenols followed the sequence 2,4-dichlorophenol (2,4-DCP) > 2,4,6-trichlorophenol (2,4,6-TCP) > 4-chlorophenol (4-CP). The sensing performance for chlorophenols was attributed to the suitable electrochemical interface of PVA/F108/Au NPs/Lac, resulting from biocompatibility, a high surface area-to-volume ratio (10.42 m^2 g^{-1}) and superior mechanical properties of the electrospun nanofibers. The biosensor exhibited good repeatabilities of 7.6%, 2.8% and 9.0% (R.S.D.) and reproducibilities of 14.9%, 10.4% and 13.7% (R.S.D.) for 4-CP, 2,4-DCP and 2,4,6-TCP, respectively. Lac retained 65.8% of its initial activity after a 30-day storage period.

Introduction

Chlorophenols (CPs) have been introduced into the environment through release from direct application as biocides, leaching from wood products, synthesis during bleaching operations and emissions from operating facilities.[1,2] Due to their carcinogenicity and persistence, many CPs have been included in the lists of priority pollutants of the US Environmental Protection Agency (US EPA)[3] and the European Union (EU).[4] Traditional analytical methods for CPs were expensive, time-consuming, sometimes required preconcentration or extraction steps and were inadequate for on-line monitoring.[5] Therefore, there is great interest in developing a simple and effective procedure for on-line determination of CPs. Electrochemical biosensors, which have the advantages of being simple, requiring minimal sample preparation, high selectivity, high sensitivity, easy miniaturization, on-line monitoring and cost effectiveness, were suitable for CP determination.[6,7] Commonly used biosensors were based on tyrosinase,[8,9] laccase[10,11] and horseradish peroxidase[12,13] for CP detection, since phenolic compounds can act as electron donors for oxidized tyrosinase, laccase and horseradish peroxidase.[14]

Laccase (*p*-diphenol: dioxygen oxidoreductases, EC 1.10.3.2, Lac) is a copper-containing oxidase, which has been reported to be widely specific and can catalyze the oxidation of *ortho*- and *para*-substituted phenol, aminophenols, polyphenols, methoxyphenols, aromatic amines, ascorbate, benzenethiols and a series of other oxidizable compounds with the concomitant reduction of oxygen to water.[15,16] So Lac has extensive applications in various fields, such as wastewater treatments,[17] green organic synthesis,[18] biofuel cells,[19] pharmaceutical synthesis[20] and so on. In a typical Lac reaction, the phenolic substrate is subjected to a one-electron oxidation, giving rise to an aryloxy radical. This active species can be converted into quinone or non-enzymatic reactions like hydration, disproportionation, and/or polymerization in the second stage of the oxidation.[21] These liberated species can be electrochemically reduced to allow convenient low-potential detection of the phenolic compounds.[22] Thus, the electrochemical behavior of Lac has received increasing attention in recent years and Lac-based biosensors have been employed for phenolic compound determination.[23,24]

The effective immobilization of enzymes on the electrode is the crucial step to construct an enzyme biosensor since it would greatly influence the performance of the biosensor.[25] Conventional methods, *e.g.* adsorption, cross-linking, covalent binding and encapsulation, have been employed to immobilize enzymes on the electrode. Among these methods, encapsulation is currently of great interest as it may permit the stabilization and tuning of the properties of the enzyme molecules[26] and protect

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the enzyme from the external environment.[27] Electrospun nanofibers are excellent candidates for encapsulating enzymes due to their extraordinary properties such as a high surface area-to-volume ratio; porous structure, which is favorable for the improvement of the mass-transfer rate of substrate to the active sites of enzymes; small diffusion resistance; superior mechanical properties and reusability of the fibrous membranes due to easy separation from the reaction mixture.[28] What is more, multiple enzymes from diverse sources can be encapsulated together.[29] In this technique, only the external surface of the electrospun fibers is utilized for enzyme immobilization; the interior of fibers which can protect enzyme molecules from harsh conditions remain inactive.[30,31]

In this work, a novel Lac biosensor based on the *in situ* encapsulation of Lac into PVA nanofibers *via* electrospinning for CP detection has been accomplished. This *in situ* encapsulation method can protect the enzyme from harmful solvents. Polyvinyl alcohol (PVA) was used as an attractive matrix for enzyme immobilization due to its biodegradability, biocompatibility and good mechanical properties in forming membranes.[32] PVA can be dissolved in water and be easily electrospun. The present biosensor was fabricated by co-electrospinning a mixture of PVA aqueous solution and Lac on a glassy carbon electrode. The aim of this present study was to develop a simple, inexpensive, stable and highly sensitive biosensor for CP determination. The surface morphology structures of the prepared biosensor were investigated. The experimental parameters (working potential, pH and enzyme loading amount) which affect the response of the fabricated biosensors were discussed in detail. Under the optimal conditions, aspects including the limit of detection, sensitivity, apparent kinetic parameters and other properties of the developed biosensor were also examined.

Experimental

Materials

All chemicals used in the experiments were reagent grade or higher and were used without further purification. 2,4-Dichlorophenol (2,4-DCP, 99.0%), 4-chlorophenol (4-CP, 99%), 2,4,6-Trichlorophenol (2,4,6-TCP, 98%), Lac (23.1 U mg^{-1}) from *Trametes versicolor* and its substrate 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS, 99%) were obtained commercially from Sigma-Aldrich (Milwakee, WI, USA). Polyvinyl alcohol (PVA-124, polymerization degree: 2400, degree of alcoholysis: 98∼99%), chloroauric acid tetrahydrate, trisodium citrate, potassium ferricyanide ($K_3Fe(CN)_6$), potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$) and other reagents for buffer solution were obtained from Sinopharm Chemical Reagent Beijing Co., Ltd. Triblock copolymer PEO–PPO–PEO (polyethylene oxide–polyoxypropylene–polyethylene oxide, F108) was supplied by BASF (Germany). 0.2 mol L^{-1} phosphate buffer solution (PBS) was prepared by disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$) and sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$), and adjusted to the desired pH with NaOH or H_3PO_4. Stock solutions of CPs were prepared daily by dissolving the appropriate amount in 0.2 mol L^{-1} PBS at pH 5.5, which was used as the supporting electrolyte. Milli-Q ultrapure water (Millipore, ≥18 MΩ cm^{-1}) was used throughout.

Preparation of fluorescein isothiocyanate-labeled Lac

Lac powder (40 mg) was dissolved in 1 mL of PBS (pH 7.2), then 1.5 mL of 0.5 mol L^{-1} carbonate buffer (CB, pH 9.0) was added and blended with each other under vigorous stirring for preparing the Lac solution. In another test tube, 10 μg fluorescein isothiocyanate (FITC) was dispersed into 0.3 mL of 0.5 mol L^{-1} CB, and when fully dissolved, the FITC solution was moved into the Lac solution, and the reaction mixture was magnetically stirred at room temperature (25 ± 1 °C) for 2 h, then centrifuged for 15 min. Subsequently, the supernatant was imbibed for Sephadex G-15 filtration and then the fluorescently-labeled Lac was collected with three centrifugal tubes. The samples were stored in a refrigerator at −20 °C for usage.

Preparation of gold nanoparticles

The preparation of gold nanoparticles (Au NPs) was carried out as reported by Frens:[33] a volume of 50 mL of 0.01% (w/v) chloroauric acid aqueous solution was heated up to 92 ± 4 °C in an oil bath, 1.0 mL of 1% (w/v) sodium citrate solution was quickly added to it with rapid stirring. Within 3 min, the solution changed to blue, then to royal purple, and finally to fuchsia. Then this solution was cooled to room temperature and the Au NPs were obtained.

Preparation of the working electrode by electrospinning

The PVA/F108/Au NPs/Lac glassy carbon electrode (GCE, 3 mm in diameter) was prepared as follows: both PVA (0.6 g) and F108 (0.03 g) were dissolved in 9.4 mL water at 85 °C for 3 h to obtain 6% (w/v) PVA solution. After cooling to room temperature, a volume of 0.4 mL of Lac solution was added to 5 mL of PVA/F108 solution. In order to enhance the electric conductivity of the electrospun membranes, a volume of 0.4 mL of the prepared Au NP solution was added to the PVA/F108/Lac solution, before being blended for 20 min to ensure a homogeneous distribution. After that, the solutions were loaded into a glass syringe equipped with a stainless-steel needle (12 mm inner diameter), which was connected with a high-voltage power supply (HB-Z503-2AC, Tianjin Hengbo High-voltage Power Supply Plant, China). Electrospinning experiments were performed at a 13.2 kV voltage, with a distance of 9 cm between the needle tip and the surface of the GCE. The flow rate of the solution was 0.2 mL h^{-1} controlled by a syringe pump (RWD Life Science Co., Ltd. China) and it took 5 h to electrospin. Finally, the fibers were collected on the surface of the GCE and the modified electrode (PVA/F108/Au NPs/Lac GCE) was prepared. Before being used, the enzyme electrode was dipped in stirred PBS for 15 min to eliminate the excess of enzymes not encapsulated, rinsed with PBS, and stored immersed in PBS at 4 °C. All electrospinning experiments were conducted at room temperature and a relative humidity of about 25 ± 2%. For the purpose of morphology comparison, the pure PVA/F108/Au NPs electrospun membrane was prepared under the same electrospinning conditions.

Characterization

A field emission scanning electron microscope (FESEM S-4800, HITACHI, Japan) was used to characterize the morphology of the surface of PVA/F108/Au NPs GCE and PVA/F108/Au NPs/Lac GCE at 5.0 kV. All samples were sputtered with gold. The fiber diameter distribution was taken on more than 100 counts randomly selected from 10 different SEM images for each sample.

To visualize the presence and distribution of Lac in the nanofibers, a thin layer of the PVA/F108/Au NPs/FITC-Lac composite nanofibers was directly collected on a microscope glass slide and then observed by laser confocal scanning microscopy (LCSM, LSM510, ZEISS, Germany). The excitation and emission wavelengths were 488 and 535 nm, respectively.

Enzyme activity measurements

To measure the enzyme activity, PVA/F108/Au NPs/Lac electrospun fibrous membranes were cut into dimensions of 2 cm × 2 cm (length × width) and weighed to about 10 mg. The specimens were washed three times with PBS firstly to elute the Lac adsorbed on the surface. Then the specimens were immersed into 5 mL of 0.5 mmol L^{-1} ABTS solution, then incubated at room temperature under constant shaking (100 rpm) for 5 min. After that, the enzymatic activity was measured spectrophotometrically by monitoring the oxidation rate of $ABTS^{2-}$ to $ABTS^{-}$ (λ_{max} = 420 nm; $\xi_{420} = 3.6 \times 10^4$ mol^{-1} L).[34] The electrospun fibrous membrane activity was defined as follows:

$$\text{Unit activity} = (AV) \times 10^{-3}/(t\xi_{420})/M_0 \quad (1)$$

where A is the absorbance at reaction time t, V is the volume of reaction solution, ξ_{420} is the molar extinction coefficient for the oxidation of ABTS at 420 nm, and M_0 is the mass of immobilized Lac.

Electrochemical measurements

All electrochemical experiments were performed on a CHI660D electrochemical workstation (CH Instrument Co., Beijing, China) at room temperature. A three-electrode system was used with the modified GCE as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum wire counter-electrode, and all of the potentials were expressed *versus* the SCE. Amperometric experiments were carried out in an electrochemical cell containing 0.2 mol L^{-1} PBS (pH 5.5) or CPs solution under constant stirring with a magnetic bar and under free access of air.

Electrochemical impedance spectroscopy (EIS) was performed in 0.1 mol L^{-1} KCl solution containing 10 mmol L^{-1} $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1 : 1). The frequency range was from 10 mHz to 100 kHz with an amplitude of 2 mV. The direct current potential was the average peak potential of the cathodic and anodic peaks.

Results and discussion

Morphology and structure of electrospun nanofibers

SEM images of PVA/F108/Au NPs (A) and PVA/F108/Au NPs/Lac (B) electrospun nanofibers are shown in Fig. 1. SEM image

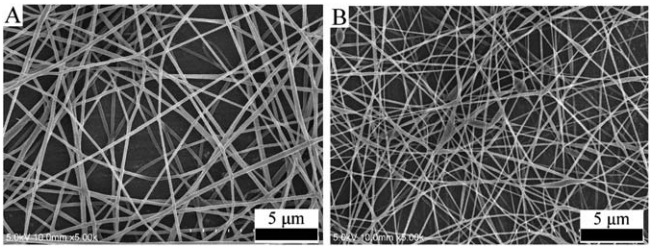

Fig. 1 SEM images of PVA/F108/Au NPs nanofibers (A) and PVA/F108/Au NPs/Lac nanofibers (B).

(A) showed that the PVA/F108/Au NPs electrospun nanofibers were smooth and uniformly arrayed with the diameter from 150 to 325 nm. But when Lac was encapsulated in PVA/F108/Au NPs electrospun nanofibers (B), the fibers became irregular and were interspersed with shuttle-sharp beads, and the fiber diameter became thinner. One possible reason is that the addition of enzyme may reduce the conductivity of the electrospinning solution, which made the electric field force difficult to overcome the surface tension of the solution. On the other hand, Lac consists of many different amino acid residues, and it is difficult for it to be electrospun alone into nanofibers due to its complex three-dimensional structure as well as strong hydrogen bonding interaction between intra-molecular and inter-molecular forces of Lac. However, the blend of Lac and PVA can interfere with the complex structure of Lac and destroy their inter-molecular forces because PVA can form secondary bonding with Lac. Therefore, the stability of the electrospinning solution weakened when adding Lac, the beads appeared and the fiber became thiner.[35,36] In addition, the PVA/F108/Au NPs/Lac nanofibers have a large BET surface area (10.42 m^2 g^{-1}). These characteristics of electrospun fibrous membranes provide a large reaction area between the enzyme and substrate, and may have a significant role toward the easy substrate infiltration into the fibers and sufficient reaction with the enzyme, thus resulting in a good amperometric response.[37]

Laser confocal scanning microscopy (LCSM) observations were carried out as they can provide evidence of the location of the enzyme within the nanofibers. The LCSM image is presented in Fig. 2. The results indicated that the PVA/F108/Au NPs/FITC-Lac composite nanofibers emitted green fluorescence (Fig. 2A) and Lac was uniformly distributed along the nanofibers. However, under the same parameters and conditions, the fluorescence could not be observed in the composite nanofibers prepared from original Lac (Fig. 2B). This phenomenon

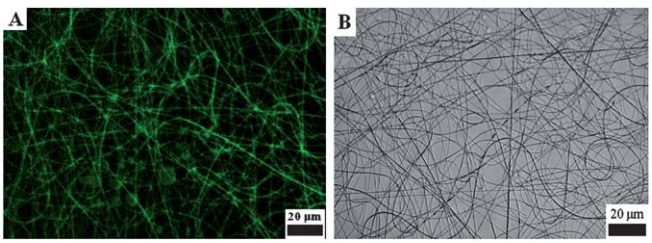

Fig. 2 LSCM images of PVA/F108/Au NPs/Lac composite nanofibers: (A) Lac labed with FITC; (B) original Lac (excitation wavelength 488 nm, emission wavelength 543 nm).

demonstrates that Lac could be encapsulated into PVA/F108/Au NPs electrospun nanofibers and it could provide an explanation for the amperometric response of the enzyme biosensor.

Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) can provide useful information on the impedance changes during the modification process of the electrode surface. To further confirm that Lac was encapsulated in the PVA/F108/Au NPs electrospun nanofibers, the EIS of the bare GCE and different modified GCEs were analyzed in $Fe(CN)_6^{3-/4-}$ probe solution. The results are shown in Fig. 3. The bare GCE exhibited an almost straight line at low frequencies corresponding to the diffusion process, and a small semicircle at high frequencies corresponding to the electron transfer limited process. The R_{et} value could be estimated 4.076×10^3 Ω from the diameter for the bare GCE. After being coated with PVA/F108 fibrous membranes and PVA/F108/Au NPs fibrous membranes, it showed an almost straight line at all frequencies which significantly facilitated the interfacial electron transfer, suggesting that the electrode reaction was controlled by diffusion. It is notable that the slope of the straight line is larger with the PVA/F108/Au NPs GCE than that of the PVA/F108 GCE, and this phenomenon was enhanced with the increasing amount of Au NPs (Fig. 4). This demonstrated that the use of Au NPs played an important role in accelerating the transfer of the electron[38] and made the electrochemical detection of the products from reactions more feasible. After Lac was encapsulated in PVA/F108/Au NPs fibrous membranes, the R_{et} values of the electrode (8.846×10^3 Ω) increased obviously. This phenomenon was attributed to the coverage by a protein layer with poor conductivity, which could hinder the interfacial electron transfer between the electrode and $Fe(CN)_6^{3-/4-}$ and enlarging the R_{et} value of the electrode.[39] The change of the EIS during the modification processes was strong evidence because the Lac was successfully encapsulated in the PVA/F108/Au NPs electrospun nanofibers. The coverage (θ) of Lac in the PVA/F108/Au NPs electrospun nanofibers could be calculated from the EIS results in terms of the equation:[40]

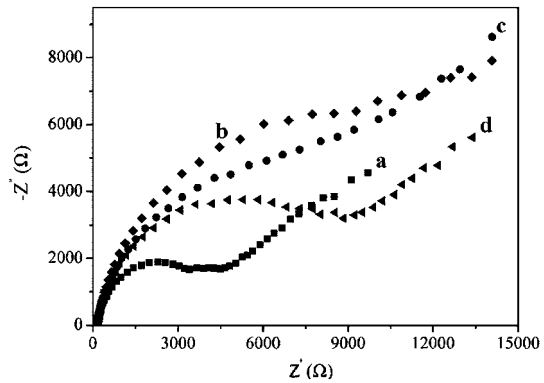

Fig. 3 EIS of different modified electrodes: bare GCE (a), PVA/F108 GCE (b), PVA/F108/Au NPs GCE (c) and PVA/F108/Au NPs/Lac GCE (d) in 0.1 mol L^{-1} KCl solution containing 10 mmol L^{-1} $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1 : 1).

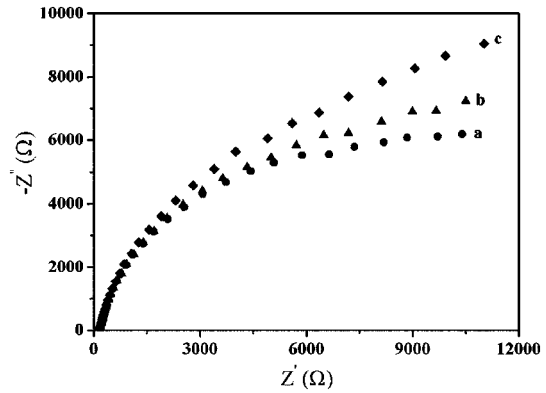

Fig. 4 EIS of PVA/F108/Au NPs GCE with different amounts of Au NPs: (a) 0.2 mL, (b) 0.3 mL and (c) 0.4 mL in 0.1 mol L^{-1} KCl solution containing 10 mmol L^{-1} $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1 : 1).

$$\theta = 1 - \frac{R_{et}^{PVA/F108/nanogold}}{R_{et}^{PVA/F108/nanogold/Lac}} \quad (2)$$

Using eq. (2), the coverage was calculated to be nearly 100%, indicating that Lac was successfully encapsulated in the PVA/F108/Au NPs electrospun nanofibers.

Influence of applied potential on Lac biosensor response

The applied potential has an important influence over the biosensor response because the sensitivity and selectivity of the system partially depends on it.[41] The effect of the applied potential on the response signal of the Lac GCE for CPs was tested over the −0.30 to +0.20 V range with 0.05 mmol L^{-1} 4-CP, 2,4-DCP and 2,4,6-TCP in PBS at pH 5.5. As shown in Fig. 5, the current increased with the decrease of the working potential from +0.2 to −0.15 V for 2,4-DCP. After −0.15 V, a slight decrease was observed. Therefore, −0.15 V was chosen as the optimal working potential for further determination experiments for 2,4-DCP. The applied potential has a rather similar behavior for 4-CP and 2,4,6-TCP. The currents increased with the decrease of the working potentials in the tested ranges +0.2 to 0.0 V and +0.2 to 0.1 V, respectively. After that, the currents began to level off. But the currents increased as the applied potentials shifted to

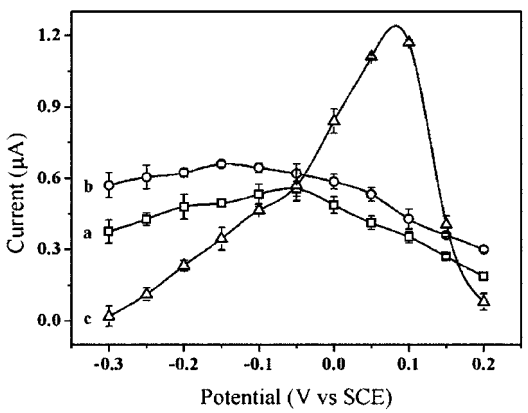

Fig. 5 Effect of working potential (−0.30 V to +0.20 V) for 0.05 mmol L^{-1} 4-CP (a), 2,4-DCP (b) and 2,4,6-TCP (c) in 0.2 mol L^{-1} PBS at pH 5.5.

more negative values finally. Thus, 0.0 V and +0.1 V were selected as the working potentials for the determination of 4-CP and 2,4,6-TCP, respectively.

Influence of pH on Lac biosensor response

The working solution pH value could affect the life time and detection limit of the biosensor as the activity of the immobilized enzyme would be affected.[42] Fig. 6 shows the change of the response currents with pH values from 2.5∼10.0 in a 0.2 mol L^{-1} PBS with 0.05 mmol L^{-1} (a) 4-CP, (b) 2,4-DCP and (c) 2,4,6-TCP. As seen from Fig. 6, the Lac GCE has optimal pH values for the three CPs: the observed optimal pH values were 5.5 for 4-CP (a) and 2,4,6-TCP (c); and 5.0 for 2,4-DCP. The response of the Lac GCE in acid conditions was higher than that in alkaline conditions for the studied CPs. The reason may be that the response of the Lac GCE is closely related to the activity of Lac. Investigation on the activity of free Lac found that free Lac showed a higher activity under acidic conditions than that under alkaline conditions. It has the highest activity at pH 5.5, indicating that the *in situ* encapsulation of the enzyme by electrospinning did not change the characteristics of the enzymes, and that the electrospun nanofibers were appropriate for immobilization of enzymes. In addition, it was found that the redox peak potentials shifted to more negative values with the increase of pH values. This might be attributed to the Bohr effect of the redox reaction:[43] electronic transfer with proton transfer. This process will alter the electrostatic interaction between the center ions of the oxidized state or reduced state among protein molecules and protonation process, thus the redox peak potentials shifted to more negative values with the increasing pH values. Accordingly, pH 5.5 was chosen for follow-up study.

Influence of enzyme loading on Lac biosensor response

The enzyme concentration is a crucial factor affecting the sensitivity of the enzyme electrode. The influences of the enzyme concentration on the reduction currents for 0.05 mmol L^{-1} 4-CP, 2,4-DCP and 2,4,6-DCP were examined. Various GCE solutions containing 1, 2, 4, 5, 8, 10 and 20 mg mL^{-1} Lac were prepared by electrospinning. As shown in Fig. 7, the enzyme concentration had a similar effect on the CPs. The response currents increased with the increase of the Lac loading amount at lower enzyme concentration. However, a sharp decrease in current response was observed at higher enzyme concentration (>5 mg mL^{-1}) due to the insulation effect of the enzyme. This insulation effect resulted from a higher protein amount in the electrospun nanofibers, which limited the diffusion and permeation of the quinones produced in the enzymatic reaction to the surface of the GCE, and thus the response current reduced.[44] The condition of 5 mg mL^{-1} Lac was chosen for further experiments.

Sensitivity performance of the Lac biosensor

The calibration curves were investigated in the range from 1 to 25 μmol L^{-1} of 4-CP, 2,4-DCP and 2,4,6-TCP. Fig. 8 shows that the reduction currents increased linearly with the increasing CP concentration. The correlation coefficients (R^2) for 4-CP, 2,4-DCP and 2,4,6-TCP were 0.9924, 0.9889 and 0.9891, respectively. Limit of detection (LOD) was calculated according to $3S_b/b$ formula, where S_b is the standard deviation of the blank measurements ($n = 6$) and b is the slope of the linear range of the respective calibration curve.[45] The calculated values of the LOD are presented with K_m^{app}, i_{max} and i_{max}/K_m^{app} in Table 1. As seen from Table 1, the LODs were 12.09, 2.70 and 9.33 μmol L^{-1} for 4-CP, 2,4-DCP and 2,4,6-TCP, respectively. The sensitivity obtained for the three substrates followed the sequence 2,4-DCP > 2,4,6-TCP > 4-CP. It can be concluded that the sensitivity for a variety of phenolic compounds not only depends on the nature of the protecting matrix, but also on the access efficiency to the active center of the enzymes of these phenols and the stability of the phenoxy radicals produced in the enzyme reaction.[45] Generally, the degradation rates of *ortho*-substituted chlorophenols catalyzed by Lac are faster than those of *para*-substituted ones. The possible reason is that the –OH on the benzene ring is an active group, activating the benzene ring and making it easy to react with an electrophilic substitute.[46] The

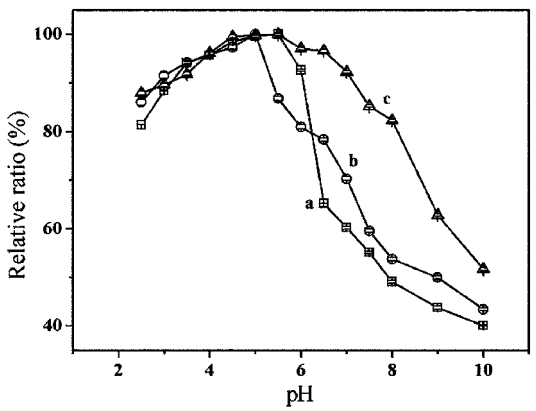

Fig. 6 Response currents of PVA/F108/Au NPs/Lac GCE in a 0.2 mol L^{-1} PBS with 0.05 mmol L^{-1} (a) 4-CP, (b) 2,4-DCP and (c) 2,4,6-TCP at different pH values (2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, 10.0).

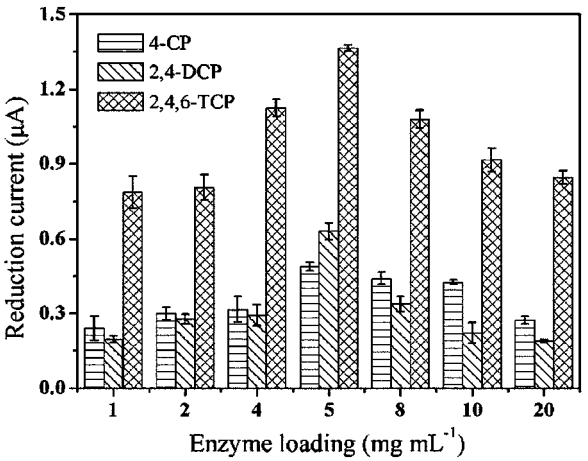

Fig. 7 Response currents of PVA/F108/Au NPs/Lac GCE in a 0.2 mol L^{-1} PBS (pH 5.5) with 0.05 mmol L^{-1} 4-CP, 2,4-DCP and 2,4,6-TCP with different amounts of enzyme loading (1, 2, 4, 5, 8, 10 and 20 mg mL^{-1}). Applied potentials for 4-CP, 2,4-DCP and 2,4,6-TCP were 0.0 V, −0.15 V and +0.1 V (*vs.* SCE), respectively.

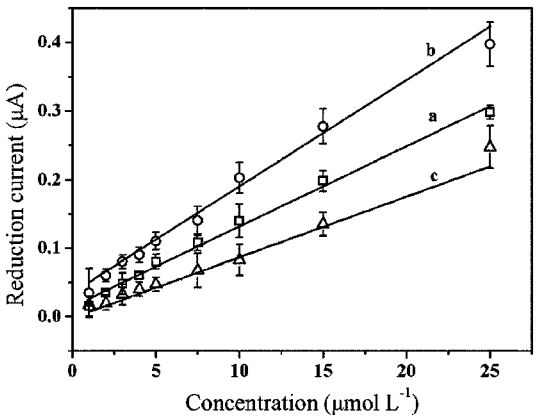

Fig. 8 Calibration curves of PVA/F108/Au NPs/Lac GCE for 4-CP (a), 2,4-DCP (b) and 2,4,6-TCP (c) in 0.2 mol L^{-1} PBS at pH 5.5.

electrophilic substitution would firstly react at the *ortho*- and *para*-positions, and then the cyclohexadienyl cation intermediates produced at the *ortho*- or *para*-positions can be stable with the unpaired electron in the ring, but reaction at the *meta*-position is unstable. The rate of a laccase-oxidised reaction has been shown to depend on the redox potential difference (ΔE^0) between the enzyme and the substrate. This will be improved with the increase of the stability of the cation, and so for the conversion efficiency of phenols.[47,48] Moreover, the key characteristic of Lac is the standard redox potential of the T1 site, and the redox potential of the T1 copper of *T. versicolor* Lac has been determined to be 767∼785 mV *versus* the normal hydrogen electrode (NHE),[49] which makes it highly active in homogeneous solutions. Additionally, because of the orienting effects of the phenolic hydroxyl group and chlorine substituents, the redox properties of CPs in the conversion reactions and the electrocatalytic properties of their products could be different from those of the other CP congeners, resulting in the selectivity in the present study.[21]

The apparent Michaelis–Menten constant (K_m^{app}), a reflection of the biological activity of the immobilized enzyme, was evaluated from the electrochemical version of the Lineweaver–Burk equation:[50]

$$1/i_{ss} = (K_m^{app}/i_{max})(1/C) + 1/i_{max} \quad (3)$$

where i_{ss} is the steady-state current after addition of substrate, C is the concentration of substrate, i_{max} is the maximum current measured under saturated substrate conditions and K_m^{app} was determined from the $1/i_{ss}$ (the cathodic current) *versus* $1/C$ curve. The K_m^{app} value displays the affinity of an enzyme towards its substrate, and a lower K_m^{app} value indicates stronger substrate binding and a higher catalytic activity. As expected, lower K_m^{app} values were obtained for the phenolic compounds exhibiting a higher sensitivity, as a consequence of the substrate recycling phenomenon confirmed for amperometric biosensors.[51,52] As can be seen in Table 1, the K_m^{app} values were estimated to be 426.06, 9.41 and 73.36 μmol mL^{-1} for 4-CP, 2,4-DCP and 2,4,6-TCP, respectively. In this present study, the K_m^{app} value is 9.41 μmol mL^{-1}, which is obviously lower than the reported values, such as 40.2 μmol mL^{-1} of the biosensor based on laccase entrapped in a Cu-OMC/chitosan (CS) film for the detection of catechol[53] and 22.4 mmol mL^{-1} of a laccase-modified graphite electrode for the detection of 4-CP.[54] The low K_m^{app} values obtained in this study indicated that Lac encapsulated into electrospun nanofibers retained its high catalytic activity and exhibited a higher sensitivity for 2,4-DCP. Thus, a novel phenol biosensor with excellent bioelectrochemical interface was successfully prepared, which presented high sensitivity and a low detection limit for 2,4-DCP.

The performance of the Lac GCE was further investigated for the repeatability, reproducibility and stability. The repeatability of Lac GCE was tested by repetitive amperometric measurements conducted on 0.05 mmol mL^{-1} CPs solutions. With a series of six successive assays, the relative standard deviations (R.S.D.) were 7.6%, 2.8% and 9.0% for 4-CP, 2,4-DCP and 2,4,6-TCP, respectively. The reproducibility of the responses was obtained with 6 different electrodes in 0.05 mmol mL^{-1} CP solution. The R.S.D. values of the responses were 14.9%, 10.4% and 13.7% obtained for 4-CP, 2,4-DCP and 2,4,6-TCP, respectively.

Stability is usually considered as one of the key factors in the construction of a biosensor, since it is not only beneficial to biosensor transport but also helps decrease the per-measurement cost.[22] Stability of the proposed electrode was conducted for measuring the Lac activity stored in pH 5.5 PBS at 4 °C. No obvious decrease in Lac activity was observed in the first few days. After a 30-day storage period, Lac retained 65.8% of its original activity which corresponded to 65% of the biosensor based on laccase chemical immobilized on glycidyl methacrylate and 3-thienylmethyl methacrylate polymeric matrix.[10] The results indicated that the prepared GCE had good storage stability and that the good storage stability is due to the biocompatible microenvironment around the enzyme molecules which was provided by the PVA electrospinning nanofibers to stabilize its biological activity to a large extent.[55] It was implied that electrospun nanofibers were appropriate for the immobilization of enzymes.

Conclusions

Trametes versicolor Lac was encapsulated in PVA/F108/Au NPs nanofibers by *in situ* electrospinning to prepare a Lac biosensor. LCSM and EIS analytical results proved that Lac was successfully encapsulated into electrospun nanofibers. The Lac biosensor was then used and optimized for CP determination. In this present study, 4-CP, 2,4-DCP and 2,4,6-TCP were chose to be target

Table 1 Initial performance characteristics of Lac GCE

Substrate	R^2	LOD/μmol L^{-1}	i_{max}/μA	K_m^{app}/μmol L^{-1}	i_{max}/K_m^{app}/nA μmol^{-1} L
4-CP	0.9924	12.09	0.66	426.06	1.5
2,4-DCP	0.9889	2.70	0.35	9.41	37.2
2,4,6-TCP	0.9891	9.33	0.77	73.36	10.4

compounds. The amperometric signal for 4-CP was the highest at the applied potential 0.0 V *vs.* SCE, pH 5.5 with 5 mg mL^{-1} Lac concentration; for 2,4-DCP was at the applied potential −0.15 V *vs.* SCE, pH 5.0 with 5 mg mL^{-1} Lac concentration; and that for 2,4,6-TCP was at the applied potential 0.10 V *vs.* SCE, pH 5.5 with 5 mg mL^{-1} Lac concentration. Under the optimal conditions, the sensitivity of the biosensor obtained in the linear range for CPs followed the sequence 2,4-DCP > 2,4,6-TCP > 4-CP. The difference could be probably caused by the stability of the phenoxy radicals produced in the enzyme reaction. The obtained K_m^{app} values were 426.06, 9.41 and 73.36 μmol mL^{-1} for 4-CP, 2,4-DCP and 2,4,6-TCP, respectively. The results indicated that Lac encapsulated into electrospun nanofibers retained its high catalytic activity and the sensing performance for CPs was attributed to the suitable electrochemical interface (*e.g.* biocompatibility, high surface area-to-volume ratio and superior mechanical properties) of PVA/F108/Au NPs/Lac. In addition, the biosensor exhibited good repeatabilities of 7.6%, 2.8% and 9.0% (R.S.D.) and reproducibilities of 14.9%, 10.4% and 13.7% (R.S.D.) for 4-CP, 2,4-DCP and 2,4,6-TCP, respectively. Lac retained 65.8% of its initial activity after a 30-day storage period, implying that the electrospun nanofibers were appropriate for the immobilization of enzymes.

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