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# Precipitated and chemically-crosslinked laccase over polyaniline nanofiber for high performance phenol sensing

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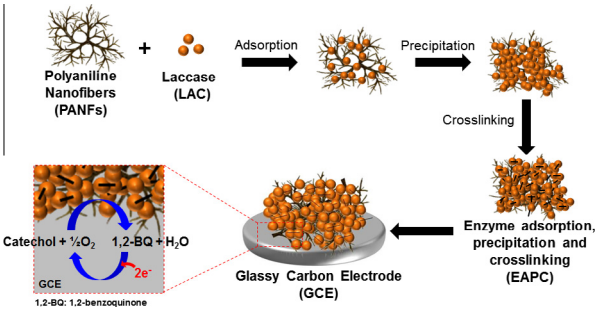
## HIGHLIGHTS

- Laccase (LAC) based amperometric biosensor for detection of phenolic compounds.
- LAC was immobilized on the matrix of polyaniline nanofibers (PANFs).
- Enzyme adsorption, precipitation, and crosslinking (EAPC) was applied to LAC on PANFs.
- EAPC-LAC on PANFs offers high enzyme loading and stability.
- EAPC-LAC shows a great promise for highly sensitive and stable phenol biosensors.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The present study aims at fabricating a laccase (LAC) based amperometric biosensor for detection of phenolic compounds. LAC was immobilized into the porous matrix of polyaniline nanofibers (PANFs) in a three-step process, consisting of enzyme adsorption, precipitation, and crosslinking (EAPC). Immobilized LAC on PANF in the form of EAPC was highly active and stable when compared to control samples of 'enzyme adsorption (EA)' and 'enzyme adsorption and crosslinking (EAC)' samples. For example, the activity of EAPC was 19.7 and 15.1 times higher than those of EA and EAC per unit weight of PANF, respectively. After 6 days at room temperature, EAPC maintained 100% of its initial activity, while EA and EAC retained only 7.7% and 11% of their initial activities, respectively. When the samples were subjected to the heat treatment at 60 °C over 3 h, EAPC maintained 74% of its initial activity, while EA and EAC retained around 1% of their initial activities, respectively. To demonstrate the feasible application of EAPC in biosensors, the enzyme electrodes were prepared and used for detection of phenolic compounds, which are environmentally hazardous chemicals. The sensitivities of biosensors with EA, EAC, and EAPC were 20.3 ± 5.9, 26.6 ± 5.4 and 518 ± 11 μA $mM^{-1}$ $cm^{-2}$, respectively. At 50 °C for 5 h, EAPC electrode maintained 80% of its initial sensitivity, while EA and EAC electrode showed 0% and 19% of their initial sensitivities, respectively. Thus, LAC-based biosensor using EAPC protocol with PANFs showed a great promise for developing a highly sensitive and stable biosensor for detection of phenolic compounds.



## 1. Introduction

Phenolic compounds, which are easily found in nature, have been considered as environmentally hazardous materials (Bruce et al., 2001). They are present in wastewater streams of the oil,

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paint, paper, polymer and pharmaceutical industries. When phenolic compounds are transferred into the food chain by wastewater streams, they can lead to dangerous and toxic effect on living organisms because phenolic compounds have poor biodegradability and high toxicity on ecological system as well as human body (Philippat et al., 2013). Thus, quick and accurate determination of phenolic compounds from the environments is of great importance (Ahmaruzzaman, 2008). Electrochemical enzyme biosensors can provide a simple and fast way for the determination of phenols (Karim and Fakhruddin, 2012; Rao et al., 2014). However, the use of such kind of enzyme biosensors have two major limitations when employed for in-situ monitoring of phenolic compounds in various environmental conditions, especially using them continuously over a long operation time. These two major limitations that hamper their successful uses for the practical applications are a low sensitivity and a short operation time, both of which corresponded to low loading and poor stability of enzymes, respectively. Low sensitivity and short operation time of enzyme biosensors can be improved via the enzyme immobilization in/on nanostructured materials by increasing enzyme loading and preventing the enzyme denaturation.

Nanobiocatalytic approaches using conductive nanomaterials have generated successful results that can potentially lead to the development of high performance enzyme electrodes with high loading and stability of enzymes for the various electrochemical biosensor applications (Kim et al., 2008; Kwon et al., 2010; Kim et al., 2011a). One of the successful strategies in nanobiocatalysis is the enzyme adsorption, precipitation and crosslinking (EAPC) method on polyaniline nanofibers (PANFs). Conductive PANFs can be synthesized via an easy and inexpensive process when compared to other nanostructured materials such as nanoparticles, electrospun nanofibers, mesoporous materials and carbon nanotubes. We previously reported biofuel cells using glucose oxidase immobilized on PANFs, showing a reasonably high power output with an exceptionally high long-term stability (Kim et al., 2011b).

In the present work, we immobilized laccase (LAC), which can oxidize phenol compounds (Solomon et al., 1996; Rao et al., 2014), in a form of EAPC on PANFs and evaluated its electrochemical sensing performance for detection of catechol as one of phenolic compounds. To our knowledge, the fabrication of such LAC-based enzyme electrode in the form of EAPC using PANFs as the support for the electrochemical phenol sensing has never been reported. For comparative studies, we also prepared the enzyme electrodes using the enzyme adsorption (EA) and the enzyme adsorption and crosslinking (EAC) methods. By comparing a performance stability of EA, EAC and EAPC, it would be possible to understand the effects of enzyme precipitation and enzyme crosslinking processes on the fabrication of LAC-based enzyme electrode and its electrochemical performances toward the phenol sensing.

## 2. Materials and methods

### 2.1. Laccase immobilization over PANF in the form of EA, EC and EAPC

LAC from *Trametes versicolor* was purchased from Sigma (St. Louis, MO, USA). Immobilized LAC samples in the forms of EA, EAC, and EAPC were prepared by following the protocols (Kim et al., 2011b). In details, the PANF were synthesized by mixing aniline and ammonium persulfate as initiator (Huang, 2006; Li et al., 2009). First, aniline monomer solution was dissolved in 1 M HCl (volume ratio of 8.5:1.5) and 0.1 M ammonium persulfate solution was prepared in 1 M HCl. Both aniline and ammonium persulfate solutions in HCl were mixed and shaken at 200 rpm and room temperature for 24 h. After the polymerization reaction, PANFs were centrifuged down, washed using DI water excessively for 3 times, suspended in DI water, and stored at 4 °C until use.

Immobilization of LAC over PANF in forms of EA, EAC and EAPC was previously reported (Kim et al., 2014) and modified protocol was carried out. To prepare the immobilized LAC in the form of EA, 2 mg of PANF were incubated in the 10 mg/ml of LAC solution in 100 mM sodium phosphate buffer (pH 6.5) under shaking (150 rpm) condition for 2 h. For EAC, the glutaraldehyde (GA) as the chemical crosslinking agent was introduced to the EA sample with a final concentration of 0.5% (w/v) under shaking (50 rpm) condition at 4 °C for 17 h. EAPC was prepared by introducing the ammonium sulfate solution with a final concentration of 50% (w/v) into the buffer solution containing both EA sample and free LAC. In the presence of the ammonium sulfate salt, the free LAC (i.e., LAC that is not adsorbed over PANF surface) was precipitated out to form the enzyme aggregates. Following this enzyme precipitation step, the GA solution with a final concentration of 0.5% (w/v) was added into the mixture to chemically crosslink the individual LAC molecule within the precipitated LAC aggregates and these aggregates over the surface of EA sample (See Fig. 1). To cap unreacted aldehyde groups, the samples were shaken at 200 rpm in 100 mM Tris–HCl buffer (pH 7.4) for 30 min, and the samples were excessively washed under shaking (200 rpm) for 3 times with the 100 mM sodium phosphate buffer (pH 6.5). EA, EAC, and EAPC were stored in 100 mM sodium phosphate buffer (pH 6.5) with the final concentration of 2 mg/ml at 4 °C until use.

### 2.2. Activity and stability measurement of LAC-immobilized PANF

The activities of the samples were measured by following the protocol found in the previous paper (Ride, 1980). The LAC activity was measured via triplicate experiments based on the oxidation of syringaldazine, which is a well-known substrate for the conventional laccase assay. To measure the activity of immobilized LAC on PANF, 100 μL of each LAC-immobilized PANF sample solution was mixed with 800 μL of 100 mM sodium phosphate buffer (pH 6.5), and the mixtures were incubated at 30 °C for 10 min. To this mixture, 100 μL of 0.216 mM syringaldazine in methanol was added. The absorbance at 530 nm was measured using a spectrophotometer (UV-1800, Shimadzu), and the activity of the LAC-catalyzed reaction was calculated from the slope of the time-dependent increase of absorbance at 530 nm. The stabilities of LAC-immobilized samples were checked by measuring their time-dependent residual activities after incubating the samples at different temperatures (40 and 60 °C) in the buffer.

### 2.3. Scanning electron microscopy (SEM) analysis

PANF, EA, EAC and EAPC were analyzed by using a scanning electron microscopy (SEM, Hitachi S-4300, Hitachi, Tokyo, Japan). For the SEM analysis, all the samples were washed by deionized water and freeze-dried for 24 h. After platinum (Pt) coating, the samples were analyzed at an accelerating voltage of 15 kV. By checking twenty randomly-chosen spots in each SEM image, the average thicknesses of PANF, EA, EAC and EAPC were determined.

### 2.4. Preparing enzyme electrodes of EA, EAC and EAPC

Nafion® solution (5 wt%) was mixed with 0.5 mg/ml of LAC-immobilized PANF sample, and the mixture was stirred at 4 °C for 1 h. The suspension (20 μL) of the mixture was applied over glassy carbon electrodes (GCE, 3 mm diameter, CH Instruments, Austin, TX, USA), and dried under ambient conditions for 1 h. The electrodes were stored in the 100 mM sodium phosphate buffer (pH 6.5) at 4 °C until use.

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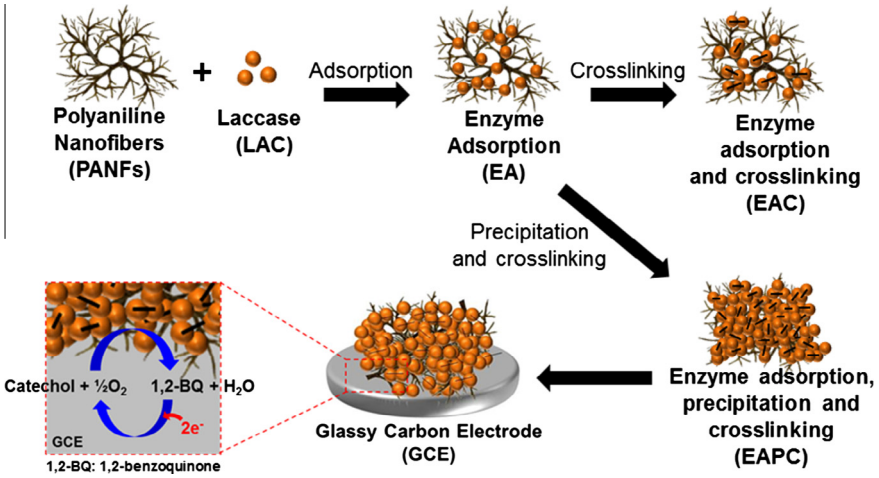


**Fig. 1.** Schematic illustration of three different enzyme immobilization methods used in this study: EA, EAC and EAPC and schematic of the electrochemical reaction occurring over EAPC electrode.

### 2.5. Electrochemical measurements in catechol sensing

The enzyme electrode, Ag/AgCl, and a platinum wire were used as the working, reference, and counter electrodes, respectively. For the amperometry sending tests, −50 mV vs. Ag/AgCl was applied to the enzyme electrodes. Their steady current responses were measured under magnetic stirring condition as a small aliquot (10 μL) of 10 mM of catechol solution was added. The measurements were performed after a time interval, which is appropriate for the stabilization of current response after each addition of aliquot. The electrochemical measurements were repeated three times in 100 mM sodium phosphate buffer (pH 6.5) at room temperature. For the thermal stability test of enzyme electrodes, the electrochemical performance of each enzyme electrode was checked by performing the amperometric sensing after incubating it in the 100 mM sodium phosphate buffer (pH 6.5) at 50 °C for 100 h.

## 3. Results and discussion

### 3.1. Immobilization of LAC on PANFs

Fig. 1 schematically shows the preparation of EA, EAC, and EAPC in PANF and electrochemical reaction on EAPC electrode. PANF is a good enzyme supporting material because it offers a porous interfiber matrix, which provides a high surface area for the effective enzyme immobilization. In the present paper, LAC was immobilized in PANF using three different approaches: EA, EAC, and EAPC. EA was prepared through a simple enzyme adsorption followed by an excessive washing, while the enzyme crosslinking process using the GA treatment was added for EAC approach. EAPC was prepared by introducing the enzyme precipitation and crosslinking to form crosslinkages between aggregated enzyme molecules. As shown in Fig. 2, the SEM images of EA and EAC showed no evident difference when compared to that of PANF, suggesting that both EA and EAC approaches for the LAC immobilization did not significantly improve the enzyme loadings on PANFs. On the other hand, the SEM image of EAPC shows thickly coated PANFs by the presence of the crosslinked LAC aggregates. By checking the nanofibers thickness, PANF, EA, EAC and EAPC showed 75 ± 12, 86 ± 14 and 90 ± 13 and 166 ± 18 nm of average nanofiber thicknesses. It can be explained that EAPC induce the thicker coating of enzymes on PANFs by ammonium sulfate precipitation and crosslinking steps compared to that of EA and EAC samples. In case of EAPC, the more SEM images in Fig. S1 show the variable thickness of EAPC during the step of enzyme precipitation and crosslinking.

According to elemental analysis, the enzyme loadings of EA, EAC, and EAPC were 0.026, 0.068, and 0.447 mg LAC per mg of PANF, respectively (Table 1). Hence, the EAPC resulted in 17-fold and 6-fold improvement of enzyme loading when compared to the EA and EAC samples. Thus, the elemental analysis data agree with their SEM images.

### 3.2. Activity and stability of LAC on PANFs

The activities per unit weight of PANFs of EA, EAC, and EAPC samples were 3.23 ± 0.28, 4.21 ± 0.30, 63.6 ± 3.0 A530 $min^{-1}$ per mg of PANF as shown in Fig. 3. There is a negligible difference in the activity between EA and EAC samples, while the activity of EAPC was 20-fold and 15-fold higher than that of EA and EAC, respectively. This enhanced activity of EAPC can be explained by its improved enzyme loading on PANFs with the additional precipitation and crosslinking steps. Consequently, SEM images, elemental analysis and activity data reveal that enzyme precipitation and crosslinking steps are critical for improving enzyme loading and achieving high activity per unit weight of PANF. The specific activities of EA, EAC and EAPC were 125, 61.9 and 142 A530 $min^{-1}$ per mg of LAC, respectively (Table 1). The lower specific activity of EAC than EA can be explained by inactivation of LAC during chemical crosslinking, while the step of precipitation and crosslinking for EAPC prevented the enzyme inactivation that leads to its higher specific activity than EA and EAC.

For the stability experiments, each sample was incubated at room temperature, 40 °C and 60 °C. The stabilities of EA, EAC, and EAPC were investigated by measuring the residual activity over the incubation times. After 6 days of incubation at room temperature, the relative activities were 7.69%, 11.2%, and 100% for EA, EAC, and EAPC samples, respectively (Fig. 4a). The relative activities of both EA and EAC samples were decreased almost down to 1% less than 18 days, which is a similar trend shown by the free LAC sample. On the other hand, the relative activity of EAPC sample was much stable over time and it still maintains the high relative activity value of 86% even after incubating it for 11 days at room temperature. Furthermore, the high thermal stability of EAPC sample was also demonstrated by checking the activity at 40 °C and 60 °C. After 10 h at 40 °C, EAPC retained 83% of the initial activity, whereas free LAC, EA and EAC retained 9.3%, 14% and 17% of initial

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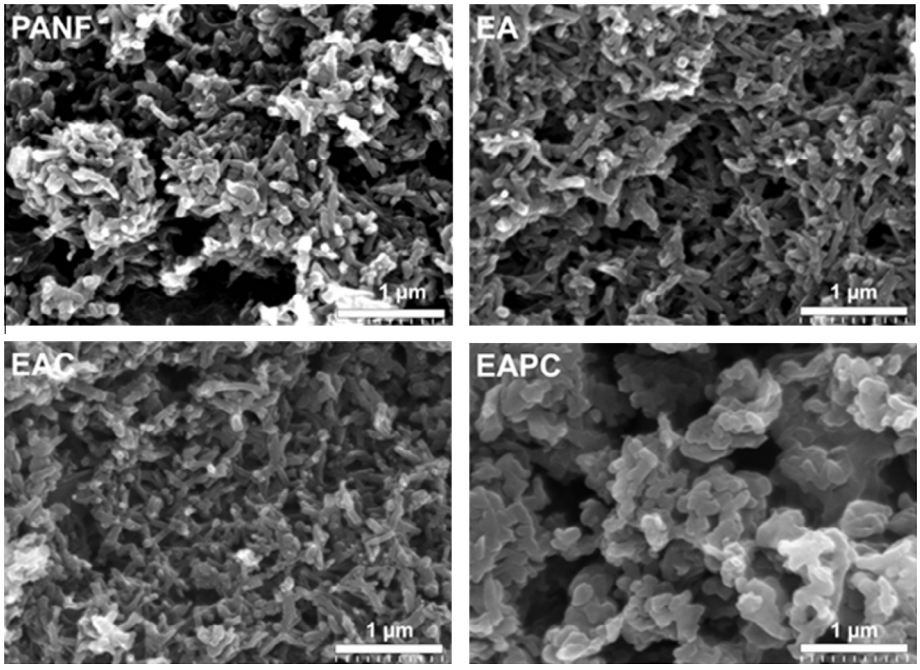


**Fig. 2.** SEM images of PANF, EA, EAC and EAPC. The scale bars in all images represent 1 μm.

**Table 1**
Enzyme loadings, activities per unit weight of PANF and specific activities of immobilized LAC on PANF in form of EA, EAC and EAPC.

| | LAC/PANF | | |
|---|---|---|---|
| | EA | EAC | EAPC |
| LAC loading[a] (mg LAC per mg PANF) | 0.0259 | 0.0680 | 0.447 |
| Activity per PANF (A530/min per mg PANF) | 3.23 | 4.21 | 63.6 |
| Specific activity (A530/min per mg LAC) | 125 | 61.9 | 142 |

[a] LAC loadings of EA, EAC and EAPC samples. The enzyme loadings were estimated from the measurement of nitrogen content in each sample via elemental analysis. PANF was used as a control.

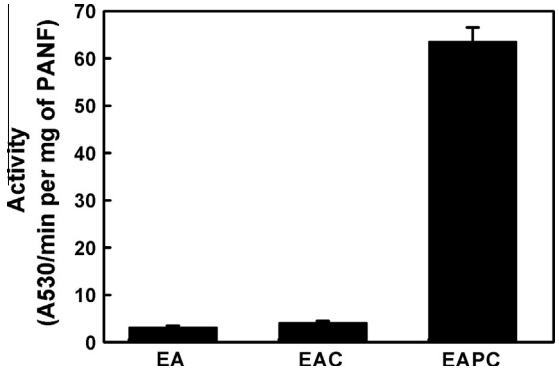


**Fig. 3.** The activity of the EA, EAC and EAPC per unit weight of PANF.

activity, respectively (Fig. 4b). In the case of 60 °C, EAPC maintained 74% of initial activity after 3 h of incubation, while relative activities of free LAC, EA and EAC were decreased around 1% within 3 h (Fig. 4c). These poor stabilities of EA and EAC samples can be explained by the denaturation and continuous leaching of enzymes from the matrix of PANFs, which also reflects on their low enzyme loadings and activities per unit weight of PANF. In the case of EA sample, the enzymes were physically adsorbed over the surface of PANF without chemical crosslinking. For EAC sample, the enzymes were chemically crosslinked to each other due to the GA treatment. However, its degree of chemical crosslinking is much lower than that of EAPC sample. EAPC sample have higher degree of chemical crosslinking due to a closer distance between enzyme molecules induced by ammonium sulfate precipitation (Martinek et al., 1977; Mozhaev et al., 1990; Roy and Abraham, 2004). Consequently, EAPC sample can significantly reduce the enzyme leaching and denaturation with its exceptionally high degree of chemical crosslinking.

### 3.3. Electrochemical biosensing performance

We prepared the enzyme electrodes by entrapping LAC-immobilized PANF in the Nafion® matrix over the glassy carbon surface. Fig. 1 describes the two main reactions that are taking place during the catechol sensing over the EAPC electrode surface. The catechol is first oxidized by reacting with the molecular oxygen over immobilized LAC to produce 1,2-benzoquinone and $H_2O$. This first reaction is non-electrochemical enzymatic reaction. For the second reaction, 1,2-benzoquinone is electrochemically reduced over the glassy carbon electrode surface at the fixed potential of −50 mV vs. Ag/AgCl (Quan and Shin, 2004). To effectively operate the electrochemical LAC-based biosensor, it should be designed such that rates of both non-electrochemical and electrochemical reactions are sufficiently high. Under such condition, the concentration of catechol is directly proportional to the current value measured from the amperometric sensing test.

Before the catechol sensing test, the background current was first measured by stabilizing the current response in the buffer solution. Once the background current is established, 10 μL aliquot of 10 mM catechol was added into 10 ml of the 100 mM sodium phosphate buffer (pH 6.5) and the current signal was obtained as shown in Fig. S2. Based on these amperometric sensing tests, the plots of current response versus the catechol concentration are plotted as shown in Fig. 5a. By measuring the slope of their linear regions, the sensitivities of EA, EAC, and EAPC electrodes were estimated as 20.3 ± 5.9, 26.6 ± 5.4 and 518 ± 11 μA $mM^{-1}cm^{-2}$, respectively (Fig. 5b). As described in Fig. 1, the performance of the

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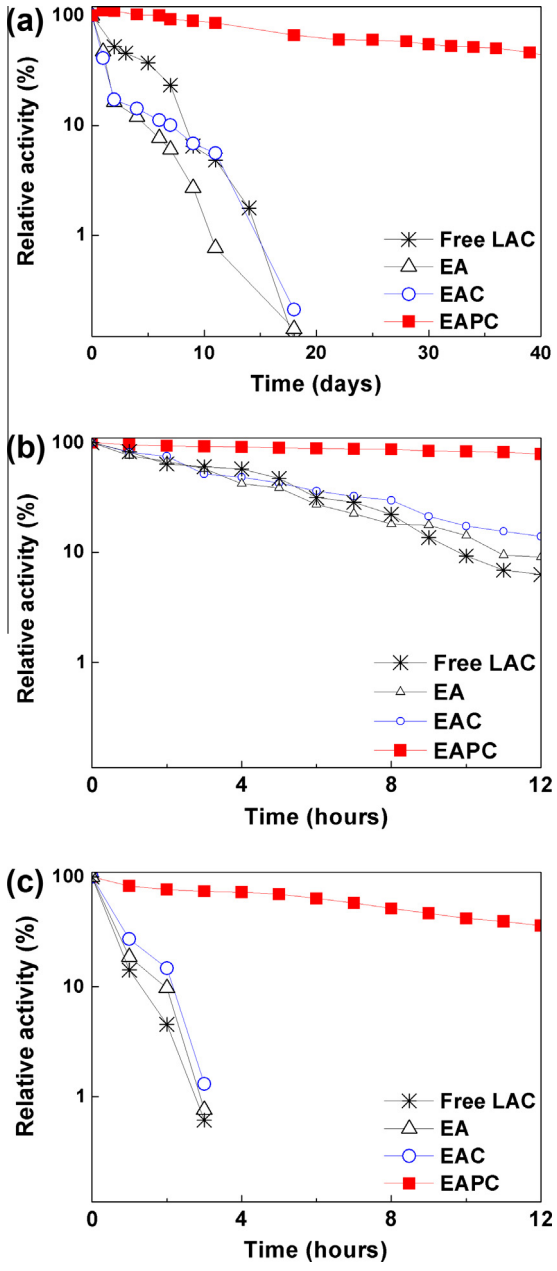


**Fig. 4.** (a) The stability of free LAC, EA, EAC and EAPC at room temperature (non-shaking). (b) Thermal stability of free LAC, EA, EAC and EAPC at 40 °C (non-shaking). (c) Thermal stability of free LAC, EA, EAC and EAPC at 60 °C (non-shaking).

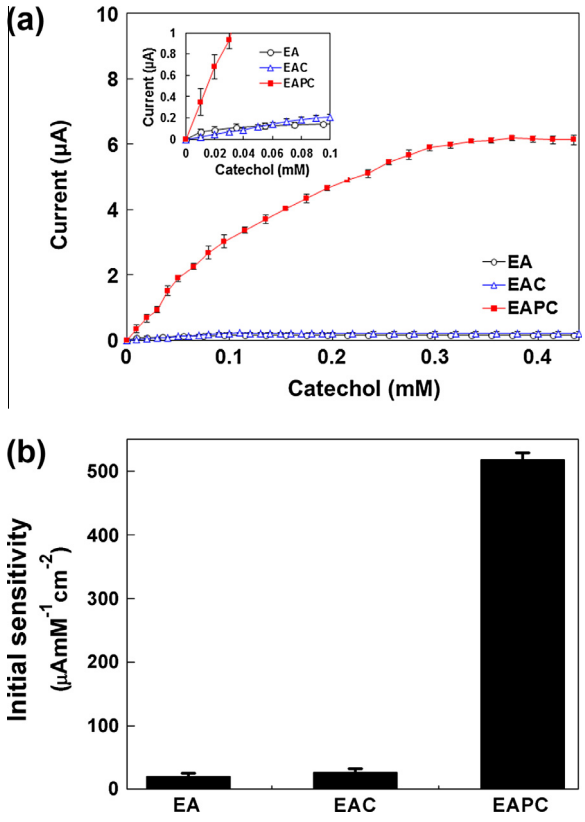


**Fig. 5.** (a) The plots of current versus catechol concentration obtained from amperometric responses. The current responses of LAC on PANF electrodes on successive additions of catechol were shown in Fig. S2. 10 μL of 10 mM catechol solution were added to 100 mM sodium phosphate buffer (pH 6.5, 10 ml). (b) The initial sensitivities of EA, EAC and EAPC electrodes. The sensitivity was calculated from the slope of the linear response region of (a).

electrochemical LAC-based biosensor is depended on both the non-electrochemical reaction over the immobilized enzyme layer and electrochemical reactions over the grassy carbon electrode surface. Since we used exactly same glassy carbon electrode for the EA, EAC, and EAPC samples, we do not expect to see any significant changes in its intrinsic electrochemical reduction activity toward the 1,2-benzoquinone regardless of what enzyme immobilization methods were used. Moreover, we are applying a bias of −50 mV vs. Ag/AgCl over our enzyme electrodes, which is strong enough to reduce the entire LAC-catalyzed 1,2-benzoquinone product without a significant electrochemical kinetic limitation. As the result, under this condition, the reduction current rate via the 1,2-benzoquinone reduction over the glassy carbon electrode would be limited by its mass transport rate. The mass transport rate of 1,2-benzoquinone is directly proportion to its production rate from the immobilized enzyme layer. Thus, for our sensing tests, the first non-electrochemical reaction over the immobilized enzyme layer would determine the reduction current and sensitivity of our enzyme electrodes. One of the most important factors that influences the rate of non-electrochemical reaction step for the production of 1,2-benzoquinone is the enzyme loading. The immobilized enzyme layer with the higher enzyme loading would provide more active sites to oxidize the larger amount of catechol. Consequently, EAPC electrode with the highest enzyme loading leads to the highest sensitivity as shown in Fig. 5b.

### 3.4. Thermal stability of EA, EAC, and EAPC electrodes

In addition to having the high sensitivity, achieving an excellent stability of enzyme electrode under the various stresses introduced by the operating environments is also an important requirement for the practical biosensor applications. For the current study, we focused on how the stability of LAC-immobilized PANF electrodes is affected by the thermal stress. To evaluate the thermal stability of our electrodes, all the enzyme electrodes were heat-treated by incubating them in an aqueous solution at 50 °C for 100 h. The relative sensitivity is defined as a ratio of residual sensitivity at each time point of measurement to initial sensitivity. The thermal stability data of EA, EAC, and EAPC electrodes are shown in Fig. 6. Both EA and EAC electrodes show relative sensitivity of less than 1% within 10 h at 50 °C. In contrast, EAPC electrode shows much improved thermal stability, which maintains 74% of its initial

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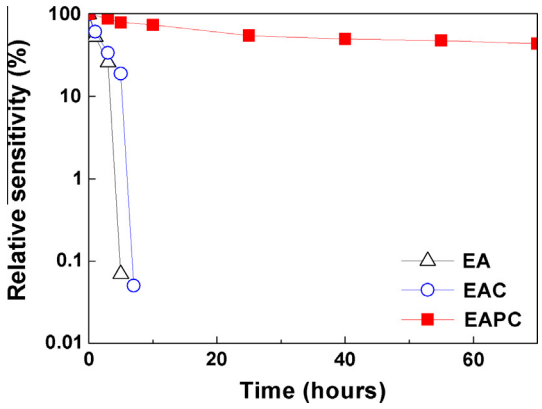


**Fig. 6.** The thermal stability tests of the EA, EAC and EAPC electrodes at 50 °C. Enzyme electrodes were incubated in an aqueous buffer solution (100 mM sodium phosphate, pH 6.5) at 50 °C. The sensitivity was calculated from the linear slope of initial amperometric responses to catechol concentration increases. Relative sensitivity is defined as the ratio of sensitivity at each measurement point to the initial sensitivity.

sensitivity after 10 h at 50 °C. Even after the 100 h of thermal treatment, the EAPC electrode is able to maintain 22% of its initial sensitivity. The large sensitivity decreases in EA and EAC electrodes are attributed to both the significant enzyme leaching and denaturation of LAC molecules over the PANF surface. On the other hand, EAPC electrode demonstrates a much improved thermal stability over 100 h due to its high degree of chemical crosslinking offered by the combination of the enzyme precipitation and crosslinking steps introduced during the immobilization process, which prevents the enzyme leaching and denaturation (Martinek et al., 1977; Mozhaev et al., 1990; Roy and Abraham, 2004).

## 4. Conclusion

Electrochemical LAC-based biosensor for detection of phenolic compounds has been successfully developed by immobilizing the LAC on PANF matrix in the form of EAPC. EAPC approach is consisted of enzyme adsorption, precipitation, and crosslinking steps. Its precipitation and crosslinking steps allow the formation of high degree of enzyme crosslinking that lead to both the high enzyme activity and stability per unit weight of PANF by increasing its enzyme loading. EAPC electrode has demonstrated its electrochemical sensing performance by showing high sensitivity for catechol detection and high thermal stability at 50 °C. When compared to EAPC electrode, both EA and EAC electrodes showed lower enzyme activity and stability per unit weight of PANF due to the lower enzyme loading and degree of crosslinking. Thus, the combination of both enzyme precipitation and crosslinking steps are critical for achieving both the high sensitivity and stability of electrochemical LAC-based biosensor. The protocol described in the present work can be applied for the immobilization and stabilization of many other enzyme systems, which would enable detection of environmentally hazardous materials.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.08.011.

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