

Tyrosinase Immobilization on ZnO Nanorods for Phenol Detection

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Directly using zinc powders as source material, ZnO nanorods were fabricated on gold wire by hydrothermal reaction without any other surfactant and stabilizing agent. The gold wire was skillfully treated to improve the nucleation for growth of ZnO nanostructures and to further improve the performance of the biosensor, which was construct by immobilizing tyrosinase (Tyr) on the ZnO nanorods for phenol detection. Electrochemical measurement, Fourier transform infrared and scanning electron microscopic analyses demonstrated that the Tyr was stably adsorbed on the ZnO nanorods surface with bioactivity for phenol oxidation. The biosensor reached 95% of steady-state current within 5s, and the sensitivity was as high as 103.08 $\mu\text{A}/\text{mM}$ at $C_{\text{phenol}} > 20 \mu\text{M}$ and was 40.76 $\mu\text{A}/\text{mM}$ at $C_{\text{phenol}} < 20 \mu\text{M}$. The detection limit of 0.623 μM was obtained at a signal/noise ratio of 3.

1. Introduction

In recent years, the nanostructural ZnO has attracted considerable interests due to its wide direct band gap, strong exciton binding energy, aesthetic morphologies, and multifunctional applications. Besides the optoelectronic properties, the nanostructural ZnO also possesses a lot of advantages for biosensing, such as high aspect ratio, polar surface along the *c*-axis, good electron communication, and nontoxicity. Notably, the isoelectric point (IEP) of ZnO is as high as about 9.5, which is suitable for immobilization of the biomolecules with low IEP, such as enzymes and proteins, assisted by electrostatic attraction in proper pH value.¹ So far, ZnO nanoparticles, porous film, nanocombs, and nanorods have been developed into biosensors to detect cytochrome *c*,^{1–3} protein,⁴ uric acid,⁵ glucose,^{6,7} and phenolic,⁸ respectively.

To fabricate the ZnO nanostructure, the hydrothermal method has gained more attention due to its simple process, low temperature, and mass product. The hydrothermal growth of ZnO nanostructures generally involves chemical reaction between a zinc salt and amine compound in water or water–ethanol.^{9–15} In the present case, we just used Zn powders as the source material and a gold wire as collection substrate to synthesize ZnO nanorods in deionized water. There are no other ions or molecules in the reaction system, so defects in the product induced from heterogeneity in ions or molecules was avoided.

In present work, Tyrosinase (Try), with a low IEP of about 4.5,¹⁶ was chosen as a model enzyme to catalyze the target phenol, which is a highly toxic contaminant in medicine, food, and the environment. The similar biosensor has been constructed in our previous report⁸ by immobilizing Tyr on the ZnO nanorods that were attached to a glassy carbon electrode by conductive nafion film. To immobilize the enzyme on the ZnO nanostructures more effectively and stably, and to further improve the performance of the biosensor, the ZnO nanorods were grown directly on the skillfully pretreated gold electrode.

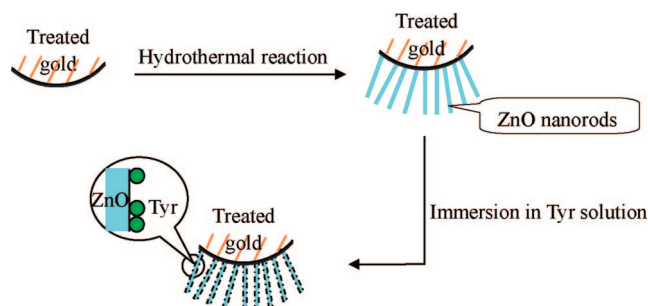


Figure 1. The procedure sketch map of immobilizing Tyr on the ZnO nanorods.

We shall present the construct method and the improved sensing characteristics in detail.

2. Experimental Section

The construct procedure of the biosensor is shown in Figure 1. The ZnO nanorods were fabricated on the treated gold sphere surface, followed by immobilization of the Tyr. The detailed experiments are described in the following.

A gold wire (99.99%, commercial) with a diameter of 0.5 mm was used as substrate for ZnO nanorod growth and further as an electrode for the electrochemical reaction. To link the ZnO nanorods stably and, further more, on the electrode, one top of the gold wire was melted into a sphere, coated with a thin layer of zinc on the ball surface by thermal evaporation, and then put into a heated tube furnace at 300 °C for 30 min to form a Zn–Au alloy. The Zn–Au alloy-covered gold wire was immersed into boiling potassium hydroxide solution (2 M) for 2 h and $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ solution with a volume ratio of 7/3 for 30 min, respectively, and then washed with deionized water. After above treatment, the gold ball presented a rough Zn–Au-coated surface. The surface was characterized by scanning electron microscopy (SEM, JSM-6360LV) and energy dispersive X-ray (EDX, Genesis2000 XM60).

Zinc powder (0.5 g, 99.95%, commercial) were put into deionized water (80 mL, $>17 \text{ M}\Omega \text{ cm}^{-2}$) in a Teflon-lined

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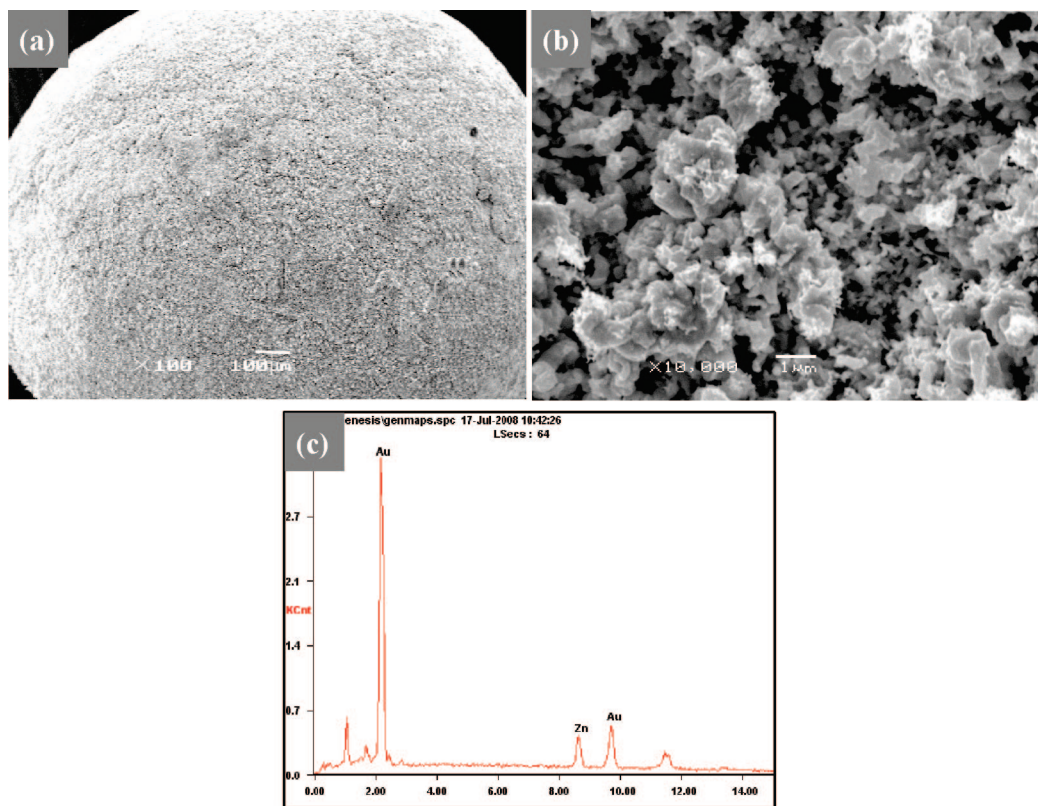


Figure 2. SEM images of the treated gold sphere surface with low (a) and high (b) magnifications and the corresponding EDX spectrum (c).

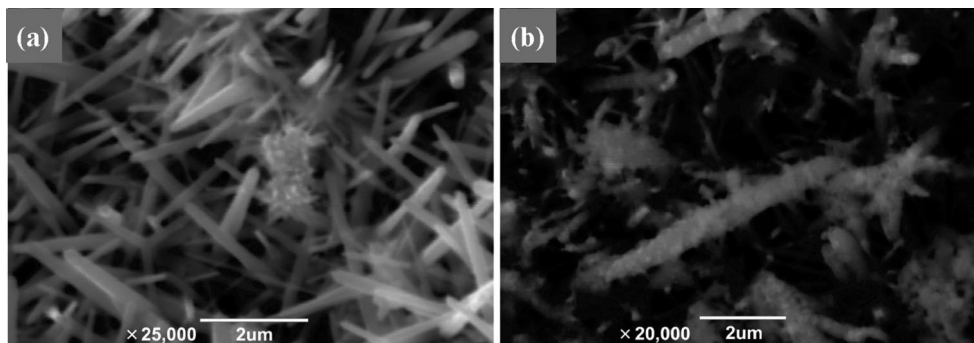


Figure 3. SEM images of ZnO nanorods before (a) and after (b) immobilization of Tyr.

stainless steel autoclave. The sphere end of the gold wire that was treated with the above procedure was hung vertically in water and sealed in the autoclave; the autoclave was then placed in an electric oven for hydrothermally growing of ZnO at 85 °C. The reaction was allowed to proceed for 20 h, after which the sample was taken out, washed with deionized water, and finally dried in air. The product was characterized by SEM and photoluminescence (PL, FLS920).

The ZnO nanorods on the gold sphere were immersed into a Tyr (EC 232–653–4, from mushroom) solution (2 mg mL⁻¹) at 4 °C for 10 h to immobilize the enzyme on the nanorods surface, based on the electrostatic attraction between the positively charged ZnO and the negatively charged Tyr in our experimental condition. Then, the modified Tyr/ZnO/Au was washed thoroughly with deionized water to remove the free Tyr. The immobilization results were examined by SEM and Fourier transform infrared spectroscopy (FTIR, Nicolet 380).

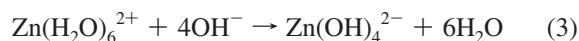
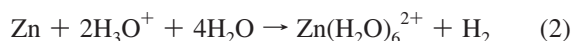
Electrochemical measurements were performed with a CHI 660C (CH Instrument Co. Shanghai) electrochemical workstation. The PBS pH value is a basic factor that influenced the Tyr catalysis capability. Our previous report¹⁷ demonstrated that

the Tyr/ZnO sensor exhibited the best performance when the pH value was 7.0. In the present case, all electrochemical measurements were carried out at the optimized pH value.

3. Results and Discussion

Figure 2 shows the SEM images and EDX spectrum of the gold ball surface after treatment. It clearly exhibits a rough porous morphology in Figure 2a and Figure 2b. The corresponding EDX spectrum in Figure 2c reveals that there is a Zn–Au alloy layer on the treated gold ball surface. In this alloy layer, the charge transfer from Zn to Au induces a decrease of electrons in the Au (5d) band as a consequence of s–d and p–d rehybridization in Au.^{18,19} The rough Zn–Au alloy surface is beneficial to nucleation,²⁰ and the good chemical activity^{21,22} is also good for growth of ZnO nanostructures.

After the hydrothermal reaction, the gold sphere surface was covered by nanostructure ZnO. The SEM image in Figure 3a shows the nanorod morphology of the as-grown product with a diameter of about 80 nm. The hydrothermal reaction mechanism from Zn to ZnO was proposed to occur in the following steps:



These equations reveal the formation process of ZnO nanorods: an H_2O molecule is ionized into H_3O^+ (H^+), which then react with Zn to form $\text{Zn}(\text{H}_2\text{O})_6^{2+}$, $\text{Zn}(\text{H}_2\text{O})_6^{2+}$ further transfer into $\text{Zn}(\text{OH})_4^{2-}$ and finally dehydrates into ZnO. The hydrothermal reaction avoided the influence of the heterogeneous ion because the ZnO nanorods grew from Zn powders in water without any other reagent. As shown in Figure 4, the PL spectrum of the ZnO nanorode on the gold sphere shows a strong near-band-edge emission band at 378 nm and a very weak defect-related green emission at about 520 nm. This shows that the defects in ZnO nanocrystal surface are low.^{23,24} Therefore, the charge state of the defect²⁴ is less influential, and the positive electricity on the ZnO nanorods surface is stronger. This is propitious to adsorb Tyr on the ZnO nanorods.

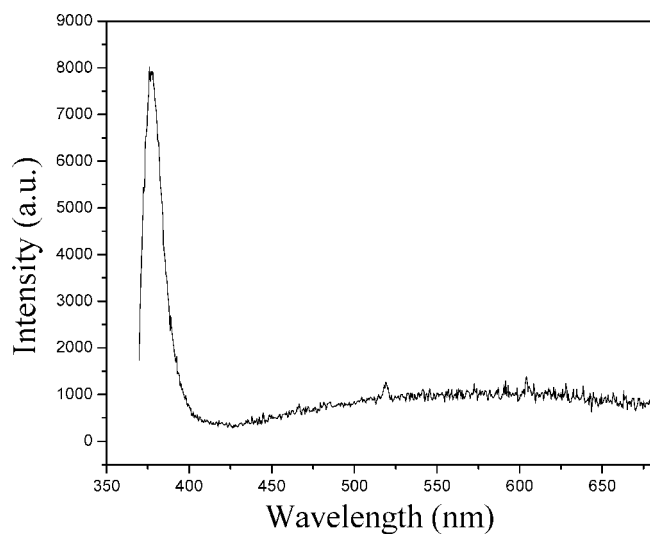


Figure 4. PL of ZnO nanorode on the gold sphere excited at 350 nm.

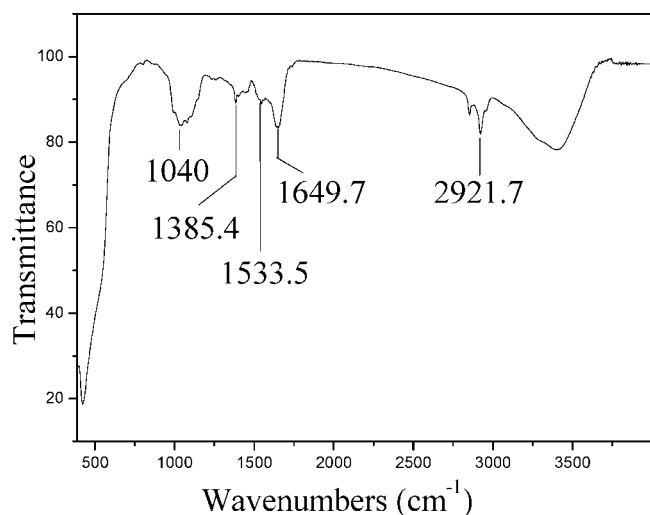


Figure 5. The FTIR spectrum of Tyr-immobilized ZnO nanorods.

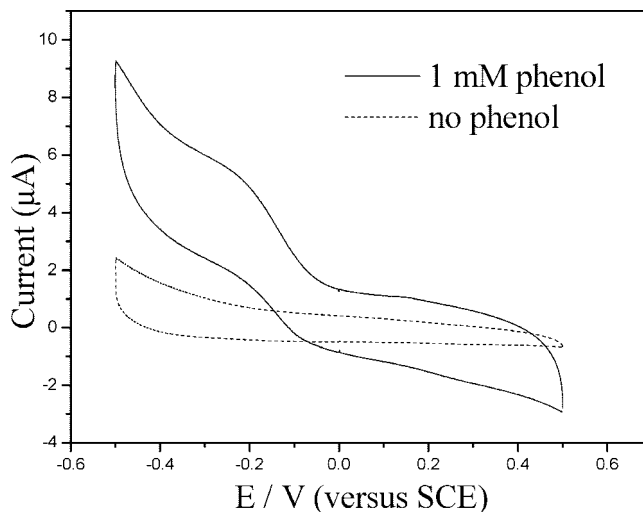


Figure 6. CV curves of Tyr/ZnO/Au electrode in 0.05 M pH 7.0 PBS with and without 1 mM phenol at 50 mV s^{-1} .

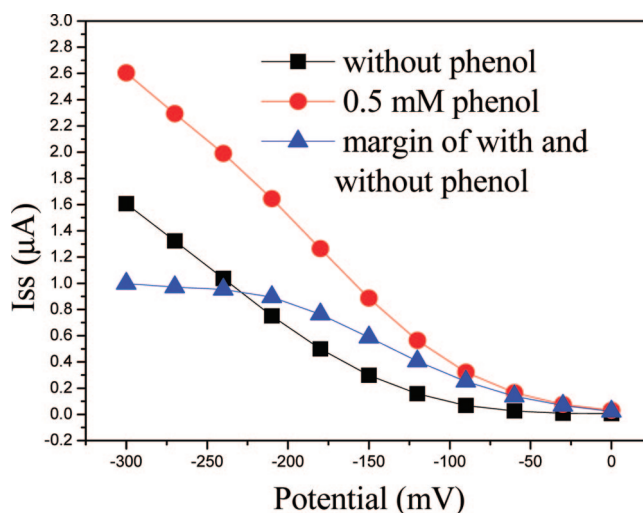


Figure 7. The points of amperometric experiment at the 120th second at series potential with and without 0.5 mM phenol, and their difference.

On the basis of the electrostatic attraction between the high IE ZnO and low IE Tyr, the enzyme molecules were effectively immobilized on the ZnO nanorods. Compared with the as-grown product, the SEM image of the Tyr-modified ZnO nanorods in Figure 3b clearly exhibits some rough cystiform tubers on the ZnO nanorods surfaces. Figure 5 is the FTIR spectra of ZnO–Tyr in reflection mode. The peaks at 2921 and 1385 are the absorption of $-\text{CH}_3$ and $-\text{CH}_2-$. The peak at 1040 cm^{-1} is the absorption of $-\text{C}-\text{OH}$ vibration. The peak at 1649 cm^{-1} for amide I is the absorption of $-\text{C}=\text{O}$ stretching vibration of peptide linkages in the protein's backbone. The peak at 1533 cm^{-1} for amide II is the absorption of $-\text{N}-\text{H}$ bending and $\text{C}-\text{N}$ stretching. The strongest peak nearby 500 is the absorption of ZnO nanorods. This demonstrated that the Tyr were successfully adsorbed on the ZnO nanorods, and the immobilized Tyr retained its bioactivity or native structure.²⁵

In a typical three-electrode electrochemical system, the Tyr-modified ZnO/Au wire was employed as working electrode, a platinum wire acted as auxiliary electrode, and a saturated calomel electrode (SCE) acted as reference against which all potentials were measured. Cyclic voltammetry (CV) experiments were carried out in a static electrochemical cell at $25 \pm 0.2^\circ \text{C}$. Figure 6 shows the CV curves in pH 7.0 phosphate

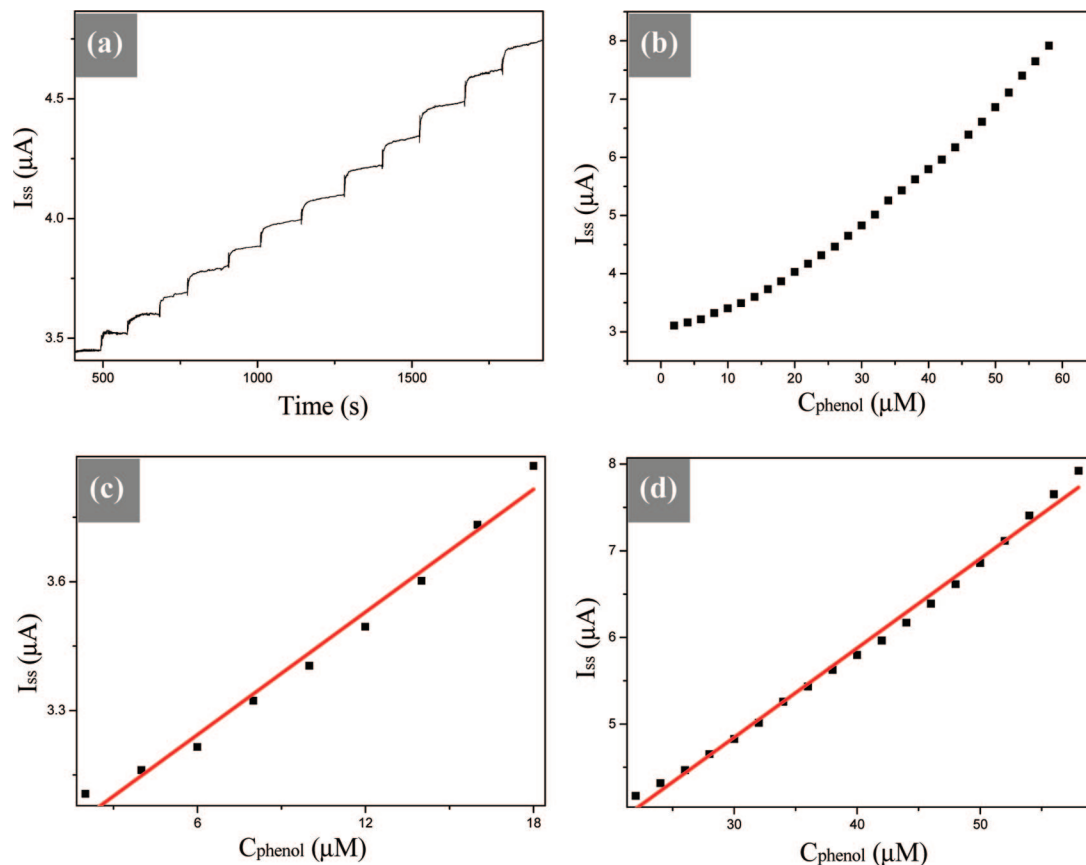
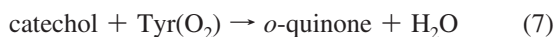
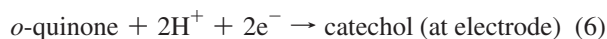
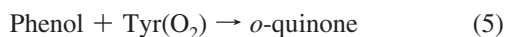


Figure 8. The current–time response (a) and the calibrated current–concentration relationship (b) for successively increasing phenol concentration in 2 μM steps at −200 mV; Panel b was separated into two linear regions corresponding to $C_{phenol} < 20 \mu\text{M}$ (c) and $C_{phenol} > 20 \mu\text{M}$ (d), respectively.

buffer solution (PBS, 0.05 M, prepared by mixing the stock standard solutions of Na_2HPO_4 and NaH_2PO_4) before and after adding the detected target of phenol. No signal was observed in the select potential windows for the PBS buffer. After adding phenol to the buffer solution, the CV curve reflected an increased reduction current at about −200 mV. This current increase resulted from the reduction of enzymatic reaction product.²⁶ The appearance of the reduction current indicates that the immobilization process retained the biological activity of the adsorbed Tyr on the ZnO nanorods. Under the catalysis of the Tyr on the electrode surface, the phenol was oxidized by dissolving oxygen to form *o*-quinone and then reduced into catechol in following steps:^{27,28}



To research the suitable potential in amperometric experiments, the cluster of amperometric curves at potentials of 0, −30, −60, ..., −510 mV, respectively, with and without phenol were worked out. We choose the points at the 120th second in every curve to analyze the optimum working potential for phenol oxidation. As shown in Figure 7, the square symbols are the points at the 120th second in the cluster curves without phenol, and the circle symbols are with 0.5 mM phenol. Both of the current signals raised up with increase of the negative potential.

TABLE 1: The Key Parameters of Tyr Biosensors for Phenol Detection

method	response time (s)	sensitivity	detection limit (μM)	K_M^{app}
Tyr–titania sol–gel films ²⁶	5	103 μA/mM	10	0.29 mM
Tyr–silica sol film ³⁰	17	23.1 μA/mM	0.1	
Tyr/silicate/Nafion ³¹	15	46 μA/mM	1	65 mM
PAA–Tyr–PAA ³²	12	150 μA mM ^{−1} cm ^{−2}		0.15 mM
Tyr–ZnO nanorods ⁸	5	12 μA/mM	15.57	0.24 mM
present case	5	103.08 μA/mM	0.623	0.178 μM

The triangle symbols in Figure 7 show their difference at the same potential correspondingly, which is regarded as the pure-catalytic current of phenol oxidation. It can be seen that the pure catalyzing current I_{ss} increases expressly from a potential of 0 to −200 mV, and trends to saturation after −200 mV. That demonstrated that the suitable potential is −200 mV for amperometric measurement.

The amperometric experiments were done in a stirred cell with a successive step addition of 10 μL of phenol (1 mM) standard solution to 5 mL pH 7.0 PBS at −200 mV. The reduction peak current increased with increasing phenol concentration in the buffer solution. Figure 8a illustrates a typical current–time plot for the sensor on successive step addition of phenol. As each 2 μM of phenol concentration was increased, the detected reduction current through the Tyr/ZnO/Au electrode rose steeply to a stable value. The response time is less than 5s, during which time the current reached 95% of steady-state value. The calibrated relationship between the reduction current and the concentration of the phenol was plotted in Figure 8b,

which can be separated into two linear regions corresponding to lower and higher concentration, as shown in Figure 8, panels c and d, respectively. It is evaluated that the sensitivity is 40.76 $\mu\text{A}/\text{mM}$ and 103.08 $\mu\text{A}/\text{mM}$ to the phenol concentration less than and more than 20 μM , respectively. The detection limit is 0.623 μM at a signal-noise ratio of 3. The calculated apparent Michaelis–Menten constant (K_M^{app}) was 0.178 μM according to the Lineweaver–Burk equation.²⁹

The performance of this Tyr/ZnO biosensor compared with other reported Tyr biosensors is summarized in Table 1.

It can be seen from Table 1 that the present ZnO/Tyr biosensor exhibits better performance than other reported Tyr biosensors. It shows faster response, higher sensitivity, lower detection limit, and smaller (K_M^{app}) constant. Compared with our previously reported ZnO/Tyr biosensor,⁸ the sensitivity and the detection limit was improved by about one order of magnitude. Different from the pasting of the ZnO nanorod on the electrode by nafion previously, the ZnO nanorods were grown directly and stably on the treated rough gold electrode surface. In this case, the uncovered ZnO nanorods were propitious to Tyr adsorption due to the large exposed surface, where the adsorbed Tyr molecules were exposed entirely in the solution, so the biocatalysis occurred more effectively and quickly. All of these advantages can be attributed to pretreatment of the gold sphere surface.

Furthermore, the biosensor has favorable stability and reproducibility. After a two-week storage period in air at 4 °C, the sensor retained 89.5% of its initial current response. The sensor retained 92.3% of its initial current response after measuring 8 times at the same experiment condition. The fabrication reproducibility is estimated for three electrodes made independently using the same condition as mentioned above. The relative standard deviation is calculated to be 7.9%, indicating good fabrication reproducibility.

4. Conclusions

The gold electrode was treated with a specific procedure to form a rough sphere head covered with an Au–Zn alloy layer, which made the ZnO nanorods grew easily and stably. The hydrothermal reaction is very simple and happened in only water using Zn powder as source material. The ZnO nanorods were had smooth surfaces and uniform size, with a diameter of about 80 nm. The SEM and PL measurements revealed the good crystal quality of ZnO nanorods, the reliable connection between the ZnO and the electrode, and the effective immobilization of Tyr on the ZnO nanorods. This effectively constructed a direct bridge for electron transfer during the Tyr catalyzing the detected phenol. SEM, FTIR, and electrochemical measurements demonstrated that the Tyr with low IEP was stably absorbed on the ZnO nanorods with high IEP and that the absorbed enzyme remained its bioactivity to a large extent. The biosensor exhibited a good performance. The response time was less than 5 s upon adding phenol to the buffer solution. The sensitivity and detection limit were improved to 103.08 $\mu\text{A}/\text{mM}$ and 0.623 μM

due to the pretreatment of the gold electrode. The favorable stability and reproducibility were befitting for the biosensor to detect the phenol.

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