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Article

Fabrication of Gold Nanoparticles/ Polypyrrole /HRP Electrode for Phenol Biosensor by Electropolymerization

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Abstract. A phenol enzyme biosensor was fabricated in this research using disposable screen-printed carbon electrode modified with gold nanoparticles (AuNPs), polypyrrole (PPy) and immobilized horseradish peroxidase (HRP) by electropolymerization method. The physical and chemical properties of the modified electrode was characterized by scanning electron microscopy (SEM), and energy dispersive X-ray spectroscopy (EDX) while electrochemical analysis was performed with amperometry. The fabrication parameters, gold precursor and enzyme concentrations, were optimized. The obtained results proved that AuNPs/PPy nanocomposite matrix could not only appropriately immobilize HRP, but also retained its bioactivity. Furthermore, the presence of AuNPs provided enhanced electrochemical responses. The proposed method was simple, and effective.

Keywords: Electropolymerization, biosensor, polypyrrole, gold nanoparticles, horseradish peroxidase.

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1. Introduction

The detection of phenols is of high necessity since phenol and its derivatives are highly toxic pollutants which are commonly used and discarded in many industrial processes e.g. resins and plastics, petroleum refining, food, pharmaceuticals, and pesticides [1]. Among several methods of analyses, biosensors are promising tools for detecting phenol and its derivatives due to their simplicity, high sensitivity, ease of use, and suitability for on-site monitoring [2]. Horseradish peroxidase (HRP) is a commonly used enzyme for phenol detection due to its high sensitivity towards a great number of phenolic compounds [3].

The design of enzyme electrode is one of the key factors in developing biosensor performances. Modification of enzyme electrodes based on combination of the advantages of screen-printed technology, nanomaterials, and conducting polymer is therefore interesting. Advancement of screen-printed technology leads to the production of disposable electrodes which are cheap, well suited for mass production, and easy to use [4]. However , direct adsorption of HRP molecules on electrode surfaces causes denaturation and loss of bioactivity including the slow electron transfer due to the active sites of enzyme have the long distance between the active sites and electrode surface [5]. Therefore, nanomaterials like gold nanoparticles (AuNPs) have been employed as a promoter to enhance the electron transfer. Furthermore, AuNPs provide good catalytic activity, biocompatibility, and large active surface area where enzymes with effectively retained activities can be strongly absorbed. In order to encapsulate both an enzyme and AuNPs, conductive polymers are used as supporting matrices while also act as electron facilitators [6].

The aim of this study was to fabricate a AuNPs/PPy/HRP modified electrode for phenol detection using an electropolymerization technique on screen-printed carbon electrodes (SPEs). In this research, we investigated the optimum parameters namely; the concentrations of AuNPs and HRP on electrochemical responses. Meanwhile, the AuNPs/PPy and AuNPs/PPy/HRP nanocomposite materials were characterized by scanning electron microscopy (SEM), X-ray energy dispersive spectroscopy (EDX), and UV-visible spectroscopy.

2. Materials and Methods

2.1. Enzyme and Reagents

Horseradish peroxidase (E.C.1.11.1.7) with an activity of 53 unit/mg (according to pyrogallol method performed by the supplier) , hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$) ,and pyrrole (98%) were purchased from Sigma Aldrich. Aqueous solution of hydrogen peroxide (30%) and potassium ferrocyanide ($K_4Fe(CN)_6$) were obtained from BDH laboratory supplies. Phenol was obtained from Carlo ERBA. Sodium chloride was bought from LBA Chemie. Potassium chloride was derived from Asia Pacific Specialty Chemical limited. Tri-sodium citrate was acquired from Rankem. Disodium hydrogen orthophosphate ($Na_2HPO_4 \cdot 12H_2O$) and Sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2H_2O$) were procured from Fisher Scientific. All chemicals used were of analytical grade and without further purification.

2.2. Preparation of AuNPs

AuNPs were prepared through the reduction of 1.0 mM $HAuCl_4$ using sodium citrate as a reducing agent. 20 mL of 1.0 mM $HAuCl_4$ solution was added to a 50 ml beaker on a stirring hot plate (IKA, Germany), and was heated to 65°C. After that, 2 mL of 1**%** solution of trisodium citrate dehydrate was added. Gold sol gradually formed as the citrate reduced Au^{3+} to Au^0. The solution was heated until a wine red color was observed then it was cooled down to room temperature, and stored in a dark bottle before being kept in a refrigerator under 4°C [7].

2.3. Fabrication of the AuNPs/PPy/HRP Modified Electrode

AuNPs/PPy/HRP nanobiocomposite film was coated onto surfaces of SPEs by electrochemical polymerization in a three-electrode cell. The polymerization medium was composed of 5 mL of phosphate buffer solution (PBS, pH 7.4) containing 0.09M pyrrole (Py), HRP (150, 250, 350, and 450 unit/ml), and 75 μL of Au^{3+} concentrations of (0.91, 2.73, 4.55, 6.36, and 8.18 mM). PBS was used as the supporting

electrolyte. Cyclic voltammetry was applied for the electropolymerization of the nanobiocomposite film by scanning the voltage between 0 and 1.0V at a rate of 10mV/s for 5 cycles.

2.4. Material Characterization

UV-visible spectroscopy (UV-2450, Shimadzu, Japan) was utilized to study the conformation of HRP enzyme in AuNPs/ pyrrole PBS. SEM-EDX (S3400N, Hitachi, Japan) was employed to investigate electrode surfaces and distribution of AuNPs in the nanocomposite film

2.5. Electrochemical Analysis

Amperometric measurements were performed with an Autolab potentiostat (Metrohm, model PGSTAT101, the Netherlands) with a Nova software version 1.5. The electrochemical cell consisted of three-electrodes which composed of a modified SPE working eletrode, a platinum wire counter electrode, and a silver/silver chloride (Ag/AgCl) reference electrode. All the measurements were done at 25°C. The AuNPs /PPy/SPEs were tested with 10 mM $K_4[Fe(CN)_6]$ and 1mM KCl in 0.1M phosphate buffer solutions (pH 7.4) at the applied potential of 0.58V (vs. Ag/AgCl) in order to study effects of the Au precursor concentration. AuNPs /PPy/ HRP/SPEs were finally investigated in 0.1M PBS (pH7.4) containing 50 μM H_2O_2/ 50 μM phenol at the applied potential of -0.05V (vs. Ag/AgCl) to assess effects of HRP concentration.

3. Results and Discussion

3.1. Structural Characterization of AuNPs/Py/HRP

UV-vis soret absorption bands were used as an effective tool to study the denaturation of heme proteins. As can be seen in Fig. 1 which shows UV-vis absorption spectra of HRP, Py, AuNPs/Py, AuNPs/Py/HRP solutions characteristic absorption bands of Py are not observed in a wavelength range between 300 and 800 nm. On the other hand, the heme bands of AuNPs/Py/HRP and HRP in PBS are detected at 402 nm which is closed to 403nm of Chen's work [8]. Moreover, a small distinctive peak of AuNPs is noted at 539 nm for AuNPs/Py, and the existence of HRP did not interfere AuNPs absorption peak. These results of UV-vis absorption bands reveal that HRP was anticipated to retain its native structure in HRP immobilization matrix containing AuNPs/PPy. Thus, AuNPs/PPy appears to be a suitable matrix.

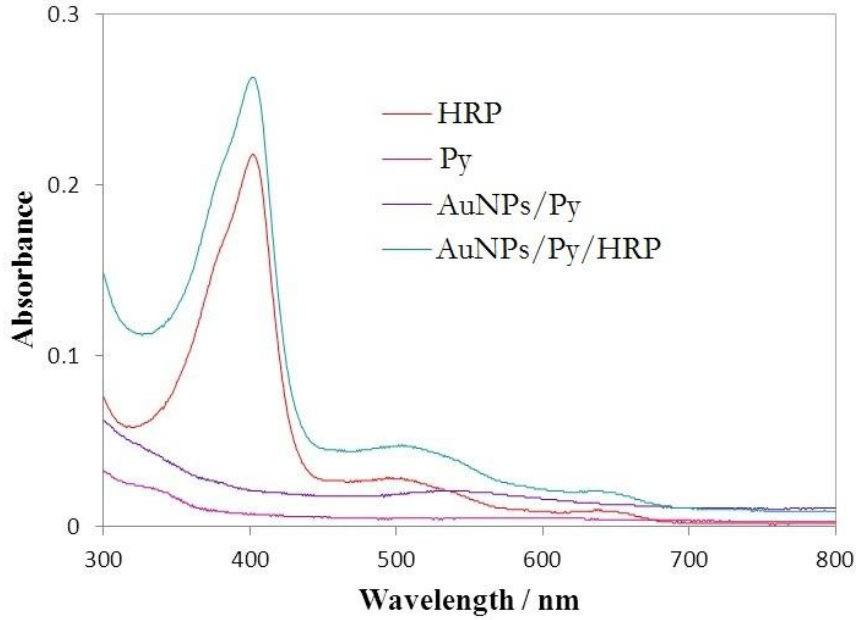

Fig. 1. UV-visible absorption bands for HRP, Py, AuNPs/Py, and AuNPS/Py/HRP in PBS (pH7.4) measured in the wavelength range between 300 and 800 nm.

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3.2. Characterization of Modified Electrode Surfaces

In order to reveal surface morphology of the modified electrodes, SEM analyses were under taken and the results are shown in Fig. 2. Bare SPE (Fig. 2(a)) was found to be of a typical rough SPE surface. Meanwhile, PPy modified SPE displays bud-like structures and smoother surface than bare SPE (Fig. 2(b)). Obviously, the bud sizes of PPy/SPE appear to be bigger than those of the bare electrode due to the growth of PPy on SPE. Moreover, Fig. 2(c) and 2(d) imply that addition of AuNPs (and HRP) to the PPy film did not cause mark differences in surface morphology in comparison to the sole PPy modified SPE. The inset of Fig. 2(c) shows TEM picture of the synthesized AuNPs which derived the spherical shape with an average diameter of 15 nm. Hence, SEM results affirmed that electropolymerization method brought the success modification of PPy/SPE, AuNPs/PPy/SPE and AuNPs/PPy/HRP/SPE.

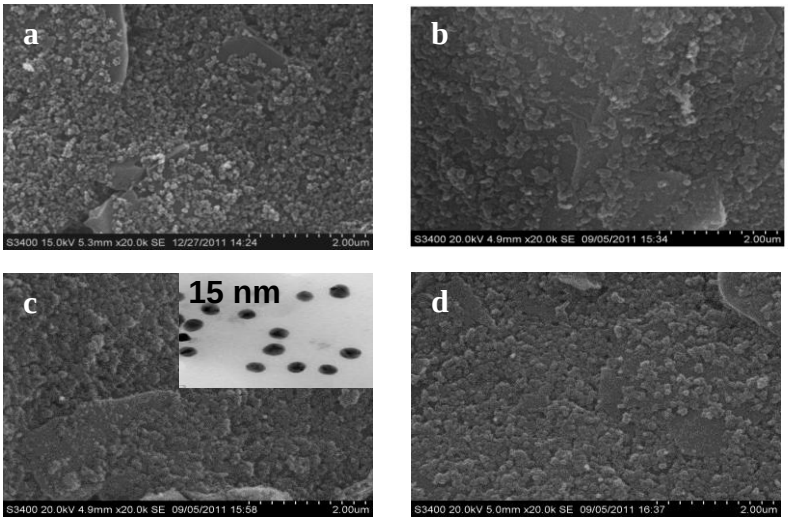


Fig. 2. SEM images of (a) bare SPE, (b) PPy, (c) AuNPs/PPy, and (d) AuNPs/PPy/HRP modified SPEs.

3.3. Elemental analysis by EDX

Energy dispersive X-ray spectroscopy (EDX) was utilized to study the elemental composition of modified SPEs (AuNPs/PPy modified electrodes of different Au precursor concentrations). Figure 3 shows the existence of AuNPs in PPy films. Homogeneous distribution of the nanoparticles was observed at all concentrations of Au precursors. However, weight percentage of AuNPs in PPy film, as shown in Fig. 4 was varied at 4.33, 4.72, 4.76, 4.74, and 4.11, respectively. These results exhibited that the percentage of AuNPs increased with Au precursor concentration until the maximum precursor concentration was reached. At high precursor concentration, the decreasing amount of incorporated AuNPs was caused by aggregation and flocculation of AuNPs in stock solutions. This observation confirms that the AuNPs was successfully entrapped during the electropolymerization process.

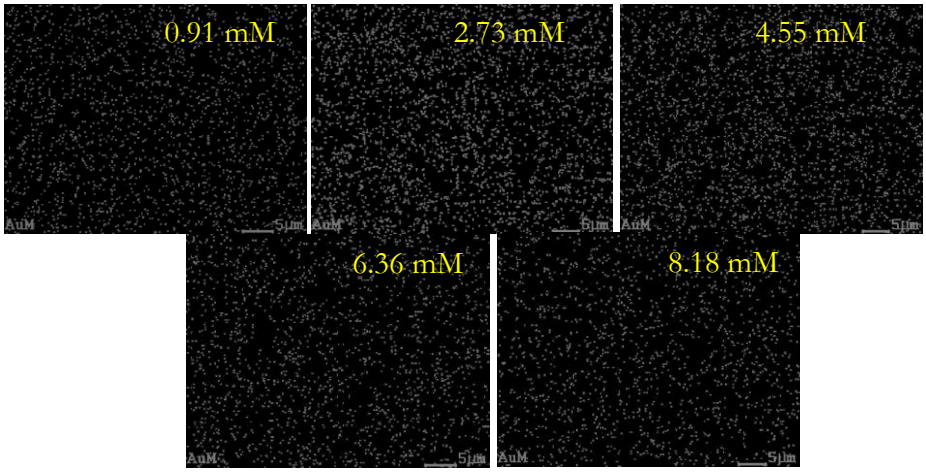


Fig. 3. The distribution of AuNPs in AuNPs/PPy nanocomposite film by EDX and the number in each picture demonstrates Au precursor concentration in the AuNPs synthesizing step.

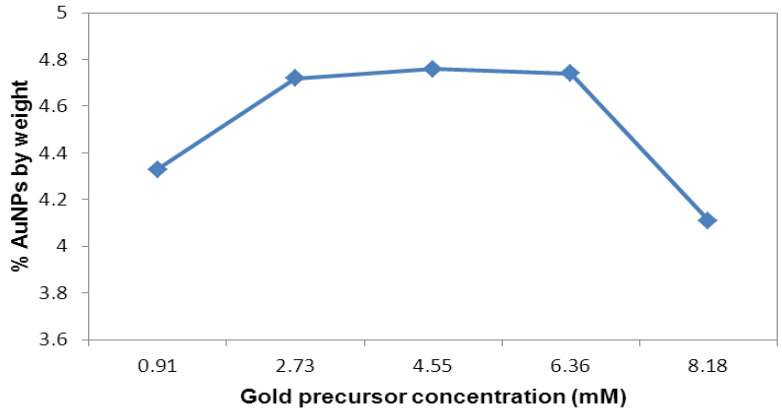
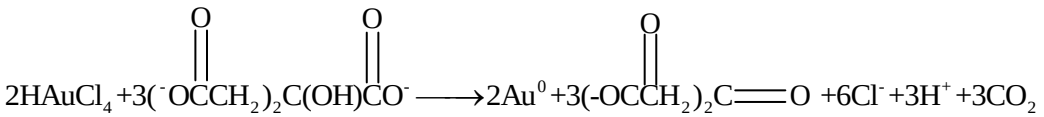


Fig. 4. The EDX determined percentages of AuNPs embedded in the PPy matrix using various Au precursor concentrations.

3.4. Electrochemical Analyses

3.4.1. Effects of Au Precursor Concentration

In this research, AuNPs were synthesized by a chemical reduction method ($[Au^{3+}]$: [citrate]=1:3.8). Due to the high ratio of reducing agent to Au^{3+} (more than two times the stoichiometric ratio), AuNPs were presumably synthesized in a complete reaction (Eq.1) [9].



$$2HAuCl_4 + 3(^{-}OCCH_2)_2C(OH)CO^{-} \longrightarrow 2Au^0 + 3(-OCCH_2)_2C{=}O + 6Cl^{-} + 3H^{+} + 3CO_2 \quad (1)$$

Figure 5 shows the relation between the current response and the concentration of Au precursor. The results exhibited that the current response increased gradually with Au precursor concentration between 0.91 to 4.55 mM. After that, it decreased. The highest current response was determined at 4.55 mM of Au precursor. This observation indicated significant current enhancement ability of AuNPs. Therefore, optimum Au precursor was determined at 4.55 Mm in the AuNPs synthesizing step.

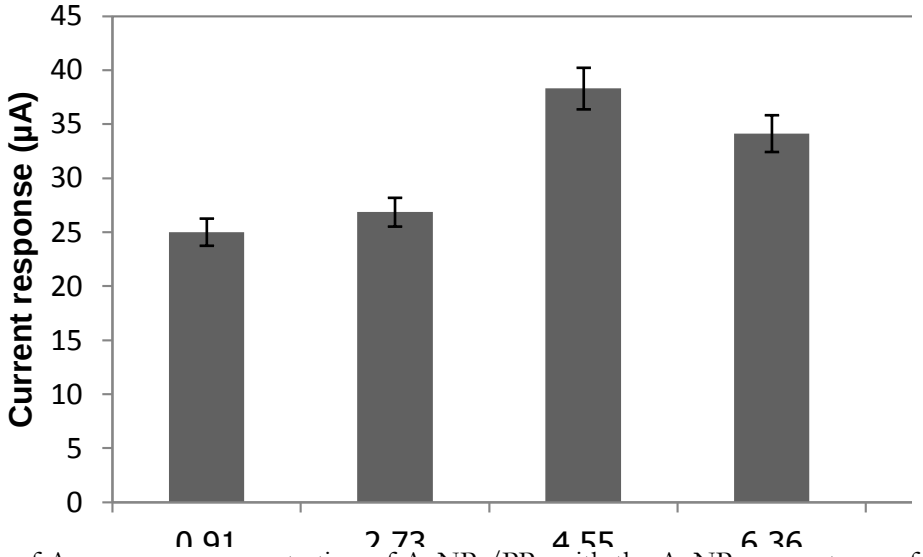


Fig. 5. Effects of Au precursor concentration of AuNPs/PPy with the AuNPs percentages of 4.33, 4.72, 4.76, 4.74, and 4.11, respectively on current response in a 0.1M PBS (pH 7.4) containing 10 mM $K_4[Fe(CN)_6]$ and 1mM KCl measured at 0.58V.

3.4.2. Effects of Enzyme Concentration

The HRP reaction mechanism involving in a phenol biosensor is a mediated electron transfer process. It consists firstly an oxidation of native HRP by hydrogen peroxide following with two reduction steps by phenols producing phenoxy radicals. These radicals are then reduced on the electrode surface as shown in Eq. (2-4). As a result, the obtained current is proportional to phenol concentration in the solution [10].

$$\text{HRP } (Fe^{3+}) + H_2O_2 \longrightarrow \text{HRP (Fe5+)} + H_2O \quad (2)$$

$$\text{HRP } (Fe^{5+}) + \text{phenol} \longrightarrow \text{HRP (Fe4+)} + \text{phenol} * \quad (3)$$

$$\text{HRP } (Fe^{4+}) + \text{phenol} \longrightarrow \text{HRP (Fe3+)} + \text{phenol} * + H_2O \quad (4)$$

Figure 6 shows current responses obtained in PBS solutions containing 50μM H_2O_2/50μM phenol at different HRP concentrations. With increased HRP concentration from 150 to 250 unit/mL, the response current was increased six-fold. However, higher HRP concentration than this point, the response current decreased with subsequent increase of HRP concentration. It is likely that the enzyme at high concentrations acted as barriers to substrate diffusion. In addition, enzyme molecules could obstruct the polymerization process which caused defects in the PPy film and therefore reduced biosensor sensitivity [11, 12]. Thus, the optimized enzyme concentration was determined at 250 unit/mL.

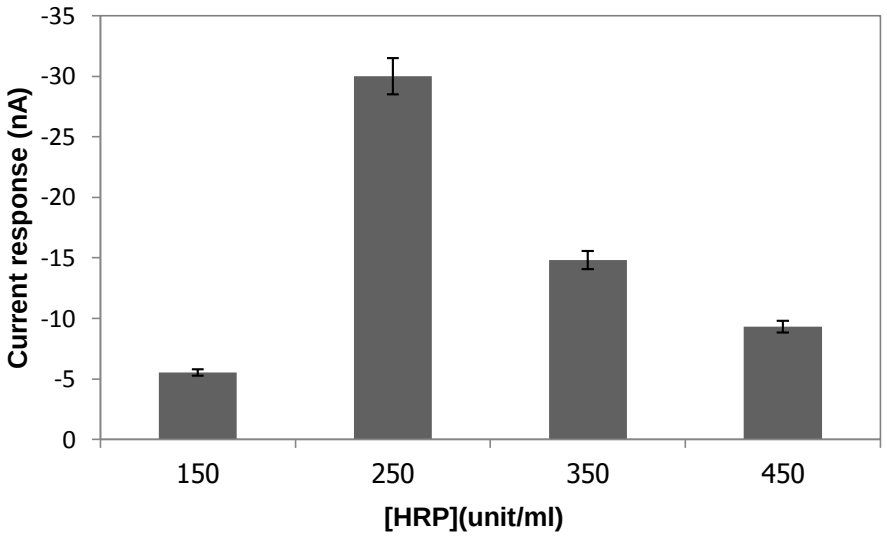


Fig. 6. Effects of HRP concentration on current response of AuNPs/PPy/HRP biosensor in a 0.1M PBS (pH 7.4) containing 50μM phenol / 50μM H_2O_2 measured at -0.05V at 4.55mM of Au precursor concentration.

3.5. Sensor characteristics

The prepared AuNPs/PPy/HRP modified SPEs performed good reproducibility of 4.0 % for five identical electrodes with a response time of 14.65 s.

4. Conclusions

In this research, the AuNPs/PPy/HRP modified disposable SPEs were successfully fabricated by means of electropolymerization which were used to detect the low concentration of phenol. The results indicated that AuNPs were homogenously incorporated with the immobilized enzyme in the PPy matrix. Optimum conditions for modified SPE fabrication were 4.55 mM of Au precursor concentration, and 250 unit/ml of HRP concentration. In the future work, we will examine the biosensor performances and kinetic parameters of this phenol HRP biosensor.

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