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# Highly Sensitive Pencil-Based Renewable Biosensor for Hydrogen Peroxide Detection With a Novel Bionanomultilayer

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A renewable graphite pencil electrode was demonstrated to be used as excellent working electrode for amperometric detection based on a novel bionanomultilayer using layer-by-layer assembly of gold nanoparticles and horseradish peroxidase. Parameters affecting the performance of the biosensor were optimized to obtain the maximal sensitivity. The resulting bionanomultilayer provided a linear range between 0.01 and 1.5 mM with the sensitivity of 149 μA $mM^{-1}$ $cm^{-2}$ and a detection limit of 0.002 mM. The biosensor was successfully applied to detect the amount of hydrogen peroxide in hair dye and disinfector samples. The results were well agreed to classical titration method. The recovery of hydrogen peroxide detection ranged from 80 to 104%.

**Keywords:** Pencil lead electrode, hydrogen peroxide, horseradish peroxidase, gold nanoparticles, bionanomultilayer

## 1. INTRODUCTION

Electrochemical detection is one of the main transducers in biosensor technique, which has become a widely concerned issue in enzyme electrode for years [1-3]. As we all known, the application of enzyme electrodes in electrochemical detection are highly selective and response quickly to the specific substrate. Commercial electrodes made from gold, platinum or glassy carbon electrodes were normally used as a working electrode. However, those electrodes are very expensive. Pencil lead is a low cost material that can be used as alternative material for handmade working electrode. Due to the composition of graphite in it, pencil lead behaves as a conducting material [4]. Therefore pencil lead has been successfully applied to be working electrode for many years [5-10].

In this paper, the application of pencil lead to be used as a working electrode was studied to construct highly sensitive biosensor based on layer-by-layer (LBL) assembly of gold nanoparticles (AuNPs) and horseradish peroxidase (HRP). The LBL technique is used to fabricate multilayer films with different materials in a reproducible way, through electrostatic force or covalent bond [11]. Normally, LBL films were produced from charged polymers such as poly(allylamine) [12], polyaniline [13], chitosan [14]. In this work, we are interested using AuNPs instant of polymers. Since, AuNPs can offer many advantages, such as high surface area, allowing the loading of a larger amount of enzyme, good biocompatibility and potentially more electron transfer [15]. Moreover, AuNPs allow a variety of functional groups, including -SH, $-NH_2$ and -CN, to form covalent bonds on their surface, which is favorable for the stable immobilization of biomolecules [16-17]. Therefore, the present work aimed to enhance sensitivity of biosensor by bionanomultilayer consisting of HRP and AuNPs for hydrogen peroxide detection. The effect of the electrode fabrication on the biosensor response and performance of amperometric detection were investigated in detail.

For the first time an amperometric hydrogen peroxide biosensor was fabricated on the basis of $(HRP/AuNPs)_n$ bionanomultilayer through LBL assembly on chitosan (CS) modified pencil electrode.

## 2. EXPERIMENTAL

### *2.1. Reagents*

Horseradish peroxidase, chitosan, sodium borohydride, hydrogen peroxide, hydrogentetrachloroaurate(III) ($HAuCl_4$) and trisodium citrate were obtained from Sigma. Glutaraldehyde was obtained from Fluka. All other chemicals were of analytical grade and all the solutions were prepared using distilled water.

### *2.2. Instrument*

All electrochemical measurements were carried out with a 663 VA Metrohm. The three-electrode system consisted of a pencil lead electrode as the working electrode, a Ag/AgCl as the reference electrode and a platinum wire as the auxiliary electrode.

### *2.3. Preparation of gold nanoparticles*

All glasswares used in the preparation of AuNPs were cleaned in freshly prepared 10% w/v of $HNO_3$ for overnight, thoroughly rinsed with distilled water and dried in air. AuNPs were obtained by reduction of gold ions with reducing agent. The AuNPs used in this work were prepared according to reported method with a slight modification [18]. Briefly, five hundred milliliters of aqueous solution containing 0.3 mM $HAuCl_4$ and 0.38 mM trisodium citrate was prepared. Then 1.2 mL of 0.125 M $NaBH_4$ solution was rapidly added into the gold solution under continuous stirring. The color of the solution turned into a purple-red solution, which indicated that the AuNPs were formed and the

prepared AuNPs were then stored in a dark glass bottle at 4 °C for further use. The resulting AuNPs was characterized with UV–vis spectroscopy (UV1601, Shimadzu) and transmission electron microscopy in a JEM 2010.

### *2.4. Preparation of pencil lead electrode*

The pencil leads (HB, 2B, 1H, 2H, 3H, 4H, 5H and 6H) were obtained from STAEDTLER, Germany. All leads had a total length of 2.0 cm and a diameter of 2.0 mm. The body of pencil lead was tightly coated with high-density polyethylene (HDPE). The electrical contact was provided by a metallic wire inserted into the pencil lead. Prior to each series of experiments, the pencil lead electrode was thoroughly polished with aqueous slurry of 0.3-0.05 μm alumina powder on a polishing cloth, and then electrochemical pretreatment of pencil electrode surface was carried out at 1.80 V for 5 min in 0.5 M acetate buffer solution (pH 4.80) containing 0.02 M of NaCl. The prepared electrodes were dried in air and immediately modified by multilayer films.

### *2.5. Preparation of multilayer films-modified pencil lead electrode*

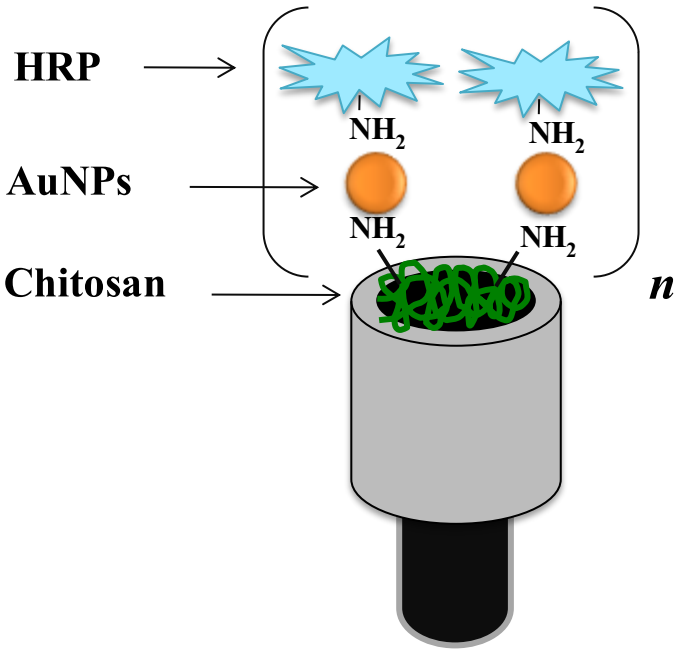


**Figure 1.** Scheme of the hydrogen peroxide biosensor.

Figure. 1 shows the schematic structure of the hydrogen peroxide biosensor based on multilayer films. For preparation of the enzyme biosensor, first cleaned pencil lead electrode was dipped into 2 mL of 0.5% w/v chitosan solution with applying a potential of 1.5 V for 5 min (denoted as CS pencil lead electrode). After the electrodeposition step, (i) modified electrode was immersed in 500 μL of AuNPs solution for 6 h (denoted as AuNPs/CS pencil lead electrode), (ii) followed by the adsorption of HRP for 12 h (denoted as HRP/AuNPs/CS pencil lead electrode), and rinsed thoroughly

with distilled water to remove some weakly adsorbed HRP which was regarded as the one layer of multilayer films modified electrode (denoted as $(HRP/AuNPs)_1$/ CS pencil lead electrode). Steps (i) and (ii) were then repeated for the required number of layers (denoted as $(HRP/AuNPs)_n$/CS pencil lead electrode). For comparison, without AuNPs modified electrode was carried out by the electrodeposition of 0.5% w/v chitosan with applying a potential of 1.5 V for 5 min. The modified pencil lead electrode was immersed in 2.5 %v/v glutaraldehyde for 20 min. Then this electrode was immersed in HRP solution for 12 h (denoted as HRP/Glu/CS pencil lead electrode). The electrode should be soaked in a pH 7.00 phosphate buffer solution at 4 ˚C until further use.

## 3. RESULTS AND DISCUSSIONS

### *3.1. Characterization of gold nanoparticle*

The spherical AuNPs were obtained from the reduction of $HAuCl_4$ using sodium citrate and $NaBH_4$ as reducing agents. The color of the solution was purple-red. AuPNs was characterized by UV–vis spectrum (data not shown) and showed a maximum absorption peak at $\lambda$ = 533 nm, which was the characteristic of surface plasma oscillation of spherical AuNPs [19-20]. The size of AuNPs was measured by transmission electron microscope (TEM) operated at high vacuum mode with the voltage of 200 kV. Sample for TEM was prepared by dropping AuNPs solution on a carbon-coated TEM copper grid and then let dry at room temperature. The TEM image of AuNPs (Figure. 2) showed that the average size of AuNPs was 4.4±0.7 nm (n=200).

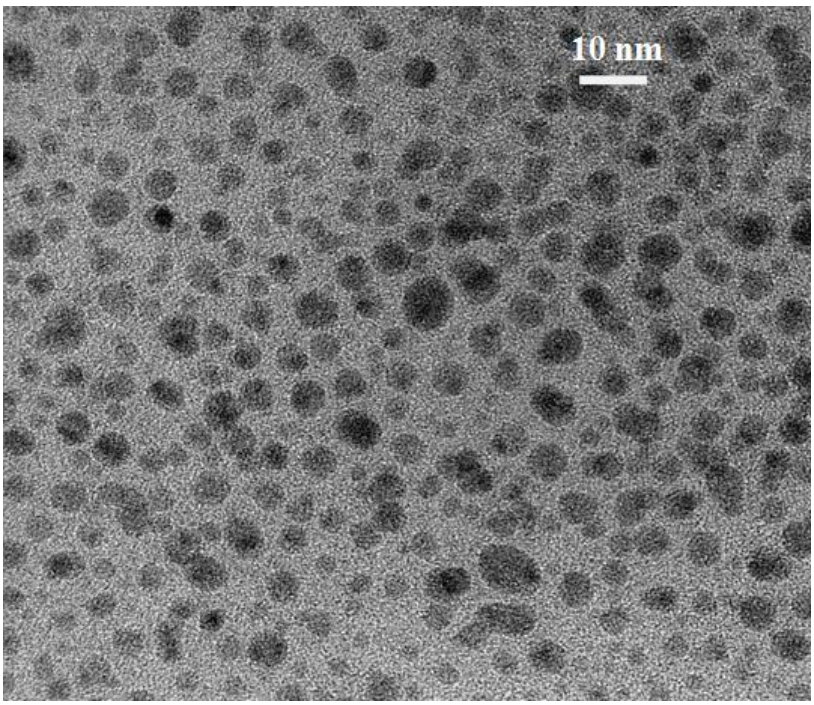


**Figure 2.** TEM image of AuNPs.

*3.2. Fabrication of the renewable pencil electrode*

To obtained the best performance of the detection, eight different hardness of pencil leads (HB, 2B, 1H, 2H, 3H, 4H, 5H and 6H) were studied using cyclic voltammetry in 10 mM of $K_4[Fe(CN)_6]$ containing 0.1 M of KCl. The voltage range was -0.2 to 0.8 V with the scan rate of 50 mV/s. Figure. 3 showed that $i_{pa}/ i_{pc}$ of $K_4[Fe(CN)_6]$ obtained from different pencil lead electrodes. The result found that the peak current ratio tend to unit for higher hardness, which indicated a good reversible redox process of $K_4[Fe(CN)_6]$. So, 6H pencil lead was selected as working electrode and was used in further studies.

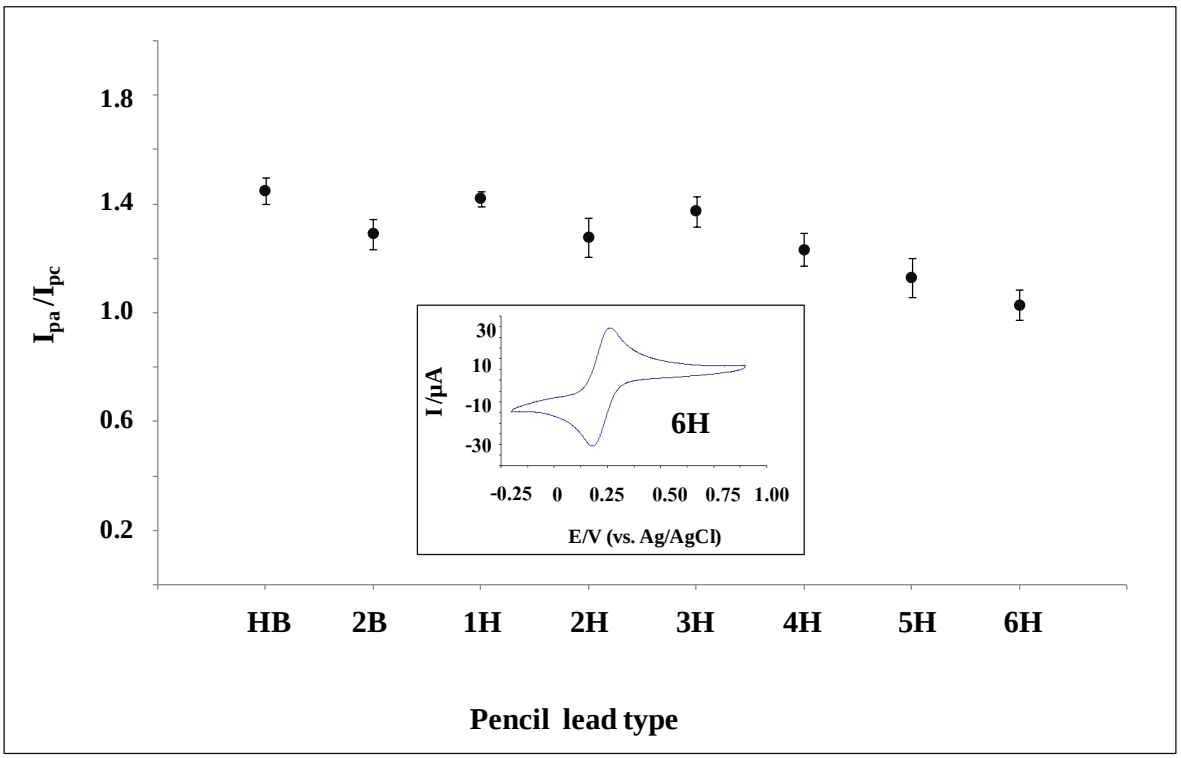


**Figure 3.** Variation of $i_{pa}/i_{pc}$ values as a function of different hardness of pencil leads. Inset showed the cyclic voltammogram of $K_4[Fe(CN)_6]_6$ obtained from 6H pencil lead electrode.

*3.3. Electrochemical deposition of chitosan*

The effect of applied voltage for electrochemical deposition of chitosan was studied. With the increasing potential from 1.2 to 1.5 V, the current response increased significantly. As the current of amperometric response obtained at higher than 1.5 V provided actually leveled off. Thus, the potential of 1.5 V was selected as the applied potential for the deposition of chitosan on pencil lead electrode.

The effect of deposition time on the properties of the deposited film was studied from 2 to 15 min. Increase the deposition time from 2 to 5 min resulted in the current response increased significantly. However, the current response of amperometric system obtained at the deposition time higher than 5 min was decreased. Therefore, 5 min of deposition time was the optimal time for

electrochemical deposition of chitosan to obtain stable film and high current response. Thus, the electrochemical deposition of chitosan on pencil lead electrode surface was carried out by using 0.5% w/v chitosan solution with the applied voltage of 1.5 V for 5 min.

### *3.4. Assembly of (HRP/AuNPs)$_n$ bionanomultilayer*

Since AuNPs allow a variety of functional groups including -SH, -$NH_2$ and -CN, to form covalent bonds on their surface, the immobilizations of HRP and AuNPs were achieved through LBL assembly via covalent interaction. This is favorable for the stable immobilization of biomolecules [21].

The influence of a number of HRP/AuNPs layers on the biosensor response was evaluated from the current response of 0.5 mM hydrogen peroxide as shown in Figure. 4a.

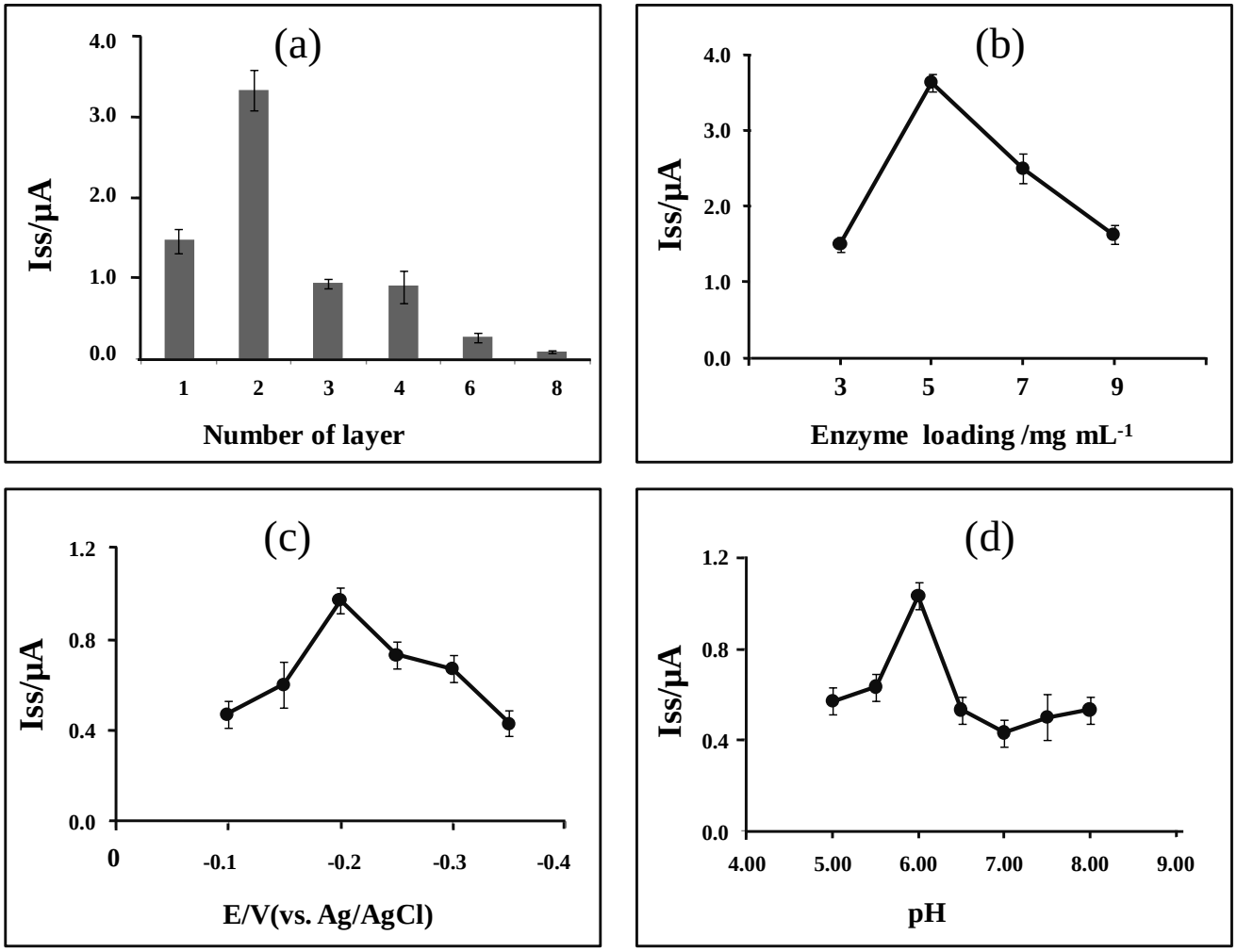


**Figure 4.** Effects of number of layer (a), enzyme loading (b), working potential (c) and pH (d) on the peak current of the response current to 0.5 mM of $H_2O_2$ at the modified enzyme electrode. $I_{ss}$ is the steady-state current after the addition of $H_2O_2$.

The response current increased with the number of assembled bionanolayer from 1 to 2 layers due to the electrode contained more enzymes to catalyze substrate and more AuNPs to promote the electron transfer. With these reasons it helps to enhance the current response and sensitivity of the biosensor. Then the response declined at above 2 layers. This is because thicker layers of assembled films would increase the electron transfer resistance and obstruct the diffusion of the substrate [22]. Therefore, two layers ($(HRP/AuNPs)_2$) of modified pencil lead electrode were chosen for ongoing experiment.

### *3.5. Effect of the enzyme loading on the biosensor response*

In the fact that, the amount of enzyme affect the sensitivity of the biosensor. Therefore, the enzyme loading on the hydrogen peroxide biosensor was investigated using different concentrations of HRP. The AuNPs/CS pencil lead electrode was immersed into 3.0, 5.0, 7.0 and 9.0 mg/mL of HRP solution. The optimized experimental result for the determination of hydrogen peroxide was shown in Figure.4b. The response increased with the concentration of enzyme increasing and reaching a maximum at 5.0 mg/mL. Thus, 5.0 mg/mL of HRP solution was selected for immobilization on AuNPs layer.

### *3.6. Effect of the working potential and the pH in amperometric detection*

In order to obtain an efficient biosensor for hydrogen peroxide detection, the influences of applied potential and pH on the response of modified electrode were optimized.

The effect of the working potential on the amperometric response of the biosensor was studied between -0.1 V and -0.35 V. The response current increased rapidly with the applied potential between -0.1 V and -0.2 V and leveled off thereafter (Figure. 4c), so a potential of -0.2 V (VS. Ag/AgCl) was selected as the applied potential for the amperometric measurements.

The effect of pH on the performance of biosensor was investigated the range from 5.00 to 8.00 in phosphate buffer. The highest current response was achieved at pH 6.00 (Figure. 4d), which was similar to the previous report by Liu et al [23]. Thus, the optimal pH value of the enzymatic reaction was pH 6.00 and was chosen for further experiments.

### *3.7. Electrochemical response to hydrogen peroxide*

Using the optimum conditions established in the above studies, the biosensor for hydrogen peroxide detection was carried out. The modified electrode with ($(HRP/AuNPs)_2$/CS pencil lead electrode) and without (HRP/Glu/CS pencil lead electrode) AuNPs were compared the amperometric response. The concentration of 0.5 mM hydrogen peroxide was used. Figure. 5 showed typical amerometric response and calibration curve (Figure 6) obtained from both electrodes. The electrode modified with AuNPs (Figure. 5a) provided current response much higher than the one without AuNPs (Figure. 5b) at the same concentration of hydrogen peroxide. Figure 6, $(HRP/AuNPs)_2$/CS pencil lead

electrode provided the sensitivity six times higher than HRP/Glu/CS pencil lead electrode. This result was confirmed by atomic force microscope (AFM) technique. The AFM is used to characterize the surface of both modified electrodes.

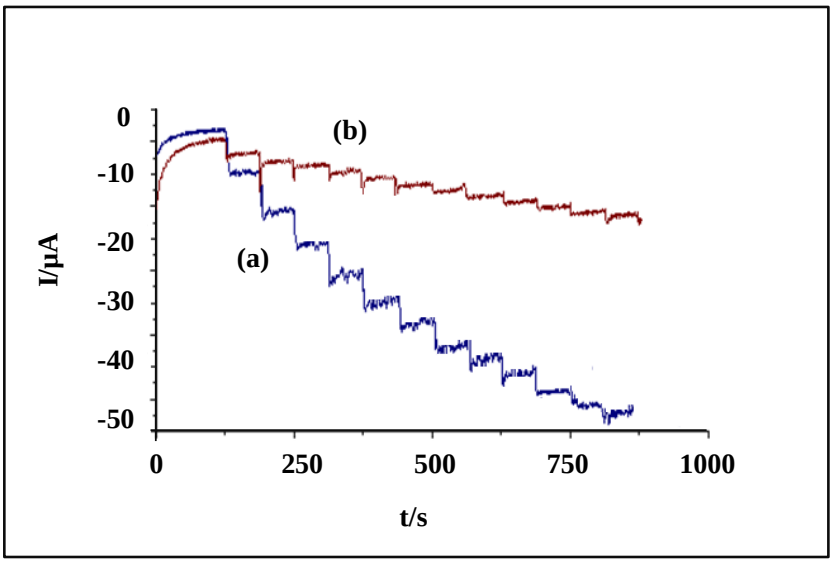


**Figure 5.** Amperometric responses of $(HRP/AuNPs)_2/CS$ (a) and (HRP/Glu/CS) (b) modified pencil lead electrode to successive addition of 0.5 mM $H_2O_2$ in phosphate buffer (pH 6.00) at the applied potential of -0.2 V.

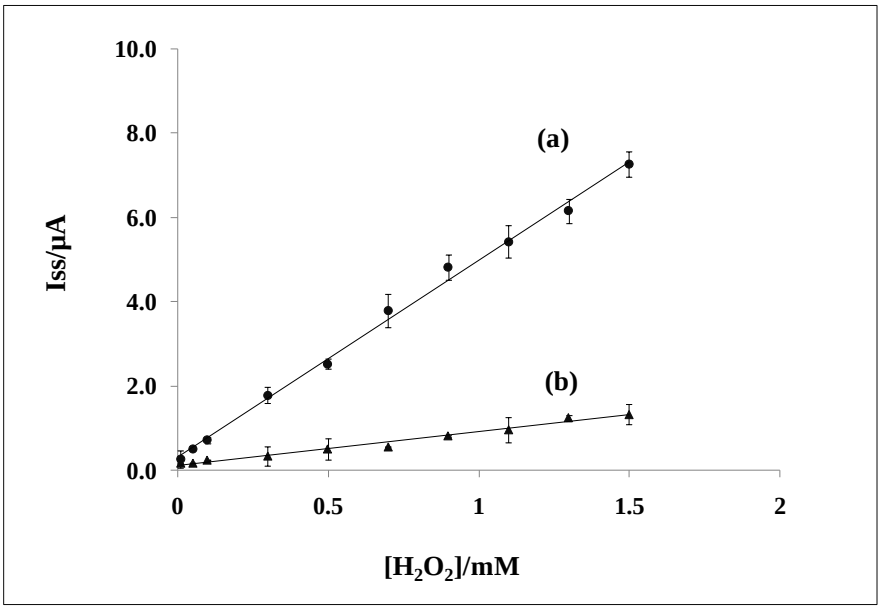


**Figure 6.** Calibration curve for $H_2O_2$ obtained by $(HRP/AuNPs)_2/CS$ (a) and (HRP/Glu/CS) (b) modified pencil lead electrode. The regression equation were expressed as $I_{ss}$/ μA= (0.312±0.005) + (4.675±0.064)[ $H_2O_2$]/mM, with the slope of (4.645±0.064)[ $H_2O_2$] μA/mM (a) and $I_{ss}$/ μA = (0.118±0.008) + (0.795±0.09)[ $H_2O_2$]/mM, with the slope of (0.795±0.09)[$H_2O_2$] μA/mM (b).

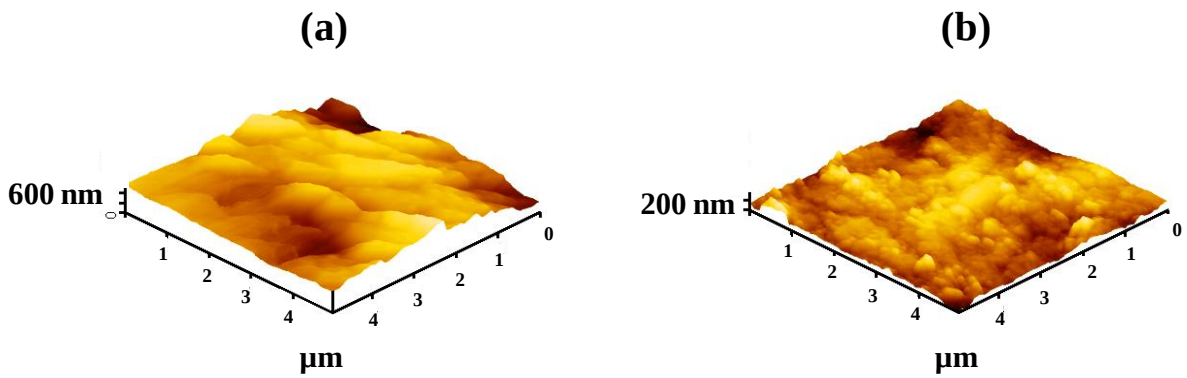


**Figure 7.** AFM images of $(HRP/AuNPs)_2$/CS (a), HRP/Glu/CS (b) modified pencil lead electrode.

Figure.7a and 7b showed surface topography images of $(HRP/AuNPs)_2$/CS pencil lead electrode and HRP/Glu/CS pencil lead electrode, respectively. AFM surface morphology characterization of $(HRP/AuNPs)_2$ CS pencil lead electrode (Figure. 6a) displayed a very rough surface causing from layer-by-layer of HRP and AuNPs. It leads to the high root mean square roughness (RMS) value of 119 nm. Whereas, the low roughness obtained from the HRP/Glu/CS pencil lead electrode caused RMS value of 57 nm. High roughness surface indicated more immobilized HRP and AuNPs on electrode surface, which played not only more enzyme reaction occurring but also AuNPs act as tiny conduction centers helping in electrons transfer efficiency.

### *3.8 Validation of developed biosensor*

#### *3.8.1. Precision and accuracy*

The precision of the method was determined through repeatability and reproducibility, commonly demonstrated by relative standard deviation (RSD). The repeatability of biosensor was examined by the detection of 0.5 mM hydrogen peroxide. The RSD was 4 % for seven determinations carried out on the same day. The electrode fabrication reproducibility was also estimated with seven different electrodes constructed by the same procedure. The RSD was 5 %. As results, the developed biosensor showed high repeatability and reproducibility.

For accuracy of method was evaluated by percentage of recovery, the recoveries of real samples were determined by the standard addition method. The results were satisfactory with the recovery in the range of 80-104%.

#### *3.8.2. Linearity and limit of detection (LOD)*

Calibration graph for hydrogen peroxide was constructed using different of concentrations. The analytical curve was linear in the concentration range of 0.01-1.5 mM with 0.9998 of the correlation coefficient (r). The detection limit of the electrode was found to be 0.002 mM based on a signal-to-noise ratio of 3.

*3.8.3 Sensitivity*

The sensitivity of the method was found to be 149 μA $mM^{-1}$ $cm^{-2}$ obtained from the slope of the analytical calibration curve. The large area of AuNPs on $(HRP/AuNPs)_2/CS$ pencil lead electrode increased the amount of immobilized enzyme on the electrode surface. Thus, higher sensitivity in electrochemical detection of hydrogen peroxide was shown in the table 1.

**Table 1.** Comparison of analytical performance of the biosensor obtained in this work with other HRP based electrodes reported in literature for $H_2O_2$ detection.

| Modified electrode | Sensitivity | LOD | reference |
|---|---|---|---|
| HRP/laponite/chitosan/GCE | 19.7 mA M-1 cm-2 | 5×10-6 M | [24] |
| HRP/RuNPs/chitosan/GCE | 0.798 μA mM-1 cm-2 | Not report | [25] |
| HRP/Bi2O3-MWCNT/GCE | 26.54 μA mM-1 cm-2 | Not report | [26] |
| HRP/Nafion–Sonogel–Carbon electrode | 12.8 nA μM-1 cm-2 | 1.6 μM | [27] |
| HRP/ DNA–Ag/PDDA–Au/DNA–Ag/Au electrode | 0.11 A M-1 cm-2 | 2.0 μM | [28] |
| (HRP/AuNPs)2/CS/pencil lead electrode | 149 μA mM-1cm-2 | 0.002 mM | This work |

GCE = glassy carbon electrode
RuNPs = ruthenium oxide nanoparticles
$Bi_2O_3$ = bismuth oxide
MWCNT = multiwalled carbon nanotube
DNA–Ag = DNA–silver nanohybrids
PDDA-Au = poly(diallyldimethylammonium chloride)- gold nanoparticles

*3.9. Stability of hydrogen peroxide biosensor*

Operational and storage stabilities were studied. The operational stability $(HRP/AuNPs)_2/CS$ pencil lead electrode was obtained by consecutively measurement within the same day. The result showed that the catalytic current response maintained more than 80% of its initial value after 50 measurements. The decreased current may be due to partly lose the bioactivity. The storage stability of the HRP biosensor was also studied in this current work. After the electrochemical measurement, the electrode was immersed in phosphate buffer and was stored at 4 °C. The electrode retained more than 90% of its original response after 1 month. The results indicated good stability of the modified electrode.

*3.10. Application to real samples*

Finally, the proposed biosensor was applied for the determination of hydrogen peroxide concentration in six different samples: three of hair dye samples and others obtained from disinfector with different brands. The dilution of samples was required in order to fit the linear range. Results

were compared with the classical titration method, which was employed as reference method. The results obtained with two methods were shown in Figure. 8. It showed a good correlation between the both methods (slope: 1.10±0.05 with r = 0.9985) and indicated that the developed biosensor showed excellent performance.

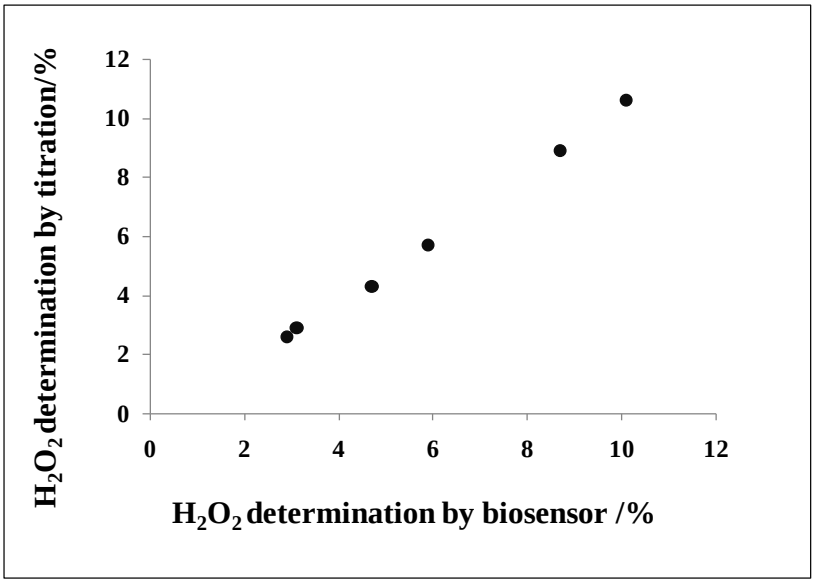


**Figure 8.** Comparison of the percentage of $H_2O_2$ in real samples obtained by the biosensor and titration method.

## 4. CONCLUSION

This work has demonstrated the attractive performance of renewable biosensor based on using pencil lead electrode and enhanced sensitivity with gold nanoparticle via LBL assembly. The developed bionanomultilayer biosensor successfully accomplishes the sensitive detection for hydrogen peroxide with a high current density. This biosensor possesses good detection precision, acceptable accuracy and long-term stability. In addition, the proposed method has good applicability for hydrogen peroxide detection to hair dye and disinfector samples. Therefore, the proposed strategy can be extended for the development of other enzyme-based biosensors.

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