

# Disposable Biosensor Based on a Hemoglobin Colloidal Gold-Modified Screen-Printed Electrode for Determination of Hydrogen Peroxide

Xiaoxing Xu, Songqin Liu, and Huangxian Ju

**Abstract**—A disposable reagentless hydrogen peroxide biosensor based on the direct electrochemistry of hemoglobin immobilized on a colloidal gold-modified screen-printed carbon electrode (Hb-Au-SPCE) was proposed. The electrochemical behavior of immobilized Hb at a SPCE was studied for the first time. The electrode reaction of immobilized Hb showed a surface-controlled process with an electron transfer rate constant of  $(0.40 \pm 0.02) \text{ s}^{-1}$  determined in the scan rate range from 25 to 200  $\text{mV s}^{-1}$ . The Hb-Au-SPCE exhibited an electrocatalytic activity toward the reduction of hydrogen peroxide with a  $K_M^{\text{app}}$  value of 1.8 mM, which was allowed to be used as a disposable sensor for determination of hydrogen peroxide with a linear range from  $1.0 \times 10^{-5} \text{ M}$  to  $3.2 \times 10^{-4} \text{ M}$ , a detection limit of  $5.5 \times 10^{-6} \text{ M}$  at  $3\sigma$ , a high sensitivity, fast response, and good selectivity, accuracy, and reproducibility. The disposable reagentless sensor was stable, low cost, and simple to use for detection of hydrogen peroxide in real samples.

**Index Terms**—Amperometry, biosensors, colloidal gold, electrocatalysis, hemoglobin (Hb), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), screen-printed electrodes.

## I. INTRODUCTION

ACCURATE and sensitive determination of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is extremely important in many fields including industry analysis, environmental monitor, biological reactions, and clinical diagnosis [1]. Electrochemical techniques have been used extensively for this purpose [2]–[8]. The direct electron transfer between an electrode and the redox protein, which catalyzes the reduction of  $\text{H}_2\text{O}_2$ , is a very promising way to prepare reagentless amperometric sensors for  $\text{H}_2\text{O}_2$  [3]–[6]. Two reagentless  $\text{H}_2\text{O}_2$  sensors have been prepared by immobilizing horseradish peroxidase on colloidal gold nanoparticle-

modified gold and carbon paste electrodes [5], [6]. These sensors have good sensitivity and stability and can be used for the fast measurement of  $\text{H}_2\text{O}_2$ . As already well known, hemoglobin (Hb) is of intrinsic peroxidase activity due to its close similarity to peroxidase. Hb immobilized on colloidal gold/cysteamine-modified gold electrode [7] and Kieselgubr film-modified pyrolytic graphite [8] exhibits a fast direct electron transfer between the Hb and electrode and an electrocatalytic behavior to hydrogen peroxide reduction with respect to its pseudoperoxidase activity, which has been used for the detection of  $\text{H}_2\text{O}_2$ .

Screen printing is a standard technology in electronics and has been used to produce disposable electrochemical sensors. Compared with conventional electrodes, screen-printed electrodes have several advantages, such as simplicity, convenience, low cost, and avoidance of contamination between samples. Various modifiers, such as mediators [9], [10], enzymes [11], and metal particles [12] have been conveniently added into the printing ink to improve the selectivity and sensitivity of the screen-printed carbon electrode (SPCE). Colloidal gold is an extensively used metal colloid, which has been used for the study of the direct electrochemistry of proteins such as HRP [5], [6], [13], cytochrome c [14], and Hb [15]. This work prepares a novel colloidal gold nanoparticles-modified SPCE by incorporating colloidal gold nanoparticles into carbon ink. Hb, as a model molecule, is chosen first to be immobilized on the electrode surface to study the direct electron transfer between immobilized proteins and the modified SPCE. Similar to polyacrylamide hydrogel [16], egg-phosphatidylcholine [17], and gluten biopolymer [18] films casted on pyrolytic graphite electrodes (PGE), the incorporated colloidal gold nanoparticles retain the bioactivity of the immobilized Hb and facilitate the direct electron transfer between Hb and carbon sensing sites. The immobilized Hb on the modified SPCE surface exhibits an electrocatalytic behavior with respect to the pseudoperoxidase activity to  $\text{H}_2\text{O}_2$  reduction, which has been used to develop a highly sensitive disposable reagentless biosensor for  $\text{H}_2\text{O}_2$ .

## II. EXPERIMENTAL

### A. Chemicals and Apparatus

Bovine Hb was purchased from Sigma (molecular weight 54 600) and used without further purification. 5  $\text{mg mL}^{-1}$  Hb stock solution was stored at 4 °C.  $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$  was purchased from Aldrich (Deisenhofen, Germany). Polyvinyl

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chloride (PVC) and  $\text{H}_2\text{O}_2$  [30% (w/v) solution] were provided by Shanghai Chemical Reagent Co. Carbon graphite powder (<325 mesh, Johnson Matthey) and cellulose diacetate (from Shanghai Chemical Reagent Co.) were used for preparation of carbon ink. Cellulose diacetate stock solution was prepared by dissolving 1.0-g cellulose diacetate in 100 mL 1:1 hexamethylene-acetone. All other chemicals were of analytical grade and all solutions were prepared with deionized water of 18-M  $\Omega$  purified from a milli-Q purification system. Colloidal gold nanoparticles with a diameter of  $24 \pm 2$  nm were prepared and stored according to [13], [14]. Unless specifically indicated, the buffer used in this work was 0.2 M pH 5.5 NaAc-HAc. 0.1-M phosphate buffer solutions (PBSs) with various pH values used to investigate the dependence of direct electron transfer of immobilized Hb on pH were prepared by mixing stock standard solutions of  $\text{K}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$  and adjusting the pH with 0.1 M  $\text{H}_3\text{PO}_4$  or NaOH.

### B. Construction of Hydrogen Peroxide Sensor

The SPCE was prepared with a screen-printed technology as follows. Silver ink was first printed onto PVC substrate to form conducting track ( $30 \times 1 \text{ mm}^2$ ). This surface was washed thoroughly with NaOH solution and then deionized water. A mixture of 10 mg of pretreated graphite powder with 20- $\mu\text{l}$  colloidal gold solution was prepared. Following evaporation of water in air, 30- $\mu\text{l}$  cellulose diacetate solution was added to the mixture to obtain colloidal gold nanoparticle-modified screen-printed carbon ink (Au-SPC). As a comparison, 10 mg of graphite powder was mixed thoroughly with 30- $\mu\text{l}$  cellulose diacetate solution without the presence of colloidal gold nanoparticles to obtain screen-printed carbon ink (SPC). The resulting inks were then printed onto the silver conducting tracks to form colloidal gold nanoparticle-modified screen-printed carbon electrodes (Au-SPCE) and screen-printed carbon electrodes (SPCE). The electrodes were insulated by overlaying a silicone rubber layer, only exposing the conductive terminal and the working surface with a diameter of about 3 mm. The immobilization of Hb was carried out by dropping 10  $\mu\text{l}$  of Hb solution onto the working surface of the SPCE or Au-SPCE and dried overnight under room temperature to obtain Hb-SPCE or Hb-Au-SPCE. The modified electrodes were rinsed with ethanol and water and stored at 4  $^\circ\text{C}$  when not in use.

### C. Electrochemical Measurements

Electrochemical experiments were carried out with a BAS-100A electrochemical analyzer at room temperature ( $18 \pm 2$ )  $^\circ\text{C}$ . A three-electrode cell was equipped with a saturated calomel electrode (SCE), a platinum wire and a SPCE, Hb-SPCE, Au-SPCE, or Hb-Au-SPCE as reference, counter and working electrode, respectively. The real geometry area of the working electrode was calculated to be 0.11  $\text{cm}^2$  from the slope of plot of the anodic peak current of 2.5-mM  $\text{K}_3[\text{Fe}(\text{CN})_6]$  in 0.1-M KCl versus the square root of scan rate. All experimental solutions were deoxygenated by bubbling highly pure nitrogen for 20 min and maintained under nitrogen atmosphere during the course of the experiment.

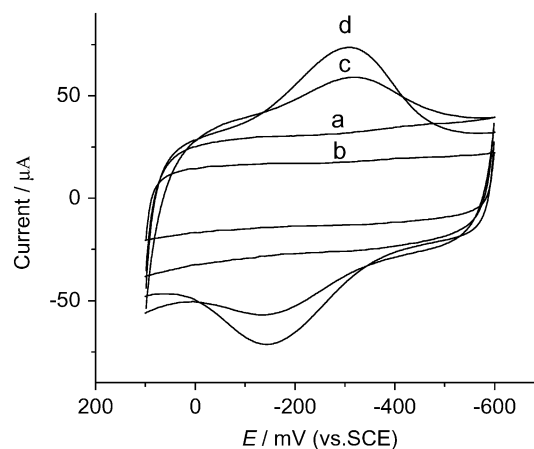


Fig. 1. Cyclic voltammograms of (a) SPCE, (b) Au-SPCE, (c) Hb-SPCE, and (d) Hb-Au-SPCE in 0.2-M pH 5.5 NaAc-HAc buffer at 100  $\text{mV s}^{-1}$ .

## III. RESULTS AND DISCUSSION

### A. Cyclic Voltammetric Properties of Hb Immobilized on the Au-SPCE

Cyclic voltammetric experiments were performed to evaluate the electrochemical behavior of Hb-Au-SPCE. When SPCE and Au-SPCE were cyclically scanned in buffer solution containing of 0.1-mM Hb, no obvious peak was observed, thus the electron transfer between these electrodes and Hb in solution was very slow or did not occur. Hb-Au-SPCE, however, gave a couple of stable and well-defined redox peaks at  $-144$  and  $-310$  mV at 100  $\text{mV s}^{-1}$  in NaAc-HAc buffer (curve d in Fig. 1), while no peak was observed at both SPCE and Au-SPCE (curves a and b in Fig. 1). Au-SPCE displayed lower background current than SPCE. Obviously, the response of Hb-Au-SPCE was attributed to the redox of the electroactive center of the immobilized Hb. In the absence of gold colloid nanoparticles, Hb-SPCE also showed the redox peaks of Hb (curve c in Fig. 1). The cathodic and anodic peak currents, however, were 2.0 and 2.9 times smaller than those of Hb-Au-SPCE, respectively. Thus, the colloidal gold nanoparticles played an important role in improving the electrochemical response and facilitating the electron exchange between the Hb and carbon particles. They gave protein molecules the conducting channels and more freedom in orientation, thus permitting proteins to orient in conformation more favorable for direct electron transfer, with the active sites closer to the conducting electrode [5], thus, facilitating the electron transfer. The formal potential of the heme Fe(III/II) couple of Hb in Hb-Au-SPCE at pH 5.5, estimated as the midpoint of the anodic and cathodic peak potentials, was  $-227$  mV (versus SCE). This value was closer that of Hb in solution [20] than both  $-0.30$  and  $-0.320$  V [16], [19], suggesting that most Hb molecules preserved their native structure after the adsorption process.

With an increasing scan rate the redox peak currents of the immobilized Hb increased (Fig. 2) and its anodic peak potential shifted to a more positive value, while the cathodic peak potential shifted in a negative direction. The anodic and cathodic peak currents ( $i_{pa}$  and  $i_{pc}$ ) were proportional to the scan rate

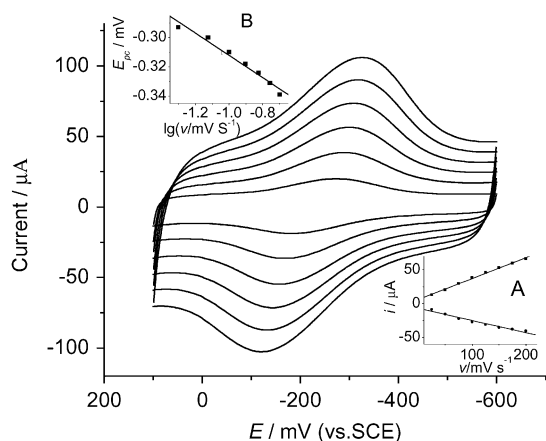


Fig. 2. Cyclic voltammograms of Hb-Au-SPCE in 0.2-M pH 5.5 NaAc-HAc buffer at 25, 50, 75, 100, 125, and 150  $\text{mV s}^{-1}$ . Inset: plots of peak currents versus  $v$  (A) and plot of anodic peak potential versus  $\lg v$  (B).

(inset A in Fig. 2), thus the electrode reaction was typical of surface-controlled quasireversible process. The kinetics of the heterogeneous electron transfer was analyzed using the model of Laviron at peak-to-peak separations less than 200 mV [21]. The plot of cathodic peak potential versus the logarithm of scan rate gave a charge transfer coefficient of 0.47. From the peak-to-peak separations in the scan rate range from 25 to 200  $\text{mV s}^{-1}$ , the average electron transfer rate constant,  $k_s$ , was estimated to be  $(0.40 \pm 0.02) \text{ s}^{-1}$ .

### B. Effects of Solution pH and Temperature on the Response of Immobilized Hb

With an increasing solution pH from 4.0 to 9.0, both reduction and oxidation peak potentials shifted negatively. Moreover, all changes in the peak potentials and currents with solution pH were reversible. The plots of the peak potentials versus pH gave two lines with the same slope of  $-46.4\text{-mV pH}^{-1}$ , which was close to the expected value of  $-57.8\text{-mV pH}^{-1}$  for the one proton and one electron participating electron transfer process at 291.2 K [16].

The immobilization of proteins and enzymes on transducer surfaces can lead to a change of their behavior compared to that observed in homogeneous solution [22], [23]. Thermal stability is a measure of the ability of biosensor to withstand elevations in temperature [24]. Both the anodic and cathodic peak currents of the Hb-Au-SPCE increased with increasing temperature from 15 to 35  $^{\circ}\text{C}$ , displaying an expected Arrhenius-type temperature dependence. After the temperature was more than 35  $^{\circ}\text{C}$ , both the anodic and cathodic peak currents decreased with increasing temperature due to the denaturation of Hb. This result was similar to the native Hb in solution, indicating that the Au-SPC matrix did not alter the optimal temperature value for electron transfer of immobilized Hb.

### C. Amperometric Response of Hb-Au-SPCE to Hydrogen Peroxide

In accordance with previously reported results, the incorporated Hb in the Kieselgubr [7] and the Prussian blue-modified electrodes [25] exhibited an electrocatalytic behavior with respect to the pseudoperoxidase activity to hydrogen peroxide re-

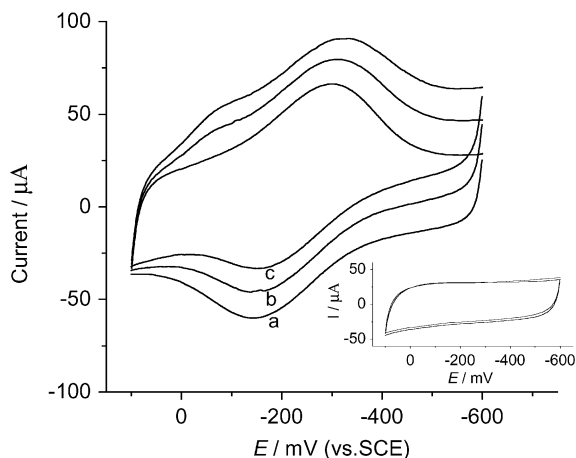


Fig. 3. Cyclic voltammograms of Hb-Au-SPCE and Au-SPCE (inset) in 0.2-M pH 5.5 NaAc-HAc buffer containing (a) 0, (b) 0.16, and (c) 0.40 mM  $\text{H}_2\text{O}_2$  at 100  $\text{mV s}^{-1}$ .

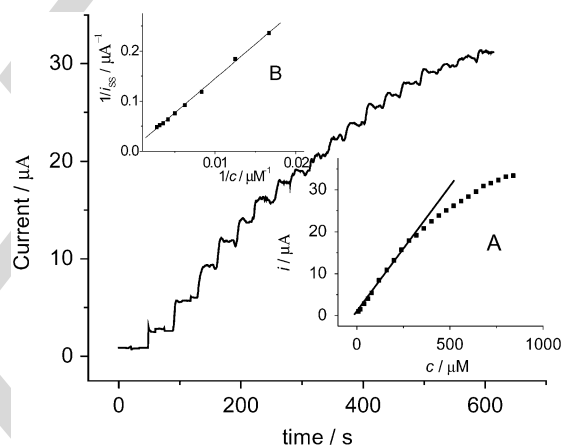


Fig. 4. Typical steady-state response of the sensor with an increasing  $\text{H}_2\text{O}_2$  concentration by 40  $\mu\text{M}$ , each step at  $-0.3 \text{ V}$ . Insets: (A) calibration curve and double-reciprocal plot of (B) steady-state current versus  $\text{H}_2\text{O}_2$  concentration.

duction. Upon the addition of  $\text{H}_2\text{O}_2$ , the shape of cyclic voltammogram for the direct electron transfer of Hb immobilized on Au-SPCE, changed dramatically with an increase of reduction current and a decrease of oxidation current [Fig. 3(a)–(c)], while no obvious change was observed at Au-SPCE without presence of Hb (inset in Fig. 3), displaying an obvious electrocatalytic behavior of the immobilized Hb to the reduction of  $\text{H}_2\text{O}_2$ . Furthermore, the reduction peak current increased with increasing  $\text{H}_2\text{O}_2$  concentration. The curves b and c in Fig. 3 showed a shoulder reduction peak. This was possibly due to the interaction between  $\text{H}_2\text{O}_2$  and the immobilized Hb to produce an intermediate that could be reduced at a more positive potential.

Fig. 4 shows a typical hydrodynamic current-time response of the Hb-Au-SPCE at  $-300 \text{ mV}$  upon successive additions of  $\text{H}_2\text{O}_2$  to 0.2-M pH 5.5 NaAc-HAc. With an increasing  $\text{H}_2\text{O}_2$  concentration, the current response for Hb reduction increased. The calibration range of  $\text{H}_2\text{O}_2$  was from 10  $\mu\text{M}$  to 840 mM with a linear relation from  $1.0 \times 10^{-5}$  to  $3.2 \times 10^{-4} \text{ M}$  ( $R = 0.998$ ) (inset A in Fig. 4). The detection limit was estimated to be  $5.5 \times 10^{-6} \text{ M}$  at  $3 \sigma$ . From the slope, a sensitivity of  $0.63 \text{ A M}^{-1} \text{ cm}^{-2}$  was obtained, which was higher than those of Prussian blue-SPCE [25], Hb-Kieselgubr-PGE [7],

TABLE I  
ANALYTICAL PERFORMANCES OF H<sub>2</sub>O<sub>2</sub> SENSORS BASED  
ON Hb-Au-SPCE AND REPORTED PREVIOUSLY

Electrodes	LOD μM	Linear range μM	Sensitivity A M <sup>-1</sup> cm <sup>-2</sup>	$K_M^{app}$ mM	Reference
Hb-Au-SPCE	5.5	10-320	0.63	1.8	This work
Metallophthalocyanines-SPCE	-	up to 1000	<0.0051	-	[10]
PB-SPCE	0.4	0.4-100	0.137	4.6	[25]
PB-Pt-SPE	5	5-50	1	-	[27]
PB-Au-SPE	-	-	2	-	[27]
PhZb-HRP-GA-BSA-CPE	0.001	0.002-40	0.127	-	[28]
Hb-kieselgubr-PGE	2.1	5-300	~0.27	0.98	[7]
Hb-egg-phosphatidyloline-PGE	3.9	10-100	0.145	-	[17]
Hb-gluten-PGE	-	2.5-95	0.65	-	[18]

and Hb-egg-phosphatidyloline-PGE [17] and much higher than those of metallophthalocyanines-SPCEs [10] (shown in Table I). The high sensitivity was due to the presence of colloidal gold nanoparticles, which improved the electrochemical response and facilitated the electron exchange. The detection limit corresponded to those of Prussian blue-modified gold and platinum screen-printed electrodes [27] and Hb-gluten-PGE [18], while this sensor had a wider linear range. Although the detection limit of this sensor was 600-fold worse than that of a sensor based on phenothiazine mediation of horseradish peroxidase proposed by Kauffmann *et al.* [28], its sensitivity was higher and the preparation of this sensor is simpler. Furthermore, this sensor was mediatorless and disposable.

When the concentration of H<sub>2</sub>O<sub>2</sub> was higher than 320 μM, the calibration curve displayed a platform, indicating a characteristic of the Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constant ( $K_M^{app}$ ) of Hb-Au-SPCE was obtained to be 1.8 mM from inset B in Fig. 4 and the electrochemical version of the Lineweaver–Burk equation [26], which was lower than those of 2.28 mM at cyt.c/Au-CPE [14], 3.69 mM at HRP-Au-CPE [6], and 2.3 mM at HRP-Au colloid self-assembled monolayer electrode [5]. Thus, Hb molecules adsorbed on Au-SPCE was of a higher affinity to H<sub>2</sub>O<sub>2</sub>.

#### D. Interferences

The effects of coexistence ions and compounds on the detection of H<sub>2</sub>O<sub>2</sub> were examined by adding them, respectively, into a pH 5.5 NaAc-HAc buffer containing  $4.0 \times 10^{-5}$  M H<sub>2</sub>O<sub>2</sub> to detect the change of electrocatalytic current. The detection results indicated that NH<sub>4</sub><sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Cu<sup>2+</sup>, CO<sub>3</sub><sup>2-</sup>, and Cl<sup>-</sup> at  $8.0 \times 10^{-4}$  M (20 times the concentration of H<sub>2</sub>O<sub>2</sub>), while Mn<sup>2+</sup> at  $6.0 \times 10^{-4}$  M, I<sup>-</sup>, S<sup>2-</sup>, and urate at  $2.0 \times 10^{-4}$  M, ascorbate and dopamine at  $1.6 \times 10^{-5}$  M led to a change less than 5% electrocatalytic current. At lower concentrations, they did not interfere the determination of H<sub>2</sub>O<sub>2</sub>. Thus, this sensor had a good selectivity.

#### E. Sample Analysis

The determination of hydrogen peroxide in tap water and serum samples were carried out by a standard addition method. 1.0-ml 1.0-M pH 5.5 NaAc-HAc buffer was added into 4.0-ml

TABLE II  
RECOVERY OF H<sub>2</sub>O<sub>2</sub> IN TAP WATER AND SERUM SAMPLES

Sample	H <sub>2</sub> O <sub>2</sub> added (μM)	H <sub>2</sub> O <sub>2</sub> found (μM)	Recovery (%)
Tap water	30	29.2	97.3
	60	61.7	102.8
	120	118.5	98.8
Serum	45	43.3	96.2
	90	93.0	103.3
	135	136.2	100.9

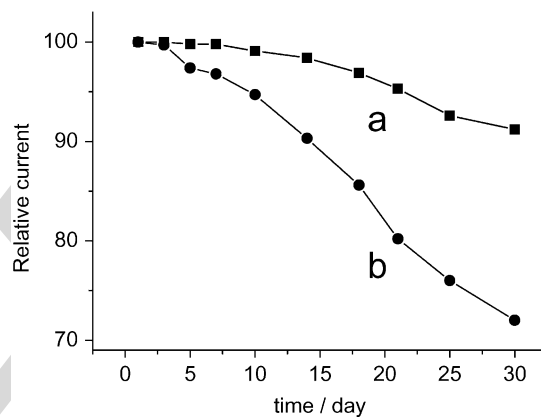


Fig. 5. Storage stability of the H<sub>2</sub>O<sub>2</sub> sensor at (a) 4 °C and (b) room temperature.

samples to control the pH value and the concentration of supporting electrolyte. After hydrogen peroxide with a known concentration was added in these prepared samples, the steady state currents were measured. Table II gives the results of recovery for the determination of hydrogen peroxide. It could be seen that the results were satisfactory. Compared with other methods, the advantages of this sensor were its simplicity, rapidity, and convenience.

#### F. Reproducibility and Stability of H<sub>2</sub>O<sub>2</sub> Sensor

The sensor had a very fast response rate. It attained 95% of the steady-state currents in less than 10 s during the calibration range of H<sub>2</sub>O<sub>2</sub> concentration. Such a fast response was attributed to the presence of the colloidal gold nanoparticles. The relative standard deviations were 3.8% and 3.5% for six successive assays at H<sub>2</sub>O<sub>2</sub> concentrations of 20 and 80 μM, respectively. The fabrication reproducibility was estimated at three electrodes made independently with the response to 20-μM H<sub>2</sub>O<sub>2</sub> in 0.2-M pH 5.5 NaAc-HAc. The relative standard deviation was calculated to be 5.3%, indicating good fabrication reproducibility.

The storage stability of the H<sub>2</sub>O<sub>2</sub> sensor was tested at 4 °C and room temperature. The results were shown in Fig. 5. After a storage period of one month, the sensor retained over 90% and 70% of its initial current response at 4 °C and room temperature, respectively. After a storage period of three months at 4 °C the sensor retained 80% of its initial current response. Thus, the

stability of the sensor was very good. At the same time, the electrode could be continuously carried out more than 100 measurements without noticeable current decrease.

#### IV. CONCLUSION

This work proposes a novel disposable biosensor for H<sub>2</sub>O<sub>2</sub> based on the immobilization and direct electron transfer of Hb on the Au-SPCE. The colloidal gold nanoparticles decrease the background current, improve the conductivity, retain the bioactivity of immobilized protein, and accelerate the electron transfer rate. The sensor shows a highly sensitive response to H<sub>2</sub>O<sub>2</sub>, satisfactory precision, good selectivity and stability, and can be used for determination of H<sub>2</sub>O<sub>2</sub> in a real sample.

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Xiaoxing Xu, photograph and biography not available at the time of publication.

Songqin Liu, photograph and biography not available at the time of publication.



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