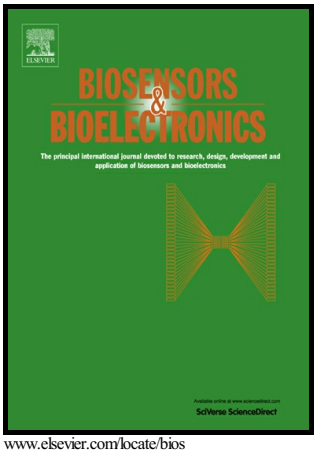

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Preparation of Hemoglobin (Hb) imprinted polymer by Hb catalyzed eATRP and its application in biosensor

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Abstract: Molecularly imprinted polymer (MIP) was prepared on the surface of Au electrode by electrochemically mediated atom transfer radical polymerization (eATRP) with hemoglobin (Hb) both as catalyst and template molecule. Firstly, the condition for eATRP such as the potential, time and Hb concentration were selected and determined to be −0.51 V, 120 min and 20 mg/mL, respectively. Further, the electrode modified with MIP (MIP/Au) was carefully examined by cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and scanning electron microscope (SEM). Finally, the MIP/Au electrode was used as a biosensor and successfully applied for the determination of Hb by differential pulse voltammetry (DPV) measurement. The results of experiments showed that the proposed biosensor displayed a broader linear range and a lower detection limit for

Hb determination when it was compared to those Hb sensors based on MIP. The linear range was from 1.0×10^{-10} to 1.0×10^{1} mg/L with a detection limit of 7.8×10^{-11} mg/L (S/N=3.3). In a word, the work of this paper established a useful way for the preparation and application of biosensor based on protein imprinted polymers.

Keywords: Hemoglobin; electrochemically mediated atom transfer radical polymerization; molecularly imprinted polymer; biosensor

1. Introduction

Hemoglobin (Hb), which is the main protein component of red blood cells, is a tetrameric metalloprotein that composed of a globular protein part and four iron-containing heme parts (Huang et al., 2015). The determination of the Hb concentration is greatly utilized for the diagnosis of many diseases such as anemia, thalassemia, heart disease, leukemia, sickle cell disease and excessive loss of blood (Pourreza and Golmohammadi 2015). Clinical immunoassay is a common method for detecting Hb, however, the processes are usually complicated and time-consuming. In addition, the antibody activity is not easy to maintain and the natural antibody is very expensive (Wang et al., 2013). Thus, it is of great scientific and practical significance to explore a method of Hb detection without antibody.

Molecularly imprinted polymers (MIPs) are artificial receptors which can recognize and specifically bind to a target (template) molecule (Altintas et al., 2015). In contrast to biological counterparts (enzymes, antibodies and hormone receptors), MIPs displayed significant advantages including chemical and thermal stability, cost-effective fabrication, reusability and high recognition capacity, and thus have been used in a variety of applications, such as chiral separation, solid phase extraction, drug controlled release and electrochemical sensor (Panagiotopoulou et al., 2015;Tan

et al., 2013; Zhang et al., 2015).Although MIP for detecting Hb had been reported (Sun et al., 2014; Zhang et al., 2015), protein imprinting was difficult for several reasons, not least their size and intrinsically poor stability under imprinting conditions (Li et al., 2013b; Poma et al., 2010).

Generally, MIPs are synthesized by the copolymerization of functional monomers and cross-linker in the presence of template， after the removal of the template, the special tailor-made binding sites that are complementary to template molecules are obtained (Wei et al., 2015). Most MIPs are currently prepared using radical polymerization (Chen et al., 2011). Controlled/living radical polymerization can greatly improve the polymeric materials and are therefore a newly emerging and highly regarded approach in the molecular imprinting area (Adali-Kaya et al., 2015). Among these, atom transfer radical polymerization (ATRP) using halogenated Cu(I) as a catalyst shows great promise as an efficient method of preparing MIP (Adali-Kaya et al., 2015; Zou et al., 2014). ATRP possesses the mild reaction conditions and can be initiated at room temperature without heating and ultraviolet radiations. These advantages make it very suitable for imprinting proteins (Gai et al., 2010). Unfortunately, the catalyst of ATRP is expensive, in environmental and often residue in the end polymers which is poisonous and hard to get rid of (Li et al., 2013c). As a result, improved ATRP was provided as a means to overcome the above drawbacks. In 2011, Matyjaszewski et al developed electrochemically mediated ATRP (eATRP) by forming the Cu(I) catalyst in situ, which conducted ATRP by a very active copper catalyst in several ppm concentration (Magenau et al., 2011). Zhou and co-workers extended the eATRP method to the fabrication of polymer brushes from an initiator-modified substrate (Li et al., 2013a; Li et al., 2012). eATRP can be carried out with low levels of catalysts, and be stopped or restarted by switching the applied potential, which has been

successfully used for the synthesis of materials with well-defined polymeric architectures (Li et al., 2013a; Li et al., 2012; Park et al., 2015). However，up to now, eATRP has not been used in the preparation of MIP.

The catalyst is an important part of the ATRP system. Except the common Cu(I), many transition metal complexes have been used as catalysts for ATRP including Ru(II), Fe(II), Ni(II), Pd(II), Rh(III) and Re(II) (Arslan et al., 2014; Ding et al., 2015). Among them, iron complex attracts particular attention in recent years owing to their low toxicity, low cost, and good biocompatibility (Sun et al., 2015a). With the development of study, a variety of iron complex have been used as ATRP catalyst (Fujimura et al., 2015; Liu et al., 2014; Nakanishi et al., 2014). Even iron-containing protein had been reported to be used as ATRP catalyst. For example, Silva et al found the catalytic activity of Hb for ATRP when methemoglobin (Hb-Fe(III)) was reduced to Hb-Fe(II) by ascorbic acid, and they successfully conducted the ATRP of N-isopropylacrylamide, (ethylene glycol) methyl ether acrylate, and (ethylene glycol) methyl ether methacrylate with Hb as catalyst (Silva et al., 2013). Sigg et al reported the ATRP of N-isopropylacrylamide catalyzed by horseradish peroxidase (Sigg et al., 2011). The iron-containing protein could provide a green, nontoxic and even edible alternative to conventional ATRP catalysts (Silva et al., 2013). These characteristics are very important for the use of ATRP in molecular imprinting area, especially for protein imprinting.

Considering the advantages of eATRP and protein catalyst, in this paper, MIP was synthesized on the surface of Au electrode by eATRP with Hb both as catalyst and template. This novel method for preparation of Hb MIP was very simple, nontoxic and saving reagent. The electrode modified with MIP (MIP/Au) was characterized by cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and

scanning electron microscope (SEM). The further experimental results showed that the MIP/Au electrode could be used as biosensor and applied for the determination of Hb by differential pulse voltammetry (DPV) measurement.

2. Materials and methods

2.1 Chemicals

Gold disk electrode (Φ= 2 mm) was purchased from Chenhua Instruments Co., Shanghai, China. Acrylamide (AM, functional monomer)， N,N'-methylene bis-acrylamide (MBA, cross-linker), copper(II) bromide ($CuBr_2$, 99.9%) and 2,2′-Bipyridine (Bpy) were obtained from Kemiou Chemical Co. (Tianjin, China). 2-Bromoisobutyryl bromide (BiBB, 98%) was from Alfa Aesar Chemical Co. 4-Hydroxythiophenol (HTP) was obtained from Acros CO. (Japan). Lysozyme (Lyz, MW 14.4 kDa), bovine serum albumin (BSA, MW 66 kDa), human serum albumin (HSA, MW 69 kDa), human immunoglobulin (HIgG, MW 155 kDa) and Hb (MW 65 kDa) were supplied by Solarbio Inc. (Beijing, China). Phosphate buffered solution (PBS, pH 7.0) was prepared using 0.02 mol/L Na_2HPO_4 and 0.02 mol/L KH_2PO_4.

All chemicals were of analytical reagent grade. All the water used in experiments was the hyperpure water (resistivity > 18 MΩ·cm).

2.2 Apparatus

CV, EIS, eATRP and DPV were carried out with a CHI660D electrochemical workstation (Chenhua, Shanghai, China). The electrochemical cell consisted of a three-electrode system with Au or modified Au as the working electrode, saturated calomel electrode (SCE) and platinum wire as the reference and the counter electrode, respectively. CV characterization was performed in PBS containing 5 mmol/L $Fe(CN)_6^{3-/4-}$ and 0.1 mol/L KCl at a scanning rate of 100 mV/s. EIS were conducted in the same solution to the CV characterization within the frequency range of 5 mHz

to 10 kHz. The surface of the modified electrode was characterized by SEM (SU8010, Hitachi, Japan).

2.3 Preparation of MIP

Initiator end with thiol was prepared by HTP reacting with BiBB according to our previously published literature (sun et al., 2015b). A cleaned gold disk electrode was immersed in thiol initiator /HTP mixed solution at room temperature for 24 h. The ratio of initiator and HTP was 20:1 in ethanol solution (Li et al., 2012). In this step mixed self-assembled monolayer (SAM) was formed on surface of electrode (Br/Au).

In order to determine the applied potential, CV measurements were performed with Br/Au as working electrode, platinum wire as counter electrode, and SCE as reference electrode. Supporting electrolyte solution of CV was PBS (pH 7.0) contained 20.0 mg/mL Hb, 0.02 mol/L MBA and 0.4 mol/L AM. The applied potential (E_{app}) was determined by the reduction peak potential of CV curve. eATRP reaction was performed at Br/Au electrode using chronoamperometry at E_{app} for 2 h at room temperature in the same solution with CV. After the eATRP reaction, the electrode modified with polymer (polymer/Au) was rinsed with PBS.

The Hb template was removed from the polymer/Au by soaking in a 10% (v/v) acetic acid solution containing 10% (w/v) sodium dodecylsulfate (SDS) for 2 h (Fatoni et al., 2014). A non-MIP modified electrode (NIP/Au) was also prepared with the similar procedure using $CuBr_2$/Bpy as the catalyst in the absence of the Hb template.

2.4 Determination of Hb

The performance of the MIP was studied by DPV method. Prior to each test, the MIP/Au electrode was incubated in Hb solution for 5 min at room temperature and washed twice with PBS. The DPV was operated from −0.2 to 0.6 V in PBS containing 5 mmol/L $Fe(CN)_6^{3-/4-}$, against the SCE reference electrode. An

increment potential of 4 mV, amplitude of 50 mV, a pulse width of 0.2 s and a pulse period of 0.5 s were set as the corresponding DPV parameters.

MIP that had adsorbed Hb was regenerated by soaking in a 10% (v/v) acetic acid solution containing 10% (w/v) SDS for 2 h at room temperature.

3. Results and discussion

3.1 Preparation of MIP

Preparation of MIP was schemed in Fig. 1. In order to improve the polymer growth, mixed SAM of initiator and HTP were self-assembled on the surface of Au electrode (Br/Au). Then the electrode was inserted to protein solution (containing Hb, AM and MBA) as working electrode. when a potential was applied to reduce Hb-Fe(III) to Hb-Fe(II) (catalyst for eATRP)，polymerization would be triggered. After the removal of Hb, MIP was successfully prepared on the surface of electrode (MIP/Au).

Fig.1

To prove the possibility that the polymerization was catalyzed by Hb, we repeated the experiment several times with thoroughly cleaned glassware and new reagent. When AM or MBA was omitted from the reaction mixture, polymer was obtained on the electrode surface. While Hb was omitted, no polymer was obtained. Natural Hb did not result in any polymerization either. These observations allowed us to conclude that, the protein Hb-Fe(II) was responsible for the polymerization.

The applied potential for eATRP was determined by CV. Fig. 2A showed the CV curves of Hb at Br/Au electrode. As expected, the CV curve of Br/Au electrode in the absence of Hb (background) was featureless. The electrode in the presence of Hb showed irreversible reduction peak at approximately －0.39 V (E_{pc}, solid line, vs. SCE). The peak current decreased with the increasing of scan cycles and gradually became stable. The results were consisted with the characteristics of electroactive

center of heme Fe(III)/Fe(II) couples in the Hb molecules (Chen et al., 2007; Wang et al., 2010; Xu et al., 2007). According to the literature (Chmielarz et al., 2015), the applied potential (E_{app} = E_{pc} − 120 mV) was determined to be −0.51 V (vs. SCE). When eATRP was conducted, a potential of −0.51 V was applied to generate the Hb-Fe(II) complex through the electroreduction of Hb-Fe(III). Zhu et al studied the secondary structural changes in Hb during an electroreduction process by in situ circular dichroism spectroelectrochemistry (Zhu et al. 2002). They found that the electroreduction process had almost no effect on the secondary structures of Hb. So, eATRP could be used for the preparation of Hb MIP.

The polymerization time was also been studied by CV. At this time, $Fe(CN)_6^{3-/4-}$ was as the probe to indicate the electron transfer ability of polymer/Au. Fig. 2B showed the variation between anodic peak current of CV curves and polymerization time. From the figure, anodic peak current decreased sharply with the time increasing from 30 to 120 min, and it was close to 0 μA when the time was 120 min. With the polymerization time increasing further, the peak current had almost no change, thus, polymerization time was selected to be 120 min.

The amount of the Hb was an important factor that affected the polymerization and the performance of MIP. In this paper, the Hb concentration was selected by measuring the sensitivity of the MIP/Au electrode (slope of calibration curve), as shown in Fig. 2C. Increasing the concentration of Hb from 5.0 to 20.0 mg/mL, the sensitivity of the MIP/Au improved because the number of imprinted cavities increased. As a consequence the ability of MIP/Au to capture Hb was extended (Fatoni et al., 2014). However, too many Hb (20.0 to 50.0 mg/mL) caused the sensitivity to decrease. This may be because when there were too many proteins, they could become close to one another or exist as an aggregate. This would result in not

ideal imprinted cavities, and the effectiveness of the MIP to capture the template significantly reduced (Fatoni et al., 2014).

Fig.2

3.2 Characterization of MIP

The stepwise modified electrodes were investigated by CV, shown in Fig. 3A. As expected, a characteristic quasi-reversible redox cycle with anodic and cathodic peak currents ratio of approximately one was observed at the bare Au electrode (Fig. 3A, curve 1). When the electrode was modified with mixed SAM, an obvious decrease of the anodic and cathodic peak current was obtained (Fig. 3A, curve 2), which implied that the monolayer perturbed the electrochemical probe toward the electrode surface. After polymer formed on the electrode surface, a more obviously decrease of the anodic and cathodic peak current was obtained (Fig. 3A, curve 3). The reasons may be that the polymer film acted as the inert electron and mass transfer blocking layer, and it hindered the diffusion of electrochemical probe toward the electrode surface. When the Hb was removed out of the polymer, an increase of the anodic and cathodic peak current was observed (Fig. 3A, curve 4). This may be attributed to the formation of imprinted cavities, which leaved channels for the penetration of probe through the MIP to reach electrode.

Fig.3

As an effective method for probing the features of a surface modified electrode, EIS was employed to characterize the modification process of the electrode. Typical nyquist plot of EIS included a semicircle portion at higher frequencies，which corresponded to the electron-transfer resistance (R_{et}) of $Fe(CN)_6^{3-/4-}$ at the electrode surface (Han et al., 2010). Fig.3B showed EIS results of differently modified electrodes. It could be seen that the bare Au electrode revealed a very small semicircle

domain (Fig. 3B, curve 1), implying a low R_{et} on the bare Au electrode (Chen et al., 2007). After being modified with mixed SAM, the EIS of the resulting electrode showed a high semicircle diameter (Fig. 3B, curve 2), implying an increase of R_{et} of the redox probe on the resulting electrode. After the formation of polymer, a more obvious increase of the semicircle diameter was obtained (Fig. 3B, curve 3). This may be attributed to the polymer film hindered the access of the redox probe to the electrode, causing the increase in R_{et}. When the Hb was removed from the polymer, the R_{et} reduced (Fig. 3B, curve 4), which was attributed to the removal of template protein resulting in the increase of electron transfer rate (Chen et al., 2012).

The surface morphology of the electrodes was observed with SEM and shown in Fig. 3, too. It could be seen from the SEM images that there was great difference in the morphologies between MIP/Au and NIP/Au electrode. The NIP/Au exhibited a relatively smooth and condensed topology (Fig. 3C), while the MIP/Au electrode showed a rough and porous topology (Fig. 3D), which may be attributed to the imprinted structures present on the MIP/Au electrode (Tiwari et al., 2012).

From the results of CV, EIS, and SEM data, MIP was successfully synthesized on the Au electrode via eATRP catalyzed by Hb.

3.3 Electrochemical responses of the MIP toward Hb

In this paper, MIP/Au electrode was used as electrochemical biosensor and applied for the determination of Hb by DPV measurement. The current change of $Fe(CN)_6^{3-/4-}$ on the MIP/Au and NIP/Au electrode was shown in Fig. 4. The peak current of the MIP/Au electrode after the removal of Hb (curve 2 in Fig. 4A) was higher greatly than that of the electrode before the removal (curve 1 in Fig. 4A). This may be attributed to the formation of imprinted sites, which allowed the diffusion of probe through the polymeric network and led to the increase of the peak current (Luo et al.,

2014). When the MIP/Au electrode was incubated in 10^{-7} mg/L Hb for 5 min, the peak current decreased evidently (curve 3 of Fig. 4A), indicating the rebound Hb blocked the penetrating of the probe. In addition, the peak current of curve 2 was still larger than that of curve 3, indicating that only a portion of binding sites was occupied. In contrast, the peak current of the NIP/Au after removal (curve 2 in Fig. 4B) was a little higher than that of the electrode before removal (curve 1 in Fig. 4B), and the immersion in the Hb solution induced quite a small change in current. The results of Fig.4 demonstrated that MIP/Au electrode exhibited selective response toward Hb.

Fig. 4

Fig. 5 demonstrated the DPV curves of the MIP/Au after rebinding with a series of concentrations of Hb solutions. As shown in Fig. 5A, the DPV peak current of the MIP/Au decreased with the increasing Hb concentrations. This may be ascribed to the rebinding of Hb to the imprinting cavities, which blocked the penetrating of the probe and thus decreased the peak current (Kan et al., 2012). The concentration of Hb was higher, the imprinted sites were more occupied, and peak current more decreased accordingly. The relation of peak current decrement (signal response, ΔI) with Hb concentration logarithm (the calibration curve) was shown in Fig. 5B. From the figure, the MIP/Au electrode gave a linear range of the concentration of Hb from 1.0×10^{-10} to 1.0×10^{1} mg/L. The linear regression equation was ΔI (μA)=0.191log C (mg/L)+2.22 with a correlation coefficient of 0.980. From the resulting calibration curve, a detection limit (LOD, S/N=3.3) of 7.8×10^{-11} mg/L Hb was estimated. When the performance of our biosensor was compared with sensor based on MIP for Hb detection including fluorescence, phosphorescence and other electrochemical method (as shown in Table S1). It could be seen that the prepared biosensor had lower detection limit and larger linear range.

Fig. 5

3.4 Selectivity of the MIP sensor

To evaluate the selectivity of the Hb (MW 65 kDa) imprinted polymer, we chose Lyz (MW 14.4 kDa), BSA (MW 66 kDa), HSA (MW 69 kDa) and HIgG (MW 155 kDa) as interferents for comparison experiments. The signal response (ΔI) of $Fe(CN)_6^{3-/4-}$ on the MIP/Au and the NIP/Au after incubating in the same concentration (10 mg/L) of each protein were determined by DPV method and shown in Fig. 6. From the figure, the ΔI value of the MIP/Au electrode toward Hb was 2.27μA, which was 5.40, 6.88, 32.43 and 34.92 times of that toward Lyz, BSA, HSA and HIgG, respectively. The results indicated that the amounts of template protein rebound on MIP/Au electrode were much more than those of comparative proteins and MIP/Au electrode had good selectivity toward the target protein. The relatively higher current response for Lyz than other proteins may be attributed to its much smaller size, which could diffuse easily into the imprinting cavities and cause some non-selective adsorption (Luo et al., 2014). On the contrary, the larger proteins were easier to be excluded from the imprinted cavities due to steric hindrance, which led to the faint signal response.

The selectivity was further evaluated by calculating β (imprinting factor), which was calculated as following: $\beta=\Delta I_{(MIP/Au)}/\Delta I_{(NIP/Au)}$ (Kan et al. 2012). As shown in Fig. 6, the values of β were 2.47, 2.54, 1.40, 1.30 and 18.92 for Lyz, BSA, HSA, HIgG and Hb, respectively. The largest β value for Hb suggested that MIP/Au possessed a much higher capacity toward Hb than NIP/Au electrode. In contrast, the much smaller β values for the other proteins showed that there was only a slight difference between MIP/Au and NIP/Au. All the above results indicated good selectivity of MIP/Au toward the target protein.

Fig. 6

3.5 Repeatability and reproducibility

To determine the repeatability of the Hb imprinted sensor, 10 mg/L Hb (in PBS) was analyzed three times by the same sensor repeatedly. The relative standard deviation (RSD) was 3.43%, indicating that the MIP/Au sensor had a good repeatability. We prepared 3 MIP/Au sensors under the same conditions and used them to detect 10 mg/L Hb (in PBS) to determine the reproducibility. The RSD was 3.32 %, implying that the reproducibility of the MIP/Au sensors was good.

3.6 Application for real samples

Real samples analysis was carried out using standard addition method in bovine blood sample according to the literatures (Luo et al. 2014; Wang et al. 2014). The samples of whole blood were firstly analyzed by a blood test meter for detecting the concentration of Hb, and then the samples were diluted and assayed using the MIP/Au electrode according to the proposed method. The results were listed and shown in Table S2. As can be seen, the prepared MIP/Au sensor could detect Hb with good recovery, which indicated that the proposed MIP/Au sensor can be used for the analysis of real samples.

4. Conclusions

Hb imprinted polymer was successfully prepared on the surface of Au electrode by eATRP with Hb both as catalyst and template molecule. The potential, time and Hb concentration for eATRP were firstly selected and determined to be －0.51 V (vs.SCE), 120 min and 20 mg/mL, respectively. Then, the electrode modified with MIP (MIP/Au) was carefully examined by CV, EIS, and SEM. Finally, the MIP/Au electrode was used as a biosensor and successfully applied for the determination of Hb by DPV measurement. The results of experiments showed that the proposed biosensor displayed a broader linear range and a lower detection limit for Hb

determination when it was compared to those Hb sensors based on MIP. The linear range was from 1.0×10^{-10} to 1.0×10^{1} mg/L with a detection limit of 7.8×10^{-11} mg/L (S/N=3.3). In a word, the work of this paper established a useful way for the preparation and application of biosensor based on protein imprinted polymers.

5. Acknowledgement

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Caption

Fig.1 Synthesis scheme of MIP on the Au electrode surface via eATRP with Hb both as catalyst and template. The functional monomer and cross-linker were acrylamide (AM) and N,N'-methylene bis-acrylamide (MBA), respectively.

Fig. 2 (A) The CV curves of Br/Au electrode scanned between －0.8 and 0.2 V in PBS (pH 7.0) contained 20 mg/L Hb or not. (B) The effect of polymerization time. (C) The effect of Hb concentration on the sensitivity of the biosensor.

Fig. 3 The CV (A) and EIS (B) characterization of the stepwise modified electrodes (1-bare Au; 2-Br/Au; 3-polymer/Au; 4-MIP/Au) in PBS (pH 7.0) containing 5 mmol/L $Fe(CN)_6^{3-/4-}$ and 0.1 mol/L KCl. The scanning rate of CV was 100 mV/s and frequency range of EIS was 5 mHz to 10 kHz. The SEM images of the surface of NIP/Au (C) and MIP/Au (D) electrode.

Fig. 4 The DPV curves of MIP/Au (A) and NIP/Au (B) in PBS (pH 7.0) containing 5

mmol/L $Fe(CN)_6^{3-/4-}$: (1) before removal of Hb from polymer; (2) after removal of Hb from polymer; (3) after incubating in 10^{-7} mg/L Hb solution.

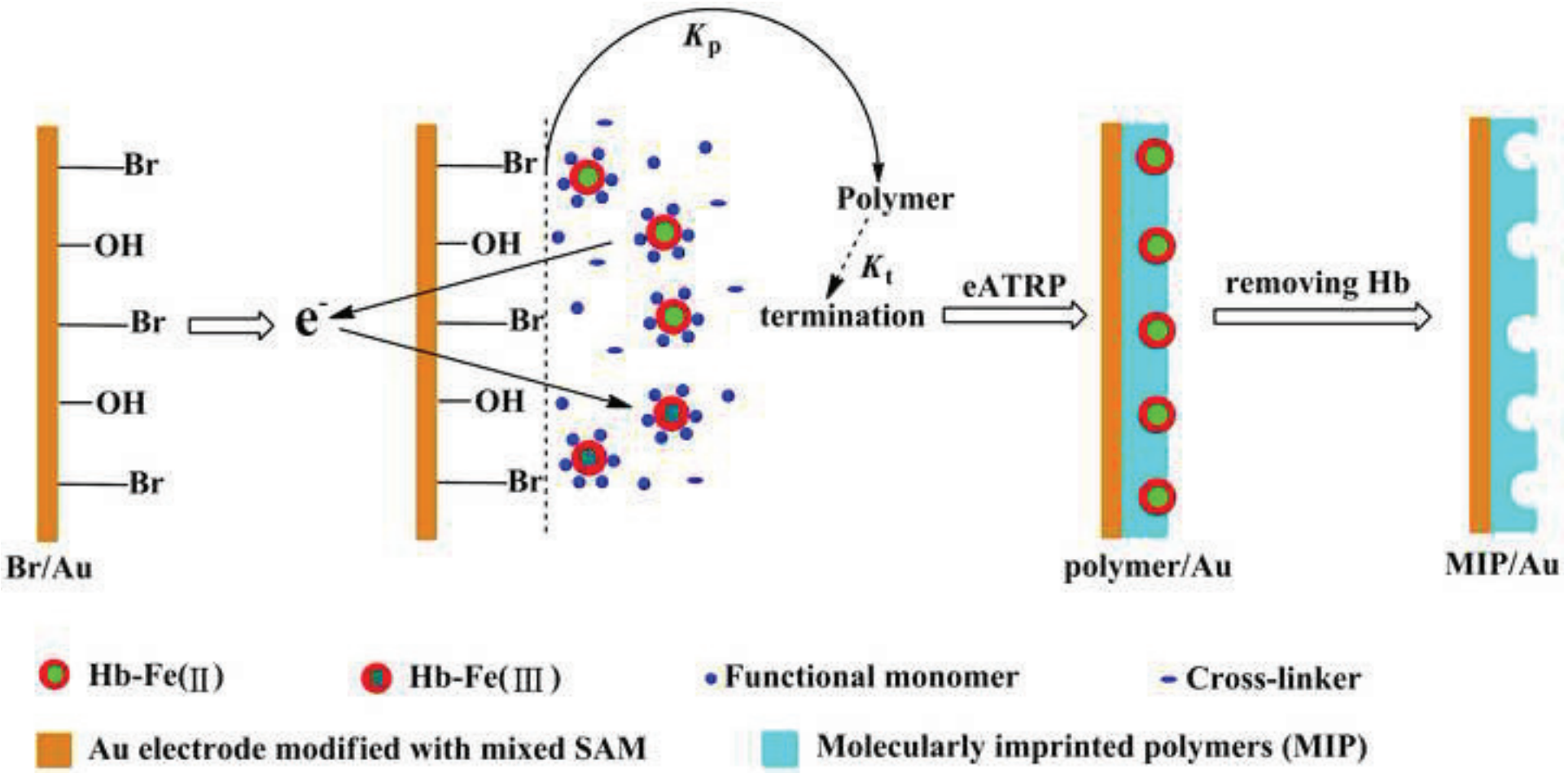
Fig. 5 (A) The DPV curves of MIP/Au electrode in PBS (pH 7.0) containing 5 mmol/L $Fe(CN)_6^{3-/4-}$ after rebinding with Hb (concentration of Hb from curve 1 to curve 13 were 0, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10 mg/L). (B) The calibration plot of MIP/Au electrode.

Fig. 6 The current response of the MIP/Au and NIP/Au electrode after rebinding with Lyz, BSA, HSA, HIgG and Hb

Highlights

- Hemoglobin (Hb) imprinted polymer was prepared by Hb catalyzed eATRP .
- The method was very simple, nontoxic and saving reagent.
- The electrode modified with Hb imprinted polymer could be used as a biosensor.
- The biosensor can detect Hb in a range from 1.0×10^{-10} to 1.0×10^{1} mg/L.

Fig 1



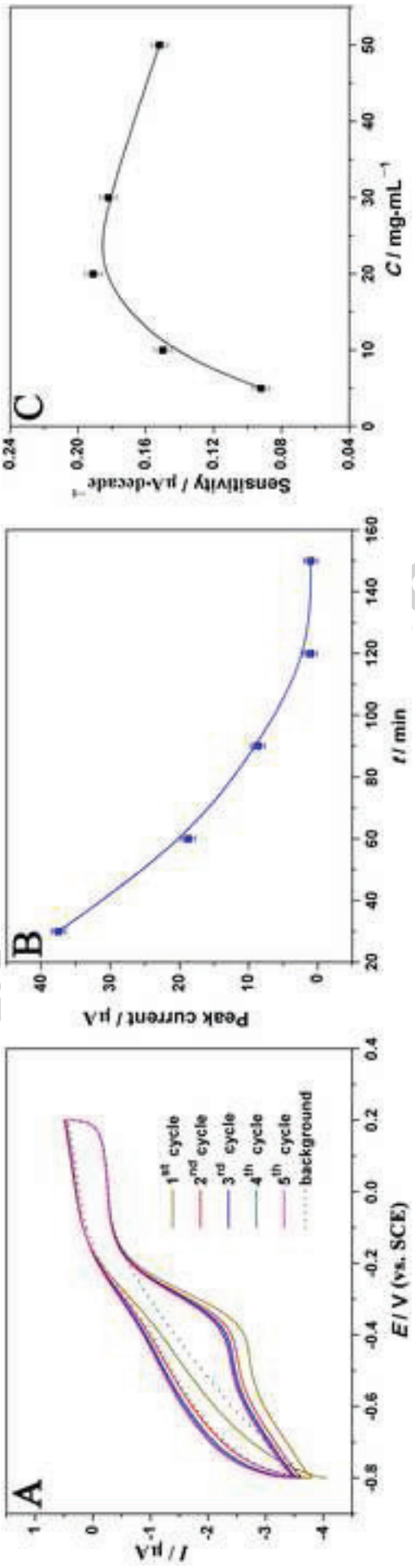


Fig 2

Fig 3

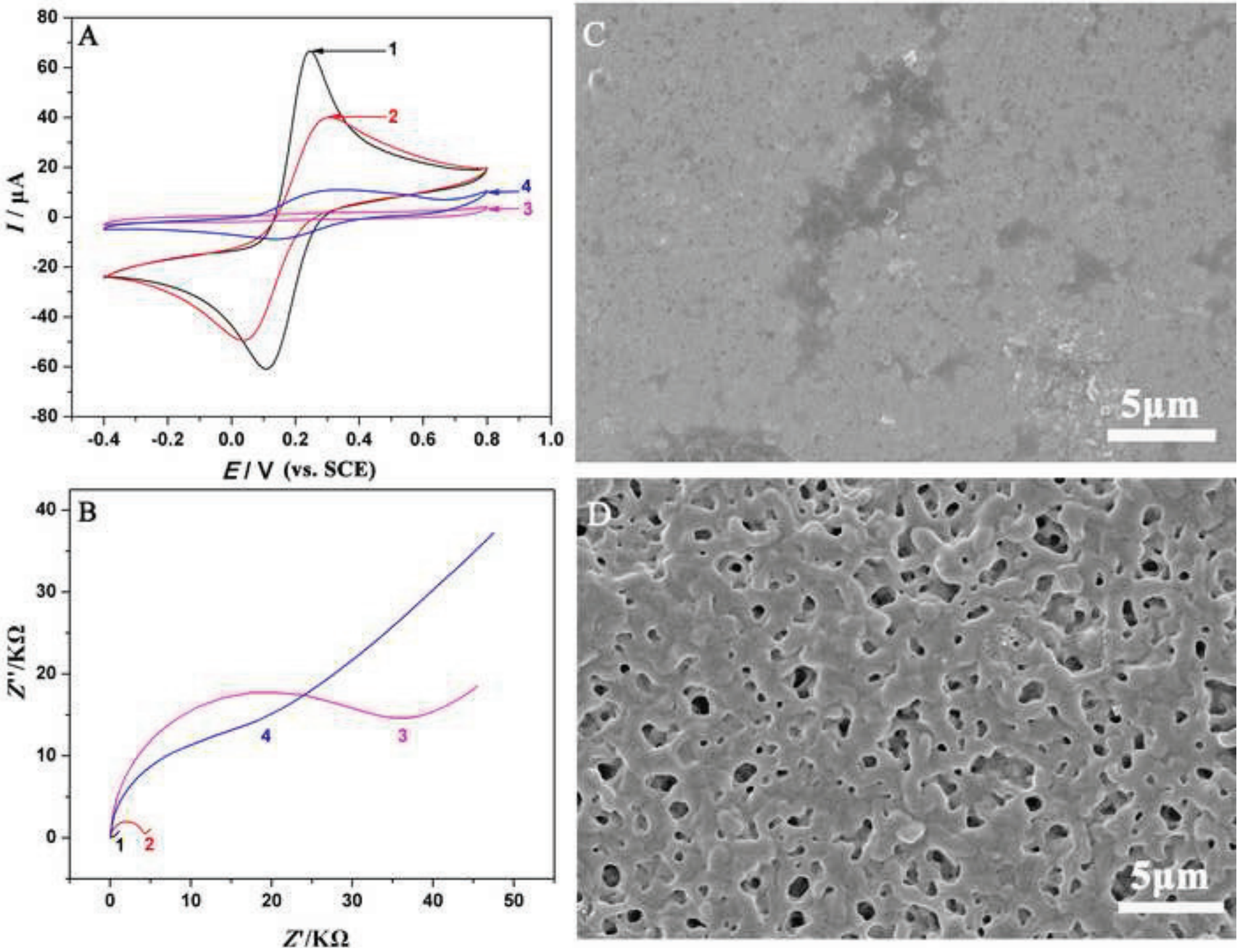
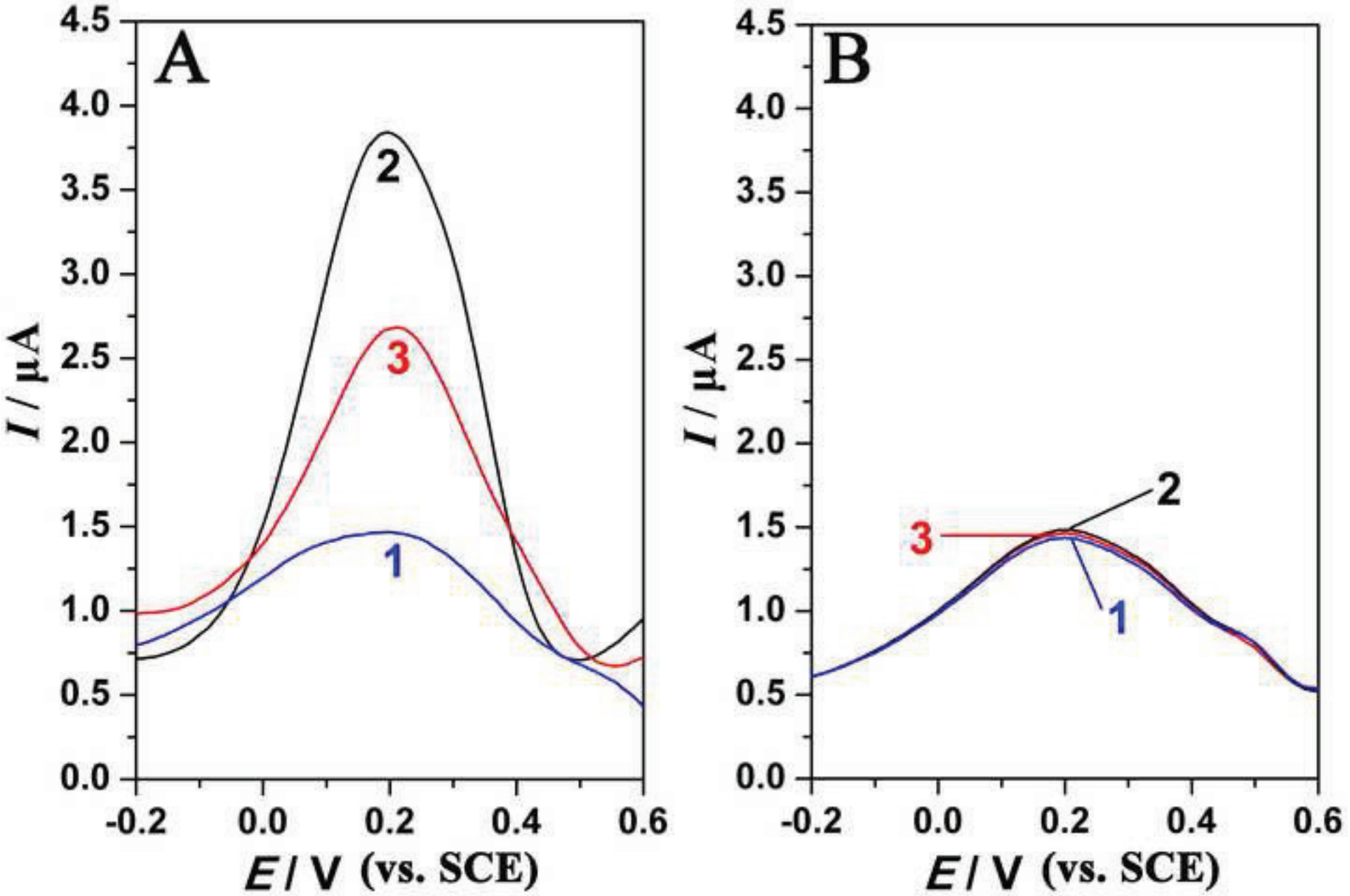


Fig 4



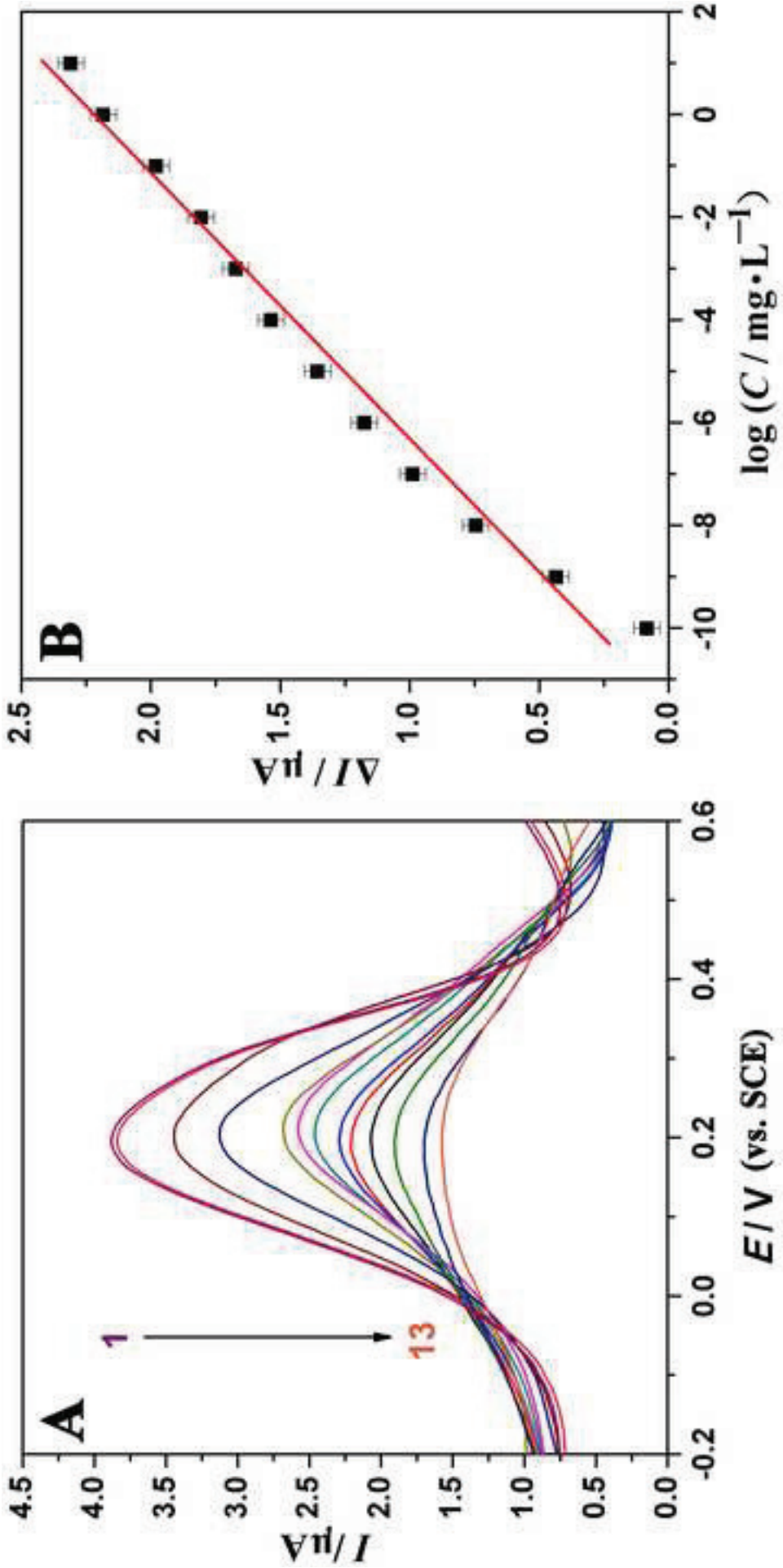


Fig 5

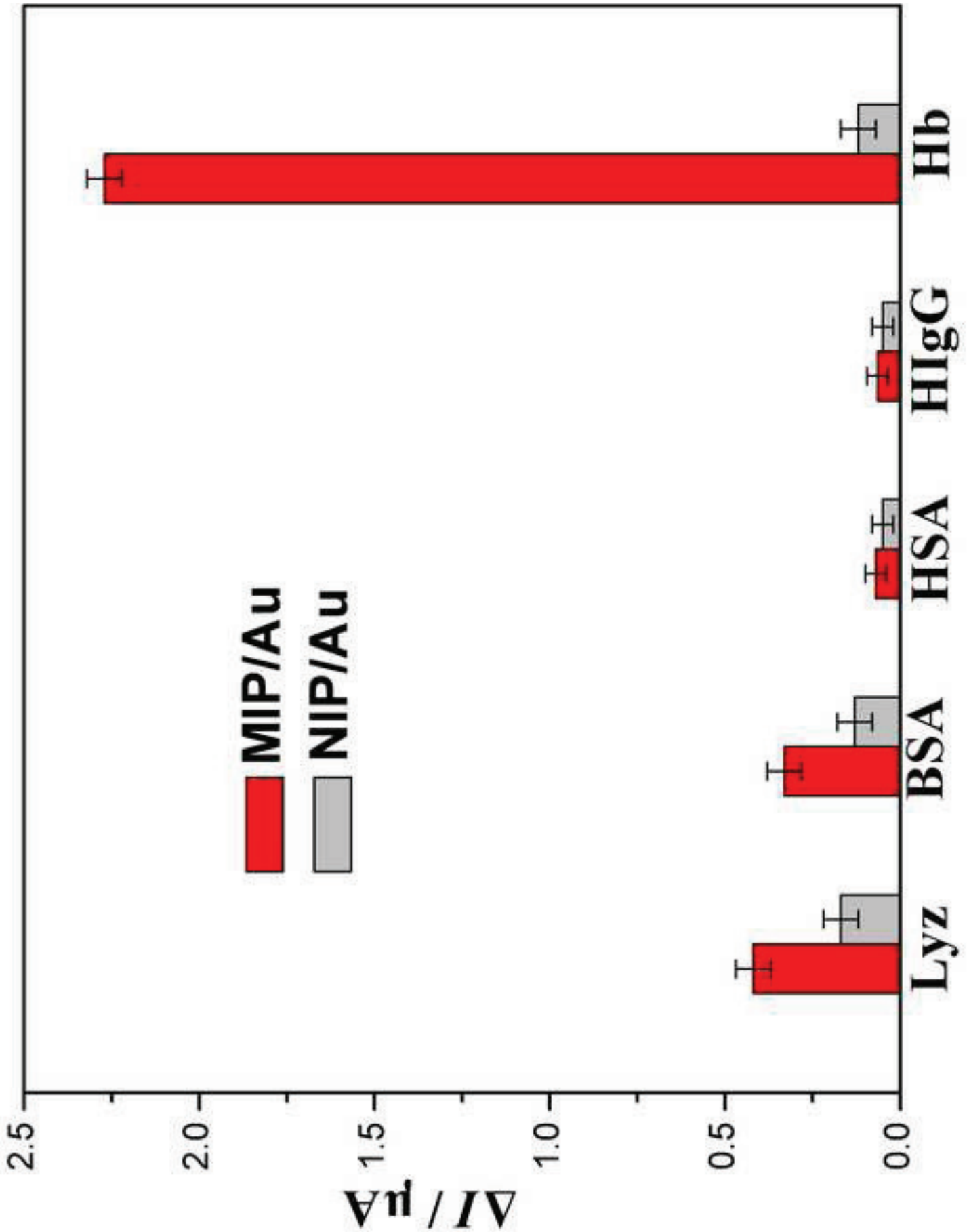


Fig 6