

*Full Paper*

## **Cysteine Function in Superoxide Dismutase Direct Electrochemistry and Superoxide Anion Sensing**

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**Abstract-** Cysteine self assembled monolayer-modified gold (Cys/Au) electrode is used to immobilize superoxide dismutase (SOD) and establish a direct electron transfer between enzyme and electrode surface. However, due to the redox activity of copper ion on Cys monolayer, there would be an ambiguity in electrochemical studies of immobilized SOD on Cys/Au electrode. We designed a series of experiments to clarify the role of Cys in this process. Comparison between voltammograms of different electrodes revealed that the current intensity was increasing by the order of  $\text{Cu}^{+2}/\text{Cys}/\text{Au} > \text{SOD}/\text{Cys}/\text{Au} > \text{Cys}/\text{Au}$ , while their electrochemical working windows were overlapping. Furthermore, for these electrodes the electron transfer rate constant were 0.77, 0.73, and 0.29  $\text{s}^{-1}$  and the surface concentration of electroactive species were  $1.05 \times 10^{-10}$ ,  $1.51 \times 10^{-11}$ ,  $1.50 \times 10^{-11}$   $\text{mol cm}^{-2}$ , respectively. When phosphate buffer solution (PBS) was prepared by ultrapure phosphate salts (copper ion free) no redox response was observed while, by deliberately addition of  $\text{Cu}^{2+}$  the Cys/Au electrode showed a redox response. EDTA as chelating agent could pick up  $\text{Cu}^{2+}$  from PBS and consequently no electrochemical response was observed for Cys/Au electrode. Comparing these results indicated that the source of the inherent electrochemical activity of Cys/Au electrode is  $\text{Cu}^{+2}$ . Finally, the Cys/Au electrode was also examined as a sensing system for determination of  $\text{O}_2^{\cdot-}$ .

**Keywords-** Superoxide Dismutase, Immobilization, Cysteine, Self Assembled Monolayer, Direct Electrochemistry

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## 1. INTRODUCTION

Superoxide dismutases (SODs) are ubiquitous metalloenzymes in aerobic organisms. They are induced during oxidative stress as part of the cellular antioxidant defense system. They protect organisms against the toxic effect of superoxide anion ( $O_2^{\cdot-}$ ) by catalyzing its rapid dismutation to  $O_2$  and  $H_2O_2$  via a cyclic oxidation-reduction electron transfer mechanism. This specific and high speed reaction (rate constant up to  $10^9 M^{-1} S^{-1}$ ) is often used for determination of  $O_2^{\cdot-}$  [1-4]. There are some reports in direct electrochemistry of SOD for development of third-generation  $O_2^{\cdot-}$  biosensor [3,5-13].

Since the metal active-site of SOD ( $Cu^{2+}$  in oxidized form) is structurally located deep in a channel with narrow width ( $<4 \text{ \AA}$ ), the direct electron transfer between the enzyme and the electrodes is blocked [3,10]. However, Iyer and Schmidt [14] observed direct and irreversible oxidation of copper-zinc SOD (Cu-Zn SOD) on a bare gold electrode in phosphate buffer solution (PBS, pH 4.0). They suggested that SOD adsorption on the electrode surface brings about a conformational change at the active sites thus facilitating direct electron transfer. Several approaches have been demonstrated to resolve the problem. Among them, the main strategy is adsorption of SOD on a modified electrode prepared by different ways: self-assembled monolayer (SAM) [4,6- 10,12,15-22], carbon nanotubes [10], silica sol-gel film [3] or silica sol-gel incorporated by gold nano-particles (GNPs) [23], nanostructured gold surfaces [11,24], carbon fiber electrode modified by GNPs-cysteine [5], nanostructured zinc oxide (ZnO) surfaces [1,13], conductive titanium oxide ( $TiO_2$ ) nanoneedles [25], silicon carbide nanoparticles [26] and nanostructured nickel oxide ( $NiO_2$ ) [27].

Preparation of SAM using either a thiol or a disulfide on metal electrode surfaces is a known method for immobilizing the enzymes. The well ordered SAM is able to close the protein molecules to the electrode surface with a high degree of control over the recognition interface. This molecular architecture can promote the electron transfer between immobilized enzyme and electrode surface [28]. L-Cysteine (Cys) is one of the interesting thiolated amino acid candidates for SAM formation on the gold electrode. Some researchers have used this architecture for SOD immobilizing and then, electrochemical studies and/or fabrication of third-generation biosensor for superoxide anion [4,6,7,8,15-23,29]. At first, Wu et al. [18] and Meng et al. [19] have immobilized SOD on the Cys SAM-modified gold electrode to study the direct electrochemistry of SOD. At the same time an identical architecture has used for SOD immobilization by Ohsaka et al. They studied the immobilized SOD behavior in the presence of superoxide [16,29]. They have also proposed a mechanism for electron transferring between  $Cu^{2+}$  (in the active center of SOD) and electrode surface. Also Tian et al. have studied electrocatalytic activity of immobilized SOD on Cys SAM and investigated the effect of some physiological interference by using voltammetry and amperometry [15]. In another work they have studied direct electrochemistry of SOD on Cys SAM in details and proposed a mechanism for electron transferring between SOD and electrode surface [4]. They

have also investigated the efficiency of thiols and disulfides SAMs with different structure on the gold electrode to immobilize SOD [8]. Mao et al. accomplished SOD immobilization using Cys SAM on GNPs deposited on carbon microfiber and used this system for amperometric determination of  $O_2^{\cdot-}$  [5]. Di et al. used silica sol-gel (SG) thin film containing Cys and GNPs, for SOD immobilization [23].

Despite the application of Cys SAM for studying the direct electrochemistry of SOD, no one has explained the precise role of Cys in such a molecular assembling. However, Qu et al. [30] have recently reported that 3-mercaptopropionic acid (3-MPA) SAM on gold electrode represents a redox behavior with a formal potential ( $E^{\circ}$ ) around 0.175 V (vs. Ag/AgCl). Thus, this SAM modified electrode exhibits a Faradaic process, rather than being inert. They have experimentally shown this Faradaic current arises from trace  $Cu^{2+}$  in buffer. Moreover, Qingwen et al. [31] noted that when Cys is used as SAM, the electrochemical working windows should be carefully controlled. Also, Cys SAM has been used for the electrochemical detection of  $Cu^{2+}$  with sub-ppb detection limit [32-36]. It was reported that the SAM of glutathione ( $\gamma$ -L-glutamyl-L-cysteinyl-glycine) on gold electrode can be used for detection of  $Cu^{2+}$  while the SAM was involved in electron transferring between copper ions and electrode surface [37].

Therefore, due to the redox activity of copper on Cys SAM there would be an ambiguity in electrochemical studies of immobilized SOD on Cys SAM in the presence of copper ions as buffer impurities. In the present work we designed a series of experiments to clarify the electrochemical behavior of Cys SAM on the gold electrode in PBS and recognize the contribution of either SOD or  $Cu^{2+}$  in a SOD/Cys/Au electrode. Finally the response of Cys SAM to  $O_2^{\cdot-}$  was properly examined.

## 2. EXPERIMENTAL

### 2.1. Materials

Bovine erythrocyte Cu-Zn superoxide dismutase (SOD, EC.1.15.1.1) and molecular sieve (4 Å) were purchased from Fluka. L-Cysteine, copper chloride ( $CuCl_2$ ), sodium hydroxide (NaOH) and dimethylsulfoxide (DMSO) were purchased from Merck and used as delivered. Also, potassium dihydrogen phosphate ( $KH_2PO_4$ ) and dipotassium hydrogen phosphate ( $K_2HPO_4$ ) in two grades: pure and ultrapure (free of  $Cu^{+2}$ ) were purchased from Merck and used to prepare phosphate buffer solution (0.02 M, pH 7.4) as supporting electrolyte (denoted as PBS and up-PBS, respectively). Ethylene-diamine-tetraacetic acid (EDTA) disodium salt (dehydrate purity > 99%) was purchased from Sigma. The solutions were prepared in deionized double distilled water (18 M $\Omega$ -cm, Barnstead, Dubuque, USA). All experiments were carried out at room temperature.

## 2.2. Apparatus

All electrochemical experiments were carried out using a computerized Potentiostat/Galvanostat (model 263-A, EG&G, USA) equipped with Power Suite software package. Electrochemical studies were performed using a single-compartment conventional three-electrode cell at  $25 \pm 1$  °C. A working modified polycrystalline gold-disk electrode ( $\Phi=3$  mm), a platinum rod auxiliary electrode (both from Metrohm), and a silver/silver chloride (Ag/AgCl, 3 M KCl) reference electrode (Azar electrode Co. Uromia, Iran) were used. Also a rotating disk electrode (RDE, model 616, PerkinElmer, USA) was used in amperometric experiments. All potentials were measured and reported versus the Ag/AgCl reference electrode.

## 2.3. Preparation of different modified working electrodes

To clean the gold disk electrode, it was mechanically polished with alumina slurry (particle sizes 10 and 0.3  $\mu\text{m}$ ) to a mirror finish. Then, it was sonicated in water for 10 min and treated electrochemically in 0.5 M sulfuric acid, cycling between -0.2 and +1.5 V at a scan rate of 0.1  $\text{Vs}^{-1}$  until the appearance of a clean gold electrode was obtained. Finally the electrode was washed with deionized double distilled water [8,15,20,38].

Clean gold disk electrode was immersed in freshly aqueous Cys solution (10 mM) for 30 min [15,20]. Finally, the Cys-modified gold (Cys/Au) electrode washed gently with ultrapure water to remove physically adsorbed Cys molecules. When the electrode was not in use, it was stored in ultrapure water at room temperature.

For SOD immobilization, a freshly prepared Cys/Au electrode was dipped into 5 mL of PBS. Thereafter, 50  $\mu\text{L}$  of the SOD solution (30 mM in PBS) was added gradually to the PBS, and after 5 min the cyclic voltammograms (CVs) of modified electrode were recorded [4]. Cyclic voltammetry was continued until the peak currents reached to a plateau.

To prepare  $\text{Cu}^{+2}/\text{Cys}/\text{Au}$  electrode, a freshly prepared Cys/Au electrode was dipped into the PBS. Thereafter, the aqueous  $\text{CuCl}_2$  (0.5 mM) was added gradually and the CVs were recorded. The addition of  $\text{CuCl}_2$  was continued until the peak currents reached to a plateau.

## 2.4. Superoxide preparation

For  $\text{O}_2^{\bullet-}$  generation [39-42] at first, DMSO was oxygenated by  $\text{O}_2$  bubbling for 5 min. Then deionized water and NaOH were added to the DMSO to reach final concentration of 10% (v/v) and 5 mM, respectively. After 30 min, the produced  $\text{O}_2^{\bullet-}$  concentration became stable for long period of time. Based on the  $\text{O}_2^{\bullet-}$  molar extinction coefficient in DMSO (2006  $\text{Lmol}^{-1}\text{cm}^{-1}$  at 271 nm) [41], the  $\text{O}_2^{\bullet-}$  concentration was estimated to be about 0.05 mM. The thus prepared solution of  $\text{O}_2^{\bullet-}$  in DMSO was stored with molecular sieve 4 Å in order to remove the excess water molecules.

### 3. RESULTS AND DISCUSSION

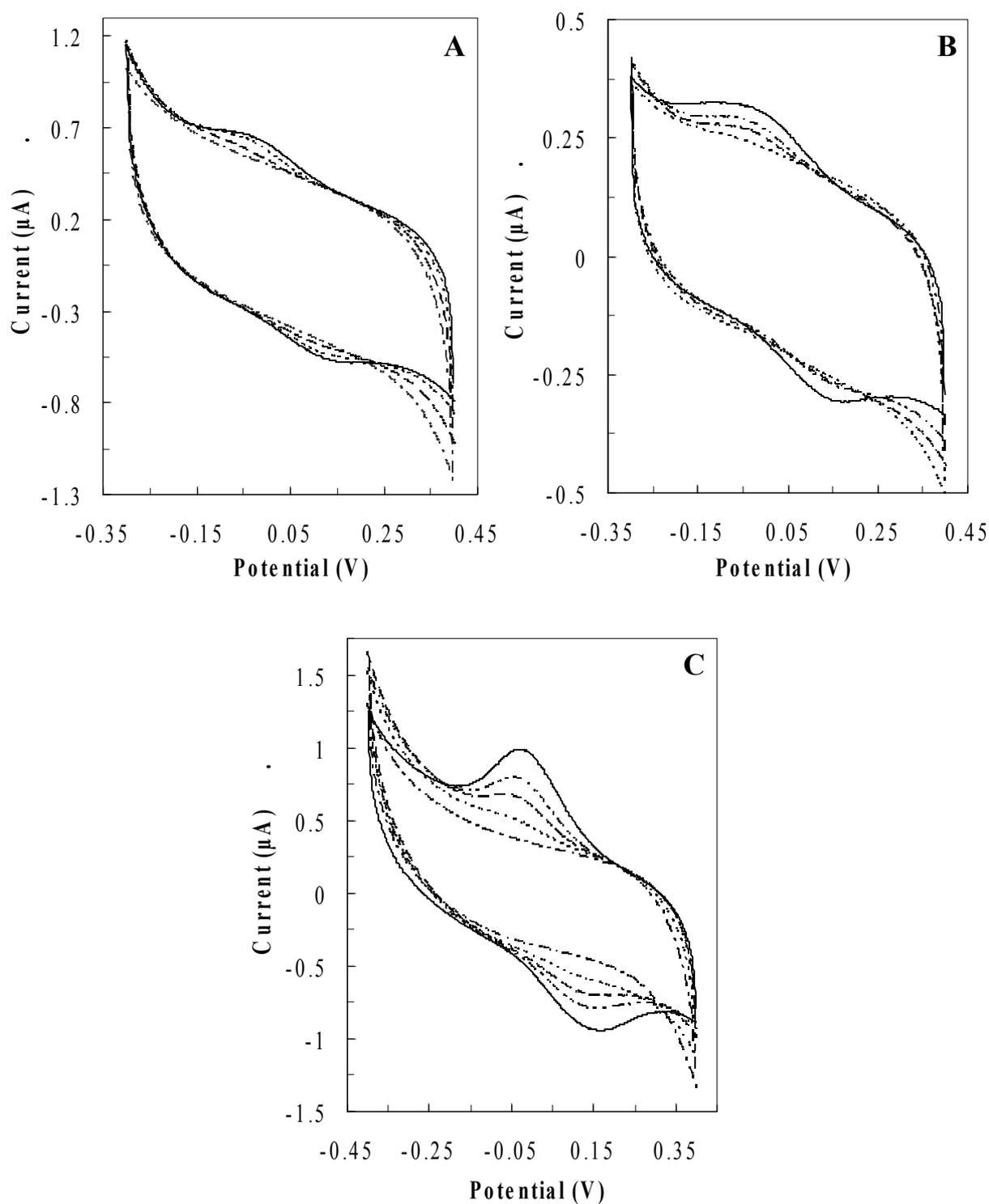
#### 3.1. Electrochemical behavior of modified Au electrode

In the first step SOD was added to the PBS while the CV of Cys/Au electrode was recorded. As shown in Fig. 1A, the peak current of Cys/Au electrode is increasing by addition of SOD concentration. Finally, the absorption of SOD on Cys monolayer is saturated at 17.41  $\mu\text{M}$  of SOD and the peak current reaches to a plateau. Many researchers have been stated that Cys SAM can promote the electron transferring process between chemisorbed SOD on Cys SAM and Au electrode [4,15]. Unlike this statement, Qingwen et al. [31] hinted for the inherent redox activity of Cys monolayer in PBS. Also Winterbourn et al., have reported that thiols oxidation occurs in aqueous solutions in the presence of Cu, Zn-SOD [38]. They observed that SOD has great effect on Cys oxidation and attributed this to the catalyzing role of  $\text{Cu}^{2+}$  as active site in Cu, Zn-SOD structure. This supposition was confirmed when Cu, Zn-SOD was replaced with Mn-SOD and in that condition no redox peak was observed.

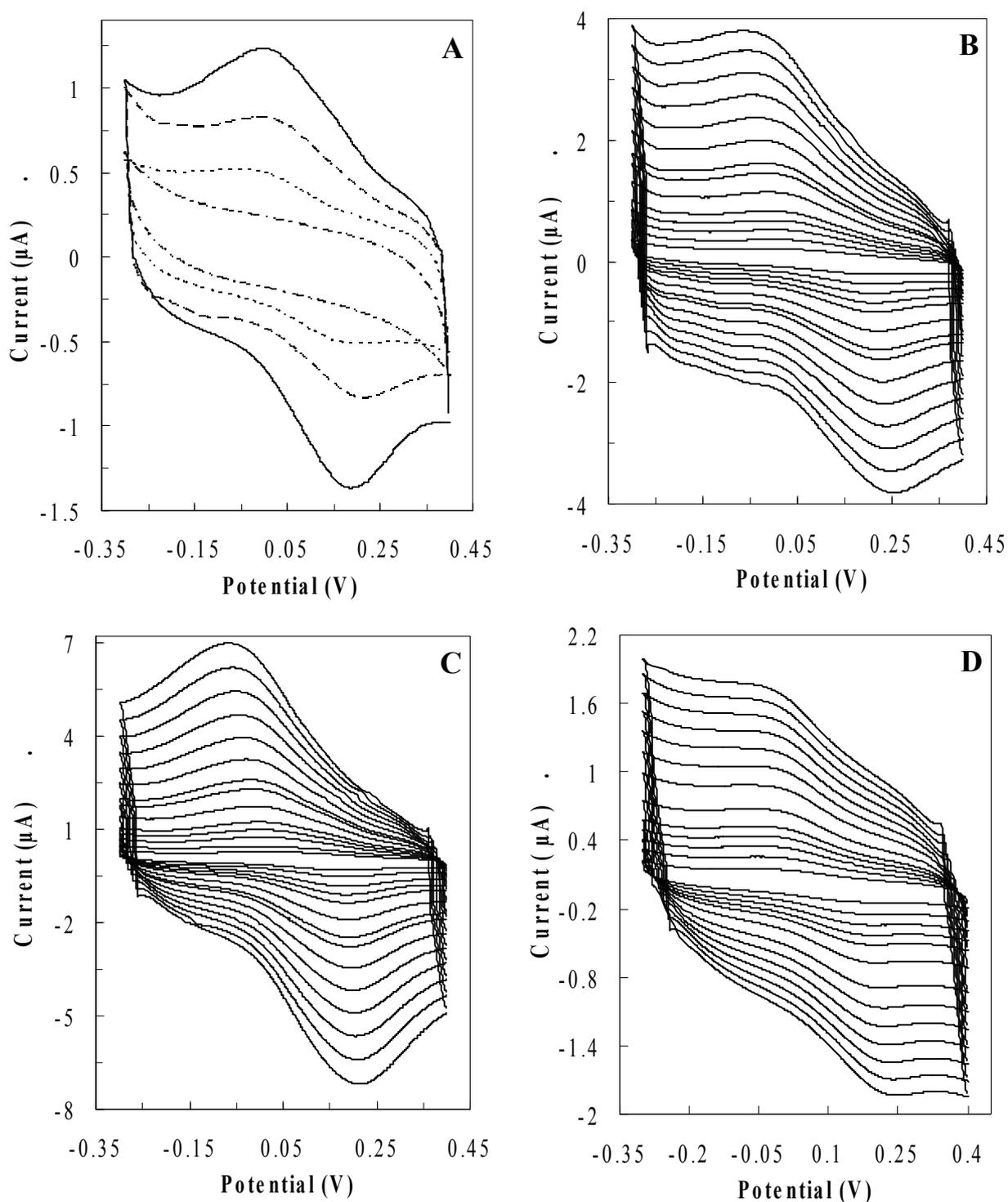
Fig. 1B shows the electrochemistry of Cys/Au electrode in PBS, in the absence of SOD. It was observed that Cys monolayer on Au electrode represents a time dependent redox behavior in which the peak currents were reached to a plateau after 90 min. The Cys/Au electrode shows a well defined quasi-reversible peaks with formal potential ( $E^{\circ}$ ) of +0.085 V vs. Ag/AgCl at scan rate of 0.10  $\text{Vs}^{-1}$ . This value is close to that observed by Qingwen and coworkers ( $E^{\circ}$ : +0.110 V vs. SCE) [31]. As seen the formal potential of Cys monolayer on Au electrode (Fig. 1B) overlaps with the redox potential of immobilized SOD (Fig. 1A). Therefore, one may conclude that the redox response of SOD could be attributed to the electrochemical behavior of Cys SAM.

#### 3.2. Origin of Cys SAM redox properties

It was reported that in physiologic media,  $\text{Cu}^{+2}$  center of Cu, Zn-SOD interacts directly with Cys and forms a 1:1 complex [38]. Also, there are some reports showing that the Cys SAM could interact with  $\text{Cu}^{+2}$  center of SOD [4,15]. Although Qingwen's group [31] did not refer to the origin of Cys/Au electrode redox behavior, but Qu. et al. [30] as mentioned previously, have recently observed similar redox response for 3-MPA SAM on gold electrode in PBS that in comparison with Cys, it doesn't have amine end group. They attributed redox behavior of 3-MPA SAM on the gold electrode to copper ion interference as buffer solution impurity [30]. Also Yang et al. showed that  $\text{Cu}^{2+}$  coordinates with amine and carboxyl functional groups of Cys and forms a 1:2 complex [32]. Therefore, to consider the role of  $\text{Cu}^{+2}$  we examined the CV of Cys/Au electrode in PBS containing different concentrations of copper ions (Fig. 1C). Comparing the results presented in Figs. 1A and 1C shows that  $\text{Cu}^{+2}$  (whether as SOD redox center or as free ions) plays a critical role for the appearance of electrochemical response by Cys/Au electrode.



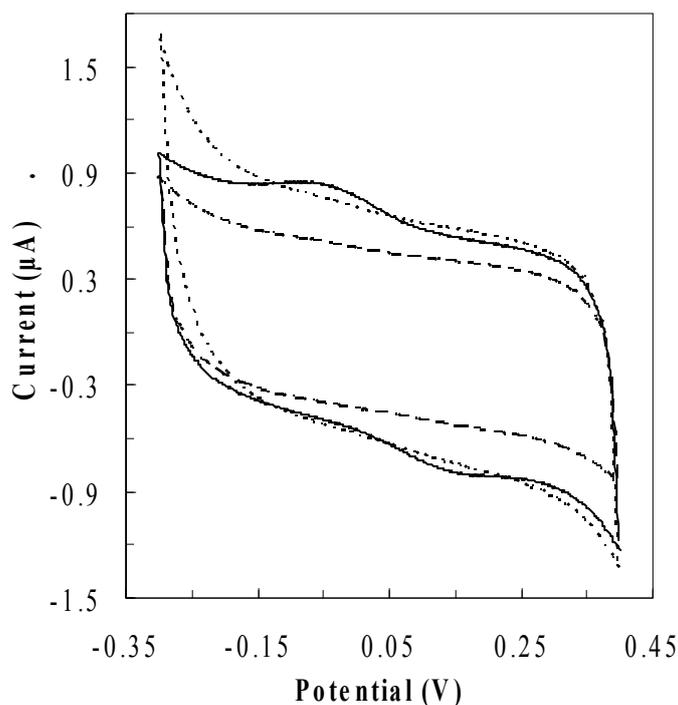
**Fig. 1.** CVs of Cys/Au electrode at different conditions: (A) in PBS containing 0, 6.03, 14.65 and 17.41  $\mu\text{M}$  SOD, (B) in PBS (without SOD) at various incubated times of 0, 20, 50 and 90 min and (C) in the PBS containing 0, 94, 156, 218 and 281  $\text{nM}$   $\text{Cu}^{2+}$ , (from inner to outer respectively). All measurements were carried out in 0.02 M PBS of pH 7.4 at the scan rate of  $0.05 \text{ V s}^{-1}$ .



**Fig. 2.** (A) Comparison of the CVs of modified electrodes at the scan rate of  $0.1 \text{ Vs}^{-1}$ . Inner to outer: bare Au, Cys/Au, SOD/Cys/Au,  $\text{Cu}^{2+}/\text{Cys}/\text{Au}$  electrodes. CVs of (B) SOD/Cys/Au, (C)  $\text{Cu}^{2+}/\text{Cys}/\text{Au}$ , and (D) Cys/Au electrodes at different potential scan rates. Potential scan rates (from outer to inner) are 0.5, 0.45, 0.4, 0.35, 0.3, 0.25, 0.2, 0.18, 0.14, 0.1, 0.08, 0.06, 0.04 and  $20 \text{ Vs}^{-1}$ , respectively. For (C) the CV was not recorded at  $0.18 \text{ Vs}^{-1}$ .

But in case of Fig. 1B it seems that the source for electrochemical activity of Cys/Au electrode is the presence of trace contaminant of  $\text{Cu}^{2+}$  in buffer solution. This conclusion is consistent with some reports [30,32] indicating that thiol SAM shows electroactivity due to the presence of  $\text{Cu}^{2+}$  as impurity. In our experiment the  $\text{Cu}^{2+}$  concentration as adventitious impurity of PBS was 112 nM which is higher than the value of reported detection limit for Cys/Au electrode toward  $\text{Cu}^{2+}$  (0.39 [34], 3.1 [43] and 80 nM [32]).

The CVs comparison of the different electrodes in Fig. 2A, revealed that the current intensity was increasing by the order of  $\text{Cu}^{2+}/\text{Cys}/\text{Au} > \text{SOD}/\text{Cys}/\text{Au} > \text{Cys}/\text{Au}$ , while their electrochemical working windows were overlapping ( $E^{\circ'}$  values were 0.100, 0.093 and 0.085 V, respectively). The proximity of  $E^{\circ'}$  indicates that in all three cases the redox peaks could be related to  $\text{Cu}^{2+}/\text{Cu}^{1+}$  couple [4,15,32,37]. These small variations could be due to the difference in microenvironments produced by either SOD or  $\text{Cu}^{2+}$  (adventitious impurity or deliberately added).



**Fig. 3.** CVs comparison of Cys-Au electrode in different solutions: up-PBS (dashed line), up-PBS and  $\text{Cu}^{2+}$  (solid line), PBS and EDTA (dotted line). Scan rate was  $0.1 \text{ Vs}^{-1}$ .

Furthermore, based on Fig. 2 B-D and using the Laviron's model [44,45], the values of electron transfer rate constant ( $k_s$ ) were calculated as 0.29, 0.73,  $0.77 \text{ s}^{-1}$  and those of surface concentration of electroactive species ( $\Gamma$ ) were calculated as  $1.50 \times 10^{-11}$ ,  $1.51 \times 10^{-11}$ ,  $1.05 \times 10^{-10} \text{ mol cm}^{-2}$  for Cys/Au, SOD/Cys/Au and  $\text{Cu}^{2+}/\text{Cys}/\text{Au}$  electrodes respectively. The

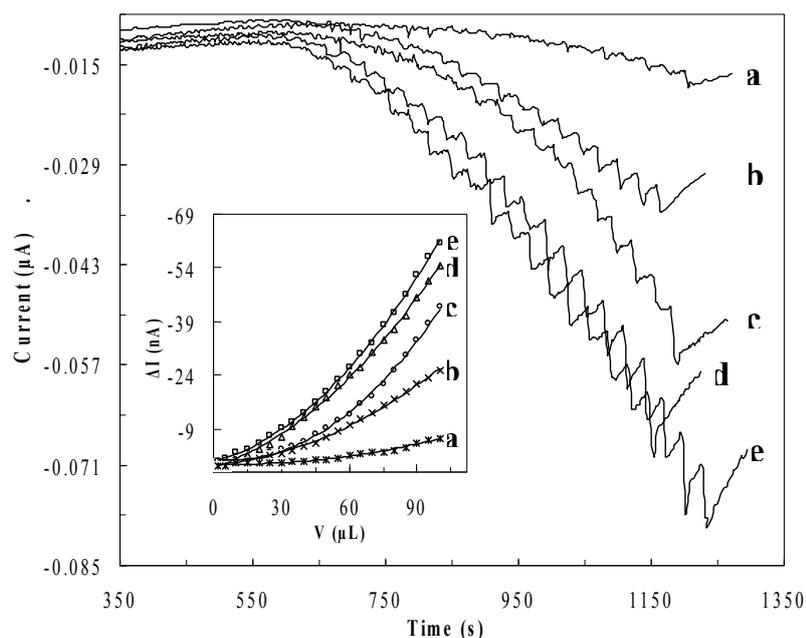
bigger  $k_s$  and  $\Gamma$  values for  $\text{Cu}^{2+}/\text{Cys}/\text{Au}$  system is due to the accessibility of  $\text{Cu}^{2+}$  ions to functional groups of Cys SAM. While in case of  $\text{SOD}/\text{Cys}/\text{Au}$ ,  $\text{Cu}^{2+}$  ions buried in the redox center of SOD and in the last case the trace  $\text{Cu}^{2+}$  is not sufficient for current production.

In the next step to control the copper ion effect, PBS was prepared by ultrapure phosphate salts (copper ion free) and used as electrolyte for electrochemical study of Cys SAM on Au electrode. As shown in the Fig. 3 (dashed line) Cys/Au electrode in ultra pure PBS (up-PBS) doesn't show any redox response. Then 2  $\mu\text{L}$  of 0.5 mM  $\text{Cu}^{2+}$  solution ( $\text{CuCl}_2$ ) was added to the solution and after 5 min the CV of Cys/Au electrode was recorded (Fig. 3, solid line). This experiment clearly indicated that the redox response of Cys/Au electrode is related to the deliberately added copper ion. In another experiment to control the copper ion effect, EDTA was added to PBS and after 5 min using the Cys/Au electrode the CV was recorded (Fig. 3, dotted line). EDTA as chelating agent could pick up copper ions from PBS consequently no electrochemical response was observed for Cys/Au electrode.

Comparing these results indicates that the source of the inherent electrochemical activity of Cys/Au electrode, is trace contaminant of  $\text{Cu}^{2+}$  in buffer solution.

### 3.3. Amperometric detection of superoxide

Chen et al. used thiols SAM with different length and head groups for  $\text{O}_2^{\bullet-}$  detection. They showed that  $\text{O}_2^{\bullet-}$  oxidation on SAM ended by amine or hydroxyl is easier than those ended by methyl or carboxyl [2]. Based on these experiences we used the Cys SAM as a bi-functional thiol to oxidize the  $\text{O}_2^{\bullet-}$  on Au electrode. The amperometric responses were recorded for bare Au, Cys/Au, SOD/Cys/Au, and  $\text{Cu}^{2+}/\text{Cys}/\text{Au}$  electrodes toward  $\text{O}_2^{\bullet-}$ -DMSO and pure DMSO as a control. As observed in Fig. 4, the pure DMSO doesn't have remarkable effect on the amperometric responses (Curve a). But in the presence of  $\text{O}_2^{\bullet-}$ -DMSO the amperometric response increased in the following order: Cys/Au >  $\text{Cu}^{2+}/\text{Cys}/\text{Au}$  > SOD/Cys/Au > bare Au (Curves b-d). To consider the reason for such an order we have to focus on the processes involved in signal generation. Basically  $\text{O}_2^{\bullet-}$  could be oxidized on the Au surface and produces a weak current as illustrated in Fig. 4b. But in case of Cys SAM on Au electrode, the  $\text{NH}_2$  terminus is positively charged at pH 7.4 which may attract the negatively charged  $\text{O}_2^{\bullet-}$  to the gold electrode surface, leading to the strong response of the Cys-modified gold electrode to  $\text{O}_2^{\bullet-}$  (Fig. 4e). According to the literature [30,32] deliberately addition of  $\text{Cu}^{2+}$  extends its coordination with Cys functional groups. Therefore, depend on the charge balance, the Cys SAM speed up the  $\text{O}_2^{\bullet-}$  absorption on Au surface (Fig. 4d). Also, SOD is a remarkably superefficient enzyme with a reaction rate approaching the diffusion limit [46]. Therefore, it makes sense that when SOD is absorbed on Cys SAM the  $\text{O}_2^{\bullet-}$  signal is decreased since the enzyme is able to eliminate superoxide anion before it reaches to electrode surface (Fig. 4c). To understand the exact mechanism further study is in current in our lab.



**Fig. 4.** Amperometric response of different modified electrodes to  $O_2^{\bullet-}$ -DMSO. Curve (a) is the same base line for (b) to (e) but in pure DMSO. (b) Bare Au, (c) SOD/Cys/Au, (d)  $Cu^{2+}$ /Cys/Au, (e) Cys/Au electrodes. Each amperometric step indicates the response of modified electrode after addition of  $O_2^{\bullet-}$ -DMSO solution (to 3 mL of PBS while stirring at 500 rpm). Amperometric tests were carried out at 0.25 V vs Ag/AgCl. Inset shows  $\Delta I$  vs. pure DMSO (a) and  $O_2^{\bullet-}$ -DMSO (b to d) volume.

#### 4. CONCLUSION

In summary, the experimental results reviewed in the literature and those carried out in this work revealed that  $Cu^{2+}$ , either as free ions or as SOD redox center or as the PBS trace impurity, plays a critical role for the appearance of electrochemical response on the Cys/Au electrode. It seems that  $Cu^{2+}$  coordinates with amine and carboxyl functional groups of Cys and forms a 1:2 complex. By this mechanism the electroactive species of  $Cu^{2+}$  shows a well defined redox peak at Cys monolayer. Due to the high affinity of Cys/Au electrode towards  $Cu^{2+}$ , the copper ion even as impurity of PBS may be absorbed on Cys monolayer and shows the electrochemical signal.

The positively charged amine groups on Cys SAM may also attract the negatively charged  $O_2^{\bullet-}$  and in this way facilitates its electron exchange toward the gold electrode. This process leads the strong current response of the Cys-modified gold electrode towards  $O_2^{\bullet-}$ .

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