



An Amperometric Biosensor Based on Cytochrome c Immobilized on a Gold Electrode for Detection of Dissolved Hydrogen Sulfide

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Abstract

Background and aims: Cytochrome c, a metalloprotein, can play the role of electron transporter electrochemically. In the present work, an amperometric biosensor for the detection of hydrogen sulfide in aqueous media has been fabricated by immobilizing cytochrome c on the surface of gold electrode.

Materials and Methods: The surface was modified by nanoparticle and self-assemble monolayer of 3-mercaptopropionic acid. 1-(3-DimethylAminoPropyl)-3-EthylCarboDiimide Methiodide (EDC) and N-hydroxysuccinimide (NHS) were applied as coupling reagents for the covalent attachment of cytochrome c to the modified gold electrode surface.

Results and Conclusion: Experimental design based on the Central Composite Design (CCD) method revealed the optimal condition for the current peak at pH 6, 5 mM phosphate buffer and 20°C. In the presence of hydrogen sulfide in electrolyte, oxidation and reduction peaks reduced and over time were completely vanished. Under the investigated aqueous conditions, the lowest tested H₂S concentration was 0.001 ppm, and a rapid amperometric response was observed. The electrode response remained observable during the investigated 15-day period.

What is “already known”:

- Immobilization of redox-active proteins on electrode surfaces enables selective, sensitive biosensing.
- Gold nanoparticles enhance direct electron transfer by minimizing the distance between protein and electrode.
- H₂S disrupts biological electron transfer, providing a detection signal for highly sensitive protein-based biosensors.

What this article adds:

- An amperometric nano-biosensor was designed for detection of H₂S.
- Cytochrome c was immobilized on modified gold electrode.
- Surface modification was done by gold nanoparticles.
- Amount of protein immobilized was calculated by cyclic voltammograms.
- The lowest H₂S concentration reached to 0.001 ppm in aqueous solution.

1. INTRODUCTION

Utilization of disparate techniques for detection of hydrogen sulfide has enticed a great interest in recent years [1,2]. Among those, design and evaluation of protein and enzymatic based biosensors do possess immense advantages due to their better response time and selectivity. Hydrogen sulfide is amongst the most hazardous gases present in nature [3]. It is produced in large scale in many industries such as gas and petroleum refining. H_2S is one of the materials disrupting the electron transfer in biological species. This interruption can be used as the detection sign of a highly sensitive protein-based biosensor [4,5]. H_2S metabolism would occur in 3 different pathways inside body: oxidation, methylation or might react with cytochrome c and other metalloproteins containing disulfides [5].

The immobilization of redox active proteins on electrode surface and evaluation of charge transfer process is one of the consequential demarches for developing more selective and more sensitive biosensors and bioelectronics devices [6,7]. In particular, development of electrodes which are functionalized with proteins from biological electron transfer systems is among the novel and marvelous research fields due to their promising applicability in innovative chemo-biosensor devices. Meanwhile, a major concern is to establish efficient electron transfer pathways between the electrode and the immobilized protein while retaining the proteins functional integrity [8–11].

By using the modified surfaces (activated surfaces) of electrodes, the study of direct and stable electrochemistry, without obstacles such as irreversible absorption of proteins on the surface, has become feasible. Thus, metalloproteins such as cytochrome c oxidase, ascorbate oxidase and cytochrome c are capable of direct electron transfer at the modified electrode surface. Hereupon, they can be utilized as the biological detector species in the biosensors [12,13]. Cytochromes and in particular cytochrome c are small proteins which are soluble in inter-membrane space of heme groups. They can act as electron transfer mediator between redox enzymes. The electron transfer between cytochrome c and

redox enzymes occur specifically. Several studies on the electrode structure and different immobilization techniques have been done [14,15]. Other researches have also stated a suitable oxido-reductase responses for cytochrome c immobilized on the thioalcanoic acids short chains, cycteamin and EDC modified gold surfaces [16,17].

Direct electron transfer is a function of electrode Nano-structure [8,18]. It has been shown that modifying the electrode surface by nanoparticles, would sequel to the most suitable position and the nearest distance between electrode and enzyme for electron transfer [19]. For instance, gold nanoparticles can efficiently provide a stable surface for immobilization of biomolecules in a manner in which the biological activity of the molecules would not alter. Therewith, these nanoparticles create conductive electrodes by which the electron can be directly transferred between redox proteins and electrode surface. Various colloidal gold features, including high surface area, high potential energy, and the ability to decline the distance between proteins and metallic particles, would preferably enhance the electron transfer between oxidation/reduction sites of the proteins and the electrode surface [20,21].

During the design of a biosensor, the stability of the biological part is of a great importance. The covalent bond is one of the most abiding immobilization methods. Its benefit lies under this fact that it only requires the modification of electrode surface. It has been suggested that the modifier agent which creates several functional groups on the surface, elevates direct and rapid electron transfer via irreversible and specific protein reactions [22]. So, this capability of the redox proteins can be implemented in detection of electron transfer disrupting agents [23].

The performance of the immobilized portion on the electrode surface is also a function of the electrolyte medium. In order to study the simultaneous effects of several parameters, statistical methods can be utilized [24,25]. The response surface method (RSM) is a set of mathematical and statistical techniques which enables modeling and analysis of problems involving several variables and implements for optimizing the whole process [26].



In this research, the bovine heart cytochrome c was immobilized covalently via the self-assembled monolayers of mercaptopropionic acid and carboxylic functional groups creation. The main purpose was to generate amide bond between the carboxylic groups and the amine groups of the protein immobilized on the gold nanoparticle-modified electrode surface. EDC and NHS were used as coupling agents in covalent bond creation. Using RSM statistical method, the optimized conditions of buffer and pH were determined. As a final point, the voltamogram changes were investigated in presence of H₂S in the electrochemical system.

2. MATERIALS AND METHODS

2.1. Materials

The materials used were of biological grade. Bovine heart cytochrome c, bovine serum albumin (BSA) 1-(3-Dimethyl aminopropyl)-3-ethylcarbodiimide methiodide (EDC methiodide) and N-Hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. 3-mercaptopropionic acid (MPA) (>99%) and pure ethanol for cytochrome c enzyme immobilization were obtained from Merck, Germany. Besides, the gold salt (HAuCl₄) made by Alfa Aesar and 3-sodium citrate made by Merck was utilized for nanoparticle synthesis.

2.2. Cyclic Voltammetry

The oxidoreductase behavior of the immobilized cytochrome c was evaluated using cyclic voltammetry in potentiostat device; all voltammetry experiments were performed using a 3-electrode system consisting of an Ag-AgCl electrode as the reference electrode, a rod-like Pt electrode as auxiliary electrode and a gold electrode as working electrode.

2.3. Gold Nanoparticles

Gold nanoparticles were synthesized by gold salt reduction procedure using 3-sodium citrate [27]. Briefly, 50 mL of HAuCl₄ · 3H₂O solution with concentration of 0.3mM was heated in sealed beaker under vigorous stirring to its boiling point. 5 mL of 3-sodium citrate solution with concentration of 5 mM was then added to the abovementioned solution. The resulting solution was heated for 15 min to its boiling

point. After observing a red color in this solution, it was cooled down to room temperature while being stirred. At last, the solution was kept in the refrigerator for further use.

2.4. Protein Preparation

Based on the Sigma-Aldrich proposed protocol, in order to obtain 10 mL reduced solution, 100 mg cytochrome c was dissolved in 8 mL of 10 mM phosphate buffer and was reduced by adding 3-5 mg L-ascorbic acid sodium, resulting in a light red solution. The ascorbic acid salt residues were removed by dialysis in phosphate buffer solution for 18 to 24h at 0-4°C and 3-time refreshment was carried out. The light absorption of the oxidized and reduced cytochrome c was compared at 550 nm using a spectrophotometry device (Ce 1010, Cecil Co.). This was accomplished after diluting the reduced solution with 10 mM phosphate buffer to achieve 10 mL final solution. The UV-spectrum for three absorption peaks in reduced form (Fe²⁺) at 550, 521 and 414 nm, as well as two adsorption peaks of reduced form (Fe³⁺) in 530 and 408 nm was considered by Hulko et al. [28].

2.5. Surface Modification

The gold electrode utilized in the present study was made out of a gold rod isolated by a plastic cover. The tip was also polished by aluminum oxide to be utilized as the immobilization surface. It was then placed inside piranha solution (containing hydrogen peroxide and sulfuric acid 1:3 volume fraction) for 5 min and washed with deionized water. In order to increase electrode surface area, the gold nanoparticles were deposited electrochemically on the surface. Since, MPA have great tendency to bind to the gold, as a result of thiol groups in its structure [29]; the gold electrode was first immersed in 0.1 M MPA for 15 min. The resulting MPA/Au electrode was then dried in N₂ after washing with water and ethanol. Finally, it was placed inside gold nanoparticle solution as electrolyte, in potential threshold of -0.2 to +1.4 with 90 scans. The final electrode was named AuNP/MPA/Au.

2.6. Protein Immobilization

The nanoparticle-modified electrode was immersed in ethanol solution of 3- mercaptopropionic acid (MPA) (pH=11) for 2h in room temperature. Afterward, the surface was washed with deionized water in order to ensure no MPA was adsorbed physically on the surface. It was then incubated in cytochrome c or BSA solution at 4°C in presence of EDC and NHS for 24h. EDC is a linker agent, which bonds the carboxylic group of MPA to the amine groups of the protein. Indeed, EDC first binds to carboxylic group, creating an unstable ester, which allows for amide bond to be generated in presence of amine groups of the protein and enzyme [30]. EDC activates the carboxyl groups of MPA, while NHS or Sulfo-NHS stabilizes the activated intermediate and improves subsequent amide-bond formation with protein amine groups; NHS/Sulfo-NHS therefore complements rather than replaces EDC. After 24h to remove weakly bonded proteins, the electrode was washed with deionized water.

2.7. Preparation of saturated H₂S solution

Depending on the temperature and pH, hydrogen sulfide would dissolve in water as H₂S, HS⁻ or S²⁻. In aqueous media, the relative fractions of H₂ S and HS⁻ depend strongly on pH and temperature; near and above neutral pH, HS⁻ becomes increasingly

important, whereas molecular H₂ S predominates under more acidic conditions. In both states the amount of S²⁻ is negligible in liquid solution [31]. The method of Vosoughi et al was used in order to acquire the desired H₂S concentrations in the solution [32].

To distinguish the optimal conditions of cathodic and anodic currents on electrode surface, the effects of 3 parameters including temperature, pH and concentration of phosphate buffer on the amount of cathodic and anodic currents was investigated.

3. RESULTS AND DISCUSSION

3.1. Gold Nanoparticles Analysis

In favor of determining the size of nanoparticles, the absorbance peak at 400-700 nm range was obtained. Fig. 1a depicts the 525 nm absorbance, which along with the color of obtained solution, demonstrates synthesis of nanoparticles with 5-20 nm diameters. Fig. 1b shows TEM image of gold nanoparticles. Surucu et al have shown that the maximum light absorption of 20 nm nanoparticles synthesized using HAuCl₄ reduction would occur in wavelength of 520 nm [33].

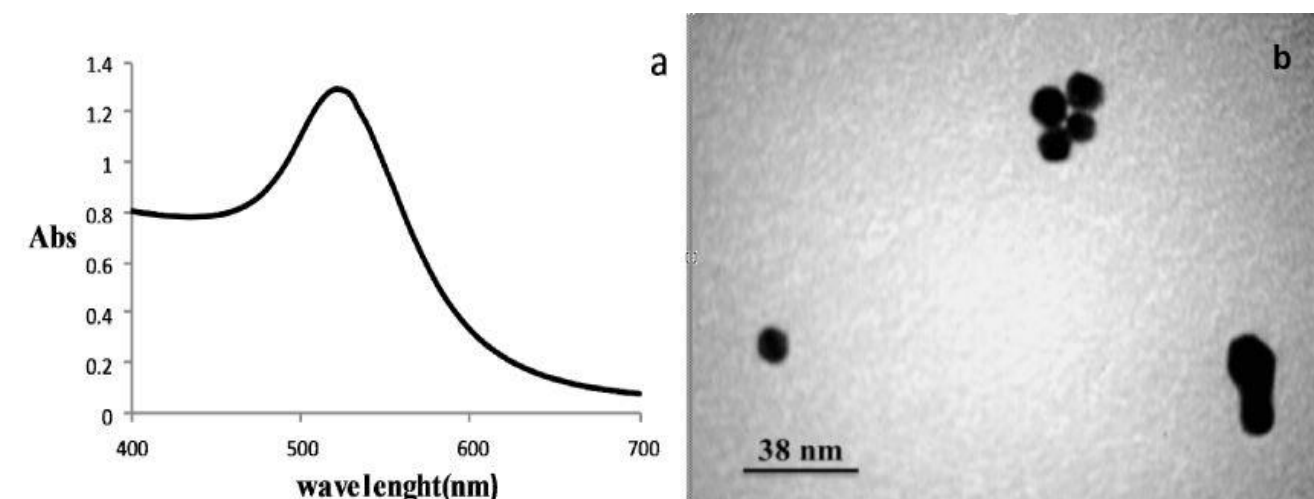


Figure 1. (a) UV-Vis absorbance spectra of gold nanoparticle, (b). TEM image of Au nanoparticle.

3.2 Surface Investigation

For increment of gold electrode surface area, based on the method presented by Surucu et al, the gold electrode was first placed in mercapto-propionic acid solution and then the gold nanoparticles were participated on them via electrochemical method [33]. The observed peak at the potential of +0.8 in voltammograms is considered. A comparison between cyclic voltammograms in gold nanoparticle modified gold electrode in 0.1 mM K₃Fe(CN)₆ and 0.1 M KCl solution as electrolyte was investigated. In voltammograms, the value of A/B demonstrates the ratio of increasing the surface. In the present study this value was equal to 1.63. The modified and unmodified electrodes with and without MPA as the binding agent, were compared after creating electrodes via cyclic voltammetry.

In an experiment performed by Kroger et al, a suitable bed for a prospering immobilization of protein on gold surface was designed using different thyoalkane self-assemble monolayers [34]. Frasca et al. have successfully immobilized human sulfide oxidase enzyme on gold nanoparticles-modified electrodes and have investigated it using cyclic voltammetry [35]. Santucci et al. confirmed direct electron transfer between gold electrode and ascorbate oxidase [36]. They extended previous methods by using cyclic voltammetry and differential pulse voltammetry (DPV). De wael et al. immobilized cytochrome c on gold electrodes with EDC as coupling agent [16]. The other researchers have implemented bonding strategies along with biological tag- affinity and electrostatic hybrid, to directly immobilize horse heart cytochrome c on gold electrode surfaces [37].

In the present study, after surface modification with gold nanoparticles and MPA as binder, the modified gold electrode was placed in a protein solution containing EDC and NHS. The results show the increase in current passing through the electrode and the effect of MPA with lower

conductivity in comparison with gold nanoparticle modified electrode.

3.3. Optimization of Electrolyte Results Considering The anodic and Cathodic Currents as the Response

By using the RSM method, the effect of 3 distinct parameters including phosphate buffer pH, concentration and temperature as well as the significance of each one in desired responses were investigated. In this model, A, B and C represents temperature, phosphate buffer pH and concentration, respectively. The p-value for each parameter represents its significance in the desired response and p values less than 0.05 was considered the significance level of each parameter. The terms containing B, AB, AC, BC, A², B² and C² was omitted by the software as a result of p values higher than 0.05; and the terms containing A, C, A² and C² with p-values less than 0.05 were reported to be significant in the first response. In order to determine the meaningful terms in other responses, the same procedure might be implemented and terms having p-values less than 0.05 will be omitted.

The obtained equation for the first response (anodic peak current) and for the second response (cathodic peak current) is as Eq. 1 & 2 :

$$R1 = 0.14 - 0.043 A - 0.041 C + 0.062 A^2 + 0.063 B$$

Eq. 1

$$R2 = 0.097 - 0.081 A - 0.040 C + 0.10 B^2$$

Eq. 2

The positive sign of any parameter, suggest an increase of current peak in higher values of the parameter; while an increase in response will be observed in lower values of a negatively signed factor. Fig.2 indicates the effects of three investigated parameters: temperature, phosphate buffer pH and concentration on the first and second response respectively and also the amount of overlap between the experimental data and the values calculated by the model for the response.

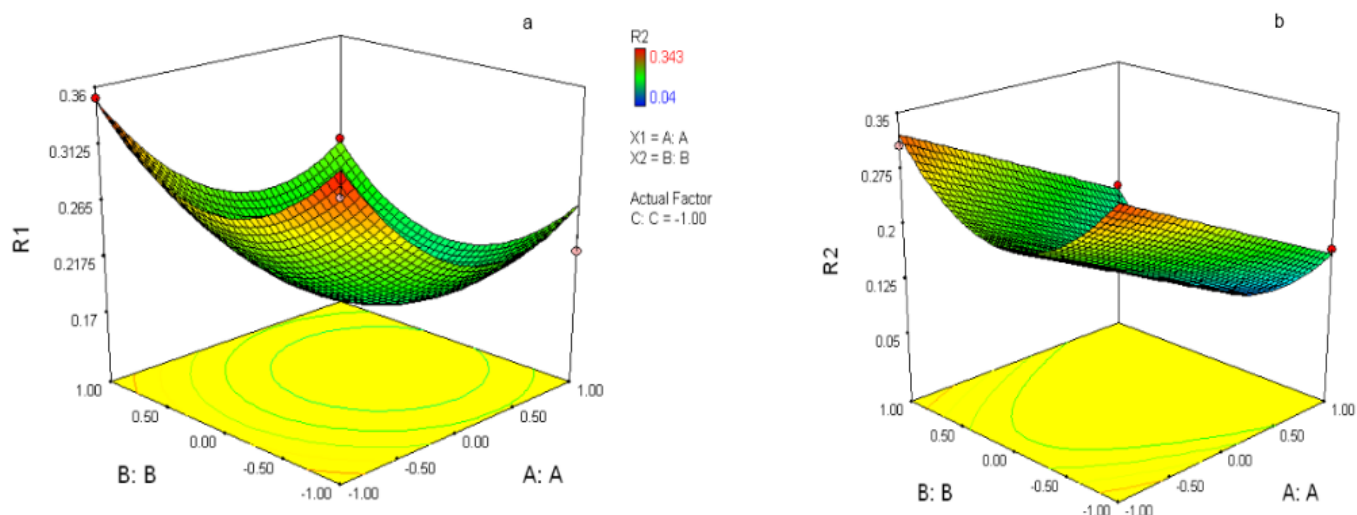


Figure 2. Three-dimensional charts of the design of experiments for: (a) anodic peak current and (b) cathodic peak current.

3.4. Quantifying the Immobilized Protein on Electrode Surface

In order to determine the amount of immobilized protein on the surface, several voltammograms of protein immobilized electrodes, with different scanning rates were recorded. Fig. 3a shows the cyclic voltammograms of the immobilized protein on the surface of gold electrode, with scanning as it can be

seen from Fig. 3b, the peak of current alterations behave linearly in scanning rates lower than 100 mV/g rates of 30, 50, 70, 90, 200, 400, 600, 800 mV/s.

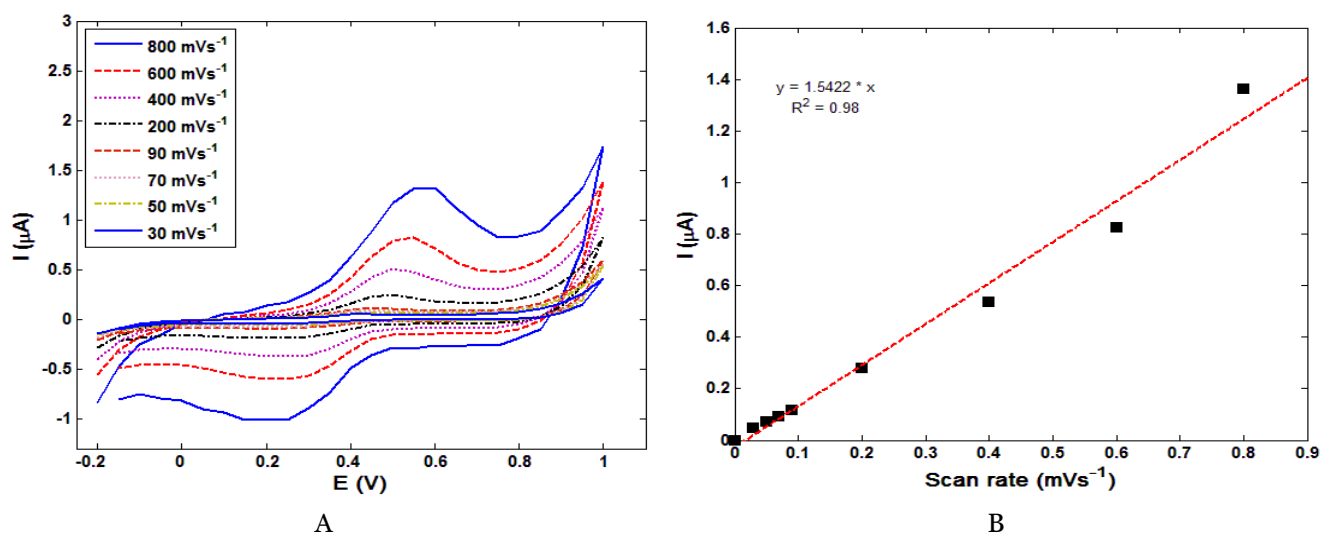


Fig. 3. (a) Cyclic voltammograms of cytochrome c covalently attached to the self-assembled monolayers of MPA on a gold nanoparticle modified electrode at different scan rates in 5 mM phosphate buffer solution (pH=6). Scan rate/mVs⁻¹:30, 50, 70, 90, 200, 400, 600, 800. (b) Relation between the anodic peak current and the scan rate.

which is consistent with surface-confined electroactive cytochrome c on the modified electrode. This scan-rate dependence is compatible with the behavior expected for a surface-confined redox protein and should not, by itself, be interpreted as proof of an ideal covalent monolayer. These are in complete agreement with the Butler–Volmer electrode kinetic model-like behavior and rapid continuous electron transfer at lower scanning rates. The surface coverage by the electroactive cytochrome c species (Γ) can be calculated using the Eq.3. [38]:

$$I_p = \frac{n^2 F^2 \vartheta A \Gamma}{4RT} \quad \text{Eq. 3}$$

where, I_p , n , ϑ , A and Γ are current peak, number of exchanged electrons, the scanning rate, the electrode surface area and the redox species concentration, respectively. Moreover, F , R and T , represent the faraday constant, gas constant and temperature respectively. Using this equation, the concentration of the immobilized protein was calculated equal to 0.114 pmol/mm².

3.5. Hydrogen Sulfide Detection

Hydrogen sulfide can interact with heme-containing redox proteins and alter their redox behavior; therefore, the response observed here is interpreted in relation to changes in the electrochemical state of immobilized cytochrome c. The oxidation-reduction peaks were disappeared in H₂S presence. This was due to the reduced protein which obtain its electron from H₂S instead of the electrode (Fig. 4a). To investigate this prediction, 1 ppm H₂S solution in deionized water was injected to (10 mL) electrolyte solution (without stirring) and repeating 20 cycles. The changes in voltammogram of Cyt c/MPA/AUNP/AU and BSA/MPA/AUNP/AU as a result of H₂S injection, and comparison of voltammograms in the first cycle and the last cycle are shown in Fig. 4b and 4c. These results were obtained at 0.1 V/s scanning rate). Amperometric real-time response of Cyt c/MPA/AUNP/AU electrode for H₂S (indicated by the arrow) shown in Fig. 4d. In this Fig., the current response profile in absence of H₂S is stable and caused by the capacitive current. Addition of 1 ppm of H₂S results in a rapid and a progressive increase of the cathodic current which provides

evidence for the presence of H₂S. This result supports the use of the developed electrode for rapid electrochemical monitoring of dissolved H₂ S under the investigated aqueous conditions. In the presence of dissolved H₂ S, the progressive attenuation of the cytochrome c redox peaks is consistent with a sulfide-induced change in the redox state of the immobilized heme protein; possible contributions from interactions at the protein/electrode interface cannot be excluded. Since in cyclic voltammetry method, the oxidation-reduction peaks are mutually coupled. When the reduction state declined by the immobilized species on the electrode surface, the oxidation peak would also disappear gradually during number of cycles [39]. Shahidi et al have used amperometry evaluation method to detected sulfur components by peroxidase enzyme immobilized via entrapment on the print screen electrode [40]. Motallebi poor et al have successfully detected H₂S gas in aqueous solution in 55 seconds, using ascorbate oxidase immobilization of the nylon bed [41]. The entrapment method has also been utilized in latter work. Mirzaei et al have also detected H₂S gas in 80s using thiobacillus thioparus bacteria entrapped in PVA bed [42]. In a lately research, Vosoughi et al were also able to craft a highly sensitive microbial H₂S biosensor which could detect in less than 72s and as low concentrations as 0.5 ppm of H₂S [32].

3.6. Calibration Curve for Different H₂S Concentrations

Different nominal sulfide concentrations (0.001, 0.01, 0.1, 1, 3, 5, and 10 ppm) were introduced into the aqueous electrolyte to construct the calibration response of the cytochrome c-based sensor (Fig. 4e). The anodic peak current alterations were recorded to plot the protein calibration curve. There was an interval of linear changes. The lowest experimentally evaluated concentration in the calibration series was 0.001 ppm. Accordingly, 0.001 ppm is reported here as the lowest tested concentration rather than as an experimentally validated detection limit.



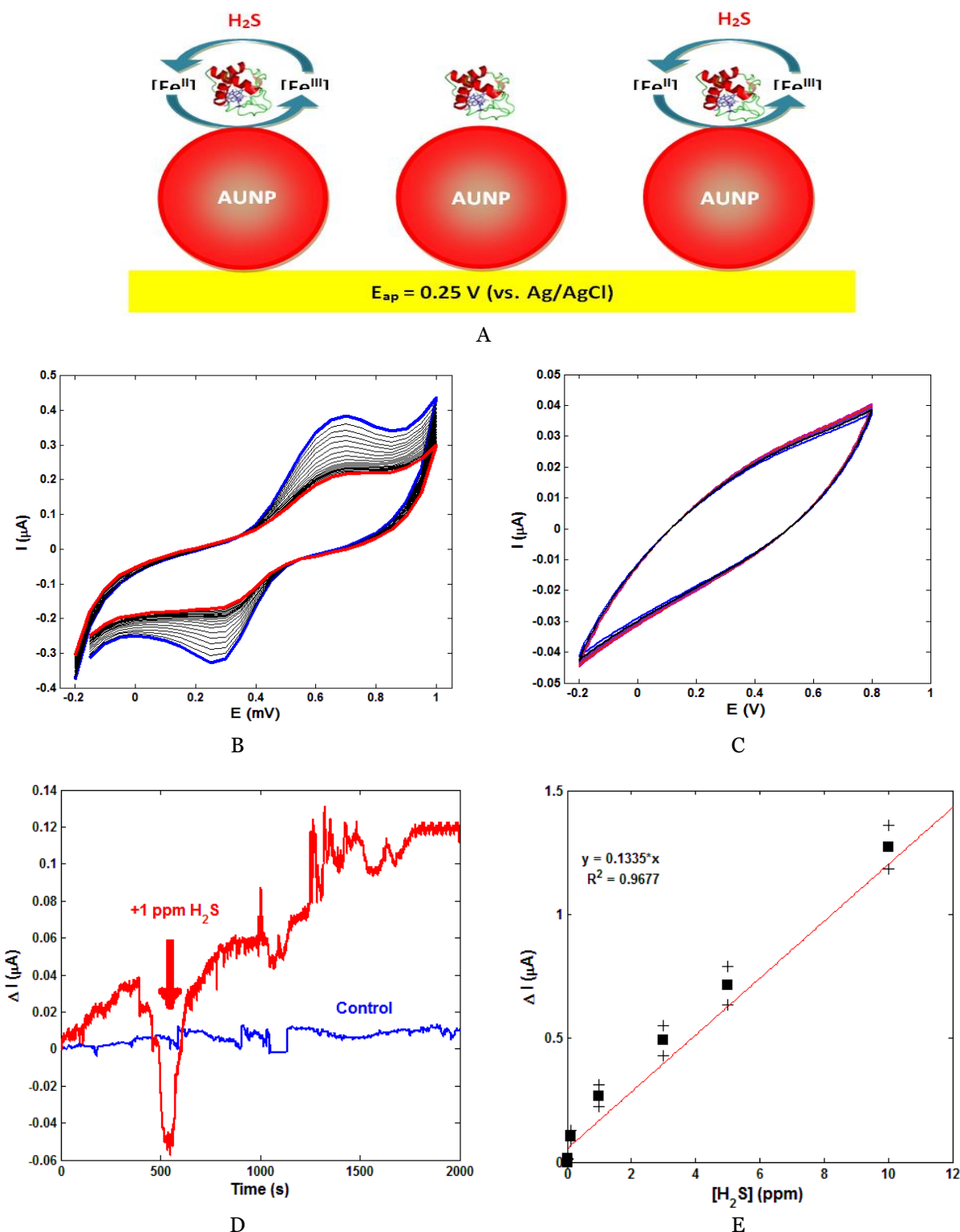


Fig. 4. (a) Schematic representation of H₂S detection by using the Cyt c/MPA/NPAU/AU. (b) Cyclic voltammograms of immobilized cytochrome c (c) BSA at the self-assembled monolayers of MPA on a gold nanoparticle modified electrode in presence of 1ppm H₂S. (d) Amperometric real-time detection of H₂S (indicated by the arrow). (e) The change of anodic peak current (ΔI) as a function of H₂S concentration (gold electrode as a working electrode and a silver-silver chloride as a reference electrode.).



4. CONCLUSION

The current experiment revealed the ability of cytochrome c as a redox protein to be implemented in H₂S detection biosensors, due to the H₂S interactions with metallic groups of metalloproteins structure. Surface modification of gold electrodes using gold nanoparticles would enhance the attachment of biological species to the surface and electron transfer between the redox protein and the electrode by increasing the electrode surface area. This modification may be achieved using several linker materials providing covalent bonds between nanoparticles and the surface; among which mercaptopropionic acid having shorter molecular chain, guarantee the lowest distance between the electrode and desired biological specie. Mercaptopropionic acid has a -SH functional group in one terminal which makes it easily bonded to the gold surface via sulfide group. Moreover, in presence of EDC, the -COOH functional group in the other terminal can provide a potential bonding site for amine groups of biological species.

In this study, the direct electrochemistry of immobilized cytochrome c enabled a rapid response to low concentrations of dissolved H₂ S under the investigated aqueous conditions. The results suggest the possibility of application of method to detect several materials disrupting electron transfer.

5. DECLARATIONS

5.1. Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

5.2. Acknowledgments

Not applicable.

5.3. Funding

No funding was received for conducting this study.

5.4. Author contributions

All authors contributed to the conception and development of the study, interpretation of the results, and critical revision of the manuscript

5.5. Ethical considerations

All ethical principles were adhered to in conducting and writing this article.

5.6. Data availability

In accordance with the principles of transparency and open research, we declare that all data and materials used in this study are available upon request.

5.7. Using Artificial Intelligence chatbots

The core ideas, conceptual framework, and original authorship remain entirely the work of the human writers themselves. In the process of designing this project, with the aim of achieving linguistically precise content, visually appealing graphic elements, and conceptually rich imagery, we utilized the advanced capabilities of language models such as ChatGPT for reviewing and refining paragraph structuring, tense consistency, and correct usage of prepositions. Simultaneously, by employing AI-powered image generation tools, we took the graphic concepts from the ideation stage to the final prototype, repeatedly redesigning and optimizing them while adhering to principles of composition, color harmony, and digital aesthetics. The final output is thus a fusion of grammatical accuracy, visual appeal, and narrative coherence—shaped through a coordinated collaboration between human expertise and machine capabilities along a gradual, controlled path.

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