



Graphite/gold nanoparticles electrode for direct protein attachment: characterization and gas sensing application

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Abstract

In this work, graphite/gold nanoparticles (G/AuNPs) were synthesized through a facile chemical method, and its potential application for direct protein attachment for electrochemical detection of carbon monoxide (CO) was investigated. The preparation of G/AuNPs electrodes was optimized by synthesizing the nanoparticles in different concentration of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ at various temperatures. The G/AuNPs electrode was subsequently modified by four types of mercaptopropionic acid, including 1-mercaptopropionic, 3-mercaptopropionic, 6-mercaptopropionic, and 11-mercaptopropionic acid, to achieve the best structure for protein attachment. Visible absorption and electrochemical studies showed that 3-mercaptopropionic acid possesses the best performance regarding the electrical conductivity between electrode and protein redox center. The cyclic voltammetry results revealed that the modified electrode has an appropriate performance for CO detection at very low concentrations while keeping a linear response. The limit of detection for the modified electrode was calculated to be about 0.2 ppb. Finally, the interactions of cytochrome C and carbon monoxides were simulated using molecular dynamics (MD), and the effect of protein conformation changes on the electrochemical signal was thoroughly examined. The simulation results suggested that the proposed electrochemical sensor has an acceptable performance for the detection of CO due to less fluctuation of amino acids near the protein chain in the presence of CO molecules.

Keywords Graphite electrode · Au nanoparticles · Cytochrome C · Carbon monoxide · Electrochemical biosensor · Molecular dynamics

Introduction

CO is a hazardous material that has many threats for human beings, even in low concentrations. For instance, exposure to CO may result in the reduction of oxygen carrying in blood

(Kakaei 2017; Mofenson et al. 1984). Inhaling CO at the concentration of 9 ppm for 8 h or 25 ppm for an hour is fatal (Weaver 1999). Therefore, fast and facile detection of CO at low concentrations is very imperative (Dolan 1985). Up to now, many efforts have been devoted to develop sensors for detection of CO through various techniques such as optical (Zayasu et al. 1997), electrical (Savage et al. 2001), biological (Blyth et al. 1995), and electrochemical sensors (Okamoto et al. 1980). Biosensors have attracted much more attention due to high specificity, selectivity, and sensitivity. Among the conventional biomarkers for the detection of CO, proteins and enzymes are widely used for CO monitoring (Dürr et al. 2006; Omid et al. 2015a; Topoglidis et al. 2003; Topoglidis et al. 1998).

Proposing new types of enzyme-based electrode for recognizing substances in the gaseous phase has been the subject of importance in multiple studies. Kuretake et al. showed that immobilized enzymes on chromatography paper could be used as electrochemical gas biosensors (Kuretake et al. 2017). Cytochrome c (Cyt c) is known as one of the most common proteins which can be used for the detection of CO

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owing to its redox nature in electrochemical biosensors (Omidi et al. 2015b). It is showed that how structural properties of Cyt c could affect the UV-Vis spectra upon reactivity with CO, NO, and O₂ providing a basis for understanding the gas binding to the heme-based sensor proteins (Hough and Andrew 2015). The main mechanism in CO detection is attributed to the reduction of Cyt c by CO molecules, which decreases the number of oxidative site in protein (Omidi et al. 2014). On the other hand, one of the important points to fabricate a biosensor electrode is the modification of electrode surface that affects the performance of biosensor (Kakaei et al. 2019). Preparing a suitable platform for decoration of nanomaterials or self-assembly of them is the first step in fabricating an electrode (Ehsani et al. 2020; Mojtaba et al. 2020). In addition, the surface treatments of electrodes such as heat or chemical treatments have been widely considered as another modification method to improve the performance of electrodes (Kakaei and Gharibi 2014). Among the conventional methods of surface treatments, chemical treatment is dominant due to facile procedure and unsophisticated equipment (Kakaei et al. 2016). The immobilization of Cyt c on the electrode surface is also significant for the detection of CO. For this purpose, linkers are usually employed for direct connection between Cyt c redox center and the electrode surface in order to facilitate the rate of electron transfer. Atomic structures play an important role on the electrical behavior of linkers (Saito et al. 1992). Various characteristics such as length, chemical nature, and type of conjugation are the main factors that might influence the conductivity of linkers (Odom et al. 1998). The electrochemical activity of Cyt c should be stable during the attachment process in order to preserve the electrical conductivity between the electrode and protein active sites. Using nanoparticles such as Au, Ag, and carbon nanostructures can maintain the electrical conductivity between biomarkers and electrode (Ehsani et al. 2019; Eshlaghi et al. 2020). Mercaptopropionic acid (MPA) is an organic acid that can be used for the attachment of Cyt c on the surface of gold electrodes. The carboxylic acid groups on MPA can react with Cyt c through covalent interaction. On the other side, Au nanoparticles can be attached on the surface through thiolation due to the presence of thiol groups. Thus, the modified electrode with Au nanoparticles significantly improves the electron transfer and sensitivity of Cyt c for the detection of CO (Aggarwal et al. 2009; Cedervall et al. 2007; Lacerda et al. 2009).

Investigating the possible interactions between CO and Cyt c has a pivotal role for designing and fabricating more applicable biosensor for CO detection. Although there are many reports on the detection of CO molecule, considering the possible interactions between this molecule and Cyt c has been ignored. Indeed, the effect of CO on unfolding or aggregation of Cyt c complex is not obvious until now. Thermodynamic properties, kinetics of protein folding, and protein structure are

three major factors which profoundly affect the unfolding of Cyt c. In this work, a facile and novel procedure for the synthesis of G/AuNPs electrode was developed. The as-prepared electrode was then modified with Cyt c for high accurate detection of CO molecules. Furthermore, the role of MPA as a linker was thoroughly studied and optimized. Finally, the molecular interaction between Cyt c and CO molecules was simulated through MD simulation.

Material and methods

Instruments and materials

All the chemical materials purchased from Merck and used without further purification and manipulation. Double distilled water was used throughout the experiments. Horse heart cytochrome c (molecular weights 12,384 g/mol) was purchased from Sigma and qualified through UV-Vis absorption at 550 nm (Abass et al. 2000; Wang and Wang 2004). All electrochemical measurements were carried out in phosphate buffer solution pH = 7.4 at room temperature using a galvanostat/potentiostat (IVIUMSTAT vertex). A conventional three-electrode electrochemical cell containing an Ag/AgCl as a reference electrode (Argentel, 3 M KCl) and a platinum wire (diameter=0.5 mm) as the counter electrode was used for all measurements. Hi-polymer graphite pencil B6 black leads (2 mm) were obtained from Cretacolor Totiens Co and utilized as the working electrode. Electrical contact with the lead was provided by soldering copper wire to the metallic parts which fixed the lead inside a pipette tip. The far-UV circular dichroism (CD) spectroscopy was obtained by an Aviv model 215 spectropolarimeter to give the content of the regular secondary structure. The UV-Vis spectra were measured by 2550 Shimadzu, Japan.

Molecular dynamic simulation

Energy minimization and molecular dynamics simulations were performed by GROMACS (5.1.4) and GROMOS53 (Abraham et al. 2015; Oostenbrink et al. 2005). The water box was created with at least 10 Å distances from protein using 10829 SPC water molecules and applying periodic boundary conditions. The 20 and 200 molecules of carbon monoxide were randomly introduced to the simulation box replacing with water molecules. The B3LYP/6-31G* carbon monoxide optimized geometry and parameters were obtained from automatic topology builder (ATB) (Malde et al. 2011).

Considering the protein net charge system, neutralization was conducted by adding 7 chloride anions. The system was subjected to 5000 steps of energy minimization to relax the structure and equilibrate the solvent around the protein while keeping at a constant temperature of 300 K (NVT) simulation.

Afterward, NPT optimization was performed for 500 ps. In order to increase the likelihood of achieving the appropriate structure, the MD simulation was performed for 1000 ps. GROMACS implemented tools were carried out for trajectory analysis. Using least-squares fitting the root-mean-square deviation (RMSD) and root mean square fluctuation (RMSF) were calculated for the protein backbone (Bitaab et al. 2015; Sefidbakht et al. 2016). The radius of gyration for cytochrome c and cytochrome in the presence of carbon monoxide was calculated. Finally, DSSP (hydrogen bond estimation algorithm) V 2.1.0 was employed to compare the probable alteration in the secondary structure of cytochrome c for both cases during MD (Joosten et al. 2011).

Preparation of graphite oxide and G/AuNPs electrode

Graphite oxide was synthesized according to the previously reported Hummer method (Hummers Jr and Offeman 1958). At the first step, graphite electrodes were immersed in $\text{HNO}_3/\text{H}_2\text{SO}_4$ (1:1) solution at room temperature in bath sonication for 2 h. After that, the electrodes were rinsed and placed in 10 ml of a solution containing 0.1 M HAuCl_4 and 40 mg NaBH_4 at 50 °C for various period of time including 30, 60, 90, and 120 min as G/Au1, G/Au2, G/Au3, and G/Au4, respectively.

Preparation of working electrode

In order to optimize the composition and concentration of MPA, four G/Au NPs electrodes were prepared and immersed in solutions containing 1-mercaptopropionic acid (1-MPA), 3-mercaptopropionic acid (3-MPA), 6-mercaptopropionic acid (6-MPA), and 11-mercaptopropionic acid (11-MPA). The concentration of MPA solutions in this step kept constant about 0.02 M, and the electrodes were immersed for 2 h at room temperature. Next, the electrodes were rinsed by water to remove excess physically adsorbed compounds. Finally, the modified electrodes were incubated at 4 °C in Cyt c solution in the presence of 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide/N-hydroxysuccinimide (EDC/NHS) with the concentration of 0.08 M and 0.1 M, respectively. The substrate was then thoroughly rinsed with deionized water to remove the weakly adsorbed Cyt c (Canterford 1975).

CO calibration solution

The saturated solution of CO with an approximate concentration of 0.98 mM was prepared at 25 °C through bubbling CO gas in 5 ml of PBS (pH 7) for 1 h. To avoid the dispersion of CO, all the measurements were performed in sealed cell. The detection of CO was carried out in different time periods

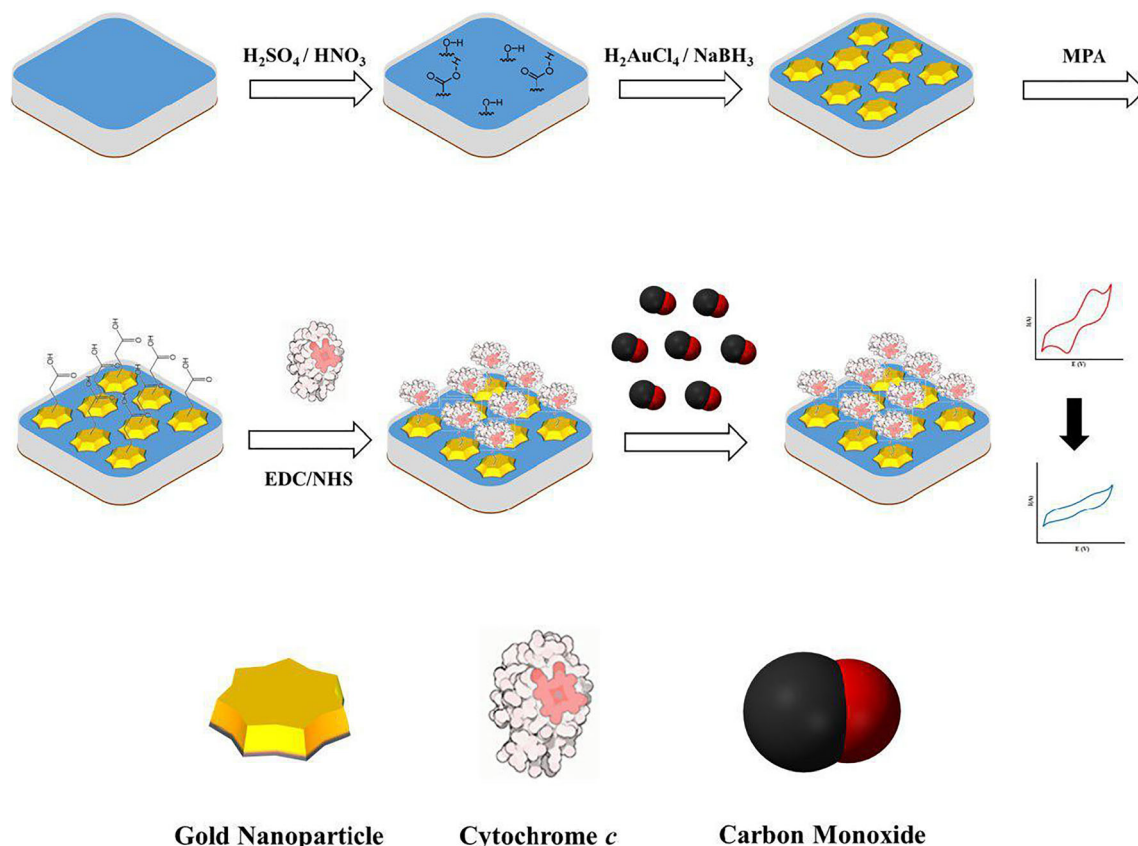


Fig. 1 Schematic illustration for the preparation of the working electrode

including 10, 20, 30, 40, and 50 s; considering that the operation is time-consuming, the time 30 s was selected for further studies. The results showed that the electrode has the best electrochemical performance after the passage of 30 s.

Result and discussions

Figure 1 shows the step-by-step procedure for the preparation of the working electrode. As illustrated, the first step is attributed to the deposition of Au nanoparticles on the surface of the electrode. For this purpose, the surface was initially modified with oxygen containing functional groups to increase the probability of AuNPs attachment. The FTIR spectra of the treated graphite electrodes with $\text{HNO}_3/\text{H}_2\text{SO}_4$ (1:1) at various times are shown in Fig. 2. As can be seen, the majority of created functional groups on the surface of the electrode alters from hydroxyl to epoxy by the passage of time. The sharp peaks at around 3400 and 1700 cm^{-1} are attributed to hydroxyl and carboxyl groups, respectively. The distinctive peaks about 1600 , 1400 , and 1050 cm^{-1} are related to the formation of carboxylic and epoxy groups on the surface of electrode, respectively. According to the FTIR spectra, it seems that the optimum time for acid treatment of electrodes is 90 min.

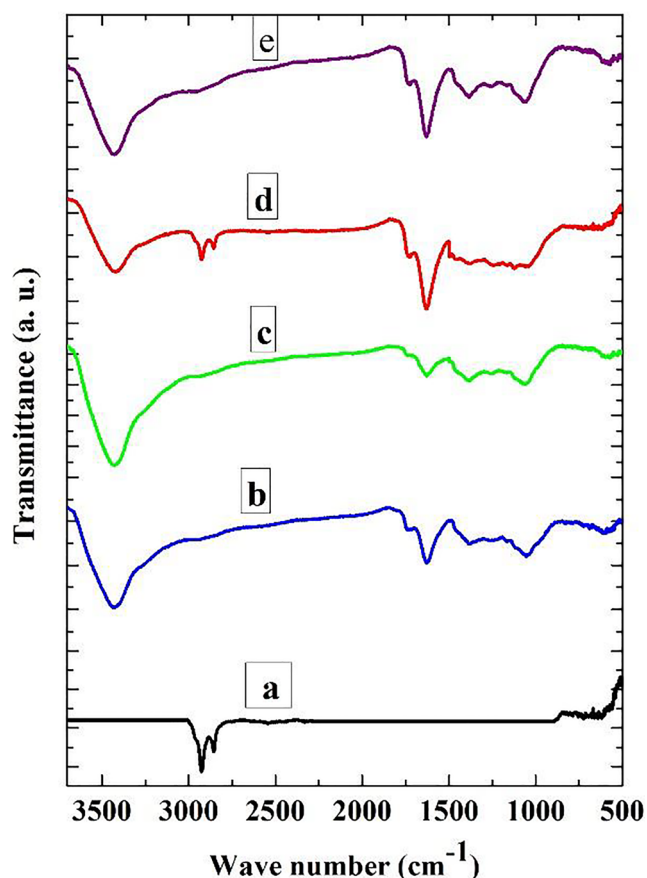


Fig. 2 FTIR spectra of graphite bare electrode (a) and acid treated at 30 (b), 60 (c), 90 (d), and 120 min (e)

The XRD patterns of the electrodes are shown in Fig. 3. As illustrated, the sharp peak at $2\theta \sim 25^\circ$ is ascribed to the (002) plane of graphite without any further diffraction characteristic of graphite. The XRD pattern of GO demonstrates that the distinctive peak at $2\theta \sim 25^\circ$ diminished and the sharp peak at $2\theta \sim 11^\circ$ appears corresponding to the formation of GO on the surface of the electrode (Salehifar et al. 2015). The small and broad peak observed at about $2\theta \sim 24^\circ$ can be associated with graphite, indicating the presence of small amounts of graphite. XRD diagram of G/AuNPs shows that the surface of the electrode was reduced and coated by Au NPs. The diffraction peaks at $2\theta \sim 38.5^\circ$, 64.3° , and 77.6° are attributed to (111), (220), and (311) planes of AuNPs, respectively. Furthermore, the additional peak at about $2\theta \sim 43.6^\circ$ is related to overlapping (200) reflection plane of Au at 44° with (100) plane of graphite match with JCPDS data card 04-0784 (Wolf and Xu 2011). Furthermore, using the Debye-Scherrer equation, the mean size of AuNPs was calculated about 60 nm.

The SEM images of as-prepared electrodes are shown in Fig. 4. As can be seen, the density of Au NPs increases by enhancing the time of immersion in the boiling reaction cell. The SEM images indicate that the deposition of Au NPs on the graphite electrode reaches the optimum condition after being immersed for 90 min (Fig. 4d). After that, by increasing the concentration of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, a noticeable agglomeration

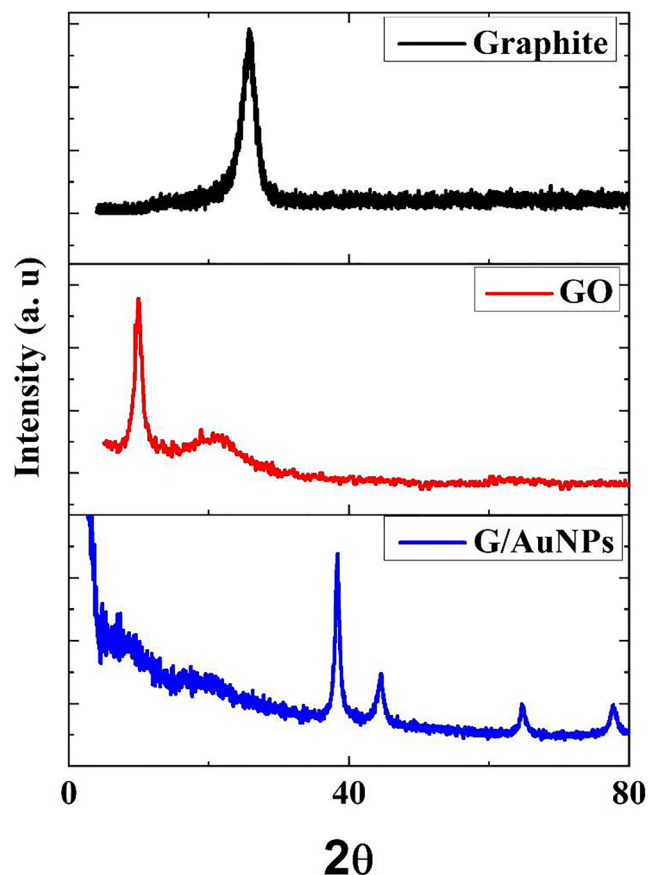


Fig. 3 XRD patterns of G, GO, and G/Au NPs electrodes

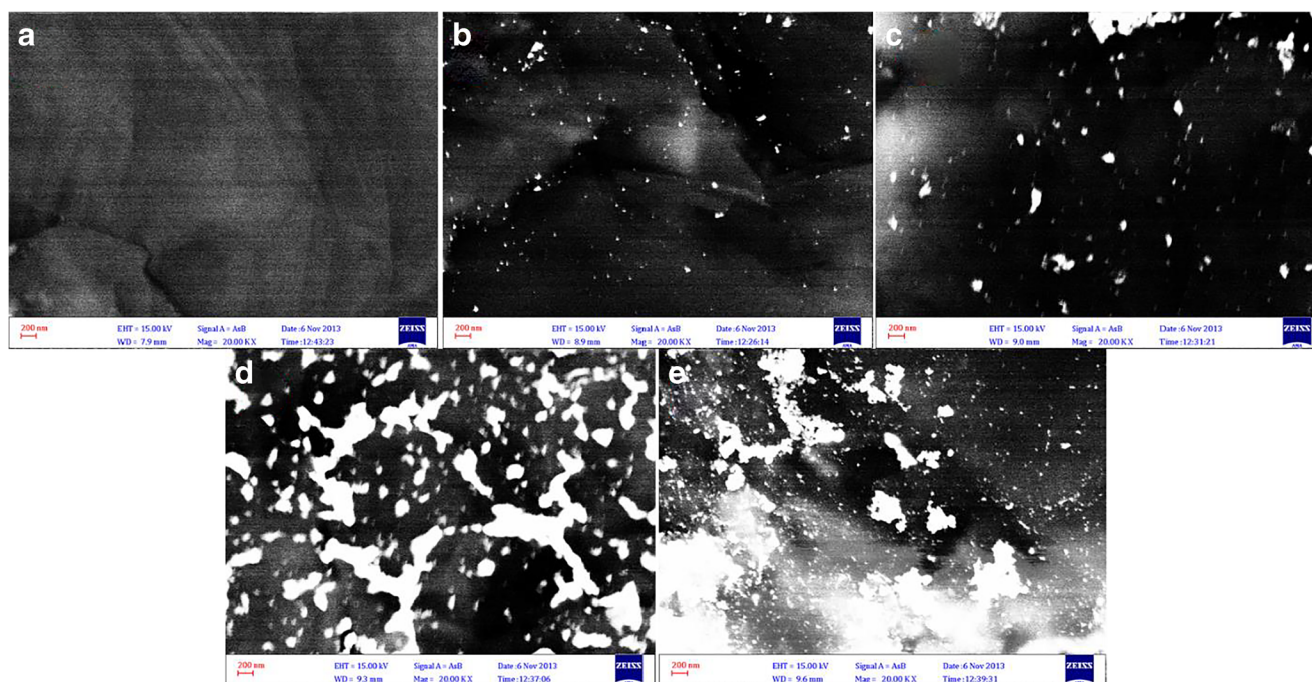


Fig. 4 SEM images of G electrodes (GE) modified with various amount of Au NPs, GE (a), G/Au 1 (b), G/Au 2 (c), G/Au 3 (d) and G/Au 4 (e)

of Au NPs has been occurred (Fig. 4e). The EDS results of the as-prepared electrodes are summarized in Table 1. As expected, the atomic percentage of Au nanoparticles increases by increasing the time of immersion in $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ solution. Although G/Au 4 electrode has more content of Au NPs, the G/Au3 electrode was indicating the optimum electrochemical behavior.

Figure 5 demonstrates the FTIR-ATR spectra of modified G/AuNPs electrodes with different types of MPA. As seen, the strong absorbance peak at 1690 cm^{-1} is attributed to carboxylate groups (curve a). The appearance of distinctive peaks at 1557 and 1403 cm^{-1} is related to C–H bending (curve b). Increasing the number of CH_2 bands in 3-MPA, 6-MPA, and 11-MPA leads to appearing the absorbance at 2845 – 2916 cm^{-1} , which can be ascribed to C–C bonds (curve c, d, and e).

Figure 6 indicates the visible spectroscopy of G/AuNPs/Cyt c and modified electrodes by different types of MPA. Accordingly, the spectroscopy of G/AuNPs/Cyt c presents an absorbance peak at about 530 nm , while the appearance of another absorbance peak at 400 nm after modifying the

electrode with Cyt c implies the successful conjugation between the surface of the electrode and Cyt c. As illustrated, the maximum absorbance of Cyt c observed in the case of 3-MPA, indicating the highest electrical conductivity between Cyt c and 3-MPA, among other electrodes. Cyclic

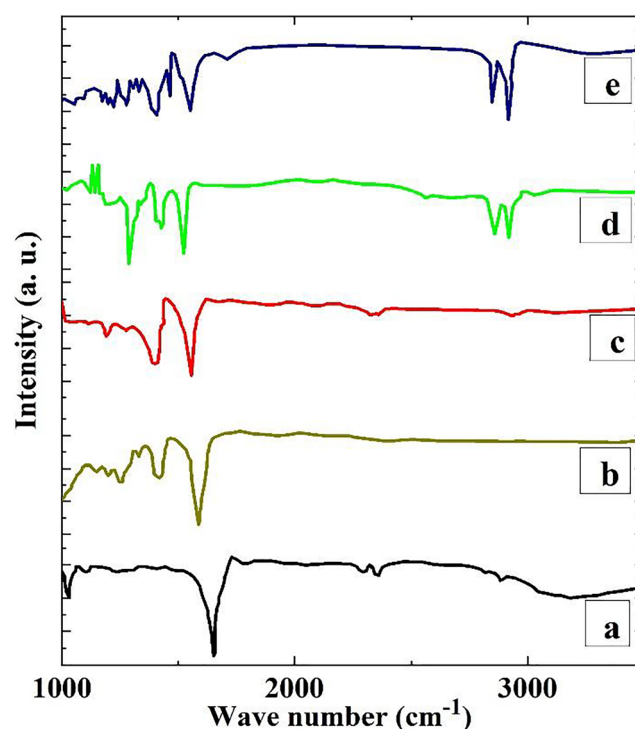


Fig. 5 FTIR-ATR diagrams of G/AuNPs electrode (a) modified by 1-MPA (b), 3-MPA (c), 6-MPA (d), and 11-MPA (e)

Table 1 Quantitative results of EDS analysis for G/Au electrodes

Electrode	Au wt%	Carbon wt%
GE	0	100
G/Au1	1.1	98.9
G/Au2	6.9	93.1
G/Au3	8.7	91.3
G/Au4	12.5	87.5

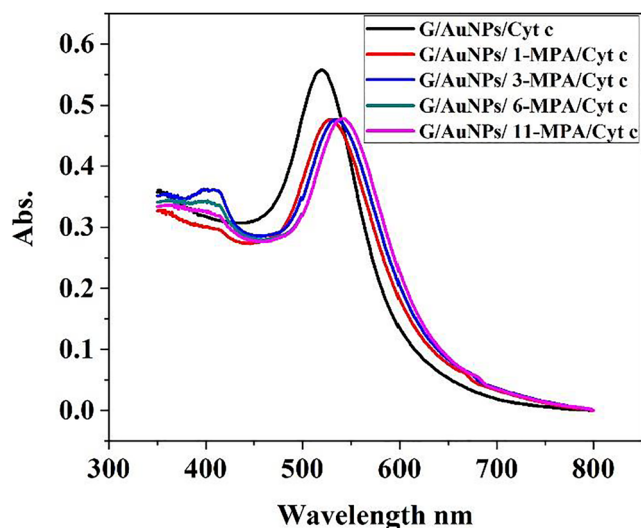


Fig. 6 Visible spectroscopy analysis of modified electrode by cytochrome c protein

voltammetry is one of the useful techniques that can be used for the study of various electrodes (Shayeh et al. 2016). Figure 7 a presents the CV curves of bare and modified graphite electrodes. As can be seen, after the modification of electrode by AuNPs, due to increasing the conductivity and active surface area of the electrode, the magnitude of non-faradic

behavior of the modified electrode increased. After MPA attachment, due to the non-conductive nature of MPA, the active surface area of the electrode diminished, and therefore, the magnitude of non-faradic behavior of the modified electrode decreased.

The second step in electrode preparation is to optimize the conjugation of Cyt c protein and electrode by using MPA. In this regard, four types of MPA were investigated, and the most conductive electrode was selected as the main working electrode for the detection of CO molecules. Figure 7 b shows the typical CVs of modified electrodes by various kinds of MPA. All the CV curves have a redox peak corresponding to oxidation and reduction of cytochrome C protein. As observed, G/AuNPs/3-MPA/Cyt c electrode has the best electrochemical performance between all the electrodes due to the highest current density and lowest potential compared with the other electrodes. The pattern shows that 3-MPA can be connected to the active center of Cyt c to the surface of the electrode, and by increasing the length of MPA chains, the electrical conductivity between the electrode surface and Cyt c active center decreased. Therefore, G/AuNPs/3-MPA/Cyt c was selected as the optimized electrode for further characterization.

According to Fig. 7c, decreasing the number of active sites on the surface of the electrode is due to the electrochemical reduction of Cyt c by CO molecules. Thus, the maximum

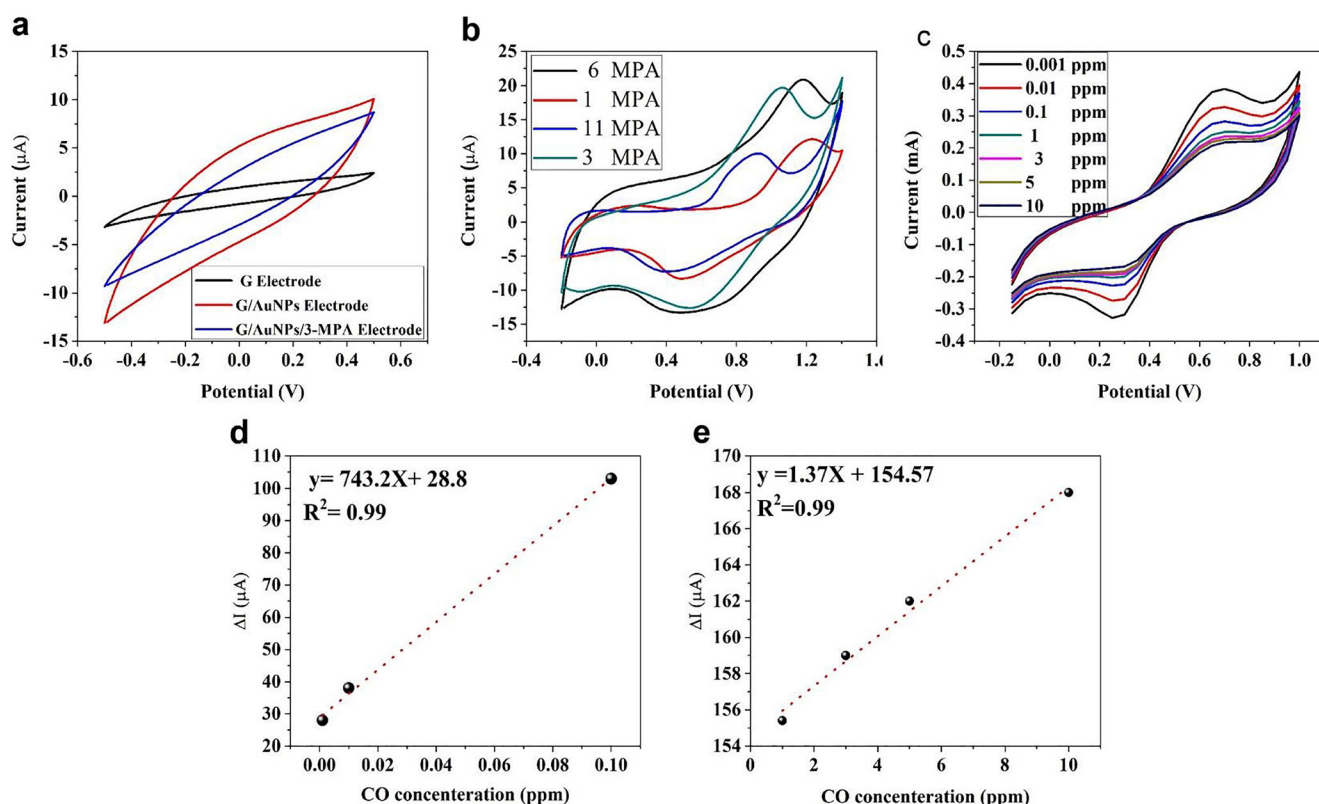


Fig. 7 CVs of graphite electrode modified with AuNPs and 3-MPA (a), CVs of G/AuNPs/MPA/Cc modified electrodes with various types of MPA connectors at the scan rate of 50 mV/s in 0.05 M PBS (b), CVs

of G/AuNPs/3-MPA/Cyt c electrode at the scan rate of 50 mV/s in various CO concentrations (c), and calibration curves of relative currents of G/AuNPs/3-MPA/Cc electrode versus CO concentrations (d, e)

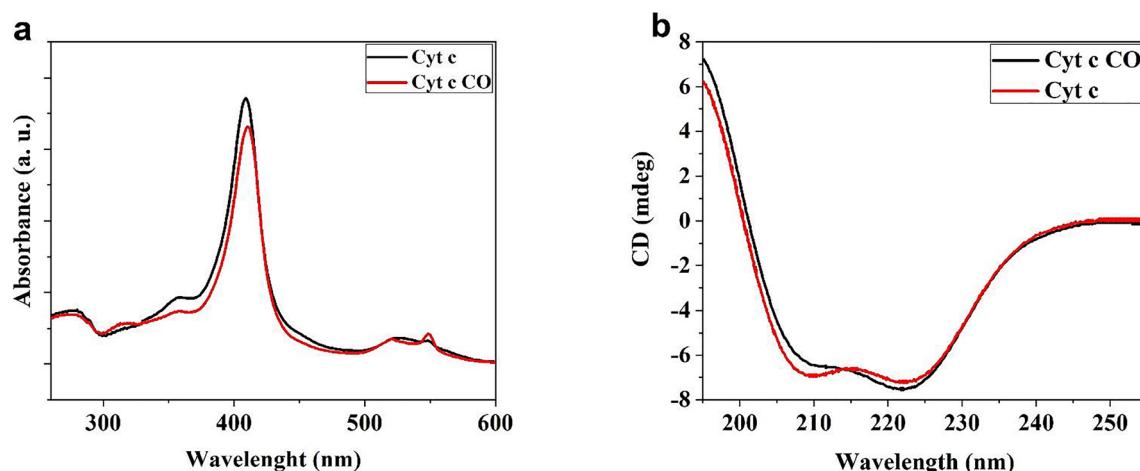


Fig. 8 **a** UV-Vis spectra of 1.25 mg/ml cytochrome c and cytochrome c in the presence of carbon monoxide of 1% at 25 °C. **b** UV-CD spectra recorded for cytochrome c and cytochrome c (red) in the presence of

carbon monoxide (blue). The corresponding silver nanoparticle-AChE system at the PB concentration of 0.20 mg/ml (pH 7.4, 25 mM) and carbon monoxide concentrations of 1% at 25 °C

current of redox peak decreases by increasing the concentration of CO owing to faster electrochemical reaction between Cyt c and CO molecules. Besides, at higher concentrations, the redox peak of Cyt c diminishes due to the consumption of all Cyt c proteins by CO molecules on the surface of the modified electrode. The proposed assembled electrode possesses an excellent linear behavior which is one of the most important features of the electrochemical biosensor (Baniasadi et al. 2014; Rashidi et al. 2013). Figure 7 d shows the relative peak currents of G/AuNPs/3-MPA/Cyt c electrode versus concentration of CO. At a lower concentration of CO, the electrode shows a linear response implying high sensitivity of the assembled electrode for CO detection. However, at higher concentration of CO (1–10 ppm), a linear correlation ($R = 0.99$) between the value of relative currents and the concentration of CO indicates

linear behavior of electrode even at high concentration (Fig. 7e). Therefore, the results show that G/AuNPs/3-MPA/Cc electrode can be used in a broad range of CO concentrations for the detection of CO molecules. The limit of detection (LOD) was also calculated to be about 0.2 ppb with S/N ratio of 3. It should be noted that the required time for CO detection was optimized. The UV-Vis spectra were also used to investigate the effect of CO molecules on Cyt c protein at room temperature (Farivar et al. 2010; Sefidbakht et al. 2012). According to Fig. 8a, the UV-Vis spectra of Cyt c in the presence of 1% CO shows four absorptions bands at different wavelengths. The distinctive peaks at around 280, 350, and 410 nm are ascribed to the aromatic amino acid region. The broad band at around 530 nm, which can be observed in both spectra, is related to oxidized protein; however, the characteristic peak near 550 nm can be attributed to the presence of CO molecules, which leads to the reduction of Cyt c (Moosavi-Movahedi et al. 2008). Hence, it can be well inferred that the reduction of Cyt c by CO can be the main reason of structural deformation. Figure 8b shows the UV-CD spectra of bare Cyt c and in the presence of CO molecules. As presented, Cyt c in the presence or absence of CO molecules has a negative peak around 210 nm suggesting the presence of high alpha-helical structures in both cases. However, the more negative pick of Cyt c in the presence of CO molecules confirms the creation of higher alpha helical structure after the addition of CO. Thus, the circular dichroism data confirms the more order in the secondary structure of Cyt c in the presence of carbon monoxide.

These observations were further investigated and approved through molecular dynamic simulations. Figure 8 shows the RMSD of Cyt c in the water and the presence of 20 and 200 molecules of carbon monoxide. The presence of CO generally reduces the RMSD changes during the MD. The radius of gyration (R_g) over trajectory was also

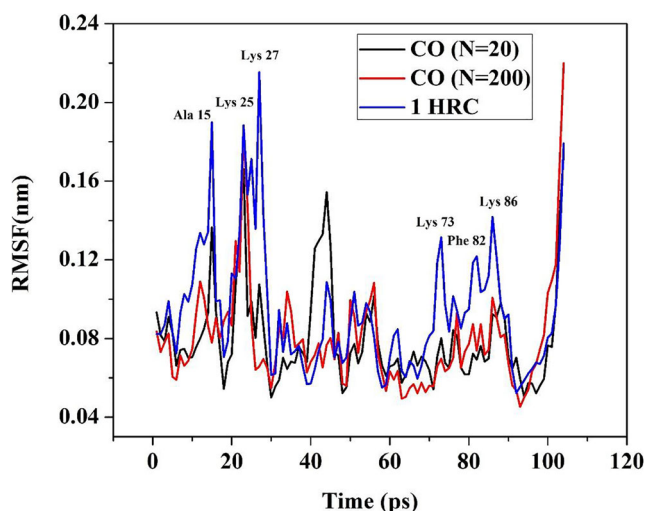


Fig. 9 The average RMSF value obtained from trajectory MD for the model (AMY1) and template (6TAA)

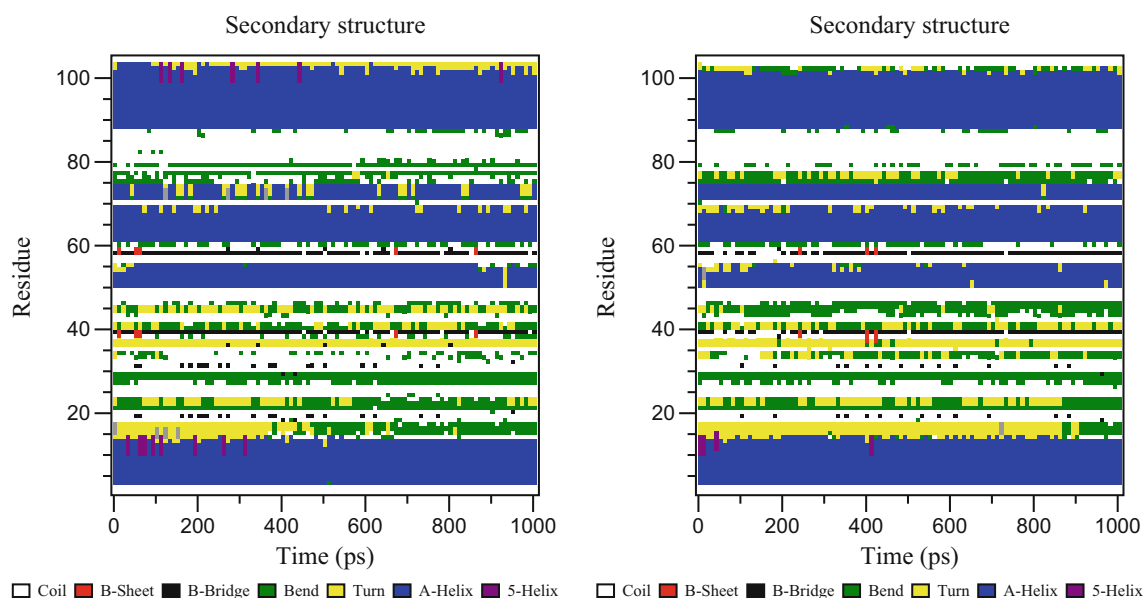


Fig. 10 The change in secondary structure over simulation for 1HRC in water and in the presence of carbon monoxide

observed to be deviating between 1.32 and 1.36 nm. Although the presence of carbon monoxide shows some variations in R_g , these effects are nearly converged after 900 ps of simulation. The molecular dynamics study of this electron transfer protein adsorption to a gold surface has shown no unfolding tendency in the secondary structure. In the real system, these particular structural changes observed here might somehow occur differently as it is investigated for heme reduction in redox proteins experimentally (Soares et al. 1998). However, it can be inferred that the flexibility of the portion of the Cyt c might affect the electron transfer rate (Siwko and Corni 2013). The RMSF is representative of the amino acids movement during the simulation (Fig. 9). These flexibilities in dynamics seem to be related to the gas concentration as can be understood from RMSF and RMSD plots represented here. As it can be well inferred, the fluctuation of various amino acids along the protein chain is reduced in the presence of carbon monoxide. Using DSSP, it would be possible to follow changes in the secondary structure content of protein from the trajectory. At the first glance, Fig. 10 represents that the Cyt c possesses five distinguished alpha helix regions (i.e., residues 4–14, 51–55, 62–70, 72–75, and 89–102). It seems that in the presence of carbon monoxide, the structural elements are more clearly formed especially in the fourth helix residues 72–75. This observation is in good agreement with experimental studies presented before.

It should be mentioned that the major effector in the electron transfer rate is related to the geometrical distance from the heme group in the active site and the electrode. However, the slight changes in the tertiary structure of heme proteins and its effect on the electron transfer are subject of study to advance the heme-based protein biosensors.

Conclusion

In this work, graphite/gold nanoparticles were chemically synthesized through immersion of graphite electrode in boiling $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ solution without any further procedure. The SEM images showed that Au NPs were uniformly dispersed on the surface of the graphite electrode, and the modified electrode is very suitable for protein attachment for electrochemical detection of CO molecules. The conjugation of Cyt c on the surface of the modified electrode was optimized by using different types of MPA. The electrochemical and spectroscopy characterizations revealed that the proposed electrochemical-based sensor has a great potential application to for detection of CO molecules in both low and high concentrations via a tremendous linear response. Molecular dynamic results suggested that the interactions between Cyt c and CO might compact the 3D structure of Cyt c, which enhances the availability of Cyt c structure for facile and rapid detection of CO molecules through an electrochemical reaction.

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