Talanta 81 (2010) 167–175

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Direct electrochemistry of hemoglobin adsorbed on self-assembled monolayers with different head groups or chain length

Zhibin Mai, Xiaojuan Zhao, Zong Dai*, Xiaoyong Zou*

School of Chemistry and Chemical Engineering, Sun Yat-Sen University, Guangzhou 510275, PR China

ARTICLE INFO

Article history:
Received 21 August 2009
Received in revised form
19 November 2009
Accepted 23 November 2009
Available online 29 November 2009

Keywords:
Self-assembled monolayer
Hemoglobin
Electron transfer

ABSTRACT

The electrochemical behaviors of hemoglobin (Hb) were studied by adsorbing Hb on the gold electrodes modified with self-assembled monolayers (SAMs) of different terminal groups and alkyl chain length. Specifically, through adsorbing the Hb molecules onto the SAMs of 3-mercaptopropanoic acid (MPA), 1-propanethiol (PT) and cysteamine hydrochloride (cys), the influences of the terminal groups of alkanethiols on the electron transfer of Hb were examined. A quasi-reversible redox process was observed when Hb was adsorbed on the MPA-modified electrode. However, an irreversible reduction process and no redox response were shown when Hb molecules were adsorbed on the SAMs of PT and cys-modified electrodes, respectively. The dependence of the direct electrochemical response of Hb on the alkyl chain length of alkanethiols (n) was further investigated on the SAMs of $HS(CH_2)_nCOOH$ (n = 1, 2, 5 and 10). When n was 1, Hb showed a weak current response. When n were 2 and 5, quasi-reversible redox processes with nearly similar electron transfer rate constants (k_s) of 0.49 and 0.47 s^{-1} were obtained, respectively. Increasing the number of methylene groups within carboxyl alkanethiol to 10 resulted in a significant decrease of the electron transfer rate and current response of Hb. The observations indicated that the SAM of MPA can provide suitable properties to keep Hb in a favorable adsorption state for direct electron transfer (DET). Furthermore, the direct electrochemistry of the Hb adsorbed on the SAM of MPA was studied in detail.

1. Introduction

The investigation of the direct electron transfer (DET) between redox proteins and electrodes has attracted great attentions in the past decades since it is not only important for constructing biosensors [1,2] and developing biomaterials [3], but also helpful for getting information on biological systems. Although lots of strategies have been reported to achieve the DET of redox proteins successfully, most of them mainly focus on the biosensing applications [4]. The understanding and manipulation of a suitable state of proteins to realize the DET still need to be studied further [5,6].

As is well know, most of the redox proteins cannot exhibit direct electrochemical response at a bare electrode since the heme groups of protein are often deeply buried in the central cavity of proteins [7]. The common way to achieve the DET of redox proteins is to adsorb them onto modified electrodes, such as on nano-scale or biocompatible materials modified electrodes [7–10]. However, the increase of the distance between the redox centers of protein and the surface of electrode, and the alterations from the optimal orientation for electron transfer can critically affect the DET of these adsorbed proteins [11]. Therefore, in order to facilitate the DET of the redox proteins, it is important to construct electrodes with suitable interfacial properties for adsorbing proteins in optimal distance and orientation.

Self-assembled monolayer (SAM) is a commonly used technique to investigate the influences of the interfacial properties of electrodes on the DET of the redox proteins, since it can easily provide relatively rigid and controllable interfaces [11,12]. Gray et al. recently studied the DET of the cytochrome *c* (cyt *c*) adsorbed on the electrodes modified with SAMs of $HS(CH_2)_nCOOH$. The electrostatic attraction between the adsorbed cyt *c* and the $HS(CH_2)_nCOOH$ plays the key role for the DET of cyt *c* [13]. Ulstrup and co-workers discussed the DET of azurin adsorbed on the alkanethiol-modified electrodes. The hydrophobic interaction between the methyl heads of alkanethiol and the hydrophobic areas around the copper atom in azurin is the main source for achieving the fast DET between azurin and the SAM-modified electrode [14].

Hemoglobin (Hb), unlike the mono-cofactor proteins such as cyt *c* and azurin, is a typical multi-cofactor protein that has two heme-containing α- and β-dimmers (αβ) [15]. Although the DET of Hb has been realized on the electrodes modified with various materials such as lipids [16–18], nanomaterials [19,20], ionic liquid [21,22] and clay [23–25], the manipulation of the orientation and

* Corresponding authors.
E-mail addresses: maizhb@mail2.sysu.edu.cn (Z. Mai), daizong@mail.sysu.edu.cn (Z. Dai), ceszxy@mail.sysu.edu.cn (X. Zou).

0039-9140/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2009.11.053

electron transfer pathway of Hb on the modified electrodes, and the comparison of DET behaviors between Hb and other mono-cofactor proteins are not investigated to date.

In this work, the electrochemical behaviors of Hb were studied by adsorbing Hb molecules onto the gold electrodes modified with the SAMs of different alkanethiols. The electrochemical responses of Hb were affected by the properties of SAMs formed from various alkanethiols with different terminal groups and alkyl chain length. The optimal conditions for promoting the DET of Hb on SAMs-modified electrodes were achieved. The current work was expected to be useful for manipulating the interfacial properties of modified electrodes to adapt the DET pathway of Hb.

2. Experimental

2.1. Reagents and materials

Bovine hemoglobin (Hb), succinic acid and mercaptoacetic acid (MAA, >98%) were purchased from Sinopharm Chemical Reagent Co., Ltd. and used as received. 3-Mercaptopropanoic acid (MPA, 99%) and 1-propanethiol (PT, 98%) were obtained from Alfa Aesar. Cysteamine hydrochloride (cys, >97%), 6-mercaptohexanoic acid (MHA, 90%) and 11-mercaptoundecanoic acid (MUA, 95%) were from Sigma–Aldrich. All other chemicals were of analytical grade. The buffer solution used in experiments was 0.1 mol L^{-1} phosphate buffer solution (PBS). The pH values of PBS were regulated with HCl or NaOH solutions. All solutions were prepared with doubly distilled water.

2.2. Preparation of apo-hemoglobin (apo-Hb)

The apo-Hb was prepared by the acid/acetone-extraction method of Rossifanelli et al. and Ascoli et al. with small modification [26–28]. Briefly, after slow addition of 5 mL of 10 mg mL^{-1} Hb solution into 10 mL of cold acetone containing 0.2% HCl and strongly stirred at −20 °C for 30 min, the apo-Hb was formed and precipitated. The heme remained in the acid/acetone phase. Further incubated at 0 °C for 10 min, the precipitated apo-Hb was separated by centrifuging the sample solution at 2500 × *g* at 4 °C. The apo-Hb was then re-dissolved in water and dialyzed against 20 mmol L^{-1} pH 7.0 PBS containing 1 mmol L^{-1} ethylene diamine tetraacetic acid (EDTA).

2.3. Preparation of SAMs-modified gold electrodes (Au/SAMs)

Gold electrodes (Au, 2 mm in diameter) were firstly treated in fleshly prepared piranha solution (the piranha solution is a mixture of sulfuric acid and 30% hydrogen peroxide (3:1, v:v)) for 30 min. (*Safety note: the piranha solution needs to be handled with extreme caution.*) After carefully washing with doubly distilled water, the gold electrodes were polished to the mirror finish mechanically with 0.3 and 0.05 μm alumina powder. The well-polished gold electrodes were cleaned in absolute ethanol and doubly distilled water by sonication for 1 min, respectively. Then, the polished gold electrodes were pretreated electrochemically in 1 mol L^{-1} H_2SO_4 by cyclic scan from +0.2 to +1.6 V for 20 cycles. The Au/SAMs were prepared by immersing the cleaned gold electrodes in 10 mmol L^{-1} desirable thiol solutions for 30 min. The resulting Au/SAMs were then washed by absolute ethanol and doubly distilled water for 1 min to remove physically adsorbed thiol molecules.

2.4. Adsorption of Hb on Au/SAMs

The adsorption of the Hb molecules on Au/SAMs was fulfilled by incubating Au/SAMs in 5 mg mL^{-1} Hb solution for 20 h at 4 °C. The final electrodes were denoted as Au/SAMs/Hb. Before measurements, the Au/SAMs/Hb were carefully rinsed with doubly distilled water.

2.5. Apparatus and measurements

All electrochemical experiments were carried out with a CHI760c electrochemical workstation (Shanghai, China) equipped with a conventional three-electrode cell. An Au/SAM/Hb was used as the working electrode. A platinum wire and a Ag/AgCl electrode (3 mol L^{-1} KCl) were used as the counter electrode and the reference electrode, respectively. The cyclic voltammograms (CVs) of Au/SAMs and Au/SAMs/Hb were obtained in 0.1 mol L^{-1} pH 7.0 PBS. Electrochemical impedance spectroscopy (EIS) was obtained in 5 mmol L^{-1} $K_3Fe(CN)_6$ containing 0.1 mol L^{-1} KCl. The polarized voltage was set as +0.2 V. The frequency range was from 0.1 to 100,000 Hz with amplitude of 5 mV, 12 points per decade. Prior to all electrochemical experiments, the buffer solutions were purged with highly purified nitrogen for 30 min and a nitrogen atmosphere was then maintained above the solution during the measurements.

UV–vis spectra were measured on a UV-3150 spectrometer (Shimadzu, Japan). Fifty milligram per liter Hb in 0.1 mol L^{-1} pH 7.0 PBS containing 1 mmol L^{-1} succinic acid was prepared and allowed to stand 20 h for interaction. For comparison, 50 mg L^{-1} Hb in 0.1 mol L^{-1} pH 7.0 PBS without succinic acid was also prepared in the same manner.

Surface plasmon resonance (SPR) measurements were performed on an Autolab Esprit SPR instrument (Eco Chemie, the Netherlands). A fresh gold disk (Eco Chemie, the Netherlands) was mounted on the SPR cell with two channels. MPA monolayer was prepared by injecting 50 μL of 10 mmol L^{-1} MPA solution into the two channels. The MPA solution was left standing for 30 min and then drained out. The gold disk was washed by absolute ethanol and then 0.1 mol L^{-1} pH 7.0 PBS until a stable baseline of SPR response was obtained. One hundred microliter of 5 mg mL^{-1} Hb in 0.1 mol L^{-1} pH 7.0 PBS with or without 1 mol L^{-1} NaCl were added into the two channels separately. The SPR response was recorded by sensorgrams.

XPS measurements were performed on a Thermo VG Scientific ESCALAB 250 spectrometer with a monochromatic AlKa X-ray source (1486.6 eV photons). The power of X-ray source was kept at 150 W. A clean gold disk was assembled by the thiol molecules and then Hb molecules were adsorbed on the resulting SAMs-modified gold disk. The S 2p and N 1s signals on the gold disk were collected. The curves were analyzed and fitted by XPSPEAK 41 software.

3. Results and discussion

3.1. The formation of SAMs and the adsorption of Hb on Au/SAMs

EIS can provide information on the impedance changes of the electrode surface during the modification process by estimating the interfacial electron transfer resistance from the semicircle diameter of the high frequency region. In this work, EIS was performed to confirm the formation of SAMs and the adsorption of Hb on the resulting Au/SAMs. As shown in Fig. 1, no obvious capacity arc can be found on the bare Au, which indicated that the bare gold electrode exhibited facile electron transfer to the solution redox species [29,30]. However, after bare gold electrodes were immersed in MPA or PT solutions for 30 min, the values of electron transfer resistance (R_{cto}) increased to 163 and 246 Ω with characteristic frequencies of 664.1 and 1758 Hz, respectively (Fig. 1A and B). The larger value of R_{cto} on Au/PT than that of Au/MPA was ascribed to the hydrophobicity of the methyl terminus, which blocked the electron transfer from bulk solution to the electrode

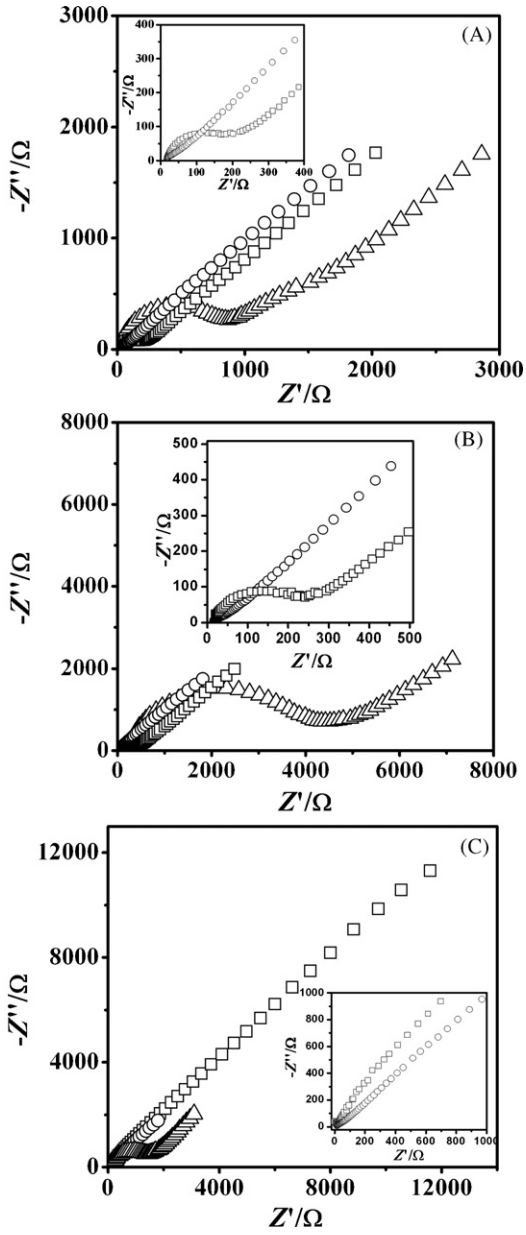

Fig. 1. EIS characterizations of the formation of Au/SAMs/Hb: (A) Au/MPA/Hb, (B) Au/PT/Hb and (C) Au/cys/Hb where symbols represented: (○) bare Au; (□) Au/SAMs and (△) Au/SAMs/Hb, respectively. Inset: comparisons of EIS of Au and Au/SAMs in high frequency region.

surface [31]. Meanwhile, no obvious semicircle can be found in the case of Au/cys, which was caused by the fact that the electrostatic attraction between $-NH_3^+$ and $Fe(CN)_6^{3-}$ would reduce the interfacial resistance (Fig. 1C) [32]. Further incubation of the Au/SAMs in Hb solutions for 20 h, an obvious semicircle can be observed and the values of electron transfer resistance (R_{ct}) were further increased by 766 Ω (Au/MPA/Hb), 4100 (Au/PT/Hb) and 1600 Ω (Au/cys/Hb) with characteristic frequencies of 175.8, 97.66 and 31.5 Hz, respectively. From the impedance spectra, the degree of surface coverage of the Au/SAMs by adsorbed protein was calculated using the following equation:

$$\theta = \frac{R_{ct} - R_{cto}}{R_{ct}} \quad (1)$$

where θ is the surface coverage of Hb on the Au/SAMs, R_{cto} and R_{ct} represents the charge transfer resistance in the absence and the presence of Hb. Accordingly, the values of surface coverage of Hb on the Au/MPA and Au/PT were calculated to be 79% and 94%, respectively. The apparent discrepancies of surface coverage of Hb molecules on Au/MPA and Au/PT were caused by the interfacial properties during the protein incubation process.

XPS was used to monitor the characteristic elements of the assembled Au/SAMs/Hb surface and to confirm formation of S–Au bonds and the adsorption of Hb. The S 2p signals with a spin doublet of 161.5 and 163.0 eV were found in Fig. 2A and C, indicating the formation of MPA and PT monolayers on the gold surfaces. And the N 1s signal of 400.4 eV in XPS spectra can be assigned to the amide linkages and to nitrogen-containing side chains such as lysine and histidine, confirmed the presence of Hb on the surface of Au/SAMs (Fig. 2B and D) [33]. In summary, both EIS and XPS results confirmed the formation of Au/SAMs/Hb.

3.2. Redox behaviors of Hb adsorbed on Au/SAMs with different terminal groups

In this study, three kinds of thiol molecules, including MPA, cys and PT, were chosen to prepare SAMs with typical interfacial properties by different terminal groups of –COOH, $-NH_2$ and $-CH_3$. Since these thiol molecules exhibited the same numbers of methylene groups, the corresponding electrochemical behaviors of adsorbed Hb on these Au/SAMs mainly relied on the head groups of Au/SAMs/Hb.

As shown in Fig. 3, no redox peaks were found on all Au/SAMs in 0.1 mol L^{-1} pH 7.0 PBS (Fig. 3A–C, dash lines). After Hb was adsorbed onto the Au/MPA (Au/MPA/Hb), a pair of well-defined redox peaks with cathodic (E_{pc}) and anodic potentials (E_{pa}) of −289 and −147 mV were observed (Fig. 3A, solid line). The peak-to-peak separation (ΔE_p) was 142 mV, indicating a quasi-reversible electrochemical process. Since no redox peaks were observed at Au/MPA/apo-Hb (Fig. S1, line b), the redox peaks of Au/MPA/Hb were attributed to the redox reaction of heme irons in Hb molecules. It should be noticed that the cathodic and anodic peaks observed on Au/MPA/Hb were asymmetric. Because SAM was a relatively inert interface [34], it cannot promote the DET of Hb to the electrode surface very effectively when compared with other layers which involved promoting factors for fast electron transfer [35]. Meanwhile, the autoxidation of Hb–Fe(II) by residual oxygen in the lower scan rates may be another possibility caused the asymmetric peaks as well [23]. However, the shape of redox peaks became poorer and the asymmetric properties of the redox peaks were not significantly changed at higher scan rates (data not shown), indicating that quasi-reversible electrochemical reaction of Hb was the dominating factor for this observation. When Hb molecules were adsorbed on Au/PT (Au/PT/Hb), only an irreversible reducing process with an E_{pc} of −329 mV was shown (Fig. 3B, solid line). And no redox peaks were observed when Hb was adsorbed onto Au/cys (Fig. 3C, solid line).

The surface concentration of electroactive Hb (Γ^*) was estimated according to the following equation

$$Q = nFA\Gamma^* \quad (2)$$

where Q is the integrated area of the cathodic peak (after background subtraction), A is the electrochemically active area (in this work, the electrochemically active areas of Au/MPA and Au/PT were obtained from the Randles–Sevcik equation: $I_p = 2.69 \times 10^5 A D^{1/2} n^{3/2} v^{1/2} C$, where n is the number of electrons participating in the redox reaction, A is the electrochemical active area of the electrode (cm^2), D is the diffusion coefficient of the $K_4Fe(CN_6)$ molecule (6.70×10^{-6} cm^2 s^{-1} in this work), C is the concentration of the probe molecule in the solution (1 mmol L^{-1} in this work), and v is the scan rate (10 mV s^{-1} in this work). The resulting electrochemically active areas for Au/MPA and Au/PT were yielded to be 0.190 and 0.121 cm^{-2},

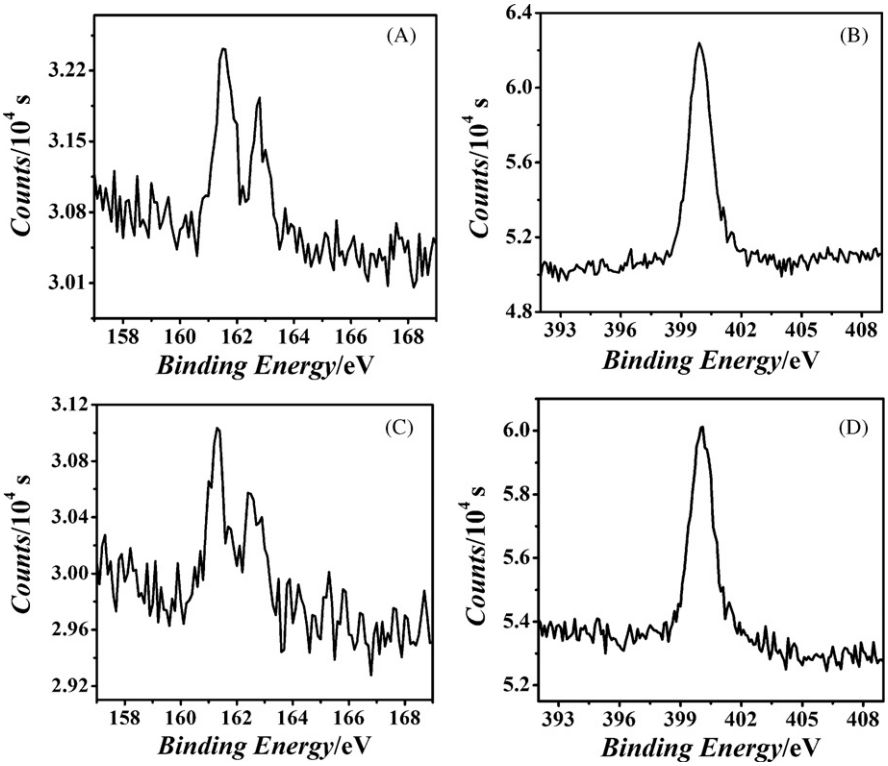

Fig. 2. XPS spectra of Au/SAMs/Hb: the S 2p signal of (A) Au/MPA and (C) Au/PT and the N 1s signal of Hb within (B) Au/MPA/Hb and (D) Au/PT/Hb.

respectively), n is the number of electron transferred, and F is Faraday's constant. Through the integration of the cathodic peak, Γ^* of Hb adsorbed on Au/MPA and Au/PT were estimated to be 17.4 and 45.5 pmol cm^{-2}, respectively.

The experimental results clearly indicated that the terminal groups of SAMs can critically affect the interfacial tropism of adsorbed Hb molecules, and therefore the routes of electron transfer. Previous reports pointed out that the interactions between the residues in protein, functional groups on substrate surface and water would cause the rearrangements of the adsorbed protein on a solid substrate [36,37]. And the degree of the loss of native structure on adsorption of globular proteins depended on the nature of the substrate surface [38]. In this work, a relatively fast DET occurred when Hb molecules were adsorbed on Au/MPA. This observation may be caused by the electrostatic attraction between the positively charged residues of the polypeptide (His-F8) which coordinated with the heme irons of Hb and the negatively charged carboxylic groups within MPA [39]. The electrostatic coupling would shorten the distance between the heme irons and electrode, alter the adsorbed orientation of Hb, promote the electron transfer and thereby increase the electroactive amount of the adsorbed Hb. On the hydrophobic SAM of PT, the adsorbed Hb may go through significant reorientation or conformational change [40] and induce higher population of exposed tyrosine residues, a large number of sulfhydryl groups of Hb, and overall more exposition of the inner hydrophobic core [36]. The possible unfolding of adsorbed Hb may destroy the microenvironment around the heme center, and slow down the electron transfer rate of heme irons to the electrode surface.

The relatively larger electroactive amount of Au/PT/Hb than that of Au/MPA/Hb was ascribed to the strong hydrophobic interaction between Hb and PT SAMs. According to the research of Jiang and co-workers [41], carboxyl-terminated SAM allowed more water molecules to penetrate into the SAM and form hydrogen bonds. Consequently, more energy was required for protein to displace the bound water molecules and then adsorb on the SAM. However, methyl-terminated SAM was highly hydrophobic and very few surface-bound water molecules needed to be replaced upon adsorption on compact or disordered SAM. Therefore, more Hb can be adsorbed onto methyl-terminated SAM under the same temperature. Although the R_{ct} was increased by 1600 Ω in the presence of Hb on cys, the resulting adsorption showed no help to the promotion of electron transfer of heme iron. Cys monolayer cannot induce the adsorption of Hb in a favorable tropism for fast electron transfer.

According to the Laviron's approach for diffusionless thin layer voltammetry [42–44]

$$\log k_s = \alpha \log(1-\alpha) + (1-\alpha)\log\alpha - \log\frac{RT}{nF\upsilon} - \frac{\alpha(1-\alpha)nF\ \Delta E_p}{2.3RT} \quad (3)$$

and taking the charge transfer coefficient α of 0.5, and the scan rate of 0.1 V s^{-1}, the k_s of Hb adsorbed on Au/MPA was 0.49 s^{-1}. However, this value was smaller than those obtained on 3-mercaptopropylphosphonic acid modified three-dimensional Au (15.8 s^{-1}) [45], colloidal clay modified glassy carbon electrode (79 s^{-1}) [23] and linoleic acid (LA) Langmuir-Blodgett (LB) monolayer modified Au (2.68 s^{-1}) [46], but larger than that on the Hb-modified carbon nanotubes powder microelectrodes (0.062 s^{-1}) [47]. The relative low electron transfer rate obtained on Au/MPA/Hb was ascribed to the relatively inert properties of SAM [34], which restricted the further enhancement of the electron transfer when compared with those interfaces with high active area [23,45] or biomembrane-like environment [46]. However, the simpler interface used in promoting the DET of Hb can provide a facile way to have a close look at the basic forces which determine the DET of Hb at the molecular level, via regulating the interfacial interactions and the electron transfer distance.

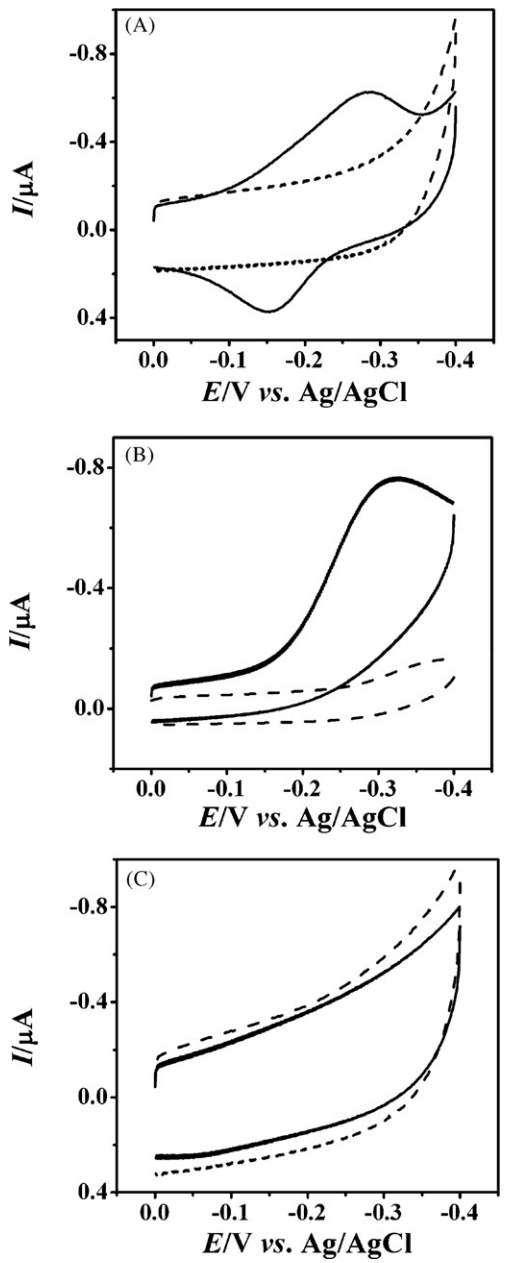

Fig. 3. CVs of Hb adsorbed on SAMs of (A) MPA, (B) PT and (C) cys. The scan rate was 0.1 V s^{-1}. Dash lines represented the background CVs of the corresponding Au/SAMs in 0.1 mol L^{-1} pH 7.0 PBS.

3.3. Electrochemical behaviors of Hb adsorbed on SAMs with carboxyl terminal groups but different methylene numbers (n)

The number of methylene groups in SAMs was another important factor that affected the DET of Hb. The alkyl chain length within thiol molecules not only roughly determined the thickness of SAMs, thus the distance of Hb to the surface of electrode, but also affected the topography and properties of SAMs, such as density and uniformity. In order to investigate the effect of the alkyl chain length within thiol molecules on the DET of adsorbed Hb, four kinds of thiol molecules, including MAA, MPA, MHA and MUA were selected to prepare Au/SAMs with the same terminal group of –COOH but different methylene numbers of 1, 2, 5 and 10.

When Hb molecules were adsorbed on Au/MAA ($n = 1$), a weak redox response with a wide peak-to-peak separation was observed (Fig. 4A). This result suggested that the adsorption of Hb on MAA resulted in a slow electron transfer from Hb to electrode. In principle, decreasing the alkyl chain length of SAMs could shorten the distance between the electron donor and acceptor, and therefore facilitate the electron transfer. However, when MAA was assembled on the surface of electrode, the van der Waals interactions among alkyl chains were relatively small due to the short alkyl chain of MAA molecules. The electron-rich sulfur atoms in MAA monolayer strongly interacted with the α carbon and the carboxylic group, causing the carboxylic group more difficult to be ionized [48]. The decrease of possible electrostatic interaction between Hb and MAA monolayer may lead to a change of the adsorbed orientation of Hb on MAA, which induced a bias from the optimal tropism for DET.

When Hb was adsorbed on Au/MPA ($n = 2$) and Au/MHA ($n = 5$), quasi-reversible redox processes were observed (Fig. 4B and C). According to the Laviron model [44], the corresponding k_s values on Au/MPA/Hb and Au/MHA/Hb were estimated to be 0.49 and 0.47 s^{-1}, respectively. Although the number of methylene groups of MHA monolayer was larger than that of MPA monolayer, the k_s values of Hb on these two SAMs were almost the same. Waldeck and co-workers had investigated the DET of horse heart cyt *c* that interacted with SAMs of carboxylic terminus. The results revealed that comparable values of k_s (ca. 10^3 s^{-1}) were obtained from DET of cyt *c* in the short chain region and such plateau region was called solvent-controlled regime [49]. Although the electron transfer of Hb to Au/MPA was much slower than that of cyt *c*, comparable plateau regime was obtained as well. The much slower electron transfer rate of Hb on the Au/MPA when compared with the case of cyt *c* may be ascribed to the more complicated molecular structure of Hb. As reported earlier [50,51], the heme irons of native Hb are deeply buried in the hydrophobic pockets of Hb with a fifth coordinative bond with the residue of the polypeptide (His-F8). In addition, there are van der Waals bindings between atoms on the porphyrin ring and about 60 atoms of the polypeptide chain in each subunit. Such large steric hindrance of native Hb resulted in the slower electron transfer of Hb to the electrode surface. When the DET of Hb was induced by the short chain SAMs with carboxylic terminus, it was expected that the sampling of the heme centers of adsorbed Hb may become the rate limiting process at short electron donor–acceptor distance, which made the k_s insensitive to the increase of the length of thiol molecules in the short chain region [49].

When Hb was adsorbed on Au/MUA ($n = 10$), an irreversible electrochemical response with a significant decrease of k_s was found (Fig. 4D). Unlike MAA molecules which was composed of short alkyl chain, the van der Waals interactions among alkyl chains in SAM of MUA were relatively strong and the resulting SAM was much compact. The longer alkyl chain length of SAM can enlarge the distance of electron transfer pathway, leading to the high resistance to the electron transfer. Meanwhile, the alternation of surface properties of carboxylic terminus within SAM can be another factor. As reported previously, the surface pK_a of $HS(CH_2)_nCOOH$ increased in concomitance with the increasing alkyl chain length ($n > 2$). When *n* was ten, the surface pK_a of MUA was estimated to be 7.3 [48]. Since the isoelectric point (p*I*) of Hb is 7.4 [52], the electrostatic attraction between MUA and Hb was weakened in the pH 7.0 PBS. The weaker electrostatic coupling of MUA and Hb may induce the adsorbing tropism of Hb bias from the optimal orientation for DET. The slow electron transfer rate of Hb on MUA made the Laviron model inappropriate to estimate the k_s. The dramatically decrease of k_s of Hb was comparable to the case of cyt *c* in the long alkyl chain SAMs [49], but the origination of such result was more complicated in the case of Hb. It was expected that elongating the alkyl chain length within SAMs can not only largely increase the distance between the electron donor and acceptor, but also alter the surface properties of SAM, accordingly decreased the corresponding electron transfer from Hb to Au/SAMs.

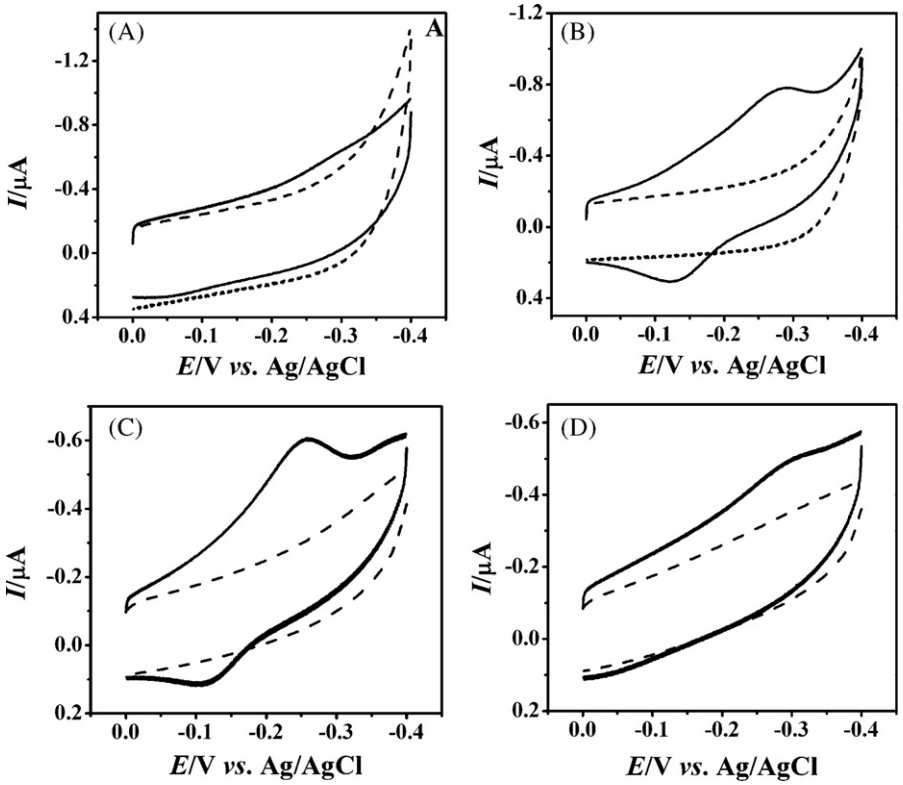

Fig. 4. CVs of Hb adsorbed on SAMs of (A) MAA, (B) MPA, (C) MHA and (D) MUA. The scan rate was 0.1 V s^{-1}. Dash lines represented background CVs of the corresponding Au/SAMs in 0.1 mol L^{-1} pH 7.0 PBS.

Based on these observations, the alkyl chain length and the terminal groups of SAMs can critically affect the electron transfer pathway of Hb on Au/SAMs. Only the SAM of MPA can provide suitable properties to keep Hb in favorable adsorption state for DET. Therefore, the electrochemical behaviors of Hb adsorbed on Au/MPA were further investigated in detail.

3.4. The electrochemical behaviors of Hb adsorbed on Au/MPA

3.4.1. The effects of the self-assembly time of MPA and the adsorption time of Hb on the electrochemical behaviors of Au/MPA/Hb

The effect of the self-assembly time of MPA on the DET of Au/MPA/Hb was investigated (Fig. 5A). The direct electrochemical response decreased with the increasing assembly time of MPA from 30 min to 31 h. The relative standard variations of the I_{pc} during the different self-assembling time of MPA (for the electrode-to-electrode) were 11, 7.3, 7.9, 9.4 and 2.9% for self-assembling time of 15 min, 30 min, 6 h, 12 h and 31 h, respectively. Previous report indicated that the formation of a monolayer for MPA on Au was rather fast at the beginning of the assembling process [53]. Extending the assembly time can lead to a well-ordered and compact SAM that can provide more coupling sites for Hb. However, unicomponent SAM will cause less restricted conformational mobility of tethered protein and thereby slow down the electron transfer of the protein [54]. When the assembly time of MPA SAM was properly short, the SAM contained lots of defects, which could decrease the density of carboxylic groups by allowing the penetration of water molecules into these defects [41]. Therefore, when the uniformity of the resulting SAM was properly decreased, the conformational mobility of tethered protein and the electroactive amount of Hb would be increased. To shorten the assembly time of MPA less than 30 min also caused the decrease of the electrochemical response of Au/MPA/Hb as well. In this case, the surface of Au may not be fully occupied by MPA molecules during such a short self-assembly time. The surface of a freshly polished gold electrode was positively

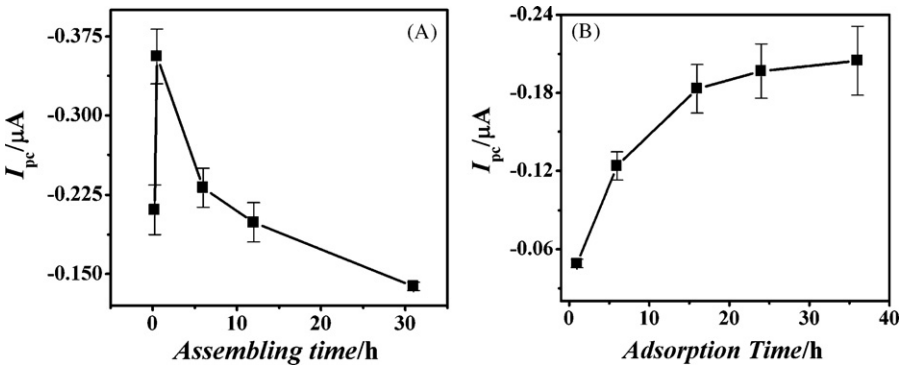

Fig. 5. The influences of (A) self-assembly time of MPA and (B) adsorption time of Hb on the current response of Au/MPA/Hb.

charged. These excess positive charges tended to prevent the electropositive heme cavity of Hb from getting close to the electrode through coulombic force, thus the heme crevice of Hb lied distal to the electrode surface [55]. Therefore, 30 min was chosen as the self-assembly time in the following work.

The changes of the direct electrochemical response of Au/MPA/Hb with the adsorption time of Hb on Au/MPA were also considered (Fig. 5B). The direct electrochemical response of Au/MPA/Hb increased from the adsorption time of 1–16 h, and leveled off after the adsorption time exceeded 16 h. The results indicated that only the Hb molecules near the electrode surface in a thin layer could undergo direct electrochemistry [56]. The increasing electrochemical response in concomitance with the adsorption time was not only ascribed to the increasing adsorbing amount of Hb and but also the probable conformational change during such process. As reported previously, adsorption of the protein onto the synthetic surface was a thermodynamically driven process, and such process was generally involved by non-covalent interactions, including hydrophobic interactions, electrostatic forces, hydrogen bonding and van der Waals forces [40]. According to the empirical models and the results of microfabricated cantilever sensors, it has been proved that the adsorbed proteins would undergo slow conformational changes (e.g., protein denaturation) as a function of time and conditions [57,58]. In this work, it was expected that the direct electron transfer was induced by the electrostatic coupling between amino residues of Hb and MPA [39]. As the interaction time increased, such conformational change would be more effective, leading to more electroactive amount of adsorbed Hb and consequently the enhancement of current response. Therefore, the Au/MPA/Hb was prepared by incubating Au/MPA in 5 mg mL^{-1} Hb solution for 20 h.

3.4.2. The interaction between Hb and MPA

The surface pK_a of the –COOH in the Au/MPA, determined by electrochemical titration method using $K_3Fe(CN)_6$ as the electrochemical probe [48], was estimated to be 6.6 (Fig. 6A, curve b). Since the isoelectric point (p*I*) of Hb is 7.4 [52], Hb has net positive charges in pH 7.0 PBS, while the –COOH groups in the Au/MPA are deprotonated. Therefore, the electrostatic attraction may occur between Hb and –COOH of the Au/MPA.

The possible electrostatic attraction between Hb and Au/MPA was also investigated by SPR. As shown in Fig. 6B, the adsorption of the Hb on the MPA monolayer from 5 mg mL^{-1} Hb in 0.1 mol L^{-1} pH 7.0 PBS caused an 828.73 m° increase of the SPR angle (Fig. 6B solid line). However, when the Hb solution was prepared by 0.1 mol L^{-1} pH 7.0 PBS containing 1 mol L^{-1} NaCl, the adsorption of the Hb on the MPA SAM only caused a 504.1 m° increase of the SPR angle (Fig. 6B dash line). The results were in agreement with those described in references [59,60], which indicated that the adsorption of Hb on MPA was predominated by electrostatic interaction.

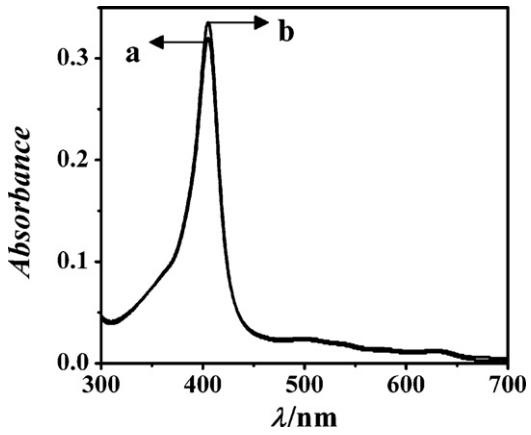

Fig. 7. UV–vis characterizations of Hb in the (a) presence and (b) absence of 1 mmol L^{-1} succinic acid.

Previous study showed that Hb can shed its metal center under certain conditions. To rule out such possibility, the possible interactions between Hb and MPA were also characterized by UV–vis spectroscopy [61]. Succinic acid, which has two carboxyl groups in its molecule, was added into the prepared Hb solution. As can be seen from Fig. 7, the absorption of Soret band showed no obvious changes before and after the addition of succinic acid into the Hb solutions. Such results clearly confirmed that Hb did not shed its iron center in the presence of carboxylic group. Therefore, Hb contacting MPA can directly exchange electrons with the electrode [62].

3.4.3. The influence of pH on the direct electrochemical response of Au/MPA/Hb

It was reported that solution pH influenced the redox potentials of Hb through modulating the accessibility of water into the heme pocket of Hb, and also the protonation of heme iron-bound proximal histidine and/or the distal histidine in the heme pocket [23]. As shown in Fig. 8A, the $E^{\circ\prime}$ of Hb shifted linearly with a slope of −69.7 mV pH^{-1} in the pH range from 5.12 to 9.36, indicating that one proton and one electron participated in the redox reaction of Hb. As the pH decreased, ΔE_p of the heme iron increased, indicating the electron transfer rate of Hb adsorbed on MPA monolayer was pH dependent (Fig. 8B). When the pH reached 5.12, the I_{pc} significantly

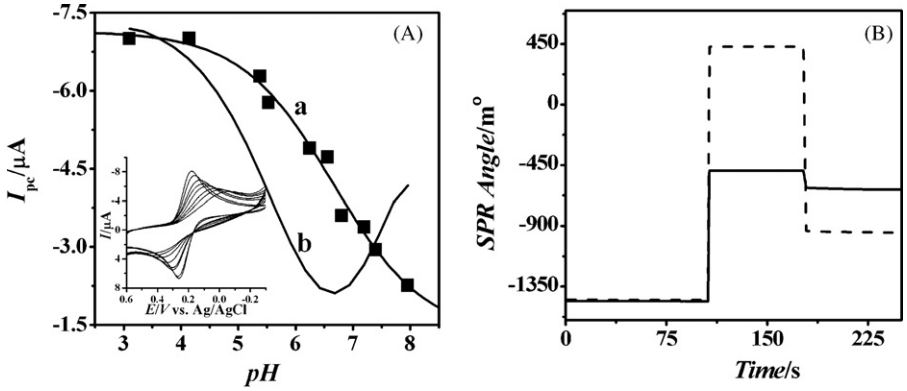

Fig. 6. The characterizations of interactions between Hb and MPA: (A) Electrochemical titration plots of Au/MPA in 1 mmol L^{-1} $K_3Fe(CN)_6$ at pH 3.1, 4.15, 5.38, 5.53, 6.25, 6.56, 6.81, 7.2, 7.4, and 7.96 at 0.1 V s^{-1}, where (a) electrochemical titration curve and (b) differential curves; (B) SPR characterizations of Hb adsorbed onto Au/MPA in the absence and presence of Cl^-: (solid line) Hb without Cl^- and (dash line) Hb with 1 mol L^{-1} Cl^-.

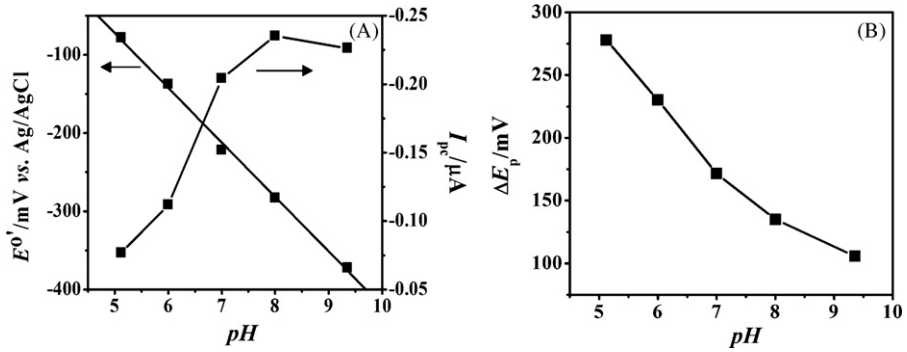

Fig. 8. The influences of pH on the electrochemical behaviors of Hb: (A) plots of I_{pc} and $E^{\circ\prime}$ vs. pH; (B) plots of ΔE_p vs. pH.

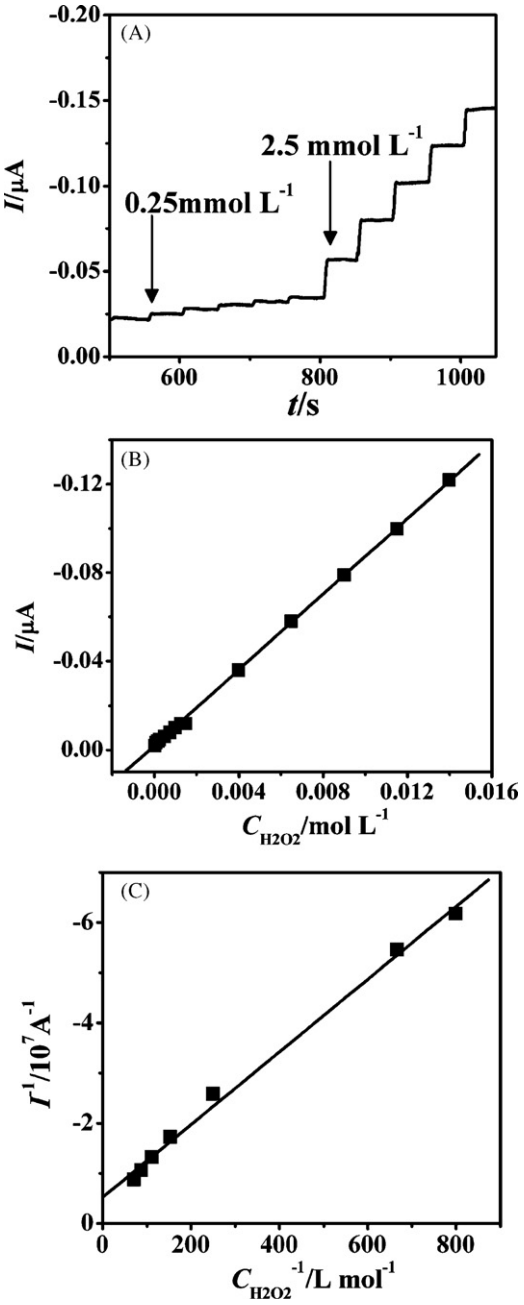

Fig. 9. Electrocatalytic activity of Au/MPA/Hb to H_2O_2: (A) steady-state current response of Au/MPA/Hb on successive injection of H_2O_2 into 5 mL stirring PBS, applied potential: −0.2 V, (B) corresponding calibration curve and (C) the calculation of Michaelis–Menten constant.

decreased. According to the modern theories of the heterogeneous electron transfer [63], the entry of highly polar water molecules to the heme cavity should increase the barrier for the electron transfer by ca. 50% [64]. Therefore, the reduction of external pH may cause the entrance of water to the heme cavity, which inhibited the electron transfer of Hb.

3.4.4. Electrocatalytic activity of the Hb adsorbed on Au/MPA toward H_2O_2

The electrocatalytic activity of Au/MPA/Hb to hydrogen peroxide (H_2O_2) was also investigated (Fig. 9). The Au/MPA/Hb was used for directly detecting H_2O_2 and a stepwise growth of reduction current on Au/MPA/Hb was observed with the successive additions of H_2O_2 (Fig. 9A). The linear range for detecting H_2O_2 was from 1.0×10^{-4} to 1.6×10^{-2} mol L^{-1} ($R = 0.9998$). The detection limit was 7.3×10^{-5} mol L^{-1} (S/N > 3) (Fig. 9B). Furthermore, the apparent Michaelis–Menten constant (K_m), according to the Lineweaver-Burk equation [65], was estimated to be 13 mmol L^{-1} (Fig. 9C).

4. Conclusions

In principle, the modulation of the electron transfer pathway of multi-cofactor protein should be more complicated to its mono-cofactor counterpart. In this work, Hb was chosen as the model protein with multi-cofactor and its electron transfer pathway was modulated by different kinds of SAMs. The terminal groups and the alkyl chain length of alkanethiols can critically affect the electron transfer pathways of Hb. The experimental results revealed that the SAMs terminated by carboxylic group can induce the direct electron transfer of Hb. Meanwhile, the electron transfer of Hb adsorbed onto carboxylic terminated SAMs with various alkyl chain lengths experienced a plateau region and a tunneling region, which was comparable to the case of cyt *c*. Further investigations confirmed that the DET of Hb obtained in Au/MPA/Hb was induced by the electrostatic interaction between Hb and the carboxylic group of MPA. The current work was expected to be useful for providing a strategy on manipulating the electron transfer pathway of Hb.

Acknowledgments

We gratefully acknowledge to the financial support by the National Natural Science Foundation of China (No. 20575082, 20805059, 20975117), the Natural Science Foundation of Guangdong Province (No. 7003714), the Project Sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholars of State Education Ministry, and the Ph.D. Programs Foundation of Ministry of Education of China (No. 20070558010).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2009.11.053.

References

[1] Y.H. Wu, S.S. Hu, Microchim. Acta 159 (2007) 1.
[2] W. Schuhmann, Rev. Mol. Biotechnol. 82 (2002) 425.
[3] S. Zhang, G. Wright, Y. Yang, Biosens. Bioelectron. 15 (2000) 273.
[4] V.V. Shumyantseva, T.V. Bulko, A.I. Archakov, J. Inorg. Biochem. 99 (2005) 1051.
[5] H.J. Yue, D.H. Waldeck, Curr. Opin. Solid State Mater. Sci. 9 (2005) 28–36.
[6] J.F. Rusling, R.J. Forster, J. Colloid Interface Sci. 262 (2003) 1.
[7] N. Li, J.Z. Xu, H. Yao, J.J. Zhu, H.Y. Chen, J. Phys. Chem. B 110 (2006) 11561.
[8] S.F. Wang, F. Xie, G.D. Liu, Talanta 77 (2009) 1343.
[9] N. Zheng, X. Zhou, W.Y. Yang, X.J. Li, Z.B. Yuan, Talanta 79 (2009) 780.
[10] W. Zheng, Y.F. Zheng, K.W. Jin, N. Wang, Talanta 74 (2008) 1414.
[11] X.X. Chen, R. Ferrigno, J. Yang, G.M. Whitesides, Langmuir 18 (2002) 7009.
[12] Y. Chen, X.J. Yang, L.R. Guo, B. Jin, X.H. Xia, L.M. Zheng, Talanta 78 (2009) 248.
[13] K. Niki, W.R. Hardy, M.G. Hill, H. Li, J.R. Sprinkle, E. Margoliash, K. Fujita, R. Tanimura, N. Nakamura, H. Ohno, J.H. Richards, H.B. Gray, J. Phys. Chem. B 107 (2003) 9947.
[14] Q. Chi, J.D. Zhang, J.E.T. Andersen, J. Ulstrup, J. Phys. Chem. B 105 (2001) 4669.
[15] L. Stryer, Biochemistry, Spektrum Akademischer Verlag, Heidelberg, 2003.
[16] E.V. Ivanova, E. Magner, Electrochem. Commun. 7 (2005) 323.
[17] Z.Q. Lu, Q.D. Huang, J.F. Rusling, J. Electroanal. Chem. 423 (1997) 59.
[18] D. Mimica, J.H. Zagal, F. Bedioui, J. Electroanal. Chem. 497 (2001) 106.
[19] L. Zhang, X. Jiang, E.K. Wang, S.J. Dong, Biosens. Bioelectron. 21 (2005) 337.
[20] H.Y. Liu, N.F. Hu, J. Phys. Chem. B 109 (2005) 10464.
[21] X.B. Lu, J.Q. Hu, X. Yao, Z.P. Wang, J.H. Li, Biomacromolecules 7 (2006) 975.
[22] A. Safavi, N. Maleki, O. Moradlou, M. Sorouri, Electrochem. Commun. 10 (2008) 420.
[23] C.H. Lei, U. Wollenberger, N. Bistolas, A. Guiseppi-Elie, F. Scheller, Anal. Bioanal. Chem. 372 (2002) 235.
[24] Y. Liu, H.Y. Liu, N.F. Hu, Biophys. Chem. 117 (2005) 27.
[25] J.M. Xu, W. Li, Q.F. Yin, H. Zhong, Y.L. Zhu, L.T. Jin, J. Colloid Interface Sci. 315 (2007) 170.
[26] A. Rossifanelli, E. Antonini, A. Caputo, Adv. Protein Chem. 19 (1964) 73.
[27] M. Marta, M. Patamia, A. Lupi, M. Antenucci, M. Di Iorio, S. Romeo, R. Petruzzelli, M. Pomponi, B. Giardina, J. Biol. Chem. 271 (1996) 7473.
[28] F. Ascoli, M.R. Fanelli, E. Antonini, Methods Enzymol. 76 (1981) 72.
[29] J. Wang, M.A. Firestone, O. Auciello, J.A. Carlisle, Langmuir 20 (2004) 11450.
[30] L.P. Lu, X.Q. Lin, Anal. Sci. 20 (2004) 527.
[31] I. Vlassiouk, C.D. Park, S.A. Vail, D. Gust, S. Smirnov, Nano Lett. 6 (2006) 1013.
[32] V. Molinero, E.J. Calvo, J. Electroanal. Chem. 445 (1998) 17.
[33] Y. Chen, B. Jin, L.R. Guo, X.J. Yang, W. Chen, G. Gu, L.M. Zheng, X.H. Xia, Chem. Eur. J. 14 (2008) 10727.
[34] Y.Y. Luk, M. Kato, M. Mrksich, Langmuir 16 (2000) 9604.
[35] F.W. Scheller, N. Bistolas, S. Liu, M. Janchen, M. Katterle, U. Wollenberger, Adv. Colloid Interface Sci. 116 (2005) 111.
[36] J. Bhattacharya, S. Jasrapuria, T. Sarkar, R.G. Moulick, A.K. Dasgupta, Nanomedicine: NBM 3 (2007) 14.
[37] A. Sethuraman, G. Belfort, Biophys. J. 88 (2005) 1322.
[38] C.A. Haynes, W. Norde, J. Colloid Interface Sci. 169 (1995) 313.
[39] X.C. Li, W. Zheng, L.M. Zhang, P. Yu, Y.Q. Lin, L. Su, L.Q. Mao, Anal. Chem. 81 (2009) 8557.
[40] K.E. Michael, V.N. Vernekar, B.G. Keselowsky, J.C. Meredith, R.A. Latour, A.J. García, Langmuir 19 (2003) 8033.
[41] L.Y. Li, S.F. Chen, S.Y. Jiang, Langmuir 19 (2003) 2974.
[42] E. Topoglidis, Y. Astuti, F. Duriaux, M. Gratzel, J.R. Durrant, Langmuir 19 (2003) 6894.
[43] M. Song, L.Q. Ge, X.M. Wang, J. Electroanal. Chem. 617 (2008) 149.
[44] E. Laviron, J. Electroanal. Chem. 101 (1979) 19.
[45] Y. Chen, X.J. Yang, L.R. Guo, J. Li, X.H. Xia, L.M. Zheng, Anal. Chim. Acta 644 (2009) 83.
[46] F. Yin, H.K. Shin, Y.S. Kwon, Biosens. Bioelectron. 21 (2005) 21.
[47] Y.D. Zhao, Y.H. Bi, W.D. Zhang, Q.M. Luo, Talanta 65 (2005) 489.
[48] Z. Dai, H.X. Ju, Phys. Chem. Chem. Phys. 3 (2001) 3769.
[49] J.J. Wei, H. Liu, K. Niki, E. Margoliash, D.H. Waldeck, J. Phys. Chem. B 108 (2004) 16912.
[50] P.H. Rogers, A. Arnone, Biochemistry 39 (2000) 15353.
[51] D.J. Wilson, G.A. Lajoie, A. Doherty-Kirby, L. Konermann, Biochemistry 43 (2004) 14792.
[52] J.B. Matthew, G.I.H. Hanania, F.R.N. Gurd, Biochemistry 18 (1979) 1919.
[53] M.J. Giz, B. Duong, N.J. Tao, J. Electroanal. Chem. 465 (1999) 72.
[54] T.D. Dolidze, S. Rondinini, A. Vertova, D.H. Waldeck, D.E. Khoshtariya, Biopolymers 87 (2007) 68.
[55] T.H. Lu, X.J. Yu, S.J. Dong, C.L. Zhou, S.Y. Ye, T.M. Cotton, J. Electroanal. Chem. 369 (1994) 79.
[56] C. Guo, F. Hu, C.M. Li, P.K. Shen, Biosens. Bioelectron. 24 (2008) 819.
[57] A.M. Moulin, S.J. O'Shea, R.A. Badley, P. Doyle, M.E. Welland, Langmuir 15 (1999) 8776.
[58] M. Mrksich, G.M. Whitesides, Annu. Rev. Biophys. Biomol. Struct. 25 (1996) 55.
[59] A. Kassab, H. Yavuz, M. Odabasi, A. Denizli, J. Chromatogr. B 746 (2000) 123.
[60] R.B.M. Schasfoort, A.J. Tudos, Handbook of Surface Plasmon Resonance, The Royal Society of Chemistry Press, Cambridge, 2008.
[61] E.V. Milsom, H.A. Dash, T.A. Jenkins, C.M. Halliwell, A. Thetford, N. Bligh, W. Nogala, M. Opallo, F. Marken, J. Electroanal. Chem. 610 (2007) 28.
[62] Y.Y. Song, W.Z. Jia, C. Yang, X.H. Xia, Adv. Funct. Mater. 17 (2007) 2377.
[63] R.A. Marcus, N. Sutin, Biochim. Biophys. Acta (BBA) Rev. Bioenerg. 811 (1985) 265.
[64] M. Fedurco, J. Augustynski, C. Indiani, G. Smulevich, M. Antalik, M. Bano, E. Sedlak, M.C. Glascock, J.H. Dawson, J. Am. Chem. Soc. 127 (2005) 7638.
[65] R.A. Kamin, G.S. Wilson, Anal. Chem. 52 (1980) 1198.