Surface-enhanced infrared absorption spectroscopy and electrochemistry reveal the impact of nanoparticles on the function of protein immobilized on mimic biointerface

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Abstract

Study of the interactions between engineered nanoparticles and biomolecules plays an important role in understanding nano-bio effect. However, most of studies focus on the interaction of protein with nanoparticle in bulk phase, the impact of nanoparticle on the function of protein immobilized on the biological interface has been ignored for a long time. In this paper, we study the effect of SiO₂ NPs on electron transfer function of cytochrome *c* (cyt *c*) adsorbed onto the 11-mercaptoundecanoic acid (MUA) self-assembled monolayer (SAM) by surface-enhanced infrared absorption spectroscopy (SEIRAS) combined with electrochemistry. Our results disclose that instead of inhibiting the electron transfer, exposure of SiO₂ NPs enhances the redox reversibility, electron transfer rate, and catalytic performance of adsorbed cyt *c*, but induces a slight negative shift in the formal potential of the adsorbed cyt *c*. Interaction of SiO₂ NPs slightly induces the structural changes of the β -sheet in the oxidized form and the change of microenvironment surrounding them, which could further change the microenvironment and the orientation of heme, facilitating the hydration of cyt *c* at the oxidized state. It could be the enhanced hydration of cyt *c* (Fe³⁺) and a smaller change in the total hydration from reduced to oxidized state that results in the slight negative shift in the formal potential and promotes the electron transfer capability. Besides, cyt *c*'s slight conformational change and the disturbance to the microenvironment of heme resulting from the adsorption of SiO₂ NPs could also be one of the reason that leads to the enhanced catalytic capability towards the reduction of H₂O₂.

Keywords: Cytochrome *c*; SiO₂ nanoparticles; Electron transfer; Surface-enhanced infrared adsorption spectroscopy

1. Introduction

Rapid translation of nanomaterial into the field of biomedicine strengthens the significance in the study of the interactions between engineered nanoparticles and biomolecules due to its relationship with the therapeutic effect and potential cytotoxicity [1]. Upon the exposure of nanomaterials into biological milieu, the formation of nanoparticle–protein corona complexes driven by high surface free energy of nanoparticles not only provides the new biological identity of nanoparticles but also disturbs the conformation and function of the proteins. Therefore, the biological impacts might be caused by both the composition of the protein corona and disturbance to the conformation of the proteins [2]. For instance, protein corona will affect the cellular binding capability of the nanoparticles [3], playing a protective role with respect to the toxic effects of the nanomaterials [4]. Nanoparticle-induced protein unfolding can lead to activation of the Mac-1 receptor and inflammation [5]. Up to now, people have tried to identify the composition of the protein corona, to quantify adsorption kinetics of proteins [6], and to study the conformational changes of adsorbed protein [7, 8]. Nevertheless, most of studies focus on the interaction of protein with nanoparticle in bulk phase where protein and nanoparticle have greater freedom to interact with each other, a long-ignored issue is the impact of nanoparticle on the function of protein immobilized on the biological interface.

Cyt *c* is a basic redox heme protein which plays an important role in the biological respiratory chain. It consists of a single polypeptide and a heme. In the native conformation, the heme moiety is covalently bound by two thioether bridges to two cysteine residues (Cys14 and Cys17) and axially liganded with His18 and Met80 [9]. To form these bonds, the heme is embedded in the hydrophobic interior of the globular polypeptide, leaving an edge exposed to solvent. The electron transfer of cyt *c* is believed to occur at the edge of the heme [10]. In mitochondria, cyt *c*

exists in the intermembrane space and transfers electrons from cytochrome bc₁ complex (III) to cytochrome *c* oxidase by transiently binding to the membrane proteins at specific adsorption sites [11]. Its electron transfer function has been extensively studied by direct electrochemistry of cyt *c* adsorbed on alkanethiols-modified self-assembled monolayer (SAM) with terminal carboxylate groups as its binding partner mimics [12]. Therefore, it is a good research system to study the influence of nanoparticles on the electron transfer of cyt *c* adsorbed onto biomimic membrane.

Surface-enhanced infrared adsorption spectroscopy (SEIRAS) is an effective detection method to study the secondary structure of proteins in a super-thin layer adsorbed onto a nano-Au film [13-15]. The vibration of the adsorbed molecules or proteins can be enhanced by more than 2 orders of magnitude, which solves the problem in poor sensitivity of conventional IR spectroscopy. Moreover, SEIRAS not only can in-situ and real-time monitor adsorption of proteins onto biomimic membranes [13, 15, 16], but also can detect the change in the adsorption properties of proteins and biomimic membranes induced by interaction with nanoparticles [17]. By combining with electrochemistry, potential-induced SEIRA difference spectroscopy can finely reflect some very small changes of the secondary structure and component groups of redox proteins during the process of electron acceptance and donation [12, 18]. It is a more advanced detection method of protein secondary structure than SEIRAS itself, since SEIRAS can only express an integrated contour of the secondary structure and some component groups of the protein. According to the surface selection rule and optical near-field effect [19], one can reason out the adsorption conformation and orientation of the protein.

Silica nanoparticles (SiO₂-NPs) have been extensively applied in food and cosmetics as additives, and in biomedical fields, including bio-imaging, bio-sensing, bio-catalysis, drug

delivery, and controlled release because of the unique properties such as easy synthesis, controllable particles scale, large surface area, available surface modification, and high drug loading efficiency [20, 21]. Such wide applications have also raised concerns with the bio-safety of SiO₂-NPs when they are released into the biological milieu. In this paper, we study the effect of SiO₂-NPs on electron transfer function and electrocatalytic behavior of cyt *c* adsorbed onto the 11-mercaptopundecanoic acid (MUA) self-assembled monolayer (SAM) by SEIRAS combined with electrochemistry. It is found that instead of inhibiting the electron transfer, exposure of SiO₂-NPs promotes the electron transfer and electrocatalytic capability of adsorbed cyt *c* by changing the adsorption orientation and microenvironment of cyt *c*'s heme.

2. Experimental

2.1. Synthesis and Characterization of SiO₂ NPs

For the preparation of silicon nanoparticles (SiO₂ NPs), 500 mL of ethanol, 20 mL of deionized water, 20 mL of ammonium hydroxide (23 wt% NH₃), and 30 mL of TEOS were mixed and then the mixture was vigorously stirred for 24 hours until white suspension can be observed. The suspension was centrifuged at a speed of 8000 rev/min for 10 min, the supernatant was discarded and the precipitate was re-dispersed with deionized water. The centrifugation and re-dispersion were repeated for 5 times. Then the precipitate was placed in a vacuum oven for drying until the white powder of silicon nanoparticles (SiO₂ NPs) was obtained [22]. The as-prepared SiO₂ NPs were characterized with transmission electron microscopy (TEM) on an H-600 electron microscope (Hitachi, Japan) and dynamic light scattering (ZEN 3600, Malvern Instrument, UK).

2.2. Electrochemical Measurements

Electrochemical measurements were performed with a CHI 830A electrochemical workstation (CH Instruments, Austin, TX) in a conventional three-electrode cell. Modified Au electrodes (2 mm diameter) were used as the working electrodes, a platinum wire as the counter electrode and an Ag/AgCl with saturated KCl solution as the reference electrode. To prepare an electron transfer biomimic membrane electrode, a cleaned Au electrode was immersed in 0.1 mM MUA ethanol solution for 90 min, forming a SAM of MUA on Au electrode (MUA/Au). After thorough rinsing with ethanol and water, the MUA/Au was incubated in 2 μ M cyt *c* dissolved in 10 mM phosphate buffer solution (PBS) at pH 7.0 for 60 min to obtain cyt *c*-adsorbed electrode (cyt *c*/MUA/Au). After rinsing with deionized water, the cyt *c*/MUA/Au was incubated in the SiO₂ NPs aqueous dispersion for 60 min, obtaining SiO₂ NPs/cyt *c*/MUA/Au. For both cyt *c*/MUA/Au and SiO₂ NPs/cyt *c*/MUA/Au, after being taken out of their incubation solution or suspension, cyclic voltammetry was performed in 10 mM PBS (pH 7.0) before and after addition of 60 μ M H₂O₂ to electrolyte solutions, respectively. The PBS solution was purged with high-purity N₂ gas for 60 min to eliminate dissolved oxygen before each electrochemical measurement.

2.3. Surface-Enhanced Infrared Absorption Spectroscopy (SEIRAS) and Potential-Induced SEIRA Difference Spectroscopy

The flat surface of a triangular Si prism was polished with 1.0 μ m Al₂O₃ slurry and then washed thoroughly with deionized water. The cleaned Si substrate was treated by immersion in a 40 wt% aqueous solution of NH₄F for 1 min. A thin gold film was prepared on the treated Si prism by chemical deposition [23]. The flat surface of the treated Si substrate was exposed to a 1:1:1 volume mixture of (1) 0.03 M NaAuCl₄, (2) 0.3 M Na₂SO₃ + 0.1 M Na₂S₂O₃ + 0.1 M NH₄Cl, and (3) 2.5 vol % HF solution for 90 s. After being rinsed with deionized water, the Si prism was transferred into a 0.1 M H₂SO₄ solution. The gold film surface was cleaned by several

oxidation-reduction cycles in the range between 0.1 and 1.4 V (vs Ag/AgCl). After the electrochemical cleaning, the Au-coated Si prism was mounted into a poly(trifluorochloroethylene) cell with a Viton O ring. A Cu plate serves as the electric contact to the gold film, and the Au/Si substrate was also used as a working electrode. The reflected signals were detected from the Si/Au thin-film/solution interface. The reference spectrum was first recorded of blank ethanol. Concomitant with the addition of 0.1 mM MUA into the ethanol, sample spectra were continuously scanned within 90 min. Upon exposure of the MUA/Au to 2 μ M cyt *c* in PBS (10 mM, pH 7.0), the adsorption of cyt *c* was monitored with 10 mM PBS (pH 7.0) subtracted as background. For each spectrum, 512 scans were collected with a spectral resolution of 4 cm^{-1} . For investigation of the SiO₂ NPs exposure, cyt *c*/MUA/Au incubated in 10 mM PBS was recorded as reference, and sample spectra were recorded upon the addition of SiO₂ NPs. For the potential-induced SEIRA difference spectra of cyt *c*/MUA/Au or SiO₂ NPs/cyt *c*/MUA/Au in 10 mM PBS (pH 7.0), a reference SEIRA spectrum was taken at -0.2 V, where the adsorbed cyt *c* is fully reduced, and a series of sample spectra were acquired from -0.1 to +0.2 V, where the adsorbed cyt *c* is gradually until fully oxidized. The whole procedure was repeated nine times, and the difference spectra were averaged to improve the signal-to noise ratio.

3. Results and discussion

3.1. Characterizations of SiO₂ NPs

The morphology and size of the as-prepared SiO₂ NPs were characterized by TEM measurements, as shown in Fig. 1a. It can be seen that the SiO₂ NPs are nearly spherical in shape with distinctly free standing and monodisperse state [24] and have an average size of 127 nm (inset). To characterize the dispersibility of the as-prepared SiO₂ NPs in aqueous solution, we measured their zeta potential and dynamic light scattering (DLS) diameters (Fig. 1b). The mean

zeta potential of SiO₂ NPs is -41 mV (blue group) and the DLS diameter is 146 nm (red group). These results reveal the good dispersibility of SiO₂ NPs in solution.

3.2. Effect of SiO₂ NPs on the Electron Transfer of Cyt *c* Adsorbed onto MUA SAM

To disclose the effect of interaction with SiO₂ NPs on the electron transport function of cyt *c*, we investigated the change in redox behavior of the protein adsorbed onto MUA SAM induced by exposure to the SiO₂ NPs using cyclic voltammetry. Fig. 2 shows cyclic voltammograms (CVs) of cyt *c*/MUA/Au and SiO₂ NPs/cyt *c*/MUA/Au in a 10 mM PBS (pH 7.0) at a scan rate of 100 mV/s. For cyt *c*/MUA/Au, a pair of well-defined redox peaks can be observed at the formal potential (E_f) of 0.016 ± 0.001 V with the peak-to-peak separation (ΔE) of 11.0 ± 1.0 mV (Fig. 2, black line). However, after the cyt *c*/MUA/Au was incubated in the SiO₂ NPs suspension for 60 min, E_f shifts negatively to 0.010 ± 0.001 V and ΔE_p decreases to 7.0 ± 0.5 mV (Fig. 2, red line). The negative shift of 6 ± 2 mV suggests that cyt *c* could suffer from some slight changes in conformation and/or orientation induced by the interaction with SiO₂ NPs [25]. The decrease in ΔE_p of 4.0 ± 1.5 mV implies the redox process of cyt *c* is promoted after cyt *c* interaction with SiO₂ NPs [26]. The SiO₂ NPs could induce cyt *c* to adopt a more proper conformation and/or orientation for electron transfer since the semiconductor SiO₂ NPs are difficult to accelerate the electron transfer of cyt *c* through electric conduction or redox within this potential window. This result is largely different from that of cyt *c* interaction with SiO₂ NPs in bulk phase, where SiO₂ NPs made greater disruption to the local microenvironment of heme and resulted in a loss of the protein's electron transfer function [27].

The change in the kinetic and electron-transport characteristics of cyt *c* induced by SiO₂ NPs was further investigated by performing cyclic voltammetry experiments with different scan rates. Fig. 3a and b show the CVs of cyt *c*/MUA/Au and SiO₂ NPs/cyt *c*/MUA/Au in 10 mM PBS (pH

7.0) at different scan rates from 50 mV s⁻¹ to 3000 mV s⁻¹. In this range of scan rate, both cathodic and anodic peak currents are found to be proportional linearly to the scan rate, which corresponds to an adsorption-controlled process of electrode [25]. In addition, from Fig. 3a and b, either before or after interaction with SiO₂ NPs, the oxidation peak of cyt *c* moves to a more positive potential as the scan rate increases, while the reduction peak shifts to a more negative potential direction. The anodic and cathodic peak potential are linearly dependent on the logarithm of scan rate (v) when $v > 1$ V s⁻¹ for cyt *c* adsorbed on MUA and when $v > 0.63$ V s⁻¹ for the cyt *c* after interaction with SiO₂ NPs (Fig. 3c and d). From the linear region of high scan rate (1000 mV s⁻¹–3000 mV s⁻¹), apparent ET rate constants (k_s) can be determined based on the method described by Laviron [28] (Fig. 3c and d). According to Laviron theory [29], the slopes of the linear part of the E_p -log v dependence are $2.3RT/\alpha nF$ and $2.3RT/(1 - \alpha)$ for cathodic and anodic peaks, respectively.

$$\log v_a/v_c = \log \left[\alpha/(1 - \alpha) \right] \quad \text{or} \quad v_a/v_c = \alpha/(1 - \alpha) \quad (1)$$

$$\log k_s = \alpha \log(1 - \alpha) + (1 - \alpha) \log \alpha - \log(RT/nFv) - \alpha(1 - \alpha)nF \Delta E_p / 2.3RT \quad (2)$$

Thus, based on the equation (1), the transfer coefficient for the redox reaction of cyt *c* adsorbed on MUA SAM and the cyt *c* after interaction with the SiO₂ NPs was 0.5 and 0.51, respectively. Then, according to the equation (2), the values of k_s can be obtained. They are 22.75±2.3 s⁻¹ and 33.57±3.8 s⁻¹, respectively. The kinetic data reveals that the electron transfer rate is enhanced for the cyt *c* adsorbed on MUA after interacting with SiO₂ NPs. Some factors can interpret the fast electron transfer of SiO₂ NPs/cyt *c*/MUA/Au electrode. The k_s depends strongly on cyt *c*'s conformation at the electrode surface [30]. Electron transfer is facilitated by an optimal

conformation of cyt *c* caused by the synergistic interaction between cyt *c* and SiO₂ NPs [31], resulting in decreased peak-to-peak separation and enhanced electrochemical reversibility.

*3.3 Effect of SiO₂ NPs on the electrocatalysis of immobilized cyt *c* towards H₂O₂.*

It has been reported that native cyt *c* could electro-catalyze the reduction of hydrogen peroxide (H₂O₂) on the “immobilized” electrodes [32, 33]. Here, we explore the effect of SiO₂ NPs on the electro-catalytic behavior of cyt *c* adsorbed on the MUA SAM towards H₂O₂ reduction. Fig. 4 and b show the comparison for the CVs of cyt *c*/MUA/Au and SiO₂ NPs/cyt *c*/MUA/Au in the presence and absence of H₂O₂ in 10 mM PBS (pH 7.0) at 100 mV s⁻¹. For cyt *c*/MUA/Au, upon addition of H₂O₂, reduction current starts to increase at the potential of *ca.* 0 V and further increases with potential scanning and the oxidation peak decreases in its intensity, suggesting the pseudo peroxidase activity of the cyt *c* immobilized on the MUA SAM [33]. The peak current of the reduction of H₂O₂ increases with increasing the concentration of H₂O₂ from 60 μM to 0.1 mM. For SiO₂ NPs/cyt *c*/MUA/Au, upon addition of H₂O₂ into buffer solution, the corresponding reduction current exhibits a larger degree of increase compared with that of cyt *c*/MUA/Au, suggesting that the interaction of SiO₂ NPs could strengthen the electrocatalytic activity of cyt *c* towards the reduction of H₂O₂ [34, 35], which might be caused by the conformational change of cyt *c* after interaction with SiO₂ NPs [36].

*3.4. SEIRAS and Potential-Induced SEIRA difference spectroscopy study the Conformation and Orientation Change of Cyt *c* Treated by SiO₂ NPs*

SEIRAS and potential-induced SEIRA difference spectroscopy were measured to further reveal the interaction between cyt *c* and SiO₂ NPs, and the mechanism of such interaction-promoted electron transfer and electrocatalytic activity of cyt *c*. We firstly monitored the formation of the SAM of MUA on the nanostructured Au film with the ethanol-incubated gold

film as reference (Fig. 5a). The abscissa is compartmentalized into two wavenumber regions, *i.e.* the fingerprint region from 1800 to 1500 cm^{-1} and the C–H stretching vibration absorption at $\sim 3000 \text{ cm}^{-1}$. Concomitant with the addition of MUA, a positive peak appears at 1714 cm^{-1} with a shoulder band at 1730 cm^{-1} and increases in intensity with adsorption time, assigning to the C=O stretching mode of the COOH of MUA with different laterally hydrogen-bonding orders [12]. In the C–H stretching region, the bands assigned to the antisymmetric $\nu_{\text{as}}(\text{CH}_2)$ and symmetric $\nu_{\text{s}}(\text{CH}_2)$ stretching vibrations of the CH_2 groups of MUA molecules appear at 2923 cm^{-1} and 2852 cm^{-1} , respectively, and the negative band at 2973 cm^{-1} belongs to the antisymmetric $\nu_{\text{as}}(\text{CH}_3)$ stretching of the desorbed ethanol induced by removing of ethanol molecules from the Au electrode upon the adsorption of MUA, which converts to positive peak with adsorption time due to the band overlap with the $\nu_{\text{as}}(\text{CH}_3)$ stretching of the MUA. The band at 2885 cm^{-1} belongs to symmetric $\nu_{\text{as}}(\text{CH}_3)$ stretching of the adsorbed ethanol molecules. After saturation coverage of MUA molecules on the Au electrode, the MUA/Au electrode was incubated with cyt *c* solution.

Two strong absorption bands appear at 1659 cm^{-1} and 1551 cm^{-1} which are assigned to the amide I and amide II modes of the protein, respectively, with 10 mM PBS (pH 7.0) as background (Fig. 5b, black lines) [37]. Then the 2 μM cyt *c* PBS (10 mM, pH 7.0) was replaced by 10 mM PBS (pH 7.0) and the cyt *c*/MUA/Au was subtracted as background. After addition of SiO_2 NPs into the solution, nearly no change can be observed at the adsorption peaks of amide I and II bands (Fig. 5b, colorful lines). It is suggested that there is no destructive extraction of cyt *c* molecules from the MUA SAM when they interact with SiO_2 NPs and that adsorption of SiO_2 NPs only induces a slight conformational change of cyt *c*.

Fig. 6a and 6b show the series of potential-induced difference spectra of cyt *c*/MUA/Au and SiO_2 NPs/cyt *c*/MUA/Au in 10 mM PBS (pH 7.0), respectively. The reference spectra were

taken at -0.2 V, where the adsorbed cyt *c* are fully reduced, while the sample spectra were acquired at successively increased potentials from -0.1 to 0.2 V, where the adsorbed cyt *c* are gradually until fully oxidized. For cyt *c*/MUA/Au, the broad water bands at 3450 cm⁻¹ increase in intensity with the sample potential and reach a plateau at 0.1 V. Since the 3450 cm⁻¹ bands in the series of difference spectra of MUA (dotted lines) shows almost no change with the sample potential, $\Delta I_{(O-H)}$ (the difference in intensity of the 3450 cm⁻¹ band between cyt *c*/MUA/Au and MUA/Au), reflects the cyt *c* redox-induced hydration change of the adsorbed film [38]. The negative nature of the broad water band in the redox-induced difference spectra suggests that the degree of hydration of native cyt *c* is decreased from the reduced to oxidized state [38]. The intensity of $\Delta I_{(O-H)}$ divided by the intensity of the 1693 cm⁻¹ vibration (ΔI_{1693} , denoting the coverage of cyt *c* since the vibration is assigned to the type III β -turns of the protein.), discloses the contribution of each adsorbed cyt *c* molecule to such a decrease in the total hydration. Upon interaction with SiO₂ NPs, the intensity ratio decreases for SiO₂ NPs/cyt *c*/MUA/Au (Fig. 6c), which indicates that such cyt *c* undergoes a smaller change in the total hydration from reduced to oxidized state. This means the oxidized cyt *c* is more hydrophilic or the reduced cyt *c* is more hydrophobic. From the energy point of view, the redox-dependent water rearrangement is the major determinant of the total reorganization energy (λ), the energy required to distort the equilibrium nuclear configuration of reactants toward the equilibrium configuration of products before electron transfer [39]. Herein, the smaller change in the degree of hydration of cyt *c* treated with SiO₂ NPs inevitably decreases the λ of its redox process, and finally results in the decrease in ΔE_p and the increase of reversibility of cyt *c* relative to those of native cyt *c*.

Fig. 6d shows the normalized potential-induced difference spectra of cyt *c*/MUA/Au and SiO₂ NPs/cyt *c*/MUA/Au in 10 mM PBS (pH 7.0) between the fully oxidized (+0.2 V, positive peaks)

and the reduced (-0.2 V, negative peaks) states. From an overall view, after cyt *c* interacted with SiO₂ NPs, the redox-induced SEIRA difference spectrum between 1800 and 1500 cm⁻¹ is somewhat different from that of native cyt *c*. In native cyt *c*, the bands at 1693 (-) and 1672 (+) cm⁻¹ and the bands at 1666 (-) and 1661 (+) cm⁻¹, are assigned to the type III β -turns and the type II β -turns and/or α -helix of the reduced and oxidized states, respectively [40]. After interaction with SiO₂ NPs, the band at 1673 (+) cm⁻¹ increases in intensity. According to the surface selection rule and optical near-field effect of SEIRAS, upon interaction of SiO₂ NPs with cyt *c* adsorbed on MUA, the type III β -turn might locate closer to the electrode surface. The band at 1636 (+) cm⁻¹ shifts to 1634 (+) cm⁻¹, both frequency being assorted to the β -sheet structure in oxidized state of cyt *c*, comprising residues 37~39 and 57~59 [37]. The shift of band frequency suggests the conformation changes of the active site and its instant environment which occurs during the redox process [37]. Thus, the structure of β -sheet in the oxidized form and the microenvironment surrounding it could change after cyt *c* (Fe³⁺) interacts with SiO₂ NPs because of the shift of frequency from 1636 (+) cm⁻¹ to 1634 (+) cm⁻¹. In native cyt *c*, there are only a short β -sheet in the ‘neck’ of the 40s Ω loop consisting of residues 37~39 and 57~59 [41]. According to the reported adsorption orientation of cyt *c* on MUA SAM (Fig. 7a), where Lys 13, 72, and 86 are the dominant electrostatic interaction sites, and the overall distribution of charge, the region including residues 37~39 and 57~59 also shows strong electropositivity in addition to the region around the electrostatic interaction sites. Most importantly, it faces the solution, and thus it is most likely that the negatively charged SiO₂ NPs interact with the positively charged region including residues 37~39 and 57~59 (Fig. 7b), which further induces the conformational changes of the β -sheet and the changes of its microenvironment indicated by the shift of band frequency. Such changes could also affect the microenvironment of heme since these residues

are connected with heme propionate, which might facilitate the coordinate of H_2O_2 , resulting in the enhanced electrocatalytic activity of adsorbed cyt *c* towards H_2O_2 reduction [42-44]. Moreover, such interaction could slightly change the adsorption orientation of adsorbed cyt *c* with heme more perpendicular to the surface as shown in Fig. 7b, facilitating electron transfer.

Based on the reported results, the magnitude of the formal potential depends mainly on exposure of heme cofactor to solvent, the nature of the axial ligand [45] and the surface charge property of adsorbed film [46]. For a partially unfolded cyt *c* [39, 47], its oxidized form always opens the heme crevice due to the change of ligand so as to have a large solvent accessibility of the heme, while its reduced form possesses a relatively compact conformation, which determines the negative shift of its formal potential relative to native cyt *c*. Since the structure and related heme microenvironment of cyt *c* treated with SiO_2 NPs were changed in the oxidized form while were kept in the reduced form relative to those of native cyt *c* as shown in Fig. 6d, a similar hydration degree could be proposed for both cyt *c* (Fe^{2+}) and a more hydrophilic microenvironment for cyt *c* (Fe^{3+}) treated by SiO_2 NPs, and thus a higher dielectric constant environment, which could result in the slight negative shift of the formal potential relative to that of native cyt *c*. In addition, the adsorption of negatively charged SiO_2 NPs could compensate the surface charge of cyt *c*/MUA/Au, which could induce the change of surface potential, resulting in slight negative shift in the formal potential of adsorbed cyt *c* [43].

4. Conclusion

In summary, electrochemistry study indicates that exposure of cyt *c* immobilized on biomimic membrane to SiO_2 NPs enhances the redox reversibility, electron transfer rate, and the catalytic performance of adsorbed cyt *c* instead of inhibiting the electron transfer. Potential-induced

SEIRA difference spectroscopy further reveals that such positive biological effect of nanoparticles comes from the modulation of heme microenvironment and adsorption orientation. The interaction of negatively charged SiO₂ NPs with the positively charged region including β -sheet residues 37~39 and 57~59 could not only compensate the surface charge of cyt *c*/MUA/Au but also induce the conformational change of β -sheet, and further change the microenvironment of heme, leading to enhanced hydration of cyt *c* (Fe³⁺) relative to that of native cyt *c*. The resulting enhanced hydration of cyt *c* (Fe³⁺) can be proposed to cause the slight decrease of hydration degree upon redox process of cyt *c*, and eventually cause the promoted electron transfer capability of adsorbed cyt *c*. In addition, cyt *c*'s slight conformational change and the disruption of its axial ligand could also lead to the enhanced catalytic ability towards the reduction of H₂O₂.

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References

- [1] C.C. Fleischer and C.K. Payne, Nanoparticle–cell interactions: molecular structure of the protein corona and cellular outcomes, *Acc. Chem. Res.* 47 (2014) 2651.
- [2] M.P. Monopoli, A. Salvati, and K.A. Dawson, Biomolecular coronas provide the biological identity of nanosized materials, *Nat. Nanotechnol.* 7 (2012) 779.
- [3] Q. Dai, C. Walkey, and W.C.W. Chan, Polyethylene glycol backfilling mitigates the negative impact of the protein corona on nanoparticle cell targeting, *Angew. Chem., Int. Ed.* 53 (2014) 5093.
- [4] C. Ge, J. Du, L. Zhao, L. Wang, Y. Liu, D. Li, Y. Yang, R. Zhou, Y. Zhao, Z. Chai, and C. Chen, Binding of blood proteins to carbon nanotubes reduces cytotoxicity, *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) 16968.
- [5] Z.J. Deng, M. Liang, M. Monteiro, I. Toth, and R.F. Minchin, Nanoparticle-induced unfolding of fibrinogen promotes Mac-1 receptor activation and inflammation, *Nat. Nanotechnol.* 6 (2011) 39.
- [6] T. Cedervall, I. Lynch, S. Lindman, T. Berggard, E. Thulin, H. Nilsson, K.A. Dawson, and S. Linse, Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 2050.
- [7] J.E. Gagner, M.D. Lopez, J.S. Dordick, and R.W. Siegel, Effect of gold nanoparticle morphology on adsorbed protein structure and function, *Biomaterials.* 32 (2011) 7241.
- [8] P. Roach, D. Farrar, and C.C. Perry, Surface tailoring for controlled protein adsorption: effect of topography at the nanometer scale and chemistry, *J. Am. Chem. Soc.* 128 (2006) 3939.
- [9] S. Zhong, D. L. Rousseau, and S.-R. Yeh, Modulation of the folding energy landscape of cytochrome c with salt, *J. Am. Chem. Soc.* 126 (2004) 13934.
- [10] K.B. Koller and F.M. Hawkrige, Temperature and electrolyte effects on the electron-transfer, *J. Am. Chem. Soc.* 107 (1985) 7412.
- [11] S.R. Solmaz and C. Hunte, Structure of complex III with bound cytochrome c in reduced state and definition of a minimal core interface for electron transfer, *J. Biol. Chem.* 283 (2008) 17542.
- [12] X. Jiang, K. Ataka, and J. Heberle, Influence of the molecular structure of carboxyl-terminated self-assembled monolayer on an Au electrode: In situ observation by surface-enhanced infrared absorption spectroscopy, *J. Phys. Chem. C.* 112 (2008) 813.
- [13] B. Jin, W. Bao, Z. Wu, and X. Xia, In situ monitoring of protein adsorption on a nanoparticulated gold film by attenuated total reflection surface-enhanced infrared absorption spectroscopy, *Langmuir.* 28 (2012) 9460.

- [14] B. Jin, G.-X. Wang, D. Millo, P. Hildebrandt, and X.-H. Xia, Electric-field control of the pH-dependent redox process of cytochrome c immobilized on a gold electrode, *J. Phys. Chem. C* 116 (2012) 13038.
- [15] J. Xu, T. Chen, W. Bao, K. Wang, and X. Xia, Label-free strategy for in-situ analysis of protein binding interaction based on attenuated total reflection surface enhanced infrared absorption spectroscopy (ATR-SEIRAS), *Langmuir* 28 (2012) 17564.
- [16] J. Kozuch, C. Steinem, P. Hildebrandt, and D. Millo, Combined electrochemistry and surface-enhanced infrared absorption spectroscopy of gramicidin A incorporated into tethered bilayer lipid membranes, *Angew. Chem., Int. Ed.* 51 (2012) 8114.
- [17] L. Wu, L. Zeng, and X. Jiang, Revealing the nature of interaction between graphene oxide and lipid membrane by surface-enhanced infrared absorption spectroscopy, *J. Am. Chem. Soc.* 137 (2015) 10052.
- [18] N. Wisitruangsakul, I. Zebger, K.H. Ly, D.H. Murgida, S. Ekgasit, and P. Hildebrandt, Redox-linked protein dynamics of cytochrome c probed by time-resolved surface enhanced infrared absorption spectroscopy, *Phys. Chem. Chem. Phys.* 10 (2008) 5276.
- [19] E. Johnson and R. Aroca, Surface-enhanced infrared spectroscopy of monolayers, *J. Phys. Chem.* 99 (1995) 9325.
- [20] D.J. Bharali, I. Klejbor, E.K. Stachowiak, P. Dutta, I. Roy, N. Kaur, E.J. Bergey, P.N. Prasad, and M.K. Stachowiak, Organically modified silica nanoparticles: a nonviral vector for in vivo gene delivery and expression in the brain, *Proc. Natl. Acad. Sci. U. S. A.* 102 (2005) 11539.
- [21] I.I. Slowing, B.G. Trewyn, and V.S.Y. Lin, Mesoporous silica nanoparticles for intracellular delivery of membrane-impermeable proteins, *J. Am. Chem. Soc.* 129 (2007) 8845.
- [22] W. Stober and A. Fink, Controlled growth of monodisperse silica spheres in the micron size range, *J. Colloid Interface Sci.* 26 (1968) 62.
- [23] H. Miyake, S. Ye, and M. Osawa, Electroless deposition of gold thin films on silicon for surface-enhanced infrared spectroelectrochemistry, *Electrochem. Commun.* 4 (2002) 973.
- [24] I.A. Rahman and V. Padavettan, Synthesis of silica nanoparticles by sol-gel: size-dependent properties, surface modification, and applications in silica-polymer nanocomposites—A review, *J. Nanomater.* 2012 (2012) 1.
- [25] A.B. Moghaddam, M.R. Ganjali, R. Dinarvand, T. Razavi, A.A. Saboury, A.A. Moosavi-Movahedi, and P. Norouzi, Direct electrochemistry of cytochrome c on electrodeposited nickel oxide nanoparticles, *J. Electroanal. Chem.* 614 (2008) 83.
- [26] H. Yue and D.H. Waldeck, The effect of ionic strength on the electron-transfer rate of surface immobilized, *J. Phys. Chem. B.* 110 (2006) 5062.
- [27] W. Shang, J.H. Nuffer, R.W. Siegel, and J.S. Dordick, Cytochrome c on silica nanoparticles: Influence of nanoparticle size on protein structure, stability, and activity, *small* 5 (2009) 470.
- [28] K. Murata, K. Kajiya, M. Nukaga, Y. Suga, T. Watanabe, N. Nakamura, and H. Ohno, A simple fabrication method for three-dimensional gold nanoparticle electrodes and their application to the study of the direct electrochemistry of cytochrome c, *Electroanalysis* 22 (2010) 185.

- [29] R. Campos and E.E. Ferapontova, Electrochemistry of weakly adsorbed species: Voltammetric analysis of electron transfer between gold electrodes and Ru hexamine electrostatically interacting with DNA duplexes, *Electrochim. Acta.* 126 (2014) 151.
- [30] A. F. Loftus, K.P. Reighard, S.A. Kapourale, and M.C. Leopold, Monolayer-protected nanoparticle film assemblies as platforms for controlling interfacial and adsorption properties in protein monolayer electrochemistry, *J. Am. Chem. Soc.* 130 (2008) 1649.
- [31] Y. Song, H. Liu, Y. Wang, and L. Wang, A glucose biosensor based on cytochrome c and glucose oxidase co-entrapped in chitosan–gold nanoparticles modified electrode, *Anal. Methods.* 5 (2013) 4165.
- [32] L. Zhang, Direct electrochemistry of cytochrome c at ordered macroporous active carbon electrode, *Biosens. Bioelectron.* 23 (2008) 1610.
- [33] Y. Zhou, J. Zhi, Y. Zou, W. Zhang, and S.-T. Lee, Direct electrochemistry and electrocatalytic activity of cytochrome c covalently immobilized on a boron-deoped nanocrystalline diamond electrode, *Anal. Chem.* 80 (2008) 4141.
- [34] A. Ranieri, D. Millo, G. Di Rocco, G. Battistuzzi, C.A. Bortolotti, M. Borsari, and M. Sola, Immobilized cytochrome c bound to cardiolipin exhibits peculiar oxidation state-dependent axial heme ligation and catalytically reduces dioxygen, *JBIC Journal of Biological Inorganic Chemistry.* 20 (2015) 531.
- [35] G. Shi, Z. Sun, M. Liu, L. Zhang, Y. Liu, Y. Qu, and L. Jin, Electrochemistry and electrocatalytic properties of hemoglobin in layer-by-layer films of SiO₂ with vapor-surface sol-gel deposition, *Anal. Chem.* 79 (2007) 3581.
- [36] A. Ranieri, D. Millo, G. Di Rocco, G. Battistuzzi, C.A. Bortolotti, M. Borsari, and M. Sola, Immobilized cytochrome c bound to cardiolipin exhibits peculiar oxidation state-dependent axial heme ligation and catalytically reduces dioxygen, *JBIC Journal of Biological Inorganic Chemistry.* 20 (2015) 531.
- [37] K. Ataka and J. Heberle, Functional vibrational spectroscopy of a cytochrome c monolayer: SEIDAS probes the interaction with different surface-modified electrodes, *J. Am. Chem. Soc.* 126 (2004) 9445.
- [38] L. Liu, L. Zeng, L. Wu, and X. Jiang, Label-Free surface-enhanced infrared spectroelectrochemistry studies the interaction of cytochrome c with cardiolipin-containing membranes, *J. Phys. Chem. C.* 119 (2015) 3990.
- [39] L. Paltrinieri, M. Borsari, A. Ranieri, G. Battistuzzi, S. Corni, and C.A. Bortolotti, The active site loop modulates the reorganization energy of blue copper proteins by controlling the dynamic interplay with solvent, *J. Phys. Chem. Lett.* 4 (2013) 710.
- [40] S. Lin, X. Jiang, L. Wang, G. Li, and L. Guo, Adsorption orientation of horse heart cytochrome c on a bare gold electrode hampers its electron transfer, *J. Phys. Chem. C.* 116 (2012) 637.
- [41] G. Balakrishnan, Y. Hu, and T.G. Spiro, His26 protonation in cytochrome c triggers microsecond β -sheet formation and heme exposure: implications for apoptosis, *J. Am. Chem. Soc.* 134 (2012) 19061.
- [42] A. Ranieri, F. Bernini, C.A. Bortolotti, A. Bonifacio, V. Sergo, and E. Castellini, pH-Dependent Peroxidase Activity of Yeast Cytochrome c and Its Triple Mutant Adsorbed on Kaolinite, *Langmuir.* 27 (2011) 10683.
- [43] J.B. Essner, R.N. McCay, C.J. Smith II, S.M. Cobb, C.H. Laber, and G.A. Baker, A switchable peroxidase mimic derived from the reversible co-assembly of cytochrome c and carbon dots, *J. Mater. Chem. B.* 4 (2016) 2163.

- [44] E. Castellini, F. Bernini, M. Berto, M. Borsari, M. Sola, and A. Ranieri, Solvent tunes the peroxidase activity of cytochrome c immobilized on kaolinite, *Applied Clay Science*. 118 (2015) 316.
- [45] S. Monari, D. Millo, A. Ranieri, G. Di Rocco, G. van der Zwan, C. Gooijer, S. Peressini, C. Tavagnacco, P. Hildebrandt, and M. Borsari, The impact of urea-induced unfolding on the redox process of immobilised cytochrome c, *J. Biol. Inorg. Chem.* 15 (2010) 1233.
- [46] J. Zhang, Y. Zhao, C.G. Yuan, L.N. Ji, X.D. Yu, F.B. Wang, K. Wang, and X.H. Xia, Donnan potential caused by polyelectrolyte monolayers, *Langmuir*. 30 (2014) 10127.
- [47] M. Patila, I.V. Pavlidis, E.K. Diamanti, P. Katapodis, D. Gournis, and H. Stamatis, Enhancement of cytochrome c catalytic behaviour by affecting the heme environment using functionalized carbon-based nanomaterials, *Process Biochem.* 48 (2013) 1010.

Figure captions

Fig. 1 The characterization of SiO₂ NPs: (a) TEM image of SiO₂ NPs and histogram of size distribution (inset), (b) the histogram of DLS diameter and zeta potential of SiO₂ NPs.

Fig. 2 Cyclic voltammograms of cyt *c*/MUA/Au electrode (black line) and SiO₂ NPs/cyt *c*/MUA/Au (red line) in a 10 mM PBS (pH 7.0) at the scan rate of 100 mV s⁻¹.

Fig. 3 CVs of cyt *c*/MUA/Au (a) and SiO₂ NPs/cyt *c*/MUA/Au (b) at different scan rates varied from 50 mV s⁻¹ to 3000 mV s⁻¹. And trumpet plots for cyt *c*/MUA/Au (c) and SiO₂ NPs/cyt *c*/MUA/Au (d): Anodic (black spot) and cathodic (red spot) potentials vs. logarithm of the applied scan rates.

Fig.4 The CVs of cyt *c*/MUA/Au (a) and SiO₂ NPs/cyt *c*/MUA/Au (b) in the absence and presence (black line) of 60 μM or 0.1 mM H₂O₂ in 10 mM PBS (pH 7.0).

Fig. 5 SEIRA spectra of MUA adsorbed on nano-Au film (a) at 0.1, 0.4, 1.1, 4.3, 5.3, 9.3, 13.3, 25.3, 50.3, 70 min, respectively (from bottom to top), of cyt *c* adsorbed on MUA/Au (b, black) at 6, 13, 20, 42, 90 min, and then treated with SiO₂ NPs at 13, 20, 90 min (b, colorful lines) with the cyt *c*/MUA/Au as reference.

Fig. 6 Series of potential-induced SEIRA difference spectra of cyt *c*/MUA/Au (a, solid lines) and MUA/Au (a, dashed lines) as well as SiO₂ NPs/cyt *c*/MUA/Au (b) in 10 mM PBS (pH 7.0) with the reduced state as reference (-0.2 V): At the oxidation potentials of -0.1 V (1-1&2-1), -0.05 V (1-2&2-2), 0 V (1-3&2-3), 0.05 V (1-4&2-4), 0.1 V (1-5&2-5), 0.2 V (1-6&2-6). The arrows correspond to an absorbance ΔA of 5×10^{-4} . (c): the ratio of $\Delta I_{(O-H)}$ to $\Delta I_{(1693)}$. $\Delta I_{(O-H)}$ is peak value of the broad water bands at *ca.* 3450 cm⁻¹. (d) Normalized SEIRA difference spectra of cyt *c*/MUA/Au (black) and SiO₂ NPs/cyt *c*/MUA/Au (red) at 0.2 V with the reference potential at -0.2 V in 10 mM PBS (pH 7).

Fig. 7 Possible adsorption modes of (a) cyt *c* adsorbed on MUA monolayers between terminal amine groups of Lys 13, 86 and 72 and the carboxylate surface of MUA, and (b) SiO₂ NPs adsorbed on cyt *c*/MUA monolayer (Taking the significantly larger particle size of SiO₂ NPs than the diameter of cyt *c* into account, the pictorial sizes of both SiO₂ NPs and cyt *c* aren't very consistent with the actual sizes of them.). The cyt *c* structure was created by Swiss PDB Viewer 3.7 with crystallographic data taken from the Protein Data Bank (PDB entry 1HRC).

Fig. 1

Fig. 2

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Fig. 6

Fig. 7