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Reversible Immobilization and Direct Electron Transfer of Cytochrome c on a pH-Sensitive Polymer Interface

Jianhua Zhou, Xianbo Lu, Jianqiang Hu, and Jinghong Li*[a]

Abstract: A pH-sensitive polymer interface has been used as a matrix for reversible immobilization of cytochrome c (Cyt c) on an Au surface through a dip-coating process. The pH-sensitive behavior of the polymer brush interface has been demonstrated by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements. The reversible immobilization and electron-transfer properties of Cyt c have been investigated by in situ UV/Vis spectrophotometry and CV. The results have shown that the poly(acrylic acid) (PAA) brush acted as an excellent adsorption matrix and a good accelerant for the direct electron transfer of Cyt c, which gave redox peaks with a formal potential of 40 mV versus Ag/AgCl in pH 7.6 phosphate buffer solution. The average surface coverage of Cyt c on the PAA film was about 1.7×10^{-10} mol cm^{-2}, indicating a multilayer of Cyt c. The electron-transfer rate constant was calculated to be around 0.19 s^{-1} according to the CV experiments. The interface was subjected to in situ attenuated total internal reflection Fourier-transform infrared (ATR-FTIR) spectroscopic analysis, in order to further confirm the immobilization of Cyt c on the surface. This polymer–protein system may have potential applications in the design of biosensors, protein separation, interfacial engineering, biomimetics, and so on.

Keywords: electrochemistry · electron transfer · pH-sensitive interface · polymer brush · reversible immobilization

Introduction

Coupled polymer–protein supermolecular structures, which provide favorable microenvironments for redox proteins to display their direct electrochemical and biomimetic functions because of the flexibility and functional groups of the polymer, have been attracting increased attention.[1] Recently, many publications have focused on the chemical or physical entrapment of redox proteins in biocompatible polymers, such as chitosan, agarose, poly(acrylamide), and poly(propyleneimine), to investigate electron transfer and thereby fabricate excellent biosensors.[2] Compared to these conventional intermixed structures or self-assembled monolayers, polymer brushes based on functional polymers are more similar to sensitive biomembranes with respect to their surface structures and functionalities.[3] Of these functional polymers, poly(acrylic acid) (PAA) is a water-soluble biocompatible polymer that has found application in controlled drug delivery because of its mucoadhesive properties.[4] Adsorption of proteins onto PAA polymer brushes has been reported.[5] However, to the best of our knowledge, no work has been reported on studies of direct electron transfer of the immobilized proteins at polymer brush interfaces.

Among the various redox proteins, cytochrome c (Cyt c) is an important heme-containing metalloprotein,[6] which is bound to the mitochondrial membrane and functions as an electron carrier in the respiratory chain. In recent decades, a substantial amount of research work has been carried out on the direct electrochemistry and electrocatalysis of Cyt c irreversibly immobilized on inert substrates.[7] From the biological point of view, however, it is particularly important to design dynamic polymer interfaces that can respond to the solution environment, alter their surface properties and, hence, reversibly regulate their interactions with proteins.[8] Considering the pioneering work in this area, modification of the interfaces with "smart" polymer brushes has been shown to constitute a useful methodology for controlling their physical and chemical properties. It also introduces a large number of functional groups on the surface layer in three dimensions, as compared to the two-dimensionality of self-assembled monolayers.[1a] Herein, we describe an elec-

[a] J. Zhou, X. Lu, Dr. J. Hu, Prof. J. Li
Department of Chemistry
Key Laboratory of Bioorganic Phosphorus Chemistry and Chemical Biology
Tsinghua University, Beijing 100084 (China)
Fax: (+86) 10-6279-5290
E-mail: jhli@mail.tsinghua.edu.cn

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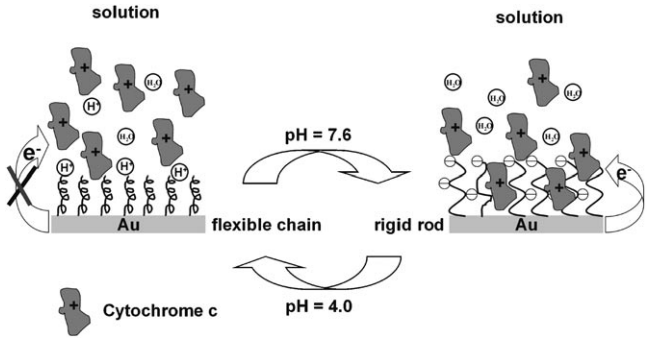
trochemical strategy to build a pH-responsive biological interface based on the biocompatible material poly(acrylic acid). The reversible immobilization and direct electron-transfer process of Cyt c at the PAA brush-modified conductive substrates, which could be effectively controlled by the environmental pH (see Scheme 1), have been successfully



Scheme 1. Reversible immobilization and direct electron transfer of cytochrome c under pH control on a switchable poly(acrylic acid) interface.

monitored by in situ UV/Vis spectrophotometry (UV), cyclic voltammetry (CV), and electrochemical impedance spectroscopy (EIS).

Results and Discussion

pH sensitivity and pH-induced switching of the PAA brush interface: The pH-sensitive interface was fabricated by means of an electrochemically induced polymerization process (see Experimental Section). Figure 1A shows cyclic voltammograms of $[Fe(CN)_6]^{3-/4-}$ on the PAA-modified electrode at various pH values. The redox probe, $[Fe(CN)_6]^{3-/4-}$, exhibited a pair of quasi-reversible peaks (peak-to-peak separation, $\Delta E_p = 191$ mV) at pH 3.6 due to the attachment of the PAA brush on the electrode. When the pH of the solution was changed from 3.6 to 10.2, an obvious decrease in the peak current and an increase in ΔE_p were observed, indicating that the electron-transfer process at the PAA-modified electrode could be greatly affected by changes in pH.

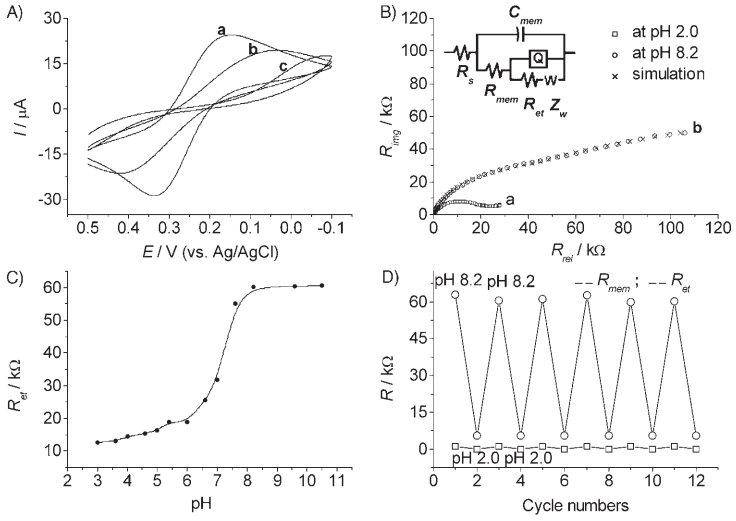


Figure 1. A) Cyclic voltammograms of $[Fe(CN)_6]^{3-/4-}$ on a PAA-modified electrode at different pH values: a) 3.6, b) 8.2, and c) 10.2; the scan rate was 50 mV s^{-1}; B) impedance spectra of the PAA brush interface at a) pH 2.0 and b) 8.2 along with the equivalent circuit used to interpret the data; C) response interfacial ETR of $[Fe(CN)_6]^{3-/4-}$ at the brush interface vs. pH; D) interfacial ETR change upon switching between pH 2.0 and pH 8.2. Electron-transfer resistance (○); resistance of PAA film (□).

It is well known that EIS is an excellent technique for investigating the surface processes of electrodes coated with films.[9] Figure 1B shows complex-plane impedance spectra (Nyquist plots) of the PAA brush-modified electrode in 5 mM $[Fe(CN)_6]^{3-/4-}$ at pH 2.0 (curve a) and pH 8.2 (curve b). The semicircular domains of the impedance spectra were derived from the electron-transfer resistance (ETR, R_{et}) of $[Fe(CN)_6]^{3-/4-}$ at the electrode surface. The fitting results are also presented in Figure 1B, along with the equivalent circuit analyzed by using a nonlinear least-squares program, where R_s, C_{mem}, R_{mem}, Q, R_{et}, and Z_w denote the electrolyte resistance, the capacitance of the PAA film, the resistance of the PAA film, the constant phase element, the electron-transfer resistance, and the Warburg impedance, respectively. At pH 2.0, R_{et} and R_{mem} were found to be about 5.5 kΩ and 1×10^{-3} kΩ, while at pH 8.2, these parameters were shifted to about 61.0 kΩ and 1.0 kΩ. A plot of the interfacial ETR of $[Fe(CN)_6]^{3-/4-}$ at the PAA brush interface versus pH is shown in Figure 1C. On increasing the pH from 3.0 to 10.5, the interfacial ETR increases markedly from about 12.5 kΩ to about 60.5 kΩ, with a dramatic increase in the region of pH 6.0–7.6. This increase in the interfacial ETR may be attributed to the ionization of carboxylate groups, which enhances the negative charge density at the surface and repels $[Fe(CN)_6]^{3-/4-}$ from the interface.[10] The variations in R_{et} and R_{mem} were recorded as the pH was repeatedly cycled between values of 2.0 and 8.2 (see Figure 1D). The observed changes can be attributed to the fact that, at low pH, the carboxylate groups are hardly ionized, and the PAA brush is in a relaxed conformation, whereas at high pH, the carboxylate groups are fully ionized, and ionic repulsion between the COO^- groups expands the polymer chain to form a stretched structure with more negative charge on its surface. This reversibility remained after the samples had been set aside without special protection over a period of months, indicating good stability of the PAA brush film. The switching of the interface demonstrates that the interfacial charge density, as well as ion permeability, can be tuned by adjusting the pH.

Reversible uptake–release cycles of Cyt c at the PAA brush interface: Cyt c is a very basic redox metalloprotein that bears several positive charges below pH 10,[11] while, as mentioned above, PAA is an electronegative polymer when the pH is higher than 7.0. It can thus be envisaged how Cyt c may be taken-up by the PAA brush membrane under weakly basic conditions, and then released once more under acidic conditions owing to electrostatic interactions. The re-

versible uptake–release process of ferric Cyt c has been monitored in situ by means of UV/Vis reflection and absorption spectroscopies. The UV/Vis spectrum of Cyt c shows a Soret band at 409 nm, and absorption peaks at 530 nm and 695 nm in 10 mM PBS at pH 7.6 or 4.0 (see Figure S-1 in the Supporting Information). The location of the Soret absorption band of an iron heme may provide information about the denaturation of the heme protein. When Cyt c is denatured, the Soret band shifts or disappears.[12] This Soret band absorption has been used as a probe to follow the uptake–release steps of Cyt c. Figure 2A, curve b,

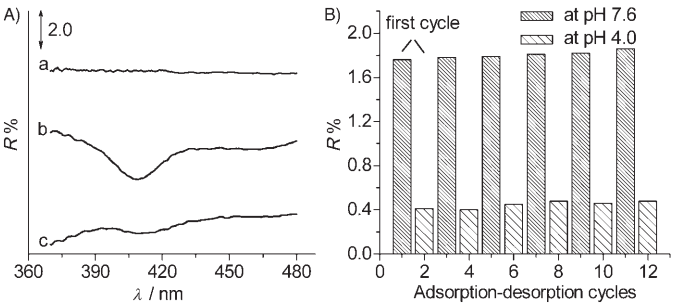


Figure 2. A) Reflection UV/Vis spectra of a) bare Au foil, and Cyt c on PAA brush-modified Au foils after dipping in b) pH 7.6 or c) pH 4.0 Cyt c solution for 5 min; B) controlled uptake–release of Cyt c by pH cycles: at pH 7.6 (dense diagonal hatching) and pH 4.0 (sparse diagonal hatching).

shows the Soret band of Cyt c immobilized on a PAA-modified Au surface at pH 7.6. The position of its Soret absorption band is very close to that in solution, indicating that the Cyt c molecule retains its biological activity in its immobilized state. To compare the absorption changes attributable to the quantity of ferric Cyt c immobilized on the PAA brush film, the absorption profiles of bare Au and PAA brush ad-Cyt c (fully loaded with Cyt c) modified Au surface at pH 7.6 and 4.0 were measured (Figure 2A). The intensity of the Soret band was seen to vary with pH. The absorption peak of Cyt c at pH 7.6 was much stronger than that at pH 4.0, suggesting that the amount of Cyt c on the PAA-modified Au surface at pH 7.6 was greater than that at pH 4.0. When the pH was varied between 7.6 and 4.0, Cyt c was taken-up and released from the PAA-modified Au surface (see Figure 2B). This phenomenon can be interpreted in terms of the electrostatic interaction between the PAA brushes and the Cyt c on the Au substrate.[13] The controlled immobilization of Cyt c on the substrate shows that the interaction between Cyt c and the PAA brush interface can be efficiently modulated by adjusting the pH of the solution.

Direct electrochemistry of the PAA brush interface in Cyt c solution: Cyclic voltammetry has been used to examine the redox properties of electroactive reactants at electrodes.[14] Cyclic voltammograms of our PAA-modified Au electrode were measured in Cyt c solution at pH 7.6 and 4.0. When the resulting PAA-modified electrode was exposed to 10 mM pH 7.6 phosphate buffer solution (PBS) containing 15 μM Cyt c, a pair of redox waves appeared between 0.3 and −0.3 V. The cathodic and anodic peak potentials were −0.021 and 0.102 V, respectively (vs. Ag/AgCl, curve a, Figure 3), and the formal potential (E°) of Cyt c was 40 mV.

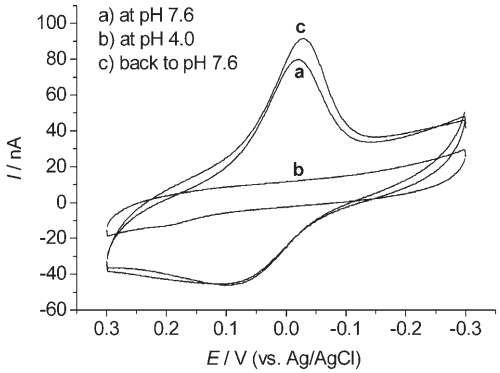


Figure 3. Cyclic voltammograms of a PAA-modified Au electrode of area 8×10^{-2} cm^2 in 10 mM PBS containing 15 μM Cyt c, a) at pH 7.6, b) at pH 4.0, c) back to pH 7.6. Scan rate: 5 mV s^{-1}.

The difference between the cathodic and anodic peaks can be attributed to the weakly adsorbed ferricytochrome c,[15] since the Cyt c in solution is mostly present in its oxidized state. A background test without the PAA brush on the Au surface was also conducted, and the results showed that there was no direct electrochemical activity at the bare Au electrode in the same solution (see Figure S-2 in the Supporting Information). This finding confirmed that the PAA brush serves to facilitate direct electron transfer of Cyt c. This behavior may be explained in terms of the electrostatic interaction between the negative charges on the PAA and the positive charges of the lysine residues around the heme site of Cyt c being sufficiently strong to allow direct electron transfer on the PAA brush-modified electrode.

On lowering the pH to 4.0, however, no redox peak was observed in the same potential range (Figure 3, curve b), suggesting that the Cyt c absorbed in the PAA brush film had been released. When the pH of solution was brought back to 7.6, the redox peaks appeared again, with a slight increase in current (curve c in Figure 3). This phenomenon may be rationalized in terms of the electrostatic interaction between the PAA and Cyt c decreasing due to protonation of the carboxylate groups of PAA below pH 6.5, and then increasing above pH 6.5 as a result of electrostatic attraction between the positively charged Cyt c and the negatively charged PAA layer. At $n\Delta E_p < 200$ mV, the electron-transfer rate constant (k_s) of Cyt c on the modified electrode can be obtained from Equation (1):[16]

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

Here, α is the charge-transfer coefficient, ν is the CV scan rate, ΔE_p is the peak-to-peak separation, and the symbols n,

F, R, and T have their usual meanings. Taking a charge-transfer coefficient, α, of 0.5, $n = 1$ (the redox process of Cyt c on the PAA-modified gold electrode is a single-electron-transfer reaction), and a scan rate of 5 mV s^{-1}, ΔE_p = 121 mV, and then one obtains an electron-transfer rate constant of about 0.19 s^{-1}, which is in accordance with that obtained by EIS (see Figure S-3 in the Supporting Information). The rate constant for interfacial electron transfer is apparently very similar to those of Cyt c on humic acid film[7a] and on a gold colloid modified electrode,[17] indicating that the PAA brush is a good promoter for the electron transfer between Cyt c and the Au electrode. The PAA brushes are envisaged as having many carboxyl groups on the polymer chains, which can easily engage in hydrogen bonds and electrostatic interactions with the carboxyl, amino, and phenol groups of Cyt c molecules and thereby immobilize them. These elastic interactions allow conservation of the natural conformations and activities of the proteins. Around the electrode surface, the highly hydrated and flexible chains also form a rather hydrophilic environment, which has been proved[18] to be necessary for direct electron transfer of Cyt c.

The coverage of electrode-addressable Cyt c immobilized on the PAA film was calculated to be about 1.7×10^{-10} mol cm^{-2} at 5 mV s^{-1}, which is about five times as much as the surface coverage calculated for a fully-packed monolayer of protein ($A_{max} = 3.4 \times 10^{-11}$ mol cm^{-2}, taking a molecule of Cyt c to be a sphere of diameter 2.5 nm[19]), thus indicating a multilayer of Cyt c on the surface of the electrode[20] (see Figure S-4 in the Supporting Information). This remarkable protein-adsorption ability may be attributed to the larger number of functional groups on the surface layer in three dimensions as compared to a self-assembled monolayer. The relationships between the peak currents (i_p) and the square root of the scan rate ($\nu^{1/2}$) remained linear from 2 to 30 mV s^{-1} (see Figure 4), denoting a diffusion-controlled

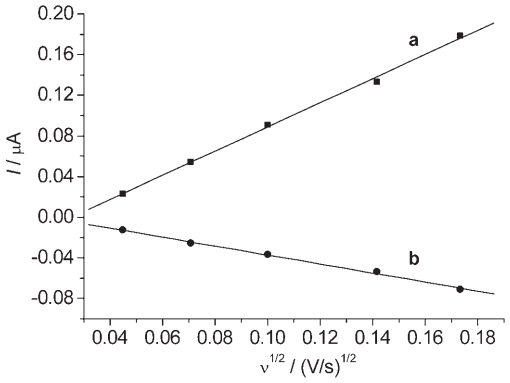


Figure 4. Calibration plots of a) cathodic and b) anodic peak currents versus the square root of the scan rate.

process at the PAA brush interface. Apparently, after the formation of the PAA brushes, the proteins diffuse into the membrane where they are immobilized, and the electron-transfer process is achieved by movement of the protein molecules or exchange of electrons between the bound proteins. Because of the strong electrostatic interaction between the negatively charged PAA brush and the positively charged protein under weakly basic conditions, the diffusion of Cyt c in the membrane is much slower than that in solution. On the same timescale, the diffusion layer in the membrane is much thinner than that in solution. Therefore, a confined diffusion behavior is exhibited in the thin confined layer. This indicates that some Cyt c is present in the free state in the PAA brush film.

Immobilization of Cyt c on the PAA brush interface at pH 7.6: Because of the negative excess charge accumulated at the interface by the PAA brush at pH 7.6, the system was found to be effective in immobilizing Cyt c through electrostatic interaction. EIS measurements were performed to confirm the adsorption of Cyt c on the PAA brush-modified interface using $[Fe(CN)_6]^{3-/4-}$ as the electrochemical probe. Curve a in Figure 5 shows a Nyquist plot of the EIS ob-

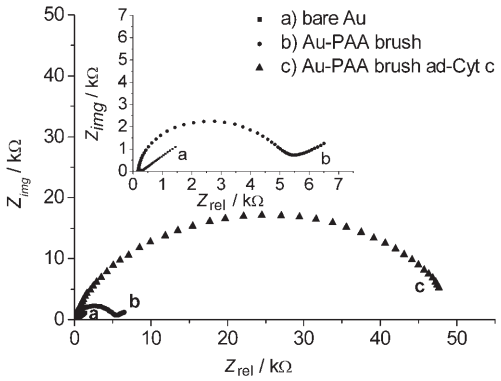


Figure 5. Nyquist plots of a) bare gold electrode, b) PAA brush, and c) PAA brush ad-Cyt c modified gold electrodes measured in 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M $KClO_4$ solution at pH 6.0. Inset: enlargement of curves (a) and (b). Electrode area: 8×10^{-2} cm^2.

tained at a bare Au electrode in $[Fe(CN)_6]^{3-/4-}$ solution. The electron-transfer resistance, R_{et}, derived from the semicircle of the impedance spectrum, was measured as 200 Ω for the bare Au electrode. After electrochemically induced polymerization of PAA in aqueous solution, the EIS of the resulting assembled PAA layer showed a higher interfacial electron-transfer resistance (R_{et} = 5.7 kΩ, Figure 5, curve b), suggesting that the PAA layer obstructed the electron transfer of the electrochemical probe through insulation of the PAA brush film at pH 6.0. The PAA brush ad-Cyt c modified electrode was finally obtained by dipping the PAA brush-modified electrode into a 15 μM Cyt c solution for 5 min. After the absorption of Cyt c, the interfacial ETR greatly increased (R_{et} = 50 kΩ, Figure 5, curve c). Thus, to sum up, Cyt c can be adsorbed on the surface of a PAA brush-modified electrode, and this increases the interfacial ETR of $[Fe(CN)_6]^{3-/4-}$.

In addition, to verify the electrostatic interaction between Cyt c and the PAA brush, in situ attenuated total internal reflection Fourier-transform infrared (ATR-FTIR) spectra were obtained for the PAA brush and PAA brush ad-Cyt c surfaces, as shown in Figure 6. The assembly of the PAA

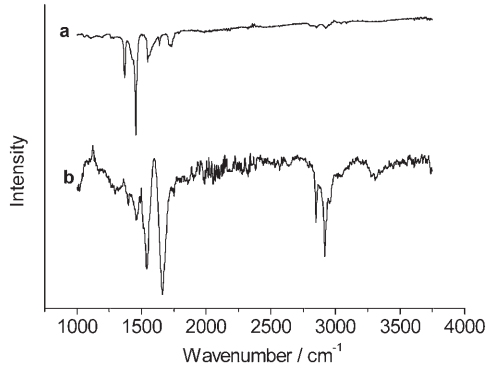


Figure 6. ATR-FTIR spectra of a) PAA brush and b) PAA brush ad-Cyt c on Au surfaces at pH 7.6.

brushes on the Au surface was confirmed by ATR-FTIR (Figure 6, spectrum a), as the spectrum obtained featured the expected absorption bands due to the carbonyl stretching (C=O) at 1723 cm^{-1}, CH_2 stretching at 1456 cm^{-1}, C–O stretching at 1264 cm^{-1}, and the symmetric and antisymmetric stretching frequencies of the carboxylate ion (COO^-) at 1420 and 1550 cm^{-1}, respectively.[21] The Cyt c was absorbed by the PAA chains simply by dipping the PAA brush-modified Au foil into a Cyt c solution for 5 min at pH 7.6. The ATR-FTIR spectrum recorded thereafter showed the characteristic absorption bands of Cyt c at 3301 cm^{-1} (N–H stretching) and 2956 cm^{-1} (C–H stretching), and bands at 1663 cm^{-1} and 1544 cm^{-1} arising from the amide (Figure 6, spectrum b). These absorption bands are characteristic of horse heart Cyt c.[22]

In addition, CV measurements were performed in order to demonstrate the immobilization and direct electron transfer of Cyt c on the PAA brush film. A voltammogram was recorded after transferring the PAA-modified electrode from Cyt c solution into pure phosphate buffer (pH 7.6). It showed the same pattern as that recorded from the Cyt c solution during potential cycling, with the same formal potential of 40 mV (see Figure 7, curve a). This result showed that the PAA-coated electrode captured Cyt c from its solution through electrostatic interaction between the Cyt c and the PAA layer. The captured Cyt c on the PAA layer was found to be stable in Cyt c-free PBS (pH 7.6) and retained the same electrochemical activity as that seen in the Cyt c solution. Only small changes in the voltammograms of the PAA ad-Cyt c electrode were observed after 15 potential sweep cycles in the same blank buffer solution or after rinsing the modified electrode with distilled water. The average surface coverage of the Cyt c immobilized on the PAA film was calculated to be about 1.2×10^{-10} mol cm^{-2} at 5 mV s^{-1}, again indicating a multilayer of Cyt c on the surface of electrode.

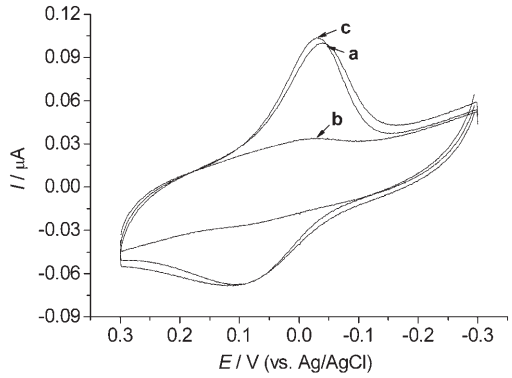


Figure 7. Cyclic voltammograms of Cyt c immobilized on a PAA-modified gold electrode in 10 mM PBS at a scan rate of 10 mV s^{-1}, a) in pH 7.6 PBS, b) in pH 4.0 PBS, c) back to pH 7.6 PBS after dipping the PAA-modified electrode in 15 μM pH 7.6 Cyt c for 5 min.

The electron-transfer rate constant obtained from the CV and EIS measurements was around 0.08 s^{-1}, slightly lower than that measured in Cyt c solution. When the PAA ad-Cyt c modified electrode was immersed in pH 4.0 PBS, the redox current of Cyt c was much smaller, indicating that most of the immobilized Cyt c had been released from the PAA brush film (curve b). The measured electrode was then immersed in 15 μM Cyt c solution (pH 7.6) for 5 min, washed with pure water, and the cyclic voltammogram was recorded in 10 mM pH 7.6 PBS. The increase in the redox peaks in curve c (Figure 7) demonstrated the reabsorption of Cyt c by the PAA brush interface. This suggested that the adsorbed Cyt c on the electrode could be renewed, and so the PAA brush-modified electrode could be reutilized as a biosensor.

Conclusion

Our results have demonstrated the viability of a new system that allows the reversible immobilization of Cyt c on a pH-sensitive polymer brush interface as part of a polymer–protein architecture. The absorption–desorption behavior and the direct electron-transfer process of Cyt c at the interface have been evaluated by spectroscopic analysis and electrochemical experiments. Reversible immobilization of proteins or enzymes at a PAA brush interface may offer some possibilities for protein separation and biosensing. The efficient and reproducible electron transfer through the polymer brush also provides an opportunity to simulate biological electron-transport systems. The integration of redox-active biomolecules into such "smart" polymer brushes could open up the way to artificial models of biological redox systems leading to bioelectronic functionalities.

Experimental Section

Materials and solutions: Cytochrome c (from horse heart, purity 97%) was purchased from Sigma-Aldrich. Acrylic acid, sodium nitrate, and sodium persulfate (Tianjin VAS Chemical Reagent Co.) were purified before use. $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]\cdot 3H_2O$, $KClO_4$, $NaH_2PO_4\cdot 2H_2O$, $Na_2HPO_4\cdot 12H_2O$, $NaHSO_3$, and HCl (Beijing Yili Chemical Reagent Co.) were analytical grade. All solutions were prepared by using Milli-Q purified water (>18.0 MΩ cm) that had been sterilized at high temperature.

A 5 mM solution of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) used as a redox probe (0.1 M aqueous $KClO_4$ solution used as supporting electrolyte) was prepared by dissolving $K_3[Fe(CN)_6]$ (0.8280 g), $K_4[Fe(CN)_6]\cdot 3H_2O$ (1.0615 g), and $KClO_4$ (6.9486 g) in purified water (500 mL), and adjusting to the desired pH using HCl. A solution of Cyt c was prepared by dissolving Cyt c powder in 10 mM phosphate buffer solution (PBS) at pH 4.0 or 7.6. All solutions were freshly prepared at 25 °C before use.

Preparation of the PAA brush-modified electrode: Prior to use, an Au disc electrode (diameter 2.0 mm) was polished on a polishing cloth with alumina of successively smaller particle sizes (1.0 and 0.05 μm diameter). The electrode was then cleaned by ultrasonication in deionized water, acetone, and ethanol, respectively. The actual area of the electrode surface, taking into account a roughness factor of about 2.58,[23] was found to be around 0.08 cm^2. In a typical process, the electrochemically induced polymerization was performed in 8.0 mL of aqueous solution, which contained acrylic acid (1.0021 g), $NaNO_3$ (0.1165 g), $NaHSO_3$ (2.1 mg), and $Na_2S_2O_8$ (9.6 mg), and had been deoxygenated by bubbling nitrogen gas through it for 40 min. The potential was then cycled (100 $mV s^{-1}$) between −0.1 and −0.7 V for 60 cycles. After the polymerization, the electrode was washed with doubly-distilled water to remove the monomers. The resulting PAA was attached to the Au surface, as confirmed by in situ ATR-FTIR spectroscopic analysis and EIS. For UV/Vis and FTIR measurements, poly(acrylic acid) was assembled on Au foils with dimensions of 1×1 cm^2 by following the same procedure.

Preparation of the PAA brush ad-Cyt c modified electrode: Immobilization of Cyt c on the PAA-modified Au electrode surface was achieved through a dip-coating method. The PAA-modified Au electrode was dipped into 15 μM Cyt c solution (10 mM pH 7.6 PBS as supporting electrolyte) for 5 min, then washed with purified water. In this way, a PAA brush ad-Cyt c modified Au electrode was obtained.

Electrochemical measurements: The electrochemical measurements were based on a conventional three-electrode system with the Au electrode as the working electrode, a platinum wire as the auxiliary electrode, and an Ag/AgCl (saturated KCl) electrode as the reference electrode. All experiments were carried out at room temperature unless otherwise noted. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were performed using a CHI 630B analyzer (CH Instruments Inc., USA) and a PARSTAT 2273 Advanced electrochemical system (Princeton Applied Research, USA), respectively.

The solutions were purged prior to the measurements by continuously bubbling highly purified nitrogen through them for at least 30 min, and a nitrogen atmosphere environment was maintained during the electrochemical measurements. To investigate the pH sensitivity of the PAA brush-modified electrode, electrochemical measurements were carried out in the presence of 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) as a redox probe (0.1 M $KClO_4$ used as supporting electrolyte). CV measurements were performed in the potential range from −0.1 to 0.5 V at a scan rate of 50 $mV s^{-1}$. EIS measurements were performed at a bias potential of 0.240 V, in the frequency range from 100 kHz to 100 mHz, using an alternating voltage of 10 mV. To assess the direct electrochemistry of Cyt c, electrochemical measurements were carried out in 10 mM phosphate buffer solution (PBS) with or without 15 μM Cyt c. CV measurements were performed in the potential range from 0.3 to −0.3 V at different scan rates. EIS measurements were performed at a bias potential of 0.042 V, in the frequency range from 100 kHz to 100 mHz, using an alternating voltage of 10 mV.

Spectroscopic analysis: UV/Vis measurements were performed with a 2100S spectrophotometer (Shimadzu, Japan) in combination with a mirror reflection accessory. A PAA-modified Au foil was dipped into 10 mM pH 7.0 PBS containing 15 μM Cyt c for 5 min, washed with pH 7.6 PBS to remove the unabsorbed Cyt c, dried in air, and then the reflection spectrum of Cyt c on the PAA-modified Au foil was recorded. The foil substrate was then immersed in 10 mM pH 4.0 PBS, also containing 15 μM Cyt c, for another 5 min, washed with 10 mM pH 4.0 PBS, and the reflection spectrum was recorded once more. The above processes were repeated for six cycles. Attenuated total internal reflection Fourier-transform infrared (ATR-FTIR) spectra were recorded by using a Spectrum One spectrometer (Perkin Elmer instruments). The spectra were collected at a resolution of 4 cm^{-1}.

Acknowledgements

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