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Covalent immobilization of horseradish peroxidase via click chemistry and its direct electrochemistry

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ABSTRACT

A simple and versatile approach for covalent immobilization of redox protein on solid surface via self-assembled technique and click chemistry is reported. The alkynyl-terminated monolayers are obtained by self-assembled technique, then, azido-horseradish peroxidase (azido-HRP) was covalent immobilized onto the formed monolayers by click reaction. The modified process is characterized by reflection absorption infrared spectroscopy (RAIR), surface-enhanced Raman scattering spectroscopy (SERS) and electrochemical methods. All the experimental results suggest that HRP is immobilized onto the electrode surface successfully without denaturation. Furthermore, the immobilized HRP shows electrocatalytic reduction for H_2O_2, and the linear range is from 5.0 to 700 μM. The heterogeneous electron transfer rate constant k_s is 1.11 s^{-1} and the apparent Michaelis–Menten constant is calculated to be 0.196 mM.

1. Introduction

Electron transfer (ET) in redox proteins plays a key role in many biological reactions, such as respiration and photosynthesis [1–3]. Moreover, the direct ET between redox proteins and contact is also of fundamental importance in life sciences and potential applications, for example, bioelectrocatalysis, bioelectronics, biosensors, and biofuel cells, where the redox protein must be immobilized on the electrode surface [4–11]. However, the direct ET is not easy to be realized, which is due to the low electronic conductivity of the surrounding amino acid chains [12], unfavorable orientation or denaturation of protein molecules on the electrode surface [13,14], and the distance-dependent heterogeneous long-range ET [15,16]. The approaches commonly used for redox protein immobilization, such as nonspecific adsorption [17,18] and some covalent attachment [19,20], are difficult to control and yield randomly bound proteins with poor orientation or inappropriate alignment of the redox center, which results in inefficient ET to electrode surfaces. Therefore, how to control the conformation, orientation and distance of redox proteins on the electrode surface are the crucial requirements for obtaining fast ET. Recently, some strategies have been proposed to promote the direct ET, such as nanoparticle–enzyme hybrid systems [1,21], monolayer-protected clusters [3,22], functional protein multilayer assemblies [23], natural or artificial electron relays [24–26] and conducting molecular wires [15,27].

On the basis of above-mentioned work, it appears that the key issue in properly investigating the direct ET of redox-active proteins is to design a biocompatible interface, which is able to immobilize the redox protein with reproducible binding, conformation preserving, distance controlling, homogeneous reparting and molecular-level orientating. In addition, in view of practical applications, the stability is also very important. Among the few protein-immobilization strategies able to meet these multiple criteria, the method based on the self-assembled technique [28–30] and click chemistry maybe open a new way to enhance the direct ET between the redox protein and under-laying electrode. It is well known that a monolayer on conductive surfaces can provide a powerful platform for redox protein immobilization with precise distance control, homogeneous repartion, and excellent conformation preservation [15,22,31]. Click chemistry, which is introduced by Sharpless and co-workers [32], winner of the 2001 Nobel Prize in chemistry, is an advanced and reliable synthetic strategy and has widespread applications, such as drug discovery [33], biomacromolecule modification [34,35], surface functionalization [36–38], and so on. One of the most popular reactions within the click chemistry is the Cu(I)-catalyzed azide alkyne 1,3-dipolar cycloaddition (CuAAC) reaction at room temperature, which is considered as the "cream of the crop". This reaction (a) has high yield and works regioselectively under very mild conditions, which can avoid the protein's denaturation, (b) proceeds irreversibly and the resulting 1,4-disubstituted

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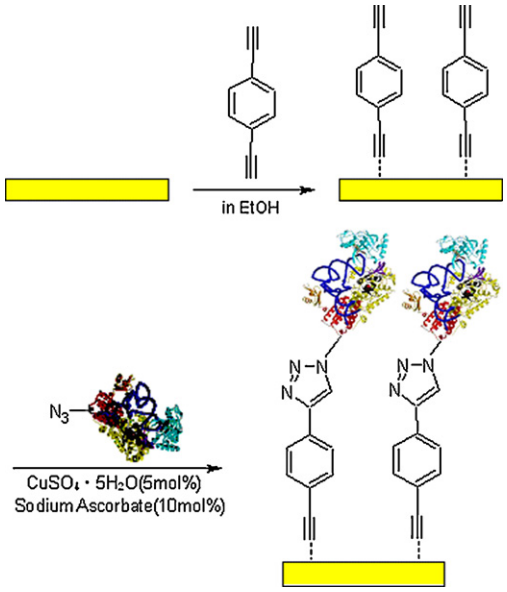

Scheme 1. Fabrication strategy of HRP/DEB modified electrode.

1,2,3-triazole ring is very stable under physiological conditions, (c) the azides and acetylenes are highly energetic and inert to biomolecules [39], which is useful for site-specifically immobilizing biomolecules on solid surface [40]. These profusive advantages make click reaction meet the critical elements of grafting proteins onto solid materials. In our previous work, alkynylation/click procedure and EDC-mediated covalent attachment were used to graft redox-active protein hemoglobin to a gold surface and the direct ET of hemoglobin was obtained [41]. In this work, click reaction was used to introduce NH_2-terminated self-assembled monolayers (SAMs) onto Au electrode surface. Then, hemoglobin was grafted onto Au electrode via carbodiimide reaction, which is hampered by a difficulty in introducing the reactive groups, a lack of specificity, or a low yield [42,43]. Based on our previous work, a new and simple strategy was proposed for covalent immobilize redox protein onto Au electrode surface for direct ET and biosensing. An alkynyl complex, 1,4-dialkynylbenzene (DEB), is chose as the self-assembled molecule since ET rate through benzene is more faster [44] and the alkynyl group could provide an unbroken π-conjugated linkage to the gold surface [45]. Then, azido group modified horseradish peroxidase (azido-HRP) was covalently immobilized onto DEB SAMs via CuAAC "click chemistry" (Scheme 1), and the direct electron transfer between the protein redox center and electrode surface was achieved successfully. To the best of our knowledge, this is the first report to make use of click chemistry for the covalent immobilization of redox proteins for direct ET and biosensing. This approach for redox protein immobilization paves a new way for fabricating the third-generation biosensors.

2. Experimental

2.1. Reagents

DEB, sodium ascorbate, and copper (II) sulfate pentahydrate were purchased from sigma. HRP (E.C.1.11.1.7) was purchased from Sinopharm Chemical Reagent Co., Ltd. Imidazole-1-sulfonyl azide hydrochloride and azido-HRP were synthesized using previously established methods [46]. Other chemicals were of analytical grade and used without further purification. All solutions were made up with twice distilled water.

2.2. Instruments

All the electrochemical experiments were carried out on a computer-controlled CHI 400A electrochemical workstation. The three-electrode cell was composed of a saturated calomel electrode (SCE) as the reference, a platinum electrode as the counter, and a modified gold electrode as the working electrode.

Reflection absorption infrared (RAIR) spectroscopy was conducted on an America-Nexus 670 Fourier transform infrared (FTIR) spectrometer. All the spectra were obtained with an average of 100 scans and 4 cm^{-1} resolution.

Surface enhanced Raman scattering (SERS) spectra were recorded using a T64000 Raman spectrometer (Jobin Yvon, France). Excitation was provided by a 785 nm He–Ne laser at the sample. The laser beam was turned to the sample at an angle of 908. All spectra were recorded by a charge-coupled device (CCD) detector. The system was operated and monitored via a computer interface.

2.3. Synthesis of azido-HRP

Firstly, imidazole-1-sulfonyl azide hydrochloride, an efficient, inexpensive, and shelf-stable diazotransfer reagent was synthesized as described by Goddard-Borger and Stick [46]. Then, azido-HRP was synthesized according to the work of Dongen et al. [47]. In briefly, to an aqueous solution of HRP (200 μL, 2.5 mg mL^{-1}), K_2CO_3 (100 μL, 2 mg mL^{-1}) and $CuSO_4 \cdot 5H_2O$ (25 μL, 1 mg mL^{-1}) were added. After mixing, a solution of imidazole-1 sulfonyl azide hydrochloride was added (15 μL, 2 mg mL^{-1}, 1.75 equiv. relative to amines in HRP) and the reaction was stirred overnight at room temperature. The mixture was transferred to a dialysis bag (molecular weight 8000–14,000), and dialyzed three times against 500 mL pH 7.0 phosphate buffered solution (PBS) for a total of 36 h.

2.4. Self-assembled of DEB SAMs

Before use, the bare gold electrode was polished with 0.05 μm Al_2O_3 slurry until a mirror-like surface was obtained, then it was sonicated in water and ethanol, cleaned with piranha solution (3:1 H_2SO_4/H_2O_2, v/v) and sonicated in water and ethanol again. Finally, the electrode was electrochemically cleaned by cyclic scans between 0 and 1.6 V in 0.1 M H_2SO_4 solution until a reduplicate cyclic voltammogram was obtained. The cleaned gold electrode was placed in freshly prepared DEB ethanol solution (10.0 mM) for 48 h. After that, the electrode was washed with water and ethanol, and then nitrogen-dried.

2.5. Covalent immobilization of HRP via click reaction

The immobilization of HRP was achieved by immersing the above electrode in azido-HRP solution (5.0 mg mL^{-1}, water) with sodium ascorbate (10.0 mol%) and copper (II) sulfate pentahydrate (5.0 mol%) at 4 °C for 24 h. The electrode was washed with water and stored at 4 °C when not used.

3. Results and discussions

3.1. FTIR

As described by Dongen et al. [47], azido-HRP was synthesized by a suitable technique for the selective modification of proteins under ambient conditions, leading to the facile introduction of azides in the side chains of lysine residues and at the N-terminus of enzymes.

RAIR spectrum is sensitive to the secondary structure of the protein, and the characteristic amide I (1700–1600 cm^{-1}) and amide

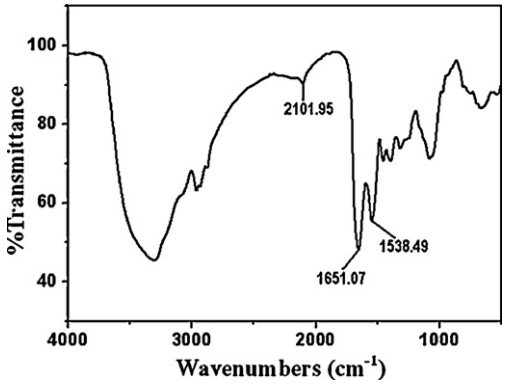

Fig. 1. RAIR spectrum of azido-HRP.

II (1600–1500 cm^{-1}) bands of HRP can provide detailed secondary structure of peptide chains. As shown in Fig. 1, the amide I and amide II bands of azido-HRP are 1651.07 cm^{-1} and 1538.49 cm^{-1}, respectively. It is similar to the native HRP (1642.12 cm^{-1} and 1531.85 cm^{-1}) [48], indicating that HRP retained the essential secondary structure after azide modification. The peak at 2101.95 cm^{-1} is corresponding to the absorption of azide [49], illustrating the successful modification of azide group.

3.2. Raman spectra

Surface-enhanced Raman scattering (SERS) has shown promise in exhibiting high sensitivity, and provides an information-rich spectrum of narrow spectral lines that is naturally suited to multiplexed analysis [50]. The SERS spectra of DEB SAMs and after HRP clicked are shown in Fig. 2. The sharp and strong peaks at 1591.75 cm^{-1} and 2128.31 cm^{-1} in Fig. 2a are attributed to the C=C group in benzene [51] and C≡C in DEB [52], respectively, which indicates the successful self-assembled of DEB SAMs on the gold surface. In Fig. 2b, after click reaction, the peak for C=C group (at 1591.17 cm^{-1}) in benzene still remains and the peak at about 2100 cm^{-1} is obviously decreased, elucidating the occurrence of click reaction. The new peaks at 1572.85 cm^{-1} and 1631.77 cm^{-1} ascribe to the amide I and amide II in HRP [53], which illuminates the success immobilization of HRP onto the terminal end of DEB SAMs. It also indicates that the immobilized HRP retains its natural conformation after click reaction. Besides this, there is a new peak at 2009.67 cm^{-1}, which might ascribe to the adsorption of excessive azide group [54]. According to the literature [47], one HRP molecule has four azides group after modification, assuming that the covalent bonding consumes one azide per HRP molecule and three azides still present in the HRP after click reaction.

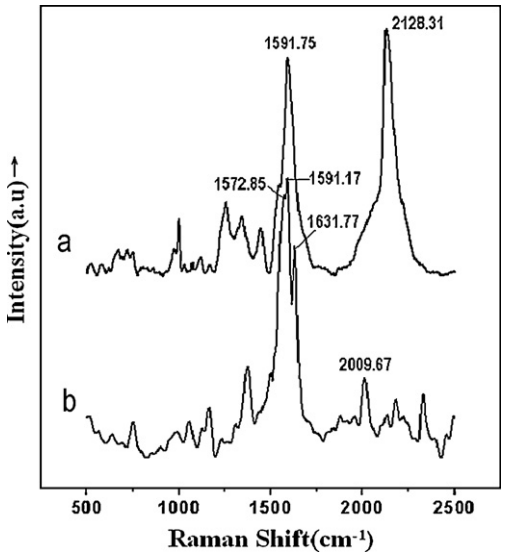

Fig. 2. SERS spectra of DEB SAMs (a) and HRP/DEB (b) on roughened gold plates.

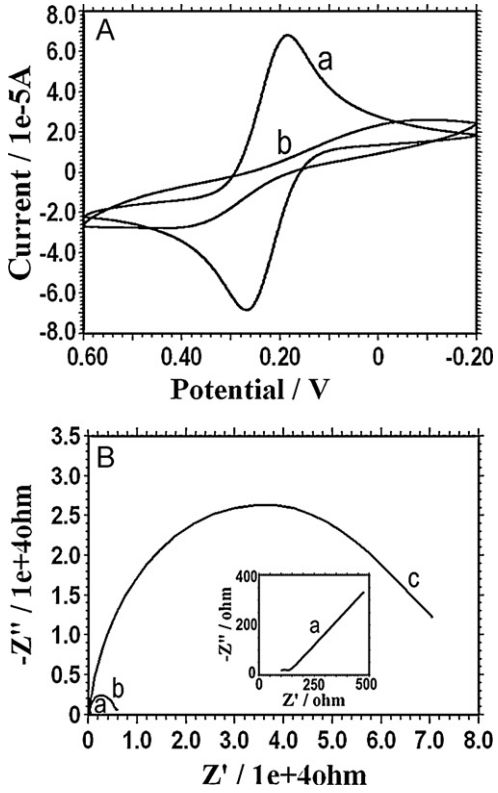

Fig. 3. CVs of 1.0 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ in 0.1 M KCl at bare gold electrode (a), DEB SAMs modified electrode (b) with scan rate of 100 mV s^{-1} (A). The EIS of bare gold electrode (a), DEB SAMs (b) and HRP/DEB modified electrode (c) in 0.1 M KCl containing 2.5 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ (B). The inset is the amplificatory EIS for bare gold electrode.

3.3. Electrochemical characterization

Fig. 3A shows the CVs of $K_4Fe(CN)_6/K_3Fe(CN)_6$ at bare electrode and alkynyl-terminated SAMs modified electrode. As shown in curve a, a good pair of reversible peaks are observed at the bare gold electrode, but after the formation of DEB SAMs, the current dramatically decreases and the potential difference (ΔE_p) increases simultaneously. It is because the DEB SAMs have no redox activity and block the electron transfer, which also confirms the successful formation of DEB SAMs on the electrode surface.

The EIS is capable of showing the impedance change at the interface of electrolyte and electrode. As displayed in Fig. 3B, the electron-transfer resistance (R_{ct}) increases obviously after the DEB SAMs was formed. It is because the DEB SAMs block the electron transfer and this also suggests the successful formation of DEB SAMs on the electrode. When HRP is immobilized on the DEB SAMs via click reaction, the R_{ct} increases further, which might ascribe to the hindrance of the macrostructure structure of HRP [55].

3.4. Direct electrochemistry of HRP

The electrochemistry of HRP/DEB electrode is shown in curve a of Fig. 4A, and a pair of quasi-reversible redox peaks at 0.039 V and 0.174 V is observed, which is corresponding to the Fe(II)/Fe(III)

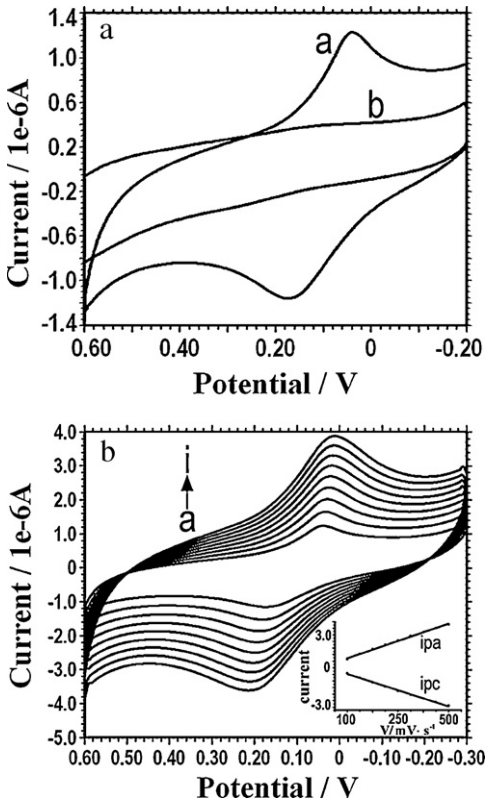

Fig. 4. CVs of DEB/Au electrode in presence of N_3-HRP with (a) and without (b) Cu (I) as catalyst in 0.1 M PBS (pH 7.0) with scan rate of 100 mV s^{-1} (A). CVs of HRP/DEB electrode in 0.1 M PBS (pH 7.0) with scan rate of 100 (a), 150 (b), 200 (c), 250 (d), 300 (e), 350 (f), 400 (g), 450 (h), 500 (i) mV s^{-1}. The inset is the plot of currents via scan rates.

redox couple in the HRP. The formal potential ($E^{\theta} = (E_{pa} + E_{pc})/2$) is 0.106 V, which is close to the value 0.08 V obtained by immobilizing HRP on the Au nanoparticles electrodeposited indium tin oxide electrode [56], and more positive than the value −0.1 V for HRP immobilized on the layered calcium carbonate-gold nanoparticles inorganic hybrid composite [57], which means that the HRP immobilized on the DEB SAMs may need less activation energy to conduct direct ET with the electrode. This may be ascribed to the favorable microenvironment for the proteins provided by the DEB SAMs. As a control experiment, while the DEB modified electrode was immersed in azido-HRP solution but without the Cu(I) catalyst, no redox peaks could be observed (curve b of Fig. 4A). It suggests that HRP is successfully immobilized on the electrode via click reaction, not by physical adsorption.

Fig. 4B displays the overlap of CVs of HRP/DEB electrode with the scan rates over the range of 100–500 mV s^{-1}. The redox peak currents are proportional to the scan rate (inset of Fig. 4B), indicating a surface-controlled electrochemical process. According to the Laviron Eq. (1)

$$I_p = \frac{nFQv}{4RT} = \frac{n^2F^2A\Gamma v}{4RT} \quad (1)$$

where n is the number of electron transferred, F is the Faraday constant, Q represents the charge amount, v is the scan rate, A stands for the area of the electrode, and Γ is the surface concentration of electroactive HRP. The number of electron transferred (n) is calculated to be 0.9, which suggests the reaction between HRP and the electrode surface is a single electron transfer process. The surface concentration of HRP (Γ) is about 2.9×10^{-11} mol cm^{-2}, which is close to the theoretical monolayer coverage (about 2.0×10^{-11} mol cm^{-2} [58]), indicating that a monolayer of immobilized HRP on the DEB SAMs participated in the electron transportation process.

The heterogeneous electron transfer rate constant k_s can be estimated by Eq. (2) when the peak-to-peak separation was less than 200 mV,

$$k_s = \frac{mnFv}{RT} \quad (2)$$

where m is a parameter related to the peak-to-peak separation, n is the number of electron transfers, F is Faraday's constant, R is the gas constant, and T is the temperature. In this system, the peak-to-peak separation was 135, 147, 159, 170, 179, 187, 192, and 198 mV at 100, 150, 200, 250, 300, 350, 400, 450 mV s^{-1}, respectively, producing an average k_s value of 1.11 s^{-1}. It is close to the value 1.15 s^{-1} obtained by immobilizing HRP on Zinc oxide nanorods [59], larger than 0.92 s^{-1} for HRP immobilized on hexagonal mesoporous silica matrix [60], and 0.66 s^{-1} for HRP coated on a sealing film covered graphite electrode [61], suggesting a relatively fast ET. The favorable orientation the HRP molecules and high electron transfer rate through the triazole linkage and benzene ring [32] may contribute to the fast ET between HRP and the electrode surface.

3.5. Catalytic activity of HRP toward H_2O_2

HRP has a heme center in the polypeptide chains, so it is able to reduce H_2O_2 electrochemically. Fig. 5 displays the typical amperometric response of the modified electrode on successive addition of H_2O_2 under the optimized experimental conditions, the linear range is from 5.0 to 700 μM, and the detection limit is 2.5×10^{-6} M (S/N = 3).

The apparent Michaelis–Menten constant K_m^{app} could indicate biologic activity of the immobilized biomolecules on the electrode surface and the affinity to the substrates. It can be calculated from the Lineweaver–Burk Eq. (3)

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_m^{app}}{I_{max}}C \quad (3)$$

where I_{ss} is the steady-state response current after the addition of substrate, I_{max} is the maximum current measured under saturated substrate conditions, C is the bulk concentration of the substrate. The K_m^{app} is calculated to be about 0.196 mM, which is lower than the value 8.0 mM obtained by electro-deposition HRP–ZrO_2 composite on gold electrode [62], 1.1 mM for HRP/Au nanoparticle/cysteine in silica sol–gel [63], and 2.6 mM of Au/graphene/HRP/chitosan biocomposites on glass carbon electrode [64]. These results indicate that the HRP/DEB modified electrode exhibits good affinity to H_2O_2 and retains their enzymatic activity.

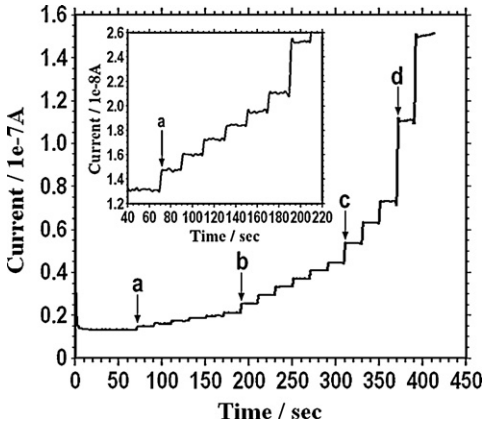

Fig. 5. i–t Response of HRP/DEB electrode to different concentration of H_2O_2 (a–d: 5.0×10^{-6}, 5.0×10^{-5}, 2.0×10^{-4}, 5.0×10^{-4} M H_2O_2) in 0.1 M PBS (pH 7.0) at 0 V. The inset is the enlargement of i–t responses for 5.0×10^{-6} M H_2O_2.

4. Conclusion

Herein, we proposed a simple and feasible methodology for redox protein immobilization based on the self-assembled technique and click chemistry. The RAIR, SERS and electrochemical characterization show that the covalent immobilized HRP retained their secondary structure and biological activity. Furthermore, the modified electrode exhibited good catalytic reduction to H_2O_2 and could be used as a H_2O_2 sensor.

Acknowledgments

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