Functionalization of carbon nanotubes for direct electrochemistry of horseradish peroxidase

Wei Zheng, Qingfen Li, Yiming Yan, Lei Su & Lanqun Mao

1School of Mechanical and Electrical Engineering, Harbin Engineering University, Harbin 150001, PR China
2Center for Molecular Science, Institute of Chemistry, The Chinese Academy of Sciences, Beijing 100080, PR China
Email: lqmiao@iccas.ac.cn

Received 9 November 2004

New strategies for functionalization of carbon nanotubes (CNTs) for facilitating direct electron transfer of horseradish peroxidase (HRP) are reported. The CNTs are covalently functionalized with hydroxyl and carboxyl groups and noncovalently functionalized with block copolymer (Pluronic P123). The functionalized CNTs are found to possess an improved solubility in aqueous solution and may have an enhanced biocompatibility with biomacromolecules such as proteins and enzymes. Moreover, all the functionalized CNTs have been demonstrated for the first time to be capable of facilitating the direct electron transfer of HRP, in which the noncovalently P123-functionalized CNTs are shown to be advantageous over those covalently functionalized with –OH and –COOH groups. The catalytic activities of HRP adsorbed onto the P123-functionalized CNTs towards the reductions of dioxygen (O₂) and hydrogen peroxide (H₂O₂) are also discussed.

Functionalization of carbon nanotubes for direct electrochemistry of horseradish peroxidase

Weizheng, Qingfen Li, Yiming Yan, Lei Su & Lanqun Mao

1School of Mechanical and Electrical Engineering, Harbin Engineering University, Harbin 150001, PR China
2Center for Molecular Science, Institute of Chemistry, The Chinese Academy of Sciences, Beijing 100080, PR China
Email: lqmiao@iccas.ac.cn

Received 9 November 2004

New strategies for functionalization of carbon nanotubes (CNTs) for facilitating direct electron transfer of horseradish peroxidase (HRP) are reported. The CNTs are covalently functionalized with hydroxyl and carboxyl groups and noncovalently functionalized with block copolymer (Pluronic P123). The functionalized CNTs are found to possess an improved solubility in aqueous solution and may have an enhanced biocompatibility with biomacromolecules such as proteins and enzymes. Moreover, all the functionalized CNTs have been demonstrated for the first time to be capable of facilitating the direct electron transfer of HRP, in which the noncovalently P123-functionalized CNTs are shown to be advantageous over those covalently functionalized with –OH and –COOH groups. The catalytic activities of HRP adsorbed onto the P123-functionalized CNTs towards the reductions of dioxygen (O₂) and hydrogen peroxide (H₂O₂) are also discussed.

The preparation and basic studies on carbon nanotubes (CNTs) are being carried out because of their unique electronic, structural and mechanical properties. These attractive properties of the CNTs enable them to be particularly useful for practical applications such as for the development of nanodevices, quantum wires, ultrahigh-strength engineering fibers and sensors. Recent studies show that the CNTs possess distinct electrochemical properties from other carbon-based materials, e.g., glassy carbon, graphite and diamond. The nanotubes have open ends and sidewalls that may respectively behave like edge and basal plane graphite electrochemically, and possess better electrocatalytic activity and higher surface area compared with other kinds of carbon-based materials. The unique properties of CNTs make them particularly useful for various electrochemical investigations, e.g., electrocatalysis, direct electrochemistry of proteins and fabrication of electrochemical sensors and biosensors. Our recent investigations on CNT electrochemistry are primarily focused on the development of CNT-based bioelectronic nanodevices such as biosensors and biofuel cells, mainly by virtue of the attractive properties of the CNTs.

A combination of the striking electrochemical properties of the CNTs with the specific enzyme-substrate reactivity of the biomacromolecules (e.g., proteins and enzymes) could yield functional nanohybrid systems. This may pave an effective way for the development of new bioelectronic nanodevices, which is largely limited by: (i) the CNTs tend to aggregate in most solvents, rendering difficulties in manipulation of the CNTs for practical applications and their integration with biomacromolecules; (ii) the electronic communication between the proteins and the CNTs, which is a critical step for developing redox protein-based bioelectronic nanodevices, is rather slow at the pristine CNTs; and, (iii) the strong interactions between the proteins and the CNTs substantially distort the proteins, leading to the loss of their biocatalytic activities toward substrates. Using horseradish peroxidase (HRP) and single-walled carbon nanotubes (SWNTs), we have tried to address these limitations by rationally functionalizing the CNTs through covalent and noncovalent ways so as to facilitate direct electronic communication between the proteins and the CNTs. These approaches may offer a straightforward way to bioelectronic nanodevices such as electrochemical biosensors and enzyme-based biofuel cells and would be very useful for investigating protein electron transfer at nanointerfaces.
Materials and Methods

CNTs (dia.30-40 nm, purity>95%, length 0.5-40 μm) were purchased from Shenzhen Nanotech Port Co., Ltd (Shenzhen, China). Prior to use, the CNTs were purified by refluxing the as-received CNTs in 2.6 M HNO₃ for 36 h. Covalent sidewall functionalization of the CNTs with hydroxyl groups was performed as already reported²⁵. Briefly, the CNTs (10 mg) and potassium hydroxide (200 mg) were weighted into a stainless steel capsule containing a milling ball. Then, the capsule was vigorously shaken for 2 h in air at room temperature. The reaction mixture was dissolved in 10 mL of distilled water and repeatedly precipitated into methanol to ensure complete removal of potassium hydroxide residue. The obtained CNTs functionalized with multiple hydroxyl groups (CNT-OHs) were highly hydrophilic and could be readily dispersed in distilled water and ethanol. The CNTs sidewall functionalized with carboxyl groups (CNT-COOHs) were received as a gift from Dr. Zhengzhong Yang at Institute of Chemistry, CAS. Structures of the CNT-OHs, CNT-COOHs and Pluronic P123 are shown in Scheme 1.

Horseradish peroxidase (HRP, m.wt. 42100) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma and used without further purification. Pluronic P123 (EO₉₄PO₇₃EO₉₄, m.wt. 5800) was purchased from Beijing Chemical Co (Beijing, China). Other chemicals were of analytical grade or higher and used as received. Aqueous solutions were prepared with doubly distilled water.

Glassy carbon (GC, 3 mm-diameter) electrodes purchased from Bioanalytical Systems Inc. (BAS, West Lafayette, IN) were first polished with emery paper (# 2000), 0.3 and 0.05 μm alumina slurry on a wooden cloth, and then cleaned under bath sonication for 10 min. and finally rinsed thoroughly with distilled water.

CNT-OHs or CNT-COOHs (1.0 mg) were dispersed into 1 mL distilled water and the mixture was sonicated to give a homogeneous and black dispersion, which was stable for at least two weeks. The dispersion (4 μL) was dip-coated onto the GC electrodes and the electrodes (denoted as CNT-OH/GC and CNT-COOH/GC) were air-dried. HRP was adsorbed onto the CNT-OH/GC electrodes by immersing the as-prepared CNT-OH/GC electrodes into 0.10 M phosphate buffer (pH 7.0) containing 20 mg/mL HRP for 8 h. After drying in air, the electrodes (HRP/CNT-OH/GC) were coated with 10 μL of 0.5% Nafion solution to improve the electrode stability. For the preparation of HRP/CNT-COOH/GC electrodes, a mixture consisting of 1.0 mg/mL CNT-COOHs, 10 mg/mL HRP and 8 mM EDC was first prepared. An aliquot (4 μL) of the resulting mixture was then coated on GC electrodes. The electrodes were finally air-dried and rinsed with distilled water.

For the preparation of HRP/CNT-P123/GC electrodes, the CNTs were dispersed into 0.40 mM aqueous solution of P123 and the mixture was sonicated to give a homogenous black dispersion containing 1 mg/mL of the CNTs. The dispersion was stable for about one month. An aliquot (4 μL) of the dispersion was dip-coated onto GC electrodes and the electrodes (CNT-P123/GC) were rinsed with distilled water and air-dried. HRP was immobilized on the CNT-P123/GC electrodes by immersing the electrodes into 20 mg/mL HRP solution containing 0.40 mM P123 for 8 h. The electrodes (HRP/CNT-P123/GC) were rinsed with distilled water prior to use. For comparison, the pristine CNTs without any functionalization were dispersed into DMF and 4 μL of the dispersion was coated on GC electrode. The electrodes (CNT/GC) were air-dried, rinsed with distilled water, and immersed into 20 mg/mL HRP solution for 8 h. The resulting electrodes (HRP/CNT/GC) were rinsed with distilled water to remove non-adsorbed HRP before electrochemical measurements.

Electrochemical measurements were carried out with a computer-controlled CHI660A Electrochemical Analyzer (CHI, Austin, USA) in a conventional and two-compartment cell. The modified GC electrodes were used as working electrode and a platinum spiral wire as counter electrode. All potentials were referred to a KCl-saturated Ag/AgCl electrode. Phosphate buffer (0.10 M, pH 7.0) was used as supporting electrolyte. The electrolyte was deoxygenated by purging pure nitrogen into the solution for more than 30 min. and nitrogen gas was kept flowing over the solution during the electrochemical measurements. All electrochemical measurements were performed at room temperature.

Results and Discussion

Solubility and biocompatibility of the functionalized CNTs

The use of carbon nanotubes to develop new bioelectronic nanodevices such as electrochemical biosensors and enzyme-based biofuel cells requires the CNTs to have a good solubility for convenient manipulation. It is known that the CNTs easily
aggregate and have poor solubility in most solvents, which makes it difficult to manipulate them for various applications\textsuperscript{26}. Homogeneously dispersed and well-separated single nanotubes are required for the development of bioelectronic nanodevices since most of the well dispersed functional nanotubes are electrochemically accessible to analyze and can be thus used for biosensing units, yielding a high signal output. We found that the strategies demonstrated here for the functionalization of the CNTs essentially solubilized the CNTs in aqueous solution and the resulting aqueous dispersions were quite stable.

On the other hand, the strong interactions between the CNTs and biomacromolecules (enzymes and proteins) essentially distort the biomacromolecules and thereby decrease their biocatalytic activity\textsuperscript{20,23}. Thus, the biocompatibility of the CNTs with the enzymes and proteins used for specific recognition toward substrate constitutes another key point to bioelectronic nanodevices. To meet such a requirement, we have tried to chemically functionalize the CNTs through both covalent and noncovalent ways. In the covalent way, we intentionally introduce oxygenated groups such as hydroxyl and carboxyl groups on the sidewall of the CNTs since the sidewall functionalization of covalently bound moieties (\(-\text{OH} \) and \(-\text{COOH} \) groups in this case) to the CNT framework inevitably destroys the inherent electronic structure of the nanotubes, i.e., change the $sp^2$ carbons into $sp^3$ hybridization, while maintaining the tubular structure of the nanotubes\textsuperscript{25}. Moreover, such covalent sidewall functionalization of the nanotubes with \(-\text{OH} \) and \(-\text{COOH} \) groups substantially improves their hydrophilic property. These changes are believed to weaken the interactions (both electronic and hydrophobic) between the CNTs and the proteins and thus may well retain the biocatalytic activity of the proteins although it is difficult to determine the activity of the proteins adsorbed onto the functionalized CNTs in the present stage.

Although the covalent functionalization of the CNTs endows the CNTs with better solubilization and possibly enhanced biocompatibility, a noncovalent strategy is still highly desired since such a strategy maintains the attractive electronic and structural properties of the CNTs\textsuperscript{27}. As a typical example, we used one of the block copolymers (i.e., Pluronic P123, structure shown in Scheme 1) to noncovalently functionalize the CNTs. The copolymer Pluronic P123 consists of two hydrophilic polymeric chains of $\text{EO}_{20}$ and one hydrophobic chain of $\text{PO}_{20}$. The hydrophobic $\text{PO}_{20}$ chain essentially interacts with the CNTs through hydrophobic interaction, leading to the adsorption of P123 onto the CNTs. The hydrophilicity of the $\text{EO}_{20}$ double chains substantially solubilizes the CNTs in aqueous solution. Indeed, our experimental results show that the CNTs are well solubilized into P123 solution; the suspension of the CNTs into P123 aqueous solution essentially gives a homogenous black dispersion that is stable for at least one month. Furthermore, the P123 is very biocompatible which is believed to retain the biocatalytic activity of the proteins adsorbed. The improved solubility and biocompatibility of the CNTs functionalized through covalent and noncovalent ways may facilitate the manipulation of the CNTs for practical applications, especially for development of bioelectronic devices such as biosensors.

Electrochemistry of HRP at the covalently functionalized CNTs

We used HRP as an example to demonstrate the possible applications of the functionalized CNTs in facilitation of direct electron transfer of homocatalytic proteins. Figure 1 depicts typical cyclic voltammograms (CVs) obtained at HRP/CNT-OH/GC (A) and HRP/CNT-COOH/GC (B) electrodes. For comparison, the CVs obtained at CNT-OH/GC and CNT-COOH/GC electrodes (without HRP) were also displayed (Inset A and B). As shown, the CVs obtained with the HRP/CNT-OH/GC electrode exhibit a pair of wave at -0.20 V with a large peak-to-peak separation of ca. 200 mV (at 100 mVs\textsuperscript{-1}) (A), which could be ascribed to the irreversible redox process of HRP at the CNT-OHs. Similarly, the irreversible direct electron transfer of HRP was also observed at the CNT-COOHs (B); a pair of redox wave was observed at -0.27 V with a peak separation of ca. 150

![Image of Scheme 1](https://via.placeholder.com/150)

**Scheme 1**

**Structures of pristine CNT (A), CNTs functionalized covalently with -OH (B) and -COOH (C) groups and P123 (D).**
mV (at 100 mVs⁻¹). The CNT-COOH/GC electrode showed a cathodic peak at -0.90 V and a pair of wave at 0.0 V, of which the wave at 0.0 V was attributed to redox process of the oxygenated groups at the CNT-COOHs. The disappearance of these peaks upon the immobilization of HRP is indicative of cross-linking of HRP at the CNTs.

Although the strategies for covalent functionalization of the CNTs with oxygenated groups, i.e., -OH and -COOH groups, essentially made it possible to realize the direct electron transfer of HRP, the facilitated redox process of HRP was yet relatively irreversible and ill-defined. Such a sluggish electron transfer process could be largely improved at the CNTs noncovalently functionalized with P123 as demonstrated below.

Electrochemistry and electrocatalysis of HRP at P123-functionalized CNTs

Figure 2 depicts typical CVs obtained at HRP/CNT-P123/GC (solid line) and HRP/CNT/GC (dotted line) and CNT/GC (dashed line) electrodes. A pair of well-defined redox peaks is observed at a formal potential of -0.25 V with a peak-to-peak separation of ca. 65 mV and a near unity of the ratio of cathodic-to-anodic peak current (at 500 mVs⁻¹), characteristic of reversible electrode process of heme Fe⁺/Fe⁺ in HRP. This observation indicates that HRP adsorbed onto the CNTs noncovalently functionalized with P123 efficiently realizes its direct electrochemistry. In comparison, the HRP/CNT/GC electrode exhibits no redox peaks which could be ascribed to the redox process of HRP, suggesting that it is difficult to realize the direct electron transfer of HRP at the pristine CNTs without any functionalization, which coincides with previous reports. Although, the mechanism for the observed facilitation of the electron transfer of HRP at the functionalized CNTs (i.e., CNT-P123, CNT-OH and CNT-COOH) still requires more experimental evidences, it is very likely that the proper orientation and/or conformation of the enzymes onto the
functionalized nanotube surface, which are believed to be favorable for interfacial electron transfer of the proteins, constitutes one of the consequences for the above results.

Figure 3 displays the CVs of the HRP/CNT-P123/GC electrode in phosphate buffer at different potential sweep rates. As shown, the peak currents are linear with potential scan rate in a range from 100 to 900 mVs$^{-1}$ (inset, using cathodic current as an example), while the potential does not change clearly, demonstrating that the electron transfer process of HRP adsorbed onto the CNT-P123 is a fast and surface-confined process.

We next investigated the electrocatalytic activities of HRP confined onto the CNT-P123/GC electrodes. Figure 4 shows typical CVs of HRP/CNT-P123/GC electrode in phosphate buffer. The presence of H$_2$O$_2$ in solution remarkably increases the cathodic peak current, and decreases the reversed oxidation peak current (solid line) as compared with the peak currents in its absence (dotted line). This observation, along with the large positive shift of the potential for H$_2$O$_2$ reduction at the HRP/CNT-P123/GC electrode compared with that at the CNT-P123/GC electrode (inset), indicate that the HRP/CNT-P123/GC electrode possesses a good catalytic activity toward H$_2$O$_2$ reduction. A similar catalytic activity has also
been observed for $\text{O}_2$ reduction as shown in Fig. 5. As shown, the $\text{O}_2$ reduction occurs at the CNT-P123/GC electrode at -0.70 V under the present experimental conditions (inset in Fig. 5). Very similar to the case of $\text{H}_2\text{O}_2$, the introduction of $\text{O}_2$ in solution clearly increases the catalytic peak current, and decreases the reversed oxidation peak current (solid line) at the HRP/CNT-P123/GC electrode compared with the peaks in its absence (dotted line). Such an excellent catalytic activity of HRP coupled with its fast and direct electron transfer property at the CNT-P123 is responsible for the large positive shift of the potential (ca. 400 mV) for $\text{O}_2$ reduction at the HRP/CNT-P123/GC electrode compared with that at the CNT-P123/GC electrode. The observed catalytic activities of HRP adsorbed onto the functionalized CNTs may be useful for the development of new bioelectronic nanodevices, e.g., electrochemical biosensors and enzyme-based biofuel cells.

Conclusions

Three strategies have been utilized here for both covalent and noncovalent functionalizing of the CNTs to facilitate direct electronic communication between the proteins (i.e., HRP in this work) and the CNTs. Functionalization of the CNTs with hydroxyl and carboxyl groups and block copolymer (i.e., P123) essentially increases the solubility of the CNTs and facilitates the manipulation of the CNTs and may enhance the biocompatibility of the CNTs with biomacromolecules such as proteins and enzymes. These properties are believed to be very useful for the development of bioelectronic nanodevices such as electrochemical biosensors and enzyme-based biofuel cells. Moreover, the functionalized CNTs have been found to be capable of facilitating the direct electron transfer of HRP. The noncovalently P123-functionalized CNTs have been found to be advantageous over those covalently functionalized with -OH and -COOH groups. The combination of the direct electron transfer property of HRP with its biocatalytic activity substantially enables electrochemical reductions of dioxygen and hydrogen peroxide to occur with a low overpotential.

Acknowledgement

Financial support from National Natural Science Foundation of China (Grant Nos. 20375043 and 20435030) and Chinese Academy of Sciences (Grant No. KJCX2-SW-H06) is sincerely acknowledged. The authors thank Prof. Zhi-Xin Guo for helpful discussions.

References