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Carbon nanodots–chitosan composite film: A platform for protein immobilization, direct electrochemistry and bioelectrocatalysis

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ABSTRACT

A novel composite film based on carbon nanodots (CNDs) and chitosan was readily prepared and used as immobilization matrix to entrap a heme protein, hemoglobin (Hb) for direct electrochemistry and bioelectrocatalysis. A modified electrode was obtained by casting Hb–CNDs–chitosan composites on the glassy carbon (GC) electrode surface. Spectroscopic and electrochemical studies showed that Hb entrapped in the composite film remained in its native structures, and CNDs in the film can greatly facilitate DET between the protein and the GC electrode. The electron-transfer kinetics of Hb in composite film was qualitatively evaluated by using the Marcus theory, and the apparent heterogeneous electron-transfer rate constant (k_s) was estimated to be 2.39($\pm$0.03) s^{-1} with Laviron equations. The modified electrode showed excellent electrocatalytic behavior to the substrate, hydrogen peroxide (H_2O_2). The linear current response for H_2O_2 was from 1×10^{-6} to 1.18×10^{-4} M with a detection limit of 0.27($\pm$0.02) μM at the signal-to-noise ratio of 3, and the apparent Michaelis–Menten constant was 0.067($\pm$0.02) mM. These important features of CNDs–chitosan film have implied to be a promising platform for elaborating bioelectrochemical devices such as biosensors and biofuel cells.

1. Introduction

Direct electron transfer (DET) between native redox-active proteins and electrodes has become a popular topic among the studies on electron transfer reactions of redox proteins since the two pioneered reports on DET between cytochrome *c* and gold electrode by Hill (Eddowes and Hill, 1977), and cytochrome *c* and tin doped indium oxides electrode by Kuwana (Yeh and Kuwana, 1977) in 1977. Studies on DET process of redox protein–electrode system are of scientific importance not only in investigating the fundamental mechanism of protein-mediated biological redox reactions such as photosynthesis, respiration and bioenergetic metabolism Voet and Voet (1993), but also in fabricating mediator-free (without redox relays) bioelectrochemical devices such as biosensors, bioreactors and biofuel cells (Flexer et al., 2011; Gao et al., 2007; Gao et al., 2011a, 2011b). However, it is generally difficult to achieve DET between redox proteins and conventional bare electrodes due to some obstacles including unfavorable orientation of protein moleculars on electrode surface, long distance between redox-active centers and electrode, adsorption denaturation of protein moleculars on electrode surface (Léger and Bertrand, 2008), and electrode passivation resulting from the adsorption of the large three-dimensional structure of protein moleculars on electrode surface (Stellwagen, 1978). Hb is probably one of the most extensively studied proteins for DET investigations and DET-based bioelectrochemical applications. However, the facilitation of DET between the heme-centers within the large three-dimensional structure and the electrode is still a challenge.

Fortunately, favorable electrode materials and stable protein immobilization could provide an opportunity to facilitate DET process of redox proteins. Thus, it is very intriguing to modify conventional electrodes with favorable materials and immobilization method to entrap proteins on electrode surface to achieve DET process, and consequently fabricate practical bioelectrochemical devices. Over the past decades, the modification of the electrode surface with varied nanomaterials composed of metal oxides (Si et al., 2011;Yang et al., 2012), and conducting polymers (Wang et al., 2009) has appeared to facilitate DET between proteins and the electrode because the unique chemical, physical, and electrical properties of nanostructured materials may

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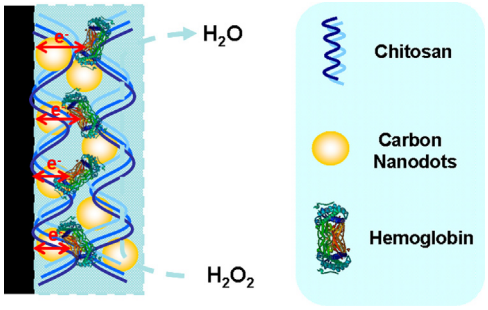
effectively shorten the electron-tunneling distance and provide an electron-mediating function. A wide variety of carbon-based nanomaterials, such as carbon nanotubes, carbon nanofibers, carbon nanochips, and graphene oxide, have shown to be ideal electrode materials and the capabilities of DET since they possess high conductivity, good biocompatibility, ease of functionalization, and large surface area (George and Lee, 2009; Gooding et al., 2003; Kumar et al., 2012; Lu et al., 2008; Shan et al., 2009; Vamvakaki et al., 2006; Wang, 2005; Wu et al., 2007; Zuo et al., 2010).

CNDs are very interesting newcomers to the world of carbonaceous nanomaterials and constitute a fascinating class of nanocarbons that comprise discrete, quasi-spherical nanoparticles with sizes below 10 nm Baker and Baker (2010). Owing to their important photoluminescent properties dependent on size and wavelength, lack of any known cytotoxicity, resistance to photobleaching, CNDs are emerging as viable alternatives to traditional semiconductor-based quantum dots, and fascinating luminescent materials as well as promising building blocks for future nanodevices (Yang et al., 2012). Currently, the immense research interests in CNDs are primarily driven by their excellent photoluminescent properties and have shown their potential applications in a broad range of areas such as optical sensing, bioimaging, and light energy conversion Baker and Baker (2010). However, similar to their popular older cousins including fullerenes, carbon nanotubes, and graphene, CNDs also possess several favorable attributes of carbon-based nanomaterials as described in foregoing paragraph. These remarkable characteristics make CNDs suitable for the supports of biocatalysts and electrochemical signal transduction. However, utilization of CNDs in this area has not been reported so far. Such limited attention is not proportional to the remarkable properties and potential merits of CNDs.

Different approaches, such as covalent attachment, adsorption, electrostatic interactions, and film entrapment have been used to immobilize proteins on the electrode surface (Lojou and Bianco, 2004). Among these approaches, film entrapment is of greater interest because the ultrathin films can probably provide a suitable microenvironment for proteins to retain their native structures and enhance DET between the proteins and electrodes. To attain this, substances with film-forming ability, such as SiO_2, Nafion, room-temperature ionic liquids are generally used to form films to entrap proteins. Chitosan, a natural polysaccharide biopolymer derived from chitin, is composed of β(1→4) linked glucosamine units together with some proportion of N-acetylglucosamine units. It is an attractive linear hydrophilic biopolymer that exhibits excellent biologic compatibility and film-forming ability (Peniche et al., 2003). Owing to its relatively poor conductivity, chitosan was usually combined with conducting materials such as carbon materials, redox mediators, metal nanoparticles, and ionic liquid to form conductive film for entrapping proteins (Lu et al., 2006; Zhang et al., 2004).

In this study, a composite film formed with chitosan and CNDs is considered as an excellent immobilization matrix for Hb entrapment, as shown in Scheme 1. The homogeneous solution containing chitosan, CNDs, and Hb can be readily made into film on GC electrode surface after it is dried. CNDs embedded in the three-dimensional chitosan network can serve as conductor due to its good intrinsic conductivity, and Hb–CNDs–chitosan composite film modified electrode (defined as Hb–CNDs–chitosan/GC) can be prepared by a very simple approach. Ultraviolet visible (UV–vis) and Fourier transform infrared (FT-IR) spectroscopy were used to identify the morphological and structural variations of Hb immobilized in the CNDs–chitosan film. Direct electrochemistry and bioelectrocatalysis of Hb toward H_2O_2 reduction were demonstrated at Hb–CNDs–chitosan/GC electrode, and thereafter a third-generation (without redox mediators) biosensor for H_2O_2 was established.



Scheme 1. Schematic illustration of Hb–CNDs–chitosan system.

2. Experimental

2.1. Materials and chemicals

Hb (from Bovine blood, MW=67,000) was purchased from Sigma. Chitosan (from crab shells, minimum 90% deacetylated), hydrogen peroxide (H_2O_2, 30% w/w), and other chemicals were all obtained from Sinopharm Chemicals Co., Ltd. (Shanghai, China). All the chemicals were of at least analytical grade and used without further purification. All solutions were prepared with Milli-Q purified water (> 18.0 MΩ).

A 50 mM pH 7.4 phosphate buffer solution (PBS) prepared by 50 mM Na_2HPO_4 and 50 mM KH_2PO_4 solution was used as supporting electrolyte. The dilute H_2O_2 solution was prepared freshly and only used daily. A 1.0 mg mL^{-1} stock Hb solution was prepared by dissolving 1 mg Hb in 1.0 mL PBS. Chitosan aqueous solution was prepared according to the previously reported protocols (Zhang et al., 2004) and is shown in Supplementary data. CNDs were prepared with candle soot as starting material according to reported methods (Li et al., 2011; Zhang et al., 2013) and is also shown in Supplementary data. The CNDs were dispersed into chitosan solution to give a homogeneous suspension with the aid of ultrasonic agitation. The mixture solution for Hb entrapment was prepared by adding an appropriate volume of chitosan solution, Hb solution, and CNDs into PBS. To obtain good cyclic voltammetric responses, a typical mixture solution containing 0.15 mg mL^{-1} chitosan, 0.25 mg mL^{-1} hemoglobin, and 0.40 mg mL^{-1} CNDs was used for preparing modified electrodes.

2.2. Apparatus

All the electrochemical experiments were carried out on CHI660C potentiostat (CHI, Shanghai) with a conventional three-electrode cell. A modified electrode was used as the working electrode, a Ag/AgCl as the reference electrode, and a platinum wire as the counter electrode. The experimental solutions were bubbled with highly pure nitrogen for 30 min to deoxygenate and kept under nitrogen atmosphere during the electrochemical measurements. The electrochemical measurements were performed at room temperature and repeated minimum three times. UV–vis absorption spectra were measured on UV-3010 spectrophotometer (Hitachi, Japan), and FT-IR 8400S spectrophotometer (Shimadu, Japan) was used for IR spectra using KBr pellet samples.

2.3. Electrode preparation

Prior to use, GC electrode (⌀=3 mm) was pretreated with conventional method. A 4 μL of resultant mixture solution for Hb entrapment was cast onto the electrode surface by using a 10-μL micro-syringe to prepare an Hb–CNDs–chitosan/GC electrode. A beaker was covered over the electrode so that solvent can be evaporated slowly in air and a uniform film can be formed. The