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ScienceDirect
Biosensors and Bioelectronics 23 (2008) 1374–1381
BIOSENSORS & BIOELECTRONICS
www.elsevier.com/locate/bios

A cytochrome *c* modified-conducting polymer microelectrode for monitoring *in vivo* changes in nitric oxide

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Received 7 September 2007; received in revised form 14 November 2007; accepted 10 December 2007
Available online 23 December 2007

Abstract

A nitric oxide (NO) microbiosensor based on cytochrome *c* (cyt *c*), a heme protein, immobilized onto a functionalized-conducting polymer (poly-TTCA) layer has been fabricated for the *in vivo* measurement of NO release stimulated by an abuse drug cocaine. Based on the direct electron transfer of cyt *c*, determination of NO with the cyt *c*-bonded poly-TTCA electrode was studied using cyclic voltammetry and chronoamperometry. Interferences for the sensory of NO by foreign species such as oxygen and hydrogen peroxide were minimized by covering a Nafion film on the modified electrode surface. Cyclic voltammograms taken using the cyt *c*/poly-TTCA electrode with NO solutions show a reduction peak at −0.7 V. The calibration plot showed the hydrodynamic range of 2.4–55.0 μM. The detection limit was determined to be 13 ± 3 nM based on S/N = 3. The microbiosensor was applied into the rat brain to test fluctuation of NO evoked by the abuse drug cocaine. The concentrations of NO levels by acute and repeated injections of cocaine were determined to be 1.13 ± 0.03 and 2.13 ± 0.05 μM, respectively, showing high sensitivity of the microbiosensor in monitoring NO concentrations in the *in vivo* intact brain.

Keywords: Cocaine stimulation; Cytochrome *c*; *In vivo* monitoring; Nitric oxide microbiosensor; Poly-TTCA

1. Introduction

Due to its unique properties, nitric oxide (NO) has been implicated in the pathogenesis of many diseases. NO is a highly diffusible and reactive molecule that plays a major role in several physiological processes, such as neurotransmission, immune response and vasodilatation (Packer, 1996; Contestabile and Ciani, 2004; Bolly, 2001). In addition, NO level is important in the control of major cell functions, including reactivity, proliferation and apoptosis (Contestabile and Ciani, 2004; Wink and Mitchell, 1998). Furthermore, in cells, NO may have coexisting beneficial or detrimental effects (Wink and Mitchell, 1998) and its metabolic interaction with other intra- or extracellular reactive molecules, such as the reactive oxygen species (superoxide, hydrogen peroxide, etc.), is quite intricate whereby NO has a half-life of 2–6 s *in vivo* (Wink and Mitchell, 1998; Lisdat, 2004). Thus, it is important to quantify the details of NO production in biological tissues, including direct measurement. Current demands include sensor element miniaturization for spatially resolved NO detection *in vivo* and high selectivity of the response signal in relation to relevant drug abuse.

For detection of NO, a variety of sensor systems have been developed. They can be divided into two classes: optical (Lim et al., 2006) or electrochemical sensors (Patel et al., 2006; Griveau et al., 2007). Among these, electrochemical sensors are most advantageous due to simplicity, speed and sensitivity, being able to perform *in vivo* measurements. Thus, most electrochemical sensors exploit the redox reaction of NO at metal or carbon electrodes (Malinski et al., 1996; Christodoulou et al., 1996; Sedlak and Blurton, 1976) due to the redox behavior of NO where it can be oxidized or reduced at the electrode surface. One of the electrochemical techniques for *in vivo* detection includes the use of carbon fiber electrodes modified with polymer films in a manner analogous to the work previously

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doi:10.1016/j.bios.2007.12.008

mentioned (Malinski and Taha, 1992; Kulagina et al., 2001; Boon and Marletta, 2006). Electropolymerization provides one of the in situ sensor preparation methods, examples of sensory material being conducting polymers such as polypyrrole (Fabre et al., 1997) and polyterthiophene (Lee and Shim, 2001). Reasoning that polyterthiophene films might also be active in the electrocatalytic reduction of NO due to direct electrochemistry at the surface-modified microelectrode, these films can also be prepared reproducibly and quite thinly (Rahman et al., 2005, 2006), ensuring a rapid and stable response of the sensor, thus terthiophene films were applied in this study.

To date, several protein complexes have been used in biosensors as a catalyst (Ge and Lisdat, 2002; Tu et al., 1999), mainly porphyrins (Lantoine et al., 1995), due to their preparation potential complementary to electropolymerization. One example, cytochrome *c*, has been known to have a binding affinity for small ligands such as oxygen and superoxide free radical. The use of cytochrome *c* to detect superoxide has aroused increasing interest since it provides a foundation for novel electrochemical biosensor construction and a model for electron transfer studies between proteins in biological systems (Ferguson-Miller et al., 1979; Moore and Pettigrew, 1990). An electrochemical sensor array system for the direct *in vitro* monitoring of superoxide production by cultured glioblastoma cells using cytochrome *c* as a sensing element has been reported (Chang et al., 2005). Alternatively, cytochrome *c*', a heme protein, has been used as a recognition element for the fluorescent-lifetime-based and radiometric detection of extra- and intracellular macrophage NO (Barker et al., 1999). Hence, this study is the first time that cytochrome *c* has been used as a sensing element, based on its electrocatalytic properties, to directly monitor the fluctuation levels of NO *in vivo*.

In the present study, we describe the preparation and characterization of cytochrome *c* on a conducting polymer-coated microelectrode and its electrocatalytic activity toward NO reduction. It was found that electropolymerized terthiophene films, in particular with covalently bonded cytochrome *c*, were quite active in the electrocatalytic reduction of nitric oxide. The experimental parameters such as pH and applied potential were optimized. We also demonstrate the microbiosensor's applicability to the *in vivo* determination of NO release in the rat brain due to stimulation brought about by the infusion of the abuse drug cocaine.

2. Experimental

2.1. Materials

A terthiophene monomer bearing a carboxylic acid group, 5,2′:5,2″-terthiophene-3′-carboxylic acid (TTCA) was synthesized according to our previous work (Lee et al., 2002). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Nafion (5 wt% solution in a mixture of lower aliphatic alcohols and water), dichloromethane (CH_2Cl_2) (99.8%, anhydrous, sealed under nitrogen gas), hydrogen peroxide (30% solution), and cocaine hydrochloride were purchased from Sigma–Aldrich (USA). Cytochrome *c* from horse heart (type VI, Sigma Co.) was used after purification by the previously described procedure (Park et al., 2001). First, cyt *c* was converted to the fully oxidized form by addition of excess $K_3Fe(CN)_6$ and then purified by ion-exchange chromatography on Whatman CM-32, eluted with 0.5 M NaCl + 10 mM PBS (phosphate buffer solution, pH 7.0). Eluent containing the purified protein was concentrated by ultrafiltration using Amicon YM-3 membranes, and then dialyzed extensively to remove phosphate (Paul and Watts, 1991). Tetrabutylammonium perchlorate (TBAP, electrochemical grade) was received from Fluka (USA), purified and then dried under vacuum at 1.33×10^3 Pa. Disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride, sulfuric acid and ethanol were purchased from Aldrich Chemical Co. (USA). A phosphate buffer saline solution (PBS) was prepared by modifying 0.1 M of disodium hydrogen phosphate with the mixture of 0.1 M of sodium dihydrogen phosphate with 0.1% sodium chloride. All other chemicals were of extra pure analytical grade and used without further purification. All aqueous solutions were prepared in doubly distilled water, which was obtained from a Milli-Q water purifying system (18 MΩ cm).

2.2. Preparation of NO standard solutions

Saturated NO solutions were prepared by bubbling a 8 mL 0.2 M phosphate buffer solution (pH 7.0) with nitrogen gas for 30 min first to purge oxygen and then with NO gas for 20 min. Standard solutions were freshly made for each experiment and kept in a glass vial sealed with a rubber septum. The NO concentration in the saturated solution was taken as 1.9 mM at 25 °C as reported (Pallini et al., 1998). Finally, the solution was stored at 0 °C for a few hours at maximum.

2.3. Microbiosensor preparation

The microelectrode was fabricated according to previous work (Rahman et al., 2005). The total length of the Pt microelectrode was ~40 mm. The diameter of the microelectrode was 100 μm. The Pt microelectrode was subsequently cleaned by cycling the applied potential between +1.4 and −0.2 V for 10 cycles at a scan rate of 500 mV/s in 0.5 M H_2SO_4 solution followed by washing with distilled water where it was then used in all subsequent experiments. The nano-particle comprised conducting polymer layer onto a microelectrode was obtained through electropolymerization following the procedure from previous works (Lee and Shim, 2001; Rahman et al., 2005, 2004). The conducting polymer layer coated microelectrode was immersed for 12 h in a 0.01 M phosphate buffer solution (pH 7.0) containing 20.0 mM of EDC to activate the carboxylic acid groups of the polymer layer. Then, the EDC treated conducting polymer-modified microelectrode was washed with buffer solution and subsequently incubated for 12 h in 5 mM PBS solution (pH 7.0) containing 6 mg/mL cyt *c* at 4 °C. By this procedure, cyt *c* was covalently bonded through its amine groups to the carboxylic groups on the poly-TTCA, forming amide bonds. The cyt *c*/poly-TTCA microelectrode was dipped in 1% Nafion solution (diluted with ethanol) for 2 min. The Nafion film was then dried for 1 h in a calcium chloride atmosphere. It was found that