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Construction of a highly sensitive non-enzymatic sensor for superoxide anion radical detection from living cells

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ABSTRACT

A novel non-enzymatic superoxide anion (O_2•$^-$) sensor was fabricated based on Ag nanoparticles (NPs)/L-cysteine functioned carbon nanotubes (Cys-MWCNTs) nanocomposites and used to measure the release of O_2•$^-$ from living cells. In this strategy, AgNPs could be uniformly electrodeposited on the MWCNTs surface with average diameter of about 20 nm as exhibited by scanning electronmicroscopy (SEM). Electrochemical study demonstrated that the AgNPs/Cys-MWCNTs modified glassy carbon electrode exhibited excellent catalytic activity toward the reduction of O_2•$^-$ with a super wide linear range from 7.00×10^{-11} to 7.41×10^{-5} M and a low detection limit (LOD) of 2.33×10^{-11}M (S/N=3). Meanwhile, the mechanism for O_2•$^-$ reduction was also proposed for the first time. Importantly, this novel non-enzymatic O_2•$^-$ sensor can detect O_2•$^-$ release from cancer cells under both the external stimulation and the normal condition, which has the great potential application in clinical diagnostics to assess oxidative stress of living cells.

1. Introduction

Reactive oxygen species (ROS) are important intracellular signaling molecules, mainly regulating DNA damage, protein synthesis, cell apoptosis, etc. (Chang et al., 2013; Roberts et al., 2011). However, the excessive amount of ROS accumulation in cells will lead to oxidative stress that causes various pathological events such as neurodegeneration, alzheimer disease, autoimmune diseases and cancer (Pagliari et al., 2012; Trachootham et al., 2009). Therefore, the selective and accurate measure of ROS are important to illuminate the mechanism of regulating signal transduction pathways and further exploit the potential application in clinical pathological diagnosis (Borgmann, 2009). Among various ROS, the superoxide anion radical (O_2•$^-$) is the most active one and involves in many physiological and pathological processes (Auchère and Rusnak, 2002; Halliwell and Gutteridge, 1984; Kaji et al., 2009; Shanlin et al., 1997). The relationship between O_2•$^-$ concentration and human health has attracted great attention. It is well-known that, under normal physiological conditions, O_2•$^-$ is in a rather low physiological concentration about 10–100 nM. However, the concentration of O_2•$^-$ may increase to 0.1 mM under some environmental stresses or illness (Deng et al., 2008). Therefore, the wide dynamic linear range is required for in vivo applications.

Up to date, various detection methods of O_2•$^-$ have been employed, such as electron spin resonance (Kaji et al., 2009), chemiluminescence (Xie et al., 2008), spectrophotometry (Haseloff et al., 1991) and electrochemical analysis (Liu et al., 2008; Rafiee-Pour et al., 2010; Rajesh et al., 2010). Among these techniques, the electrochemical method is considered as one of the most promising strategies due to its simplicity, fast response, low detection limit, and easy of use. Especially, enzyme-based electrochemical biosensors (such as superoxide dismutase (SOD), a specific enzyme for O_2•$^-$ dismutation, immobilized in the various kinds of functionalized electrodes) have always been the research highlights. For example, Tian et al. prepared a ZnO/SOD modified microelectrode for in vivo detection of superoxide anion in bean sprout (Deng et al., 2008), and Wang et al. constructed O_2•$^-$ biosensors based on three kinds of SOD (Cu/ Zn-SOD, Fe-SOD, Mn-SOD) modified gold electrodes via 3-mercaptopropionic acid (MPA) (Tian et al., 2004). Nevertheless, the relatively high cost, limited lifetime and the critical operating situation limit their applicability (Li et al., 2010; Yuan et al., 2008). Therefore, non-enzymatic O_2•$^-$ sensors have received keen interest and need to be developed rapidly recently.

Nowadays, with the development of nanotechnology, metal nanoparticles (NPs), such as PtNPs (Li et al., 2014), AuNPs (Li et al., 2012) and AgNPs (Cui et al., 2008; Yu et al., 2012; Li et al., 2013), play important roles in improving sensor performance, which have become excellent substitutes for enzymes to catalyze the O_2•$^-$. Among these materials, AgNPs possess the excellent catalytic activity in a variety of applications (Cui et al., 2008; Welch et al., 2005). Recent studies found

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that performance of the sensor depended strongly on the size, shape and distribution of AgNPs on the electrode (Chen et al., 2011). Hence, it is very important to prepare the matrix for dispersed AgNPs highly.

Carbon nanotubes (CNTs), as another typical nanomaterial, have been widely used in electrochemical sensors since they possess relatively large surface area, excellent electric conductivity, high chemical stability and strong adsorptive ability (Nie et al., 2011). More and more researchers focused on utilizing CNTs as the templates for supporting metal nanoparticle catalysts. Nevertheless, van der Waals interactions between the pristine tubes cause poor water solubility, restricting their applications (Cao et al., 2013). Therefore, it is strongly desired to find pathways for overcoming above obstacle. Reported showed that CNTs functionalization is an effective way to resolve this problem (Kim et al., 2011; Star et al., 2001; Zhou et al., 2009; O'connell et al., 2002; Singh et al., 2009). In this work, we utilize L-cysteine functionalized MWCNTs to disperse MWCNTs effectively and load large amounts of AgNPs at the same time. As the modified material of the sensor, AgNPs/Cys-MWCNTs nanocomposites showed excellent electric conductivity and high catalytic activity.

The aim in this article is to fabricate a novel non-enzymatic sensor for the determination of $O_2•^-$ by utilizing Cys functionalized MWCNTs as the matrix for electrodepositing of AgNPs. By combining the advantages of MWCNTs and AgNPs, the designed sensor exhibited excellent performance towards $O_2•^-$ with super wide linear range, low detection limit and excellent reproducibility. More importantly, it can detect the $O_2•^-$ release from living cells under both the external stimulation and the normal condition. Thus, this work has the great potential application in clinical diagnostics to assess oxidative stress of living cells.

2. Experimental section

2.1. Apparatus

The surface morphology of AgNPs/Cys-MWCNTs composite was characterized by JSM-6701F scanning electron microscopy (SEM, Japan). Electrochemical measurements were performed on a CHI660C electrochemical workstation (Austin, TX, USA) with conventional three-electrode system. A bare or modified glassy carbon electrode (GCE, d=3.0 mm) was employed as working electrode. A platinum electrode and a saturated calomel electrode (SCE) were served as the auxiliary and reference electrode. All potentials given in this paper were referred to the SCE. Electrochemical impedance spectroscopy (EIS) experiments were performed on Multi-potentiostat (VMP2, Princeton Applied Research, USA). Ultraviolet-Visible spectroscope (UV–vis, EVOLUTION 220, Thermo Scientific) was used to detect the concentration of $O_2•^-$ obtained from the KO_2 stock solution. Before each electrochemical measurement, solutions were thoroughly deoxygenated by bubbling nitrogen through the solution for at least 20 min to remove dissolved oxygen.

2.2. Reagents

The multi-walled carbon nanotubes (MWCNTs) used (diameter: 20–40 nm, length: 1–2 μm, purity: ≥95%) came from Shenzhen Nanotech Port Co. Ltd. (Shenzhen, China). KO_2 was obtained from Aladdin Industrial Inc. DMSO was purchased from Beijing Chemical Works (Beijing, China). 18-crown-6 was bought from Energy Chemical (Shanghai Chemical Industries, Ltd.). 4 Å molecular sieve was obtained from Tianjin Kermel Chemical Industries, Ltd (Tianjin, China). Superoxide dismutase (SOD) was bought from Sigma (USA). $AgNO_3$ and KNO_3 were purchased from Xi'an Chemical Reagent (Xi'an, China). PBS (pH 7.0) was prepared by mixing suitable amounts of 0.2 M NaH_2PO_4/Na_2HPO_4. Other chemicals were all of analytical grade, and the solutions were prepared by doubly distilled water.

2.3. Fabrication of Cys-modified multiwalled carbon nanotube nanocomposite

Preparations of functionalized MWCNTs were carried out according to the procedure described elsewhere (Pilehvar et al., 2014). In brief, MWCNTs were refluxed with H_2SO_4: HNO_3 (3:1, v/v) at 70 °C to obtain carboxylic acid functionalized MWCNTs and then washed with distilled water several times and allowed to dry under vacuum.

The synthesis of Cys-MWCNTs was performed in the following manner (Wang et al., 2014). 10 mg MWCNTs, 10 mg L-cysteine were dispersed into 10 mL ultrapure water, then EDC and NHS were used as coupling reagents for combining the carboxyl bond of MWCNTs with the amido of L-cysteine to obtain Cys functionalized MWCNTs (Cys-MWCNTs). After continuous stirring overnight at room temperature, the product Cys-MWCNTs was obtained. Then centrifugation at 10,000 rpm for 15 min and washed several times by doubly distilled water is used to remove excess reagents. The sediment of resulted Cys-MWCNTs was resuspended in ultrapure water (1 mg mL^{-1}) and stored at 4 °C for further use.

2.4. Preparation of the $O_2•^-$ sensor

A glassy carbon electrode (GCE) was polished with 1.0, 0.3 and 0.05 μm alumina slurry to a mirror-like, respectively, followed by rinsing thoroughly with doubly distilled water. The electrode was successively sonicated in 1:1 nitric acid, acetone and doubly distilled water, and then allowed to dry at room temperature. Then 6 μL of Cys-MWCNTs aqueous solution (1.0 mg mL^{-1}) was dropped on the surface of GCE and dried in air, and the Cys-MWCNTs/GCE was obtained. Next, the electrode was electrodeposited in the solution containing 1.0 mM $AgNO_3$ and 0.1 M KNO_3 for 200 s at 0 V to obtain AgNPs/Cys-MWCNTs modified electrode.

2.5. Generation of superoxide anion

The chemical generation of $O_2•^-$ was performed by dissolving KO_2 in dimethyl sulphoxide (DMSO) solution (containing 18-crown-6), and stored together with 4 Å molecular sieve. The solubility of KO_2 in DMSO can be increased by adding 18-crown-6. After sonicating the solution for 5 min, additional 4 Å molecular sieve was added to remove traces of H_2O. According to the molar absorptivity of $O_2.^-$ in DMSO (2006 M^{-1} cm^{-1} at 271 nm), the concentration of $O_2•^-$ could be estimated by a UV–Vis spectroscope (Thandavan et al., 2013).

2.6. Cell culture

PC12 (rat adrenal medulla pheochromocytoma) cells were obtained from Institute of Hematology, Chinese Academy of Medical Sciences. They were maintained in DMEM (high glucose, Gibco) medium supplemented with 10% heat-inactivated fetal calf serum (Evergreen, China), 100 U/mL penicillin (Sigma, USA), and 100 mg/mL streptomycin (Sigma, USA) at 37 °C with 5% CO_2 in a 95% humidified atmosphere.

2.7. Electrochemical detection of $O_2•^-$ released by cells

PC12 cells were seeded in a 6-well plate (1.5×10^5 cells per well) for 24 h for electrochemical experiments. The media was removed and the cells were washed three times with phosphate-buffered saline, and then 3 mL phosphate-buffered saline was added for real sample measurements. The amperometric detection of the release flux of $O_2•^-$ from PC12 cells was performed using the AgNPs/Cys-MWCNTs/GCE at −0.6 V (vs. SCE). When the current went to a stable level, ascorbic acid (AA) was injected to the cells suspension, which can motivate cells generation of $O_2•^-$. The experiments were conducted in the water bath at 37 °C.