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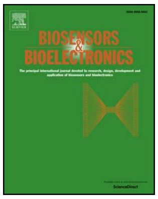

A facile way to fabricate manganese phosphate self-assembled carbon networks as efficient electrochemical catalysts for real-time monitoring of superoxide anions released from HepG2 cells

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ABSTRACT

Quantification of superoxide anions ($O_2^{\bullet -}$) is significant in the monitoring of many serious diseases and the design of enzyme-mimic catalysts plays the main role in the development of non-enzymatic $O_2^{\bullet -}$ sensors. Herein, we proposed a facile self-assembly process to synthesize manganese phosphate modified carbon networks using three kinds of widely-used carbon materials (MWCNTs, NGS and GO) as pillar connectors. Characterizations demonstrate that manganese phosphate is widely dispersed inside and on the surface of carbon networks without visible morphology. Meanwhile, all three kinds of synthesized catalysts were successfully immobilized on the screen-printed carbon electrodes to evaluate the electrochemical performance of fabricated sensors. The results indicate that sensors based on $Mn_x(PO_4)_y$ modified MWCNTs exhibit high sensitivity with an extremely low detection limit of 0.127 μM (S/N = 3) and a wide liner range of 0–1.817 mM (R^2 = 0.998). We further employed the recommended sensors in the real-time monitoring of HepG2 cells released $O_2^{\bullet -}$ under the stimulating of Zymosan (20 mg/mL). Noticeably, the proposed sensors exhibit not only sensitive response but also stable current steps upon different addition of Zymosan. The calculated concentrations of cell-released $O_2^{\bullet -}$ vary from 6.772 to 24.652 pM cell^{-1} for the Zymosan amount used in this work. The established novel sensors display low background current and signal noises, thus holding unique advantages in the trace analysis of $O_2^{\bullet -}$ in biological samples and in vivo environment.

1. Introduction

Superoxide anion ($O_2^{\bullet -}$) is one of the most crucial reactive oxygen species (ROS) which was mainly generated during the produce of ATP inside human body. Since its discovery in last century, the biological function and metabolic pathway of $O_2^{\bullet -}$ has been widely studied (Sadeghian et al., 2016). The involvement of $O_2^{\bullet -}$ in many serious diseases including cancer, cardiovascular illness and aging (Uttara et al., 2009), has aroused great passion of scientists from different fields for the determination of $O_2^{\bullet -}$, which could improve the diagnosis and medical treatment of ROS-induced diseases in the future (Luo et al., 2011; Xiao et al., 2017). There are several methods for determination of $O_2^{\bullet -}$ including high-performance liquid chromatography (HPLC), electron paramagnetic resonance (EPR), florescence sensors and electrochemical sensors. Among these techniques, electrochemical sensors, which can complete the detecting process within several seconds and give a real time data, is preferred as an ideal method to detect ROS like $O_2^{\bullet -}$ which is extremely unstable (Crulhas et al., 2017; Tian et al., 2005). Meanwhile, owing to its high sensibility, excellent selectivity and non-destructive detecting process, electrochemical sensor have extended its application in the real sample analysis such as the detection of $O_2^{\bullet -}$ in extracellular environment and in vivo (Liu et al., 2017c).

Concerning previous researches, most of $O_2^{\bullet -}$ electrochemical sensors are based on enzymatic materials such as superoxide dismutase (SOD) and cytochrome c due to their high catalytic activity and satisfying selectivity toward $O_2^{\bullet -}$ sensing. However, the inactivation tendency of enzymes has made the response of enzymatic sensors vary along with environmental conditions such as temperature and pH, thus depressing the stability of established sensors (Nissim and Compton, 2014). Therefore, scientists have been devoted on the development of

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non-enzymatic electrochemical sensors, which exploring novel enzyme-mimic nanomaterials as alternative to natural enzyme for the determination of $O_2^{\bullet-}$.

Natural SOD consists of protein structures and transition metal center (Cu, Zn, Mn and Fe) which can catalyze the dismutation of $O_2^{\bullet-}$. Recent research has discovered that transition metal ions coupled with phosphate groups could serve as efficient SOD-mimic (Kumar et al., 2015; Shen et al., 2016). However, the synthesis of metal phosphate without multilayer structures such as nanocubics and nanotubes has been a challenge for scientists and there are few researches concerning this issue (Liu et al., 2017b; Sadeghian et al., 2016). Recently, Shen and coworkers came up with a method for obtaining MnSOD mimic using phosphate group modified SiO_2 nanoparticles as template and assembled Mn^{2+} with SiO_2-PO_4, forming SiO_2-$Mn_2(PO_4)_3$ with controllable morphology (Shen et al., 2016). Based on this research, here we propose a self-assembly method to modify carbon materials with phosphate groups and assemble metal ions on carbon substrates. As is known to us all, carbon materials such as multi-walled carbon nanotubes, reduced graphene and carbon spheres exhibit high conductivity which can efficiently enhance the electron transfer between analyte and electrode, improving the current response of electrochemical sensors. This novel synthesis method, which has many advantages such as mild reaction condition and short reaction time, can approach $Mn_x(PO_4)_y$ layer with large surface-to-volume ratio and numerous active sites which allows more adsorption of target analyte on its surface.

In this work, three kinds of manganese phosphate based catalysts were successfully obtained and employed to fabricate electrochemical sensors. Multi-walled carbon nanotubes (MWCNTs), which exhibit high conductivity and contain abundant oxygen-containing groups, was used as pillar connector for constructing carbon networks. The recommended sensor we proposed exhibited promising performance on the online monitoring of $O_2^{\bullet-}$ released from HepG2 cells with both stable current response and obvious liner dependency between the concentration of cell released $O_2^{\bullet-}$ and the amount of stimulating drug. Also, the established sensors have wide liner range, low detecting limit, excellent long-term stability and satisfying sensitivity for the determination of $O_2^{\bullet-}$. Moreover, two other kinds of widely-used carbon materials include nitrogen-doped graphene sheet (NGS) and graphene oxide (GO) were also employed to construct $Mn_x(PO_4)_y$ self-assembled carbon networks, and the performance of sensors based on these materials were systematically evaluated. We assume that the designed self-assembly process can be applied as a general method to fabricate transition metal phosphate on carbon materials which contain oxygen-containing groups on the surface, approaching catalysts for different uses in all kinds of fields.

2. Experimental

2.1. Chemicals and apparatus

Multi-walled carbon nanotube powder with a mean width of 15 nm and a mean length of 1.5 μm was pursued from Shenzhen Nanotech Port Co. Graphene powder, $MnSO_4{\cdot}H_2O$, KH_2PO_4, K_2HPO_4, KCl, $NaNO_3$, K_2SO_4, $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$ and melamine were provided by Sinopharm Chemical Reagent Co., Ltd. Ammonia, $KMnO_4$, H_3PO_4, H_2SO_4, and HNO_3 were obtained from Shanghai Lingfeng Chemical Reagent Co., Ltd. Phytic acid solution (50%), anhydrous dimethyl sulfoxide (DMSO), 18-crown-6 and KO_2 were purchased from Aladdin Industrial Inc. Zymosan, D-glucose, uric acid (UA), ascorbic acid (AA) and L-Glutathione (GSH) were provided by Sigma-Aldrich. Dulbecco's modified Eagle's medium (DMEM) cell culture medium, penicillin/streptomycin, trypsin and fetal bovine serum (FBS) were bought from Gibco Invitrogen. Ultrapure water used in all experiments was home-made by a Laboratory Water Purification System (18.2 M cm). All the above chemicals were directly used without any further purification.

Zeta potential of all synthesized materials was evaluated by a Delsa Nano C particle analyzer. TEM images were obtained through a JEM-1400 transmission electron microscope (JEOL, Japan) with an accelerating voltage of 100 kV. The EDS and elemental mapping patterns were obtained using a XM-2 energy disperse spectrometer (EDAX, USA) equipped to a Tecnai G2 F30 S-TWIN field emission transmission electron microscope (FEI, USA). The XPS measurement was carried out on an ESCALAB 250Xi X-ray photoelectron spectrometer (Thermo Fisher, USA). Ultraviolet-visible spectroscope (UV–Vis, CARY 300, Agilent Technologies) was used to detect the UV–vis absorption of $O_2^{\bullet-}$ released from KO_2 solution. All electrochemical measurements were carried out on a CHI660D working station based on the three-electrode system consisting of a home-made screen printed carbon electrode (SPCE) working electrode, a platinum counter electrode and an Ag/AgCl reference electrode.

2.2. Preparation of carbon materials

Multi-walled carbon nanotubes (MWCNTs) were commercially pursued and shortened using a similar method as reported in references (Gooding et al., 2003; Liu et al., 2000; Zhu et al., 2014). In detail, 1 g of MWCNTs were dispersed in 30 mL mixture of concentrated HNO_3 and H_2SO_4 (v/v, 1:1) under ultrasonication. After refluxing for 2 h at 80 °C, the MWCNTs were shortened and carboxyl functionalized during the acid treatment. The products were washed with ultrapure water and freeze-dried overnight.

The graphene oxide (GO) was prepared by using the Hummers method (Marcano et al., 2010; Li et al., 2017) with some modification. Typically, 1.5 g of natural graphite power was mixed with 130 mL concentrated H_2SO_4 and 20 mL concentrated H_3PO_4 in a 500 mL flask. Next, 9 g $KMnO_4$ were slowly added into the mixture under vigorous stirring and the temperature was strictly controlled below 50 °C. The above mixture was subsequently heated to 50 °C and refluxed for 12 h. After cooling down in an ice bath, the products were slowly transferred into 450 mL of ultrapure water under stirring. The remaining $KMnO_4$ in the solution was reduced by 1.5 mL H_2O_2 (30%, v/v) and the mixture turned yellow. After standing overnight, the precipitated products were washed with hydrochloric acid (10%, v/v) and ultrapure water for several times until the supernatant turned neutral. The result solid was freeze-dried overnight.

The synthesis of nitrogen-doped graphene sheets (NGS) was introduced in the literature (Du et al., 2013). In brief, 25 mg GO was dispersed in 26 mL 2 wt% ammonia aqueous solution and kept stirring for 6 h at 80 °C. The product was washed with water for several times and freeze-dried overnight.

2.3. Preparation of Mn-MPSA-C catalysts

Carbon network coupled with melamine-phytic acid supermolecular aggregate (MPSA) was prepared according to the literature (Zhang et al., 2016) with some modification. Typically, 10 mg MWCNTs was dispersed in 100 mL ultrapure water by sonicating for 30 min, followed by the addition of 0.25 g melamine and 1 mL phytic acid under magnetic stirring to form MPSA-MWCNTs precipitate. Next, the solid was washed and collected through centrifugation and then dispersed in 10 mL ultrapure water. After the mixture was heated to 60 °C, 20 mL 48 mM $MnSO_4{\cdot}H_2O$ aqueous solution was added under continuously stirring for 1.5 h to form Mn-MPSA-MWCNTs through a self-assembly process. Finally, the product was washed with ultrapure water and freeze-dried overnight.

Mn-MPSA-NGS and Mn-MPSA-GO were prepared through the same process with NGS and GO, respectively, as the pillar connectors. For comparison, MPSA-MWCNTs, MPSA-NGS and MPSA-GO were also synthesized using the above mentioned method without the cooperative assembly of Mn^{2+}.