



Enzyme- and metal-free electrochemical sensor for highly sensitive superoxide anion detection based on nitrogen doped hollow mesoporous carbon spheres



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ABSTRACT

In this work, a highly sensitive enzyme- and metal-free electrochemical method for superoxide anion ($O_2^{\bullet-}$) detection has been developed by employing screen-printed carbon electrodes (SPCE) modified by nitrogen doped hollow mesoporous carbon spheres (N-HMCS). For comparison, solid carbon spheres (SCS) and hollow mesoporous carbon spheres (HMCS) were also synthesized to fabricate the modified SPCE. Compared with SCS/SPCE and HMCS/SPCE, N-HMCS/SPCE displayed a higher electrochemical performance. When applied for electrochemical detection of $O_2^{\bullet-}$, N-HMCS/SPCE exhibited a high sensitivity of $1.49 \mu A cm^{-2} \mu M^{-1}$, better than SCS/SPCE and HMCS/SPCE and many of enzyme- or metal-based superoxide anion sensors. N-HMCS is expected to become a new generation of sensing materials for electrochemical analysis of $O_2^{\bullet-}$.

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1. Introduction

Superoxide anion ($O_2^{\bullet-}$), as one kind of important reactive oxygen species (ROS), has a close relationship with some diseases such as neurodegeneration, atherosclerosis, diabetes and cancer [1–3]. For the detection of $O_2^{\bullet-}$, the enzyme-based electrochemical sensors have been developed as a favorable strategy. Among them, Cu, Zn-superoxide dismutase (Cu, Zn-SOD) [4–6] and cytochrome c [7–9] as the main enzymes were employed to fabricate $O_2^{\bullet-}$ enzymatic sensors. However, enzyme is vulnerable to the external conditions, such as pH, temperature and humidity, which have negative impact on the stability and reusability of enzyme-based sensors. In order to avoid these weaknesses, developing enzyme-free sensors for determination of $O_2^{\bullet-}$ is an attractive approach. At present, a few enzyme-free superoxide sensors have been reported based on naringin-copper complex [10], Pt nanoparticles covalently bonded to multi-walled carbon nanotubes [11] and copper-zinc

alloy nanoparticles [12]. These good results encouraged us to explore alternative non-toxic and low-cost materials as enzyme-free sensors for determination of $O_2^{\bullet-}$.

Recently, hollow mesoporous carbon spheres (HMCS) with superior structure features have gained extensive attention with widespread applications in the fields of catalysis [13,14], adsorption [15–18], drug delivery [19], and energy storage [20,21] due to their chemical inertness, high specific surface area, good electrical conductivity and biocompatibility. Moreover, the presence of mesoporous channels on the carbon shells of HMCS is beneficial for the mass transport and/or charge transfer between sensors and analytes [22]. Mesoporous carbon materials also have a high density of edge-plane-like defective sites which can promote the electron transfer to analytes and thereby enhance electrochemical activity at the electrodes [23,24]. Nevertheless, using HMCS for electrochemical applications has received little attention [25]. In addition, it is well known that incorporation of heteroatoms, such as N, B, P, and S atoms [26–29], into the carbon matrix can largely improve their physicochemical properties [30]. Among these, N-doped is a promising strategy because the strong electron donor nature of N can supply negative charges to delocalized π bond of sp^2 hybridized carbon skeleton so as to enhance electron transport properties and chemical reactivity [31–33]. Until now, a variety of N-containing carbons including

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graphene-based materials [34], carbon nanotubes [35], microporous or mesoporous carbons [36,37] and carbon nanofibers [38] have been successfully utilized in many fields. N-doped hollow mesoporous carbon spheres (N-HMCS) also have been synthesized by various methods [39–41], but they were mainly employed as an efficient catalysis for oxygen reduction reaction [42–44], and their applications in the field of electrochemical sensors for $O_2^{\bullet-}$ detection have been scarcely reported.

In this work, we focused on employing N-HMCS as an efficient enzyme-free sensing material for directly electrochemical detection of superoxide anion. The solid carbon spheres (SCS) and HMCS were applied to investigate the impact on the determination of $O_2^{\bullet-}$ from the different morphology. Based on the unique features of HMCS, such as good conductivity, large pore size/volume and high specific surface area, HMCS/SPCE exhibited much better detection performance for $O_2^{\bullet-}$ than SCS/SPCE. Further, owing to nitrogen doping, N-HMCS/SPCE showed extremely high sensitivity for quantification of $O_2^{\bullet-}$ even superior to many of metal-based or enzyme-based sensors.

2. Experimental

2.1. Reagents and apparatus

Tetrapropyl orthosilicate (TPOS), potassium superoxide, eighteen-crown-6 and anhydrous dimethyl sulfoxide (DMSO) were purchased from Aladdin Industrial Inc. Ascorbic acid (AA), dopamine (DA), glucose (Glu), uric acid (UA), 4-acetaminophen (AP) and glutathione (GSH) were obtained from Sigma-Aldrich. All other chemicals were in analytical grade and used without further purification.

Nitrogen adsorption-desorption isotherms were measured on an ASAP 2020 (Micromeritics). A scanning electron microscope (SEM, JSM-6360LV, JEOL) was utilized to observe the surface morphology of carbon spheres. The transmission electron microscope (TEM, JEM-1400, JEOL) was used to capture the morphology of carbon spheres. The surface composition of carbon spheres was recorded by X-ray photoelectron spectrometer (XPS, ESCALAB250Xi, Thermo Fisher). Raman spectra were obtained with an InVia Reflex Raman microscope. All electrochemical experiments were performed at room temperature on a CHI660D workstation equipped with a conventional three-electrode system consisting of a screen-printed working electrode, a platinum wire counter electrode, and an Ag/AgCl reference electrode.

2.2. Preparation of HMCS, N-HMCS and SCS

The HMCS was synthesized according to a report [45]. Typically, TPOS (3.46 mL, 12 mmol) was mixed with solution containing ethanol (70 mL), H_2O (10 mL) and aqueous ammonia (3 mL) under constant stirring for 15 minutes. Then, formaldehyde (0.56 mL) and resorcinol (0.4 g) were added and the system was kept stirring for 24 h at room temperature. The products were collected by centrifugation, washed with deionized water and ethanol for several times, and dried at $50^\circ C$ in the vacuum oven. HMCS were finally obtained after carbonization at $700^\circ C$ ($5^\circ C min^{-1}$) for 5 h under the N_2 atmosphere, followed by an etching process with 2 M NaOH at $80^\circ C$ to remove silica. Further, a half of prepared HMCS was dispersed in aqueous ammonia (50 mL) and then the mixture was reacted for 9 h at $180^\circ C$ in the 100 mL Teflon-lined autoclave to gain the N-HMCS.

The synthesis of SCS was a similar process as followed: resorcinol (0.24 g) and formaldehyde (0.36 mL) reacted with the mixture containing ethanol (70 mL), ultra-pure water (10 mL) and aqueous ammonia (3 mL) for 12 h in ambient temperature with constant stirring. After the above mixed solution was centrifuged

and washed by water and ethanol for several times then dried it, the obtained solid product was carbonized at $700^\circ C$ ($5^\circ C min^{-1}$) under N_2 for 5 h.

2.3. Fabrication of the modified electrodes

The home-made screen-printed carbon electrodes (SPCE) with a working area of $0.071 cm^2$ were served as the substrate according to our previous work [46]. 5 mg N-HMCS was added into 5 mL ultrapure water and sonicated to form a well-dispersed mixture. Then, 10 μL of the mixture and 2 μL of 0.05 wt% Nafion were drop-casted onto SPCE successively, and the N-HMCS modified electrodes (N-HMCS/SPCE) were finally formed after dried. The HMCS modified electrodes (HMCS/SPCE) and SCS modified electrodes (SCS/SPCE) were fabricated with a similar method, and the bare SPCE was only treated with 2 μL of 0.05 wt% Nafion.

2.4. Generation of superoxide anion

The superoxide anion was generated from the KO_2 -DMSO system according to our previous work [47]. Briefly, a stock solution of KO_2 was prepared by adding KO_2 into anhydrous DMSO, containing 18-crown-6 that can increase the solubility of KO_2 . After sonicating the solution for 2 min, KO_2 was dissociated and produced $O_2^{\bullet-}$. The concentration of $O_2^{\bullet-}$ was determined to be $8 \mu M \mu L^{-1}$ by UV-vis spectroscope with the molar absorptivity of $O_2^{\bullet-}$ in DMSO ($2006 M^{-1} cm^{-1}$ at 271 nm) [48].

2.5. Electrochemical measurement

The electrochemical behaviors of modified electrodes were investigated using cyclic voltammetric (CV), chronoamperometric, chronocoulometric, and electrochemical impedance spectroscopy (EIS) techniques, respectively. CV measurements were performed in stationary electrolyte solution. The chronoamperometric experiments were implemented by successively adding the analytes into 5 mL 0.1 M phosphate buffer solution (PBS, pH 7.4) under constantly stirring. Chronocoulometric measurements were carried out in the 0.1 mM $K_3[Fe(CN)_6]$ containing 1 M KCl. And EIS was gained in the 5 mM $K_3[Fe(CN)_6]$ (0.1 M KCl) solution and the equivalent circuit of Nyquist plots was simulated using the ZSimpWin software.

For real samples analysis, the recovery of the sensor was assessed using a calibration curve method in the Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The real time monitoring of $O_2^{\bullet-}$ by N-HMCS/SPCE in living cells was also evaluated. The proposed electrodes were tried to capture $O_2^{\bullet-}$ released from living cells L929 suspension stimulated by 30 μL Zymosan solution (5 mg/mL) at $-0.15 V$.

3. Results and discussion

3.1. Characterization of carbon spheres

The morphology of SCS, HMCS and N-HMCS was characterized by SEM and TEM. Three prepared carbon materials all exhibited a uniform spherical morphology with a mean size of 270 nm (Fig. 1A, C and D). Compared to SCS with a solid structure (the inset of Fig. 1A), a characteristic hollow structure was observed for HMCS (inset of Fig. 1C) with an interior hollow cavity and a porous shell. The morphology of HMCS was not changed after hydrothermal treatment at $180^\circ C$ by comparing Fig. 1C and B.

The textural properties of SCS, HMCS, and N-HMCS were measured by nitrogen adsorption/desorption isotherms. The typical isotherm of SCS (Fig. 1D) belongs to type I curve, suggesting

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