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A superoxide anion biosensor based on direct electron transfer of superoxide dismutase on sodium alginate sol–gel film and its application to monitoring of living cells

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ABSTRACT

The direct electron transfer of superoxide dismutase (SOD) was greatly facilitated by sodium alginate (SA) sol–gel film with the formal potential of 0.14 V, which was just located between $O_2^{\bullet-}/O_2$ and $O_2^{\bullet-}/H_2O_2$. The preparation of the SOD/SA modified electrode was simple without any mediators or promoters. Based on bimolecular recognition for specific reactivity of SOD/SA toward $O_2^{\bullet-}$, the SOD modified electrode was utilized to measure $O_2^{\bullet-}$ with good analytical performance, such as low applied potential (0 V), high selectivity (no obvious interference), wide linear range (0.44–229.88 μM) and low detection limit (0.23 μM) in pH 7.0 phosphate buffer solution. Furthermore, it could be successfully exploited for the determination of $O_2^{\bullet-}$ released from living cells directly adhered on the modified electrode surface. Thus, the proposed $O_2^{\bullet-}$ biosensor, combining with the properties of SA sol–gel film, provided a novel approach for protein immobilization, direct electron transfer study of the immobilized protein and real-time determination of $O_2^{\bullet-}$ released from living cells.

1. Introduction

Superoxide anion ($O_2^{\bullet-}$), one of the most abundant reactive oxygen species (ROS), is involved in a lot of physiological and pathological processes [1–4]. The normal amount of ROS is benefit for immune response, but the existence of excessive ROS is related to the occurrence of aging, heart disease, cancer and progressive neurodegenerative diseases such as Parkinson's disease [5–10]. Therefore, a selective and sensitive method for reliable and durable determination of $O_2^{\bullet-}$ with a low detection limit and wide linear range is essential. It is useful to gain a full understanding of the role which $O_2^{\bullet-}$ plays in pathology and physiology.

The methods commonly used to determine $O_2^{\bullet-}$ are electron spin resonance [11–13], conductometric analysis [14], mass spectrometry [15], spectrophotometry [16] and so on. Recently, considerable efforts have been paid on the development of electrochemical method for determination of $O_2^{\bullet-}$ because the electrochemical method is simple, selective, cost-effective, and can offer a direct, real time measurement in biological systems. The electrochemical measurements of $O_2^{\bullet-}$ based on the direct oxidation of $O_2^{\bullet-}$ mediated by biomimetic enzymes such as Mn^{2+} [17], and by enzymes such as cytochrome *c* [18–20] and superoxide dismutase (SOD) [21–23] have been reported. The third generation of $O_2^{\bullet-}$ biosensors based on the direct electron transfer of SOD has been well addressed due to the excellent selectivity and high sensitivity.

SOD is an important anti-oxidant enzyme in living organism [2,4]. It can catalyze the dismutation of the $O_2^{\bullet-}$ to O_2 and H_2O_2 efficiently and specifically, which was first reported by McCord and Fridovich [24]. Recently, great efforts have been made to enhance the direct electrochemistry of SOD and constructed selective and sensitive $O_2^{\bullet-}$ biosensors, because it is very useful to determine $O_2^{\bullet-}$ in vitro and in vivo models. However, the direct electron transfer of SOD is very difficult due to its deeply buried redox centers and the unfavorable orientations of SOD. To solve this problem, many immobilized methods and materials were used, such as dispersing gold nanoparticles onto poly(methyl methacrylate) (PMMA)–polyaniline (PANI) core–shell electrospun nanofibers [25], immobilizing SOD on silicon carbon (SiC) nanoparticles [26], silica sol–gel matrix [27,28], high conductive TiO_2 nanoneedles [29], ZnO nanodisks [30,31], gold nanostructures [32], carbon fiber [33], and self-assembled monolayer on gold electrodes [22,34].

Most biological macromolecules are highly efficient in recognizing specific molecules or catalyzing reactions in aqueous biological

Abbreviations: SOD, superoxide dismutase; SA, sodium alginate; ROS, reactive oxygen species; O_2^-, superoxide anion; RNS, reactive nitrogen species; H_2O_2, hydrogen peroxide; NO, nitric oxide; DA, dopamine; AA, ascorbic acid; $ONOO^-$, peroxynitrite; PBS, phosphate butter solution; SCE, saturated calomel electrode; FESEM, field emission scanning electron microscopy; FTIR, Fourier transform infrared; i_{pa}, anodic peak current; i_{pc}, cathodic peak current; $E^{o\prime}$, formal potential.

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media. Recently, sol–gel films have been widely used to construct biosensors such as sodium alginate (SA) [35–37]. SA is a natural anionic polysaccharide and consists of unbranched copolymers of 1–4 linked D-mannuronic acid and L-guluronic acid which is from marine brown-algae. It owns many properties such as non-toxicity, biocompatibility, hydrophilic property and low cost. It is an important biopolymer used to make microcapsules for drug delivery and immobilization of biocatalysts, wound dressing, tissue engineering [38]. Also it has high water absorbing ability which keeps a moist environment.

Macrophages (i.e. raw 264.7) are known to play an important role in host protection against a wide range of tumors and microorganisms. They are the first cells to participate in the immunological response, with the production of ROS and reactive nitrogen species (RNS) involved in the destruction of pathogens [39]. The major ROS and RNS produced within the macrophages are $O_2^{\bullet -}$, hydrogen peroxide (H_2O_2) and nitric oxide (NO) [40,41]. However, excess ROS and RNS production have been implicated in many inflammatory diseases [42–44]. In this paper, a novel $O_2^{\bullet -}$ sensor based on the direct electron transfer of SOD on SA sol–gel film was described. The obtained sensor exhibited excellent electrocatalytic response to $O_2^{\bullet -}$ and it had good stability and sensitivity. The monitoring of $O_2^{\bullet -}$ released from raw 264.7 macrophage cells using the proposed sensor was also demonstrated.

2. Experimental

2.1. Reagents and materials

SOD (3000 U, 3730 U mg^{-1}) was purchased from Sigma and used without further purification. SA was purchased from Shanghai Guoyao Chemical Reagent Co., Ltd. Dopamine (DA), ascorbic acid (AA) and peroxynitrite ($ONOO^-$) were purchased from Sigma. $OH^{\bullet}$ was generated in 10 μM H_2O_2 catalyzed by Fe^{2+}. Phosphate butter solution (PBS, 0.1 M) was prepared by mixed stock standard K_2HPO_4 and KH_2PO_4 solutions, and pH of PBS was adjusted by pH meter. Other reagents were of analytical grade and used as received. A stock solution of KO_2 was prepared by adding KO_2 to dimethyl sulfoxide (stored together with molecular sieve 4 Å (Sinopharm Chemical Reagent Co., Ltd.)), sonicating the solution for 2 min. The stock solutions were stored at 4 °C. All solutions were prepared with doubly distilled water.

2.2. Preparation of the modified electrodes

The bare gold electrodes were polished to a mirror-like finish with 1.0, 0.3, and 0.05 μm alumina slurry (Beuhler) followed by rinsing thoroughly with doubly distilled water. The electrodes were successively sonicated in 1:1 nitric acid, acetone and doubly distilled water, and then allowed to dry at room temperature. SOD (3000 U mL^{-1}) and SA (2 mg mL^{-1}) were mixed (1:1, v/v). Then 2 μL of the mixture of SOD and SA was applied to the pretreated gold electrodes surface, stored in refrigerator at 4 °C until it was dry.

For control experiment, 2 μL SA (2 mg mL^{-1}) sol–gel or 2 μL SOD (3000 U mL^{-1}) was dripped on the gold electrode and dried at 4 °C. Then the SA modified electrode or SOD modified electrode was formed, respectively.

When the modified electrodes were not in use, they were stored in pH 7.0 PBS at 4 °C.

2.3. Apparatus and measurements

Electrochemical experiments were carried out on CHI 660B electrochemical working station, with the modified gold electrode as a working electrode, platinum wire as a counter electrode and saturated calomel electrode (SCE) as a reference electrode. All measurements were carried out at room temperature (about 25 °C). The experimental solutions were deoxygenated by bubbling highly pure nitrogen for 15 min, and a nitrogen atmosphere was kept over the solutions during measurements. The morphologies of the samples were characterized by LEO1530 VP field emission scanning electron microscopy (FESEM) at an acceleration of 15 kV. Fourier transform infrared (FTIR) spectra were obtained in the range of 2500–1200 cm^{-1} on a NEXUS 670 (Nicolet) FTIR instrument at room temperature.

The $O_2^{\bullet -}$ solutions were prepared by the addition of aliquots of KO_2 stock solution (N_2 saturated). The concentrations of $O_2^{\bullet -}$ were determined by recording the reduction of ferricytochrome *c* spectrophotometrically and using the extinction coefficient (21.1 mM^{-1} cm^{-1}) of ferrocytochrome.

2.4. Cell culture

Raw 264.7 macrophage cells grew at 37 °C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin in a 5% CO_2 environment. After growing to 90% confluence, the cells were then washed with pH 7.4 PBS and the cell number was estimated by a hemocytometer.

2.5. $O_2^{\bullet -}$ released from living cells

Raw 264.7 macrophage cells were incubated with zymosan (250 μg mL^{-1}) in DMEM complete medium in the presence of nitroblue tetrazolium (NBT). For cell adhesion, 0.5 mL of cells at a concentration of 1×10^5 cells mL^{-1} was directly plated on the modified electrode for the electrochemical experiments. The adhered cells were fixed with 2% glutaraldehyde for 20 min at room temperature.

3. Results and discussion

3.1. Characterization of SOD/SA modified gold electrode

The FESEM images of SOD, SA and SOD/SA film were shown in Fig. 1. Significant differences in the surface morphology of the three samples could be observed. The SEM image of SOD with more uniform surface could be observed in Fig. 1A. The surface of the SA was formed by isolated and irregularly shaped enzyme biomolecular flakes (Fig. 1B). However, the image of the SOD/SA (Fig. 1C) showed the aggregates of the trapped enzyme biomolecular with a relatively uniform film, which indicated the formation of specific interface.

It could be seen from Fig. 2 that the FT-IR spectra of the SA exhibited its asymmetric stretching vibration of carboxylate O—C—O at 1646 cm^{-1}, the C—OH deformation vibration with contribution of O—C—O symmetric stretching vibration of carboxylate group at 1536 cm^{-1}, and the C—O stretching vibration of pyranose rings at 1228 cm^{-1}, respectively [45] (curve a). The IR spectra of SOD also showed the adsorption bands at 1633 and 1542 cm^{-1} which were attributed to amide I and amide II, respectively [46] (curve b). Upon adsorption of the SOD on SA, the bands at 1633 cm^{-1} shifted to 1594 cm^{-1}, and the band corresponding to amide II and adsorption bands at 1228 cm^{-1} disappeared, implying an interaction between SA and SOD (curve c).

3.2. Direct electrochemical behavior of SOD

Fig. 3 shows the cyclic voltammograms (CVs) of different electrodes in 0.1 M pH 7.0 PBS at 500 mV s^{-1}. No peak was observed at both bare gold electrode and SA modified gold electrode, which

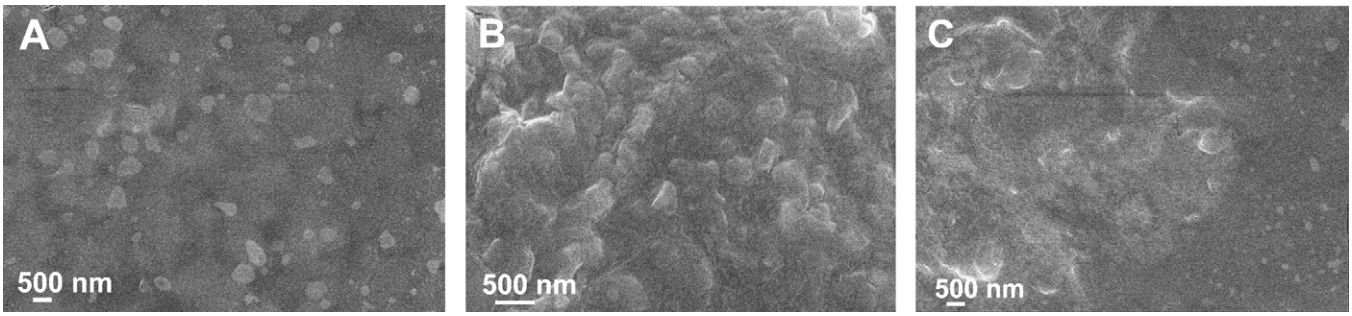

Fig. 1. FESEM images of SOD (A), SA (B) and SOD/SA (C) film.

showed SA was electroinactive in the potential window. At SOD modified gold electrode, a slight couple of broad redox peak of SOD appeared with the formal potential of SOD at 0.16 V with a scan rate of 500 mV s^{-1}. While the SOD/SA modified gold electrode exhibited a couple of stable redox peaks with the formal potential of 0.15 V and the peak-to-peak potential separation of the redox peaks was about 0.16 V. Furthermore, the response was much larger than that of the SOD modified gold electrode. The experimental results demonstrated that SA could provide a biocompatible microenvironment for SOD and supply a necessary pathway for its direct electron transfer. CV curve at SOD/SA modified electrode at lower scan rate (100 mV s^{-1}) was listed in inset in Fig. 3. The curve also exhibited a similar shape with that of SOD/SA modified electrode at higher scan rate (500 mV s^{-1}). Thus, SOD gave good electrochemical responses at the SOD/SA modified gold electrode because SA provided a microenvironment for SOD to undergo facile electron transfer reaction. The surface concentration of electroactive SOD at SOD/SA modified electrode was estimated to be 6.0×10^{-11} mol cm^{-2} which was higher than that of the monolayer coverage of hemoglobin on bare electrode (1.89×10^{-11} mol cm^{-2}) [47].

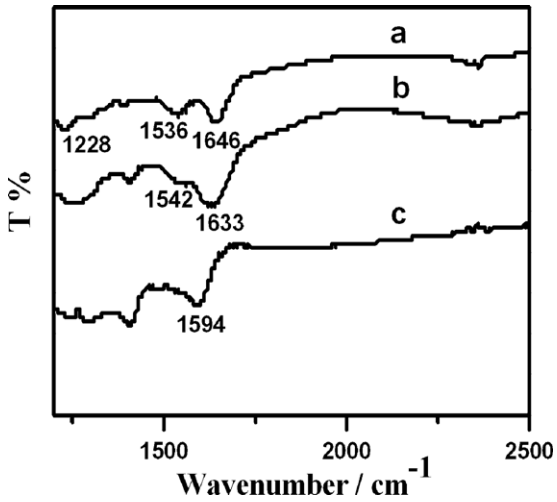

Fig. 2. IR spectra of SA (a), SOD (b) and SOD/SA (c).

The effect of scan rate on the response of the immobilized SOD was shown in Fig. 4. The CVs remained essentially unchanged on consecutive potential cycling, demonstrating that SOD was stably confined in SA. In addition, we found that both the anodic peak current (i_{pa}) and cathodic peak current (i_{pc}) varied linearly with the potential scan rate (ν) in the range of 70–500 mV s^{-1} (inset in Fig. 4) which indicated the surface-controlled process. The ratio of i_{pa}/i_{pc} at a given ν was nearly unity.

3.3. Effect of solution pH on direct electron transfer of SOD

The effect of pH values on electrochemical behavior of SOD was studied. The slop of the formal potential ($E^{o\prime}$) versus pH was estimated to be −0.054 V pH^{-1}. This indicated one proton and one

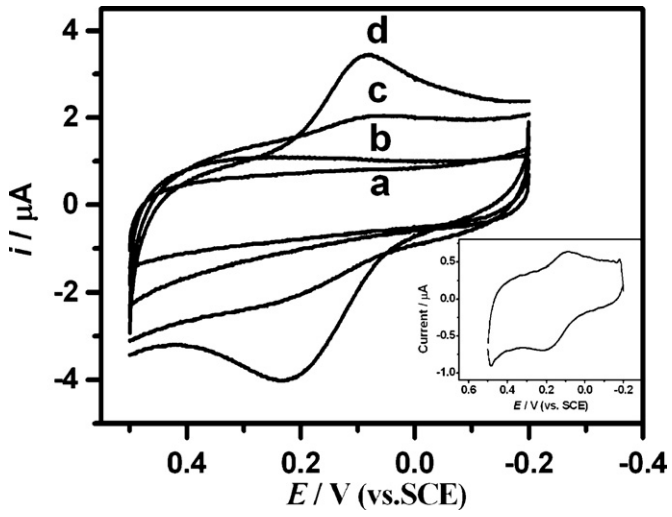

Fig. 3. CVs of bare (a), SA (b), SOD (c) and SOD/SA (d) modified gold electrodes in 0.1 M pH 7.0 PBS. Scan rate: 500 mV s^{-1}. Inset: CVs of SOD/SA modified gold electrodes in 0.1 M pH 7.0 PBS. Scan rate: 100 mV s^{-1}.

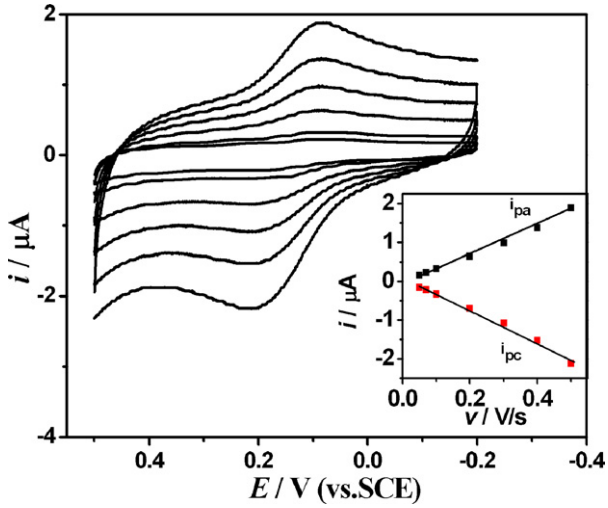

Fig. 4. CVs of SOD/SA modified gold electrode in 0.1 M pH 7.0 PBS at 70, 100, 200, 300, 400, and 500 mV s^{-1} (from inner to outer). Inset: plot of peak current versus scan rate.

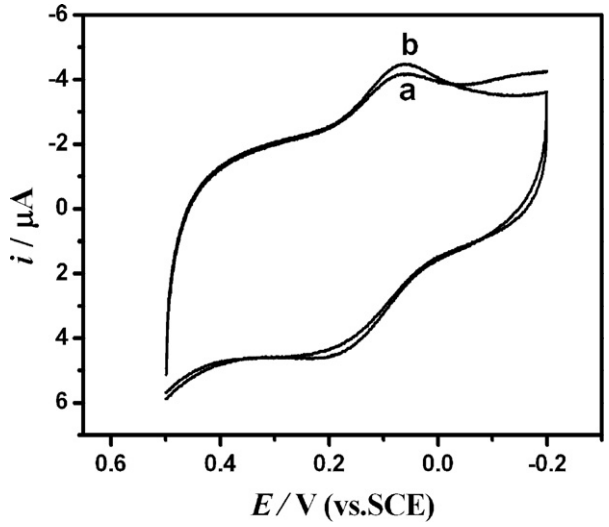

Fig. 5. CVs of SOD/SA modified gold electrode in the absence (a) and presence (b) of 50 μM $O_2^{\bullet-}$ in 0.1 M pH 7.0 PBS. Scan rate: 500 mV s^{-1}.

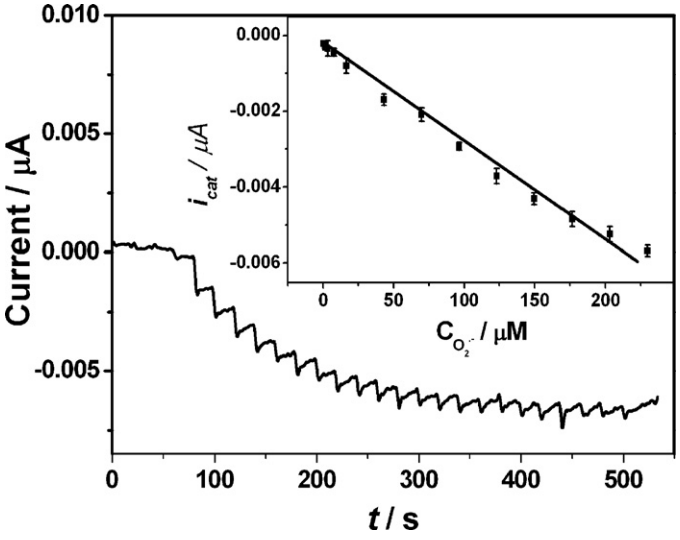

Fig. 6. Typical amperometric responses of the SOD/SA modified electrode to successive addition of KO_2 at applied potentials of 0 mV in 6 mL 0.1 M pH 7.0 PBS. Inset: calibration plots of steady-state currents versus concentration of $O_2^{\bullet-}$.

electron were involved in the electrode reaction of SOD, which was similar to the previously proposed scheme for the enzymatic catalytic mechanism of the SOD [23,48,49].

3.4. Electrocatalysis of SOD/SA modified gold electrode to reduction of $O_2^{\bullet-}$

As demonstrated above, SA sol–gel film facilitated the electron transfer of SOD, and SOD was stably confined in SA sol–gel film. This result formed a strong basis for the development of a third-generation biosensor for $O_2^{\bullet-}$ because the metal ions were the active site for the catalysis to the reduction of $O_2^{\bullet-}$. The electrocatalytic activity of electrode toward $O_2^{\bullet-}$ was investigated by CV. Fig. 5 displays CVs obtained at SOD/SA modified gold electrode in the absence (a) and presence (b) of 50 μM $O_2^{\bullet-}$ in 0.1 M pH 7.0 PBS. The addition of 50 μM $O_2^{\bullet-}$ to 0.1 M pH 7.0 PBS increased the cathodic current of the SOD confined in SA modified gold electrodes, indicating the good electrocatalytic activity of the immobilized SOD for the reduction of $O_2^{\bullet-}$.

3.5. Analytical performance of the $O_2^{\bullet-}$ biosensor

The typical amperometric responses of SOD/SA modified gold electrode to successive concentration changes of $O_2^{\bullet-}$ were examined at the applied potential of 0 mV, and the corresponding current–time responses were shown in Fig. 6. Upon addition of an aliquot of $O_2^{\bullet-}$ to the buffer solution, the reduction current increased steeply to reach a stable value. The enzyme electrode achieved 95% of the steady-state-current in less than 5 s. The results demonstrated clearly that the electrocatalytic response was very fast. Well defined steady-state current responses were obtained at the modified electrode, and the currents decreased stepwise with successive additions of $O_2^{\bullet-}$. The steady-state currents at the modified electrode were proportional to $O_2^{\bullet-}$ in the examined range of 0.44–229.88 μM with a correlation coefficient of 0.995 ($n = 12$). The linear range was wider than that obtained on SOD–ZnO nanodisks surfaces [26,50]. The sensitivity of SOD/SA was estimated to be 7.82×10^{-4} μA(cm^2 μM)$^{-1}$ which was higher than those on SOD/cysteine-modified gold electrode [51] and on SOD/NiO_x modified GC electrode [52]. The detection limit was evaluated on the basis of a signal-to-noise ratio of 3:1 and calculated to be 0.23 μM which was lower than those on SOD/Cys/Au at carbon fiber microelectrode [33] and on SOD/NiO_x modified GC electrode [52].

In control experiment, no response was observable at SA modified electrode and SOD modified electrode showed a much smaller response to $O_2^{\bullet-}$ than that of the SOD/SA modified electrode at the same $O_2^{\bullet-}$ concentration.

When the concentration of $O_2^{\bullet-}$ was higher than 230 μM, a plateau was observed, showing the characteristics of the Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constant (K_M^{app}), which gives an indication of the enzyme–substrate kinetics, can be obtained from the electrochemical version of the Lineweaver–Burk equation: $1/I_{ss} = 1/I_{max} + K_M^{app}/(I_{max}C)$. In this work, the K_M^{app} value for the SOD/SA modified electrode was estimated to be 0.10 mM. The low value of K_M^{app} indicated that SOD immobilized on the SOD/SA had a high affinity to $O_2^{\bullet-}$.

3.6. Interference

The interference of a variety of relevant analytes including DA, AA, H_2O_2, O_2, $OH^{\bullet}$ and $ONOO^-$ which exists extensively in biological system was also investigated for control experiments (Fig. 7). It was found that no obvious interference was observed with $O_2^{\bullet-}$ detection at 0 V which indicated the sensor had a good selectivity.

3.7. Stability and reproducibility of the $O_2^{\bullet-}$ biosensor

A storage period of a week in 0.1 M pH 7.0 PBS at 4 °C, the SOD/SA modified electrode did not change the responses to $O_2^{\bullet-}$. After a month, the sensor retained 92% of its initial response to $O_2^{\bullet-}$. Thus, SA sol–gel film was very efficient for retaining the bioactivity of immobilized SOD and preventing it from leaking out of the sensors. The fabrication reproducibility of six electrodes, made independently, showed an acceptable reproducibility with the average relative standard deviation of 5.8% for the current determinations of 4.0 μM $O_2^{\bullet-}$.

3.8. Measuring the $O_2^{\bullet-}$ released from raw 264.7 cells

As mentioned above, the sensor exhibited a good sensitivity, selectivity, stability and reproducibility which can be used in vitro determination of $O_2^{\bullet-}$ released from living cells. Fig. 8 shows the

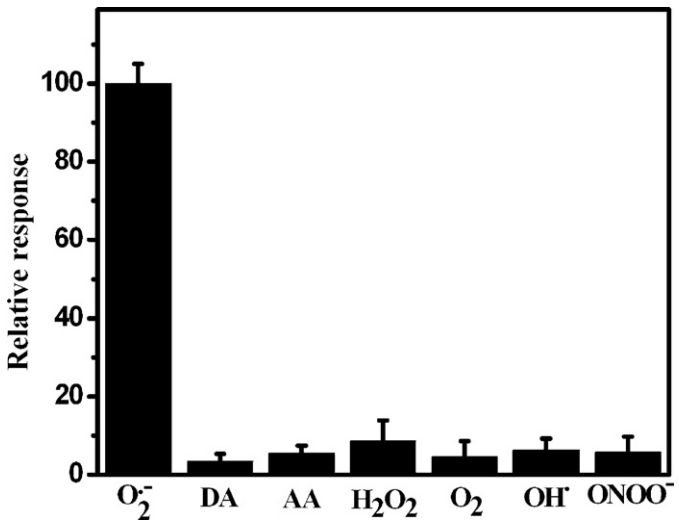

Fig. 7. Detecting specificity in a solution containing DA, AA, H_2O_2, O_2, OH• and $ONOO^-$ at SOD/SA modified electrode. It is an average value of the response of three measurements for each sample.

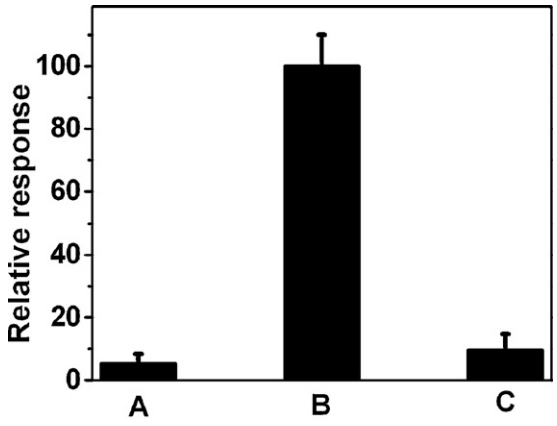

Fig. 8. Electrochemical response of SOD/SA modified electrode toward raw 264.7 at an applied potential of 0 V in the absence (A) and presence (B) of 10 mM zymosan with the addition of 3000 U mL^{-1} SOD solution (C).

electrochemical response obtained at SOD/SA surface and toward rat 264.7 macrophage cells adhered on the electrode surface at the applied potential of 0 V in the absence (A) and presence (B) of zymosan, which was reported to generate $O_2^{\bullet -}$ from cells [53]. A much higher increased cathodic current was observed after the addition of zymosan. After injection of 10 μL 3000 U mL^{-1} SOD, a selective scavenger of $O_2^{\bullet -}$, to the electrode surface, the response decreased rapidly (C). Thus, the increased response was attributed to the reduction of $O_2^{\bullet -}$ released from living cells.

4. Conclusions

The SOD/SA modified gold electrode was prepared and applied to the electrochemical determination of $O_2^{\bullet -}$. SOD was immobilized by SA sol–gel thin film on gold electrode surface. The preparation procedure of the modified electrode was simple. The direct electron transfer of SOD in the thin sol–gel film was realized at the gold electrode without any mediators or promoters. Therefore, a third-generation amperometric biosensor for the measurement of $O_2^{\bullet -}$ was developed by the sol–gel method. The sol–gel film also provided a biocompatible microenvironment around the protein and was very efficient to stabilize the enzyme activity. More interestingly, it could be successfully exploited for the determination of $O_2^{\bullet -}$ released from living cells which was expected to have the potential application of cellular biology.

Acknowledgements

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