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COMMUNICATION

Sensitive electrochemical detection of superoxide anion using gold nanoparticles distributed poly(methyl methacrylate)–polyaniline core–shell electrospun composite electrode

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In the present communication, a novel composite nanofibrous electrode is developed for the detection of superoxide anion ($O_2^{\cdot-}$) in phosphate buffered saline (PBS). The composite fiber electrode is fabricated by dispersing gold nanoparticles onto poly(methyl methacrylate) (PMMA)–polyaniline (PANI) core–shell electrospun nanofibers. The constructed architecture is proven to be a favorable environment for the immobilization of the enzyme, superoxide dismutase (SOD). Direct electron transfer is achieved between SOD and the electrode with an electron transfer rate constant of 8.93 s^{-1} . At an applied potential of +300 mV, PMMA/PANI–Au_{nano}/SOD–ESCFM shows highly sensitive detection of $O_2^{\cdot-}$. In addition to this, quantification of different activities of SOD is realized at PMMA/PANI–Au_{nano}/SOD–ESCFM. These analytical features offer great potential for construction of the third-generation $O_2^{\cdot-}$ biosensor.

Introduction

Superoxide anion radicals ($O_2^{\cdot-}$) are produced as the transient reduction product of molecular oxygen in mitochondria and are involved in the important cellular processes like mitochondrial electron transport, signal transduction, *etc.*¹ The excessive presence of $O_2^{\cdot-}$ along with other reactive oxygen species than the antioxidant capacity (aided by superoxide dismutase, catalase, glutathione peroxidase, glutathione, ascorbate, tocopherol and thioredoxin) can result in inhibition of cell growth, mutagenesis and cell death.² Hence, detection of $O_2^{\cdot-}$ is of paramount importance and challenging owing to the high reactivity, short life and rather low concentration in biological fluids.³

Electrochemical biosensors have been developed for the detection of $O_2^{\cdot-}$ using either superoxide dismutase (SOD) or cytochrome *c* as the recognition element.^{4–6} The selectivity and quantification of $O_2^{\cdot-}$ in biological fluids using cytochrome *c* based biosensors is hampered

because the heme protein resembles peroxidase and is not specific for $O_2^{\cdot-}$. However, SOD based biosensors are much more specific and sensitive towards $O_2^{\cdot-}$. SOD specifically catalyzes the dismutation of $O_2^{\cdot-}$ into O_2 and H_2O_2 via a cyclic oxidation–reduction mechanism.³ The first-generation $O_2^{\cdot-}$ biosensors are based on the direct measurement of the electrochemical response from the enzymatic product, H_2O_2 , at the electrode.⁷ However, the high over-potential required for the oxidation of H_2O_2 causes simultaneous electrochemical signals from the endogenous or exogenous compounds present in biological samples, leading to a high level of interference for the quantification of $O_2^{\cdot-}$. The second-generation $O_2^{\cdot-}$ biosensors involve specific ‘redox mediators’ that are able to shuttle electrons between the electrode and the reaction site of SOD.⁸ The third-generation $O_2^{\cdot-}$ biosensors exploit direct electron transfer (DET) between the redox site of the enzyme and electrode without any mediator. Such biosensors involving DET recently received research interest. The DET of SOD has been realized with a self-assembled monolayer,⁵ electroactive sol–gel film⁹ and ZnO nanodisc film.¹⁰

The performance of the third-generation $O_2^{\cdot-}$ biosensor depends mainly on enzyme loading and the rate of electron transfer between SOD and the electrode. Moreover, the low concentration and momentary existence of $O_2^{\cdot-}$ necessitate an extremely high enzyme loading to realize a sensitive electrochemical response from the analyte. The functional properties of the support matrix that is used for enzyme immobilization play a crucial role in the fabrication of high performance $O_2^{\cdot-}$ biosensors. In the present work, we have developed an $O_2^{\cdot-}$ biosensor using the electrospun composite functional membrane (ESCFM). Gold nanoparticles dispersed poly(methyl methacrylate)–polyaniline core–shell nanofibrous membrane (PMMA/PANI–Au_{nano}–ESCFM) is used as a matrix for the immobilization of SOD. The new ESCFM provides high surface area and porous structure for effective SOD immobilization as well as offers excellent biocompatible microenvironment for SOD.

PMMA/PANI–Au_{nano}–ESCFM possesses distinct features due to nanoscale fiber diameter and large surface area to mass ratios and these features are exploited for effective enzyme loading and biosensor application.^{11,12} Au nanoparticles provide an adequate platform for enzyme immobilization and facilitate DET between the active site of the enzyme and electrode surface.^{13,14} The high ratio of surface atoms with free valences to the cluster of total atoms gives rise

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to its high catalytic activation, which has been exploited in many of the electrocatalytic reactions.^{15–17} PANI is a unique conducting polymer owing to its mixed electronic conductivity upon doping and has shown ability as a transducer in electrochemical devices. PANI provides a low ohmic drop and limits mass transfer for the electroactive species to reach the catalytic sites. Besides, PANI serves as the matrix for the dispersion of metal nanoparticles.¹⁵ The dispersion of Au nanoparticles over PANI can augment the sensitivity and selectivity of the biosensors due to the synergistic influences from Au nanoparticles and PANI. In this work, a novel nanofibrous composite electrode, PMMA/PANI–Au_{nano}–ESCFM, is developed and the transduction ability of the electrode towards O₂^{•−} is assessed. Also, the quantification on the scavenging effect of SOD is evaluated.

Experimental

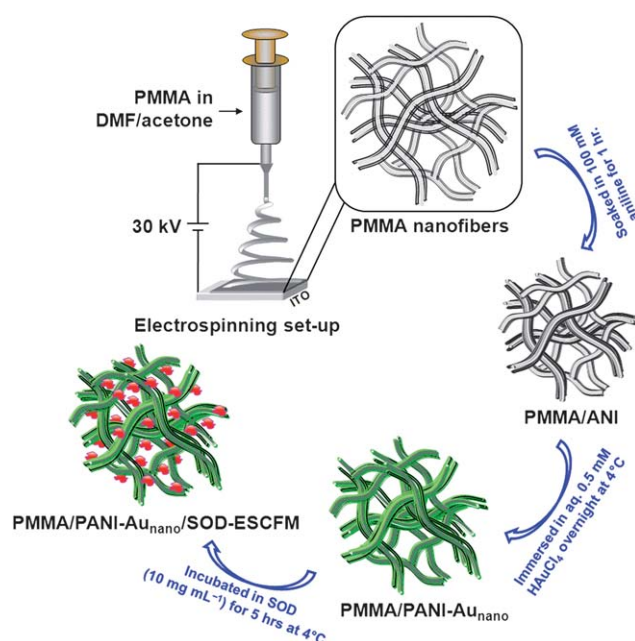
Superoxide dismutase (Cu–Zn SOD; E.C. 1.15.1.1) from bovine erythrocytes, xanthine oxidase (XOD; E.C. 1.17.3.2) from bovine milk, xanthine, poly(methyl methacrylate) (M_w : 350 000 g mol^{−1}), aniline, 2-naphthalene sulfonic acid (NSA), gold(III) chloride hydrate, ascorbic acid, uric acid and 3,4-dihydroxyphenylacetic acid were purchased from Sigma-Aldrich. Unless otherwise stated, reagents were of analytical grade from Sigma-Aldrich and used as received. Indium-doped tin oxide (ITO)-coated glass plate was used for making the sensor electrode. Before performing each of the experiments, the ITO plate was rinsed with acetone and washed with distilled water. Aqueous solutions of the analyte were prepared in 0.1 M phosphate buffered saline (PBS; pH 7.4) afresh at the time of experiments.

The PMMA/PANI–Au_{nano}/SOD–ESCFM modified ITO electrode was fabricated using the methodology detailed elsewhere.¹⁸ Briefly, an adequate amount of PMMA was dissolved in DMF/acetone mixture (7 : 3, v/v). Electrospinning of the PMMA was performed at a flow rate of 1 mL h^{−1} under an electric field of 30 kV. A distance of 20 cm was kept between the syringe tip and collector. PMMA nanofibers were collected over the ITO-coated glass plate (5 mm × 5 mm). The PMMA nanofibrous electrode was soaked in 100 mM aniline (in 1 M NSA solution) for 1 h at 25 °C. The electrode was subsequently immersed in an aqueous solution of hydrogen tetrachloroaurate (0.5 mM) and kept overnight at 4 °C. The resulting electrode was thoroughly washed again with water and placed in a SOD solution (10 mg mL^{−1}) and incubated for 5 h at 4 °C for immobilization of SOD. PMMA/PANI–Au_{nano}/SOD–ESCFM was washed with PBS and dried in a vacuum oven. The stages involved in the fabrication of PMMA/PANI–Au_{nano}/SOD–ESCFM are depicted in Scheme 1.

Field emission scanning electron microscopy (FESEM; Hitach-530) was used to study the morphology of the electrode. All the electrochemical measurements were performed using EG&G PAR Electrochemical Analyzer. A conventional three-electrode cell assembly was used (WE: PMMA/PANI–Au_{nano}/SOD–ESCFM, CE: platinum wire and RE: Ag/AgCl). For amperometric experiments, the buffer solution is stirred using magnetic stirrer at a rate of 400 rpm. O₂^{•−} is generated by the addition of aliquots of XOD in oxygen saturated PBS containing defined quantity of xanthine.

Results and discussion

The PMMA/PANI–Au_{nano}/SOD–ESCFM electrode was fabricated by a combination of electrospinning technique and *in situ*



Scheme 1 Fabrication methodology of PMMA/PANI–Au_{nano}/SOD–ESCFM.

polymerization of aniline with simultaneous formation of Au nanoparticles. From the FESEM image (Fig. 1A), the average diameter of the PMMA fibers was found to be 300 nm. The surfaces of the fibers are fairly smooth and are randomly oriented. After the *in situ* polymerization of aniline and Au nanoparticles formation, the diameters of the fibers were increased to 400–500 nm and the surfaces of the fibers became rough (Fig. 1B). Further, Au nanoparticles were found to be evenly distributed over the fibers. The increase in diameters of the fibers indicates the formation of a PANI layer over the surface of PMMA. It is worth mentioning that we have neither used a conventional oxidizing agent like ammonium persulfate for polymerization of aniline nor used a reducing agent for generating Au nanoparticles from HAuCl₄. Polymerization of aniline and formation of Au nanoparticles were simultaneously achieved over the surface of PMMA nanofibers. This is feasible due to the fact that the redox potential of aniline is negative than that of standard reduction potential of gold salt. The oxidation of aniline supplies the necessary electrons for the reduction of AuCl₄[−] to form Au nanoparticles.¹⁹ Also, the adsorbed protonated aniline molecules could effectively bind AuCl₄[−]. As a result, the AuCl₄[−] anions are preferentially reduced to Au particles concomitantly with the formation of PANI.¹⁵ The formed PANI chains act as electronically conducting micro-reactors for the formation of Au nanoparticles without any agglomeration. The EDX spectrum (Fig. 1C) shows the existence of Au particles in the nanofibers. The dc conductivity of PMMA/PANI–Au_{nano}/SOD–ESCFM was found to be 50 ± 15 S m^{−1}. It is anticipated that PMMA/PANI–Au_{nano}/SOD–ESCFM provides a high surface area and biocompatible environment for the immobilized SOD.

DET for SOD is apparent at PMMA/PANI–Au_{nano}/SOD–ESCFM through the comparison of cyclic voltammograms (CVs) recorded at ITO, PMMA/PANI–Au_{nano}–ESCFM and PMMA/PANI–Au_{nano}/SOD–ESCFM in PBS at a scan rate of 10 mV s^{−1}, Fig. 2A. Well defined redox peaks were observed in PBS ($E_p^c = +95$

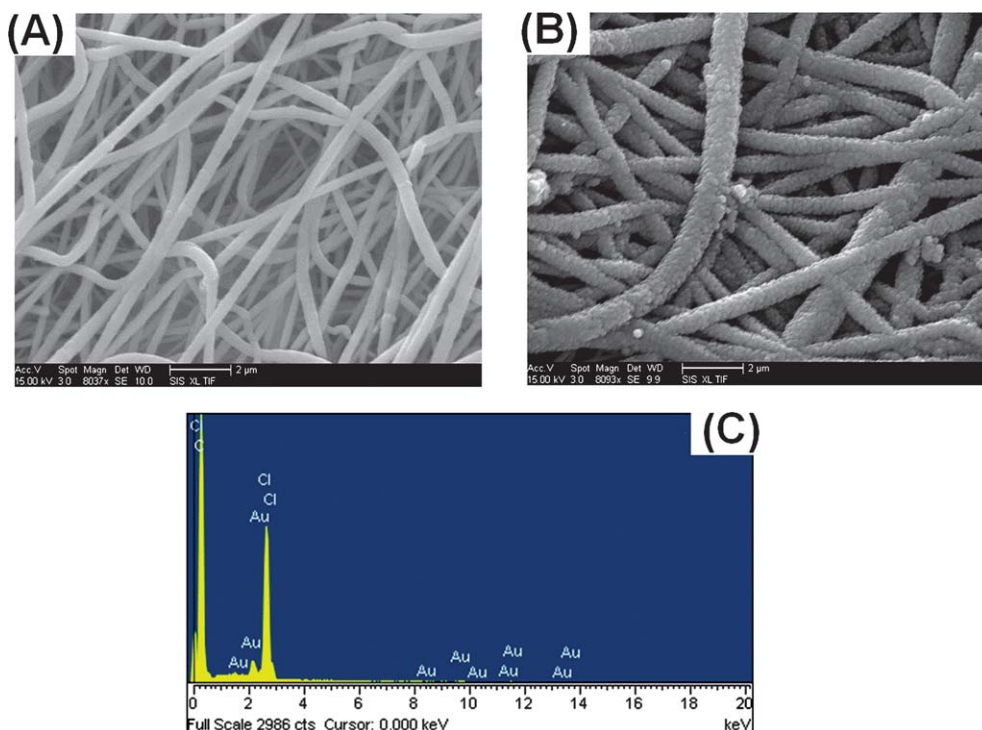


Fig. 1 FESEM images of (A) PMMA-ESCFM, (B) PMMA/PANI-Au_{nano}-ESCFM (scale bar: 2 μ m) and (C) EDX spectrum of PMMA/PANI-Au_{nano}-ESCFM; accelerating voltage, 15 kV.

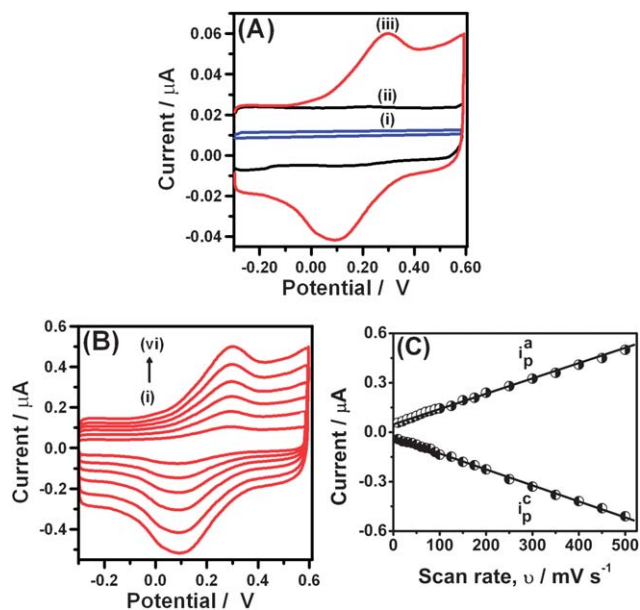


Fig. 2 (A) Cyclic voltammograms of (i) ITO, (ii) PMMA/PANI-Au_{nano}-ESCFM and (iii) PMMA/PANI-Au_{nano}/SOD-ESCFM in PBS; scan rate: 10 mV s^{-1} . (B) Cyclic voltammograms of PMMA/PANI-Au_{nano}/SOD-ESCFM in PBS at various scan rates (i $\rightarrow$ vi: 50, 100, 200, 300, 400 and 500 mV s^{-1}). (C) Plot of anodic and cathodic peak currents vs. scan rate.

mV and $E_p^a = +300$ mV) and the peaks are attributed to the redox reaction of the active site (SOD-Cu^{2+/1+}) of SOD (curve (iii)). However, no appreciable peaks were observed at either bare ITO

electrode or PMMA/PANI-Au_{nano}-ESCFM (curves (i) and (ii), respectively). The Au nanoparticles dispersed onto PANI provide a pathway to shuttle the electrons from the active site of SOD to the electrode.

The formal potential $E^{\circ'}$ of DET (+190 mV vs. Ag/AgCl) is closer to $E^{\circ'}$ reported in the literature.²⁰ The surface coverage (Γ) of SOD in the electrode (0.96×10^{-9} mol cm^{-2}) is higher than for SOD immobilized onto Au nanoparticles (1.04×10^{-10} mol cm^{-2}) and TiO₂ nanoneedles (3.1×10^{-11} mol cm^{-2}).^{20,21} The higher Γ value may be due to the high surface area of PMMA/PANI-Au_{nano}/SOD-ESCFM for SOD immobilization. Further, the redox peak currents of DET were found to be proportional to the scan rate (ν) between 10 and 500 mV s^{-1} (Fig. 2B and C), characteristic of surface-controlled electrode process. The rate constant (k_s) of the heterogeneous electron transfer for the DET at PMMA/PANI-Au_{nano}/SOD-ESCFM and charge-transfer coefficient (α_c) were calculated to be 8.93 s^{-1} and 0.64, respectively using the Laviron model. The presence of Au nanoparticles provides adequate electron transfer tunneling between the active site of the enzyme and the electrode and accounts for the higher rate constant (8.93 s^{-1}) compared with other modified electrodes.^{21,22}

It has previously been demonstrated that electrochemically active SOD immobilized onto the electrode surface offers bi-functional enzymatic catalytic activity^{5,6} and can be used as a sensing element for the detection of O₂^{•-}.^{22,23} To ascertain this, hydrodynamic voltammograms were recorded in the potentials between -600 mV and $+700$ mV (interval of 50 mV) for 2 μM O₂^{•-} in PBS at PMMA/PANI-Au_{nano}/SOD-ESCFM. A substantial rise in the current was observed at a potential higher than $+300$ mV. Further increase in the potential resulted in drastic increase in the current, presumably due to the oxidation of the enzymatically formed uric acid from the XOD/xanthine reaction. A similar trend was witnessed when the potential

was scanned cathodically. The reduction current increased with the applied potential and reached a plateau around -100 mV. A high current was observed at potentials higher than -300 mV, due to the reduction of O_2 and H_2O_2 . These results indicate that the SOD possesses bi-functional enzymatic catalytic activity for the detection of $O_2^{\cdot-}$ through redox cycling of the active site ($SOD-Cu^{2+/+}$). Besides, a sensitive detection (cathodically and anodically) could be realized by polarizing the electrode PMMA/PANI-Au_{nan}/SOD-ESCFM at -0.50 mV and $+300$ mV, respectively.

To validate the utility of PMMA/PANI-Au_{nan}/SOD-ESCFM as $O_2^{\cdot-}$ sensor, we have studied the amperometric response of PMMA/PANI-Au_{nan}/SOD-ESCFM to $O_2^{\cdot-}$ generated *in situ* by enzymatic reaction of XOD with xanthine in the presence of oxygen. Fig. 3A presents the dynamic amperometric response of PMMA/PANI-Au_{nan}/SOD-ESCFM at a working potential of $+300$ mV with different concentrations of XOD in PBS (saturated with oxygen) containing $200\ \mu M$ xanthine. A highly sensitive and steady-state current response was observed within 4 s upon the injection of XOD. The addition of XOD results in the formation of $O_2^{\cdot-}$ and causes the generation of superoxide-dependent oxidation current at the electrode. It is possible that the $O_2^{\cdot-}$ generated by XOD/xanthine would concurrently undergo dismutation. However, under the optimized conditions, we presume that there may be a counterbalance between the generation of $O_2^{\cdot-}$ and dismutation causing a steady-state current response at PMMA/PANI-Au_{nan}/SOD-ESCFM. After a certain

period of operation, a gradual decrease in the response current was observed at PMMA/PANI-Au_{nan}/SOD-ESCFM towards the baseline. Fig. 3B shows the calibration curve for $O_2^{\cdot-}$ obtained at PMMA/PANI-Au_{nan}/SOD-ESCFM and the sensitivity was found to be $42.5\ mA\ cm^{-2}\ M^{-1}$ ($R^2: 0.9944$). The steady-state concentration showed a square root dependence on the activity of the XOD enzyme ($R^2: 0.9842$).

We also performed the quantification of different (scavenging) activities of SOD, Fig. 3C. PMMA/PANI-Au_{nan}/SOD-ESCFM responded sensitively to the oxidation of $O_2^{\cdot-}$ in the PBS containing XOD/xanthine. As can be seen in Fig. 3C, a plateau in current response was observed immediately upon the addition of $50\ mU\ mL^{-1}$ XOD. After 50 s, SOD of $20\ mU\ mL^{-1}$ was injected into the electrochemical cell. A sudden decrease in the current response was observed, probably due to the decomposition of $O_2^{\cdot-}$, facilitated by SOD in the solution. Subsequent additions of SOD to the solution resulted in decrease of current response almost to the baseline current. SOD of different activities results in different rates of $O_2^{\cdot-}$ decomposition. Due to the lower production of $O_2^{\cdot-}$ and higher sensitivity of the electrode, the curve starts to level off and reaches a plateau after $150\ mU\ mL^{-1}$ SOD, Fig. 3D.

The electroactive interferences such as ascorbic acid (AA), uric acid (UA), 3,4-dihydroxyphenylacetic acid (DOPAC) and H_2O_2 were tested for their potential effect during the amperometric detection (cathodic and anodic) of $O_2^{\cdot-}$ in PBS. At -50 mV, the addition of physiological extracerebral fluid levels of AA, UA and DOPAC ($500\ \mu M$, $50\ \mu M$ and $10\ \mu M$, respectively) has a negligible ($>0.5\%$) effect on the current of $1\ \mu M\ O_2^{\cdot-}$, while at $+300$ mV, AA and UA yielded a large deviation ($>40\%$) in the $O_2^{\cdot-}$ current response. For H_2O_2 , no current response was observed when the electrode was polarized at $+300$ mV. However, a small current response (2%) was observed at -50 mV.

The reproducibility of the current responses at the PMMA/PANI-Au_{nan}/SOD-ESCFM was evaluated. A RSD value of 8.64% was noticed for five electrodes fabricated concurrently. Besides, the repeatability was evaluated using three electrodes and by performing successive $O_2^{\cdot-}$ calibration curves for each electrode. Results showed a RSD value of $<5\%$. The shelf life (stability) of the electrode was evaluated for a period of 5 days. The electrode maintained a constant response ($\pm 6\%$) for $O_2^{\cdot-}$ during the first 50 h and retained about 68% of its original response for the remaining days. Note that the electrode was stored in PBS at $4\ ^\circ C$ while it is not used. Though a high sensitivity and stability are realized at this electrode, further measurements and optimization are required to address the interferences, especially during the anodic detection of $O_2^{\cdot-}$ to make this composite fiber electrode a complete practical antioxidant assessment device.

Conclusions

A facile methodology for the fabrication of an electrospun core-shell composite functional nanofibrous bio-electrode is presented. The nanofibrous electrode provides a biocompatible platform for the immobilization of the enzyme, SOD. Direct electron transfer for SOD was witnessed at the bio-electrode without any mediators or promoters. The SOD immobilized at the electrode retains its bi-catalytic property and offers bi-functional enzymatic catalytic activity for the detection of $O_2^{\cdot-}$ in PBS. The PMMA/PANI-Au_{nan}/SOD-ESCFM responded rapidly to $O_2^{\cdot-}$, generated *in situ* in PBS and

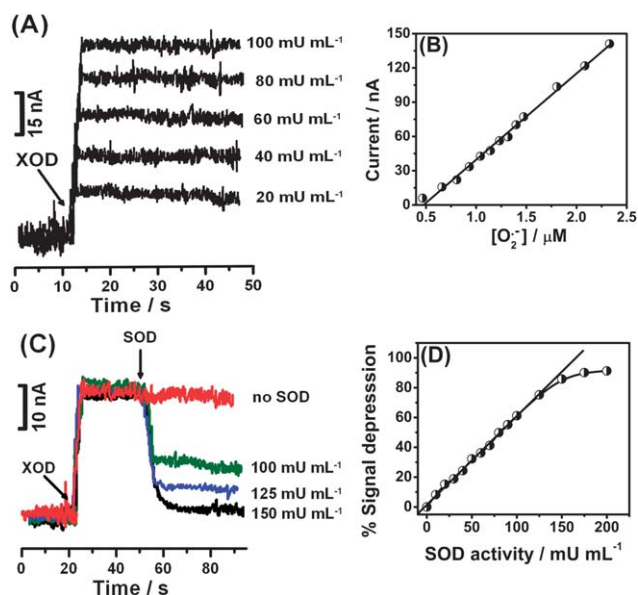


Fig. 3 (A) Amperograms obtained at PMMA/PANI-Au_{nan}/SOD-ESCFM in PBS (saturated with oxygen) containing $200\ \mu M$ xanthine upon addition of different concentrations of XOD; $E = +300$ mV (vs. Ag/AgCl). (B) Calibration curve; the concentration of $O_2^{\cdot-}$ was estimated theoretically using $[O_2^{\cdot-}] = (k_1/2k_2)^{1/2}[XOD]^{1/2}$, where k_1 and k_2 are the rate constants for the XOD-catalyzed xanthine oxidation ($k_1 = 1\ s^{-1}$) and the disproportionation of $O_2^{\cdot-}$ ($k_2 = 2.3 \times 10^5\ M^{-1}\ s^{-1}$), respectively,²⁴ assuming that $O_2^{\cdot-}$ was generated under steady-state conditions. (C) Amperometric response of PMMA/PANI-Au_{nan}/SOD-ESCFM at $E = +300$ mV (vs. Ag/AgCl). The first arrow indicates the start of $O_2^{\cdot-}$ generation by addition of $50\ mU\ mL^{-1}$ XOD in PBS containing $100\ \mu M$ xanthine. The second arrow indicates the removal of $O_2^{\cdot-}$ from the same solution by the addition of SOD. (D) The plot of SOD activity vs. % signal depression.

highly sensitive steady-state currents were observed when the electrode was polarized at -0.50 mV and $+300$ mV. Further, PMMA/PANI-Au_{nano}/SOD-ESCFM has demonstrated good analytical properties, such as low detection limit ($0.3\text{ }\mu\text{M}$ vs. XOD: 5 mU mL^{-1} and xanthine: $200\text{ }\mu\text{M}$), shorter response time (4 s), good stability and reproducibility. This novel composite nanofibrous electrode holds great promise for real-time *in vivo* detection of $\text{O}_2^{\cdot-}$. Moreover, this study offers ample scope to construct the third generation biosensors for the detection of various analytes.

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