



A novel carboxymethylcellulose–gelatin–titanium dioxide–superoxide dismutase biosensor; electrochemical properties of carboxymethylcellulose–gelatin–titanium dioxide–superoxide dismutase

Emel Emregül*, Ozge Kocabay, Burak Derkus, Tugrul Yumak, Kaan Cebesoy Emregül, Ali Sinag, Kamran Polat

Ankara University, Science Faculty, Department of Chemistry, Tandoğan, Ankara, 06100, Turkey

ARTICLE INFO

Article history:

Received 4 July 2012

Received in revised form 3 September 2012

Accepted 17 September 2012

Available online 3 October 2012

Keywords:

Carboxymethylcellulose–gelatin

TiO₂ nanoparticles

Superoxide dismutase

Impedance spectroscopy

Biosensor

ABSTRACT

A novel highly sensitive electrochemical carboxymethylcellulose–gelatin–TiO₂–superoxide dismutase biosensor for the determination of O₂^{•−} was developed. The biosensor exhibits high analytical performance with a wide linear range (1.5 nM to 2 mM), low detection limit (1.5 nM), high sensitivity and low response time (1.8 s). The electron transfer of superoxide dismutase was first accomplished at the carboxymethylcellulose–gelatin–Pt and carboxymethylcellulose–gelatin–TiO₂–Pt surface. The electron transfer between superoxide dismutase and the carboxymethylcellulose–gelatin–Pt without Fe(CN)₆^{4−/3−} and carboxymethylcellulose–gelatin–Pt, carboxymethylcellulose–gelatin–TiO₂–Pt with Fe(CN)₆^{4−/3−} is quasireversible with a formal potential of 200 mV, 207 mV, and 200 mV vs Ag/AgCl respectively. The anodic (*k*_s_a) and cathodic (*k*_s_c) electron transfer rate constants and the anodic (α_a) and cathodic (α_c) transfer coefficients were evaluated: *k*_s_a = 6.15 s^{−1}, α_a = 0.79, and *k*_s_c = 1.48 s^{−1}, α_c = 0.19 for carboxymethylcellulose–superoxide dismutase without Fe(CN)₆^{4−/3−}, *k*_s_a = 6.77 s^{−1}, α_a = 0.87, and *k*_s_c = 1 s^{−1}, α_c = 0.13 for carboxymethylcellulose–superoxide dismutase with Fe(CN)₆^{4−/3−}, *k*_s_a = 6.85 s^{−1}, α_a = 0.88, and *k*_s_c = 0.76 s^{−1}, α_c = 0.1 carboxymethylcellulose–gelatin–TiO₂–superoxide dismutase. The electron transfer rate between superoxide dismutase and the Pt electrode is remarkably enhanced due to immobilizing superoxide dismutase in carboxymethylcellulose–gelatin and TiO₂ nanoparticles tend to act like nanoscale electrodes.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Superoxide dismutases (SOD) which play an important role in cell protection mechanisms against oxidative damage from reactive oxygen species are ubiquitous metalloenzymes in oxygen-tolerant organisms [1–3]. Oxygen radicals has attracted considerable attention due to their harmful interaction with biological molecules and their involvement in signaling pathways. Superoxide radical (O₂^{•−}), the primary species of the reactive oxygen species (ROS), plays a central role in physiological processes [4–6]. Under normal metabolic conditions, O₂^{•−} is produced at a rate that is matched by the capacity of tissue to catabolize them [7]. When its production exceeds the body's natural ability to deal with the potentially cytotoxic species, a variety of pathological conditions may result including cancer, stroke, and neurodegeneration [8].

In plants, O₂^{•−} is commonly produced in illuminated chloroplasts by the occasional transfer of an electron from an excited Chl molecule or PSI components under conditions of high NADPH/NADP ratios to molecular O₂ [9]. Various environmental perturbations, such as hyperoxia, herbicides, pathogens, ozone, temperature fluctuations, and other stresses are known to induce O₂^{•−} formation in most aerobic

organisms [9]. In order to understand the role of O₂^{•−} in pathology and physiology and the relationship between O₂^{•−} and environmental stresses, it is essential to determine O₂^{•−} in a variety of *in vitro* and *in vivo* models. Therefore, the quantitative determination of O₂^{•−} concentrations and the beneficial effects of antioxidant compounds is of great interest to the medical community. Due to its low concentration, high reactivity, and short lifetime, it is still an analytical challenge to detect the local concentration of O₂^{•−}, especially in the biological systems. Determination of free radicals is usually carried out with spectrometry, fluorometry, chemiluminescence, and electron spin resonance [10–18]. Electron transfer reactions from redox enzymes, to electrodes have been widely studied, due to their significant assignments in physiological reactions, biotechnology and also in the development of biosensors and bioelectronic devices [19–23]. Protein electrochemistry has been shown to allow real-time, on-line quantification of radical concentrations. Recent attempts have concentrated on electrochemical methods due to their direct, real-time measurements and capability for *in vivo* detection [24–27]. Mostly, copper, zinc-superoxide dismutase (Cu, Zn-SOD)-immobilized electrodes have paved an elegant way to detect O₂^{•−}. SODs catalyze the dismutation of O₂^{•−} to O₂ and H₂O₂ via a cyclic oxidation–reduction electron-transfer mechanism and are widely distributed among aerobic organisms and show high rate constants, up to the order of

* Corresponding author.

E-mail address: eemregul@yahoo.com (E. Emregül).

$10^9 \text{ M}^{-1} \text{ s}^{-1}$. They are distinguished by a highly uncommon specificity to $\text{O}_2^{\cdot-}$ [28,29]. Therefore, SOD, a specific enzyme for $\text{O}_2^{\cdot-}$ dismutation, offers great potential for the sensitive and selective quantification of $\text{O}_2^{\cdot-}$ in the development of biosensors. Various studies related to direct electron transfer of Cu, Zn-SOD at electrode surface have been performed [30–42].

Electrochemical impedance spectroscopy (EIS) is a powerful, non-destructive and informative technique, which can be used to study the electrical properties of almost any sensing device interface [43]. In recent years, the potential application of this technique to the immunosensors for a broad set of applications has been examined [44–47]. EIS is capable of investigating electrode processes and determining diffusion kinetics as well as mass-transport parameters [48]. The electrochemical complex impedance (Z) can be represented as a sum of the real (Z_{re}) and imaginary (Z_{im}) components that originate generally from the resistance and capacitance of an electrolytic cell, respectively. Bode plot and Nyquist plot are the commonly used two types of impedance graphs. While semi-circle part of the nyquist plot gives information about capacitance and resistance, linear part shows diffusive effects.

In recent years, nanomaterials have attracted extensive interest for their unique properties in various fields (such as catalytic, electronic, and magnetic properties) in comparison with their bulk counterparts. Oxide nanoparticles are often used to immobilize biomolecules due to their biocompatibility, while semiconductor nanoparticles are often used as labels or tracers for electrochemical analysis [49]. TiO_2 nanoparticles, the best known semiconductor with its 3.2 eV band gap energy, were used for several electrochemical applications [50–52]. Due to its good biocompatibility, high conductivity and low cost, TiO_2 in various forms such as nanoparticles, nanoneedles and nanotubes, has become an attractive electrode material for electrochemical sensors and biosensors applications [52–54].

Previously highly sensitive SOD biosensor for the simultaneous determination of $\text{O}_2^{\cdot-}$ was developed by immobilization of superoxide dismutase using glutaraldehyde within carboxymethylcellulose–gelatin on a Pt electrode surface by our self [55]. The parameters affecting the performance of the biosensor were investigated in detail. The response of the previously developed CMC–G–SOD biosensor was proportional to $\text{O}_2^{\cdot-}$ concentration and the detection limit was $1.25 \times 10^{-3} \text{ mM}$ at a signal-to-noise ratio of 3 with 2 s response time. The objective of this study was the development of a novel highly sensitive electrochemical CMC–G– TiO_2 –SOD biosensor for the determination of $\text{O}_2^{\cdot-}$ by immobilization of SOD within a CMC–G– TiO_2 on a Pt electrode surface. The other aim of this study was to determine the electrochemistry of immobilized SOD within CMC–G using and without using $\text{Fe}(\text{CN})_6^{4-/3-}$ and CMC–G– TiO_2 biosensor. Electrochemical properties of CMC–G–SOD Pt and CMC–G– TiO_2 –SOD electrode were determined using cyclic voltammetry (CV) and EIS. The surface morphologies of the CMC–G, CMC–G–SOD, CMC–G– TiO_2 , CMC–G– TiO_2 –SOD electrode surfaces were investigated with SEM.

2. Materials and methods

2.1. Materials

Superoxide dismutase (EC. 1.15.1.1, 4733 U mg^{-1} , from bovine erythrocytes, xanthine oxidase (XOD; EC 1.1.3.22, 0.3 U mg^{-1} , from milk), xanthine (2,6-dihydroxypurine) sodium salt, gelatin, carboxymethylcellulose and chemicals used for preparation of buffers and cross-linking agents were purchased from Sigma (St Louis, MO, USA). The chemicals titanium isopropoxide (TTIP) and isopropyl alcohol (IPA) were purchased from Sigma–Aldrich and used without further purification. Evonik Degussa P-25 TiO_2 nanoparticles were used to compare. All measurements were performed with a Gamry Framework Version 5.50.

2.2. Synthesis of TiO_2 nanoparticles

The hydrothermal synthesis of TiO_2 nanoparticles was performed in an autoclave. The autoclave has an internal volume of 75 mL and the temperature can be controlled by an external apparatus. The reactor was loaded with appropriate amount of TTIP and IPA, and the autoclave was put on a heater and temperature kept constantly at 150°C for 6 h. Then the autoclave cooled to room temperature. The product was washed by distilled water and ethanol and centrifuged. The obtained product was dried in an oven at 70°C for 4 h.

2.3. Electrolysis cell and electrodes

A three-electrode electrochemical cell comprising of a working, counter, and reference electrode was used in all experiments. The working (area 0.5 cm^2) and counter electrodes were platinum (Pt); their surfaces were polished using $0.05 \mu\text{m}$ alumina slurry. The reference electrode was a Ag/AgCl electrode, introduced into the cell via Luggin capillary.

2.4. Preparation of SOD biosensor

Polymer blends (CMC and gelatin) were prepared to obtain a final weight of 3.75 mg polymer in final solution by dissolving them in phosphate buffer (0.05 M, pH 7.4). Followed by addition of TiO_2 (0.375 mg) to the polymer solution superoxide dismutase (4733 U, 1 mg) and glutaraldehyde (0.005 M) were then added to the support system solution to obtain a final volume of $50 \mu\text{L}$ at 32°C . The platinum foil electrode was covered with a total of $50 \mu\text{L}$ immobilization gel, $25 \mu\text{L}$ on each side. The carboxymethylcellulose–gelatin– TiO_2 –superoxide dismutase electrode (CMC–G– TiO_2 –SOD) was first left at room temperature for 48 h to ensure a stable dry surface. Enzyme leakage tests were performed for all electrodes by washing with phosphate buffer (0.05 M, pH 7.4), three times, for 1 h each, at 25°C . Enzyme leakage tests were performed for all CMC–G– TiO_2 –SOD biosensors obtained. The process of the CMC–G– TiO_2 –SOD biosensor was shown in Scheme 1.

2.5. Principle of method

The biosensor used to determine the $\text{O}_2^{\cdot-}$ was obtained by coupling an amperometric electrode for hydrogen peroxide with the superoxide dismutase enzyme immobilized on G–CMC support system.

The capacity of the SOD biosensor was determined as follows: The $\text{O}_2^{\cdot-}$ is produced in aqueous solution by oxidation of xanthine to uric acid in the presence of xanthine oxidase:



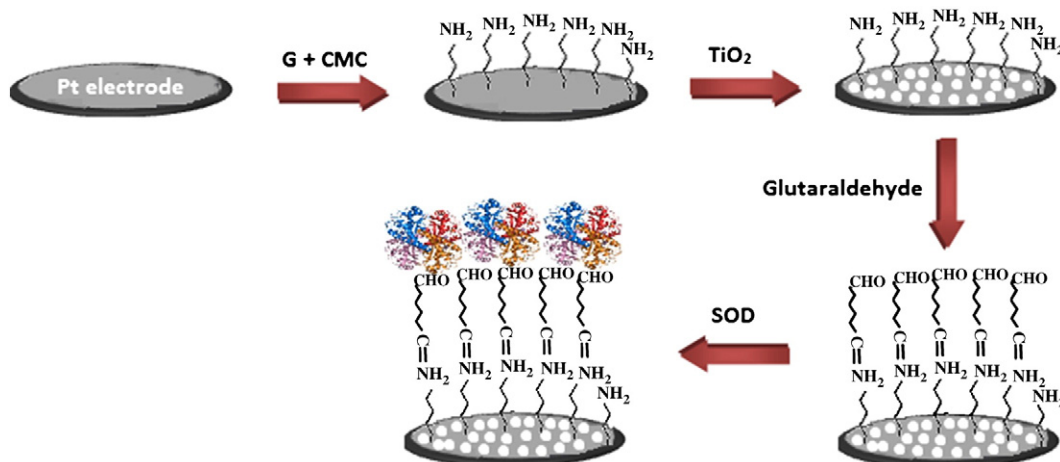
Superoxide dismutase immobilized on the Pt electrode catalyzes the dismutation reaction of the $\text{O}_2^{\cdot-}$ with release of oxygen and hydrogen peroxide according to Eq.



The H_2O_2 can be detected at the electrode surface in accordance with Eq.

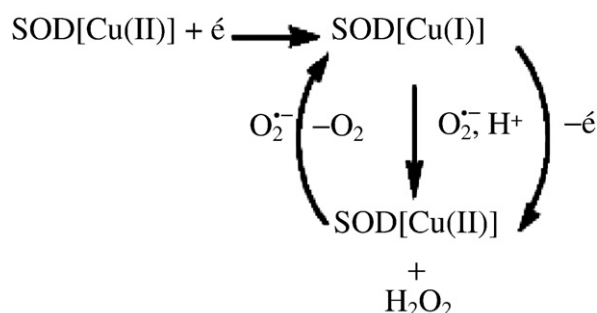


The current generated by oxidation of hydrogen peroxide at the working electrode held at 650 mV relative to the Ag/AgCl electrode is proportional to the concentration of $\text{O}_2^{\cdot-}$ in solution.



Scheme 1. The process of the CMC-G-TiO₂-SOD biosensor.

The reaction scheme on the electrode surface is as follows:



2.6. Measurement of procedure

All electrochemical measurements were performed in a standard three-electrode thermostated Pyrex cell containing 15 mL 0.05 M phosphate buffer, pH 7.4, at 25 °C. The working electrode was placed in the cell and allowed to stabilize with a constant stirring rate of 1000 rpm. After addition of the XOD enzyme (0.7 U), different concentrations of xanthine (1.25×10^{-3} –2 mM) were added to the solution. The EIS measurements were recorded within the frequency range of 0.01 Hz to 100 kHz at 220 mV open circuit potential (E_{oc}). All the experiments were performed at least three times to ensure reproducibility.

2.7. Test on healthy and diseased tissue

The biosensor response was investigated on healthy and cancerous brain tissue, meningioma (grade I, WHO 2000). Healthy or cancerous brain tissue (0.5 g) was homogenized in distilled water (3 mL) using a Bandalin homogenizer. The biosensor was left to stabilize in a cell containing phosphate buffer (0.05 M, pH 7.4) with magnetic stirring, until the response became constant. A solution of homogenized healthy or cancerous brain tissue (500 μ L) was added and the biosensor response was recorded. The tissues were stored at -20 °C before use.

3. Results and discussion

The development of highly sensitive superoxide dismutase biosensor for the simultaneous determination of superoxide radicals by immobilization of superoxide dismutase within carboxymethylcellulose–gelatin on a Pt electrode surface has previously been accomplished [55]. The

parameters affecting the performance of the biosensor were investigated in detail. The response of the CMC-G-SOD biosensor was proportional to $\text{O}_2^{\cdot-}$ concentration and the detection limit was 1.25×10^{-3} mM with a correlation coefficient of 0.9994 at a signal-to-noise ratio of 3. The response time of the developed biosensor was 2 s [55]. In this study a new CMC-G-TiO₂-SOD was developed and the electrochemistry of immobilized SOD within CMC-G and CMC-G-TiO₂ biosensor was studied. The parameters affecting the performance of the CMC-G-TiO₂-SOD biosensor were investigated in detail using CV and EIS. The surface morphologies of the CMC-G, CMC-G-SOD, CMC-G-TiO₂, CMC-G-TiO₂-SOD electrode surface were investigated with SEM.

3.1. SEM characterizations of the biosensor

The surface morphologies of the CMC-G, CMC-G-SOD, CMC-G-TiO₂, CMC-G-SOD-TiO₂ films were investigated with SEM and the images are shown in Fig. 1. The surface morphological images of CMC-G show a typical highly microporous structure on the Pt electrode surface (Fig. 1A). The image obtained from the CMC-G-SOD shows that SOD are distributed onto the microporous structure of CMC-G (Fig. 1B). When SOD is assembled onto the films of CMC-G polymer, the microporous structure of the CMC-G is seen blur, indicating that SOD biomolecules have been immobilized successfully on the surface of electrodes. The surface area was increased after the seeding of TiO₂ nanoparticles to the polymer (Fig. 1C). The enhancement of surface area with TiO₂ nanoparticles increased the SOD immobilization (Fig. 1D).

3.2. Optimization of CMC-G-TiO₂-SOD biosensor

Different parameters affecting the biosensor performance namely CMC-G ratios (0.05–0.25 w/w), glutaraldehyde concentration (0.0025–0.01 M), TiO₂ nanoparticle (0.01–0.5 mg) and SOD concentration (2400–9500 U) during the fabrication process of the biosensor was investigated. The optimum values obtained for CMC-G ratio, glutaraldehyde concentration, TiO₂ nanoparticle, SOD concentration were 0.1 w/w, 0.005 mM, 0.375 mg, and 4733 U respectively. The optimization experiments for each parameter were performed using both EIS and CV.

3.2.1. Cyclic voltammetry of CMC-G-SOD, CMC-G-TiO₂-SOD, CMC-G-Degussa-SOD biosensors

CMC-G-SOD electrode was used to investigate the electron transfer between SOD and electrode surface. The CVs of CMC-G-Pt electrodes in the presence and absence of SOD, and CMC-G-SOD biosensor in 0.05 M PBS (pH 7.4) in the applied potential range from -0.5 to $+0.5$ V at a scan rate of 50 mV s^{-1} are shown in Fig. 2A. The electron transfer to

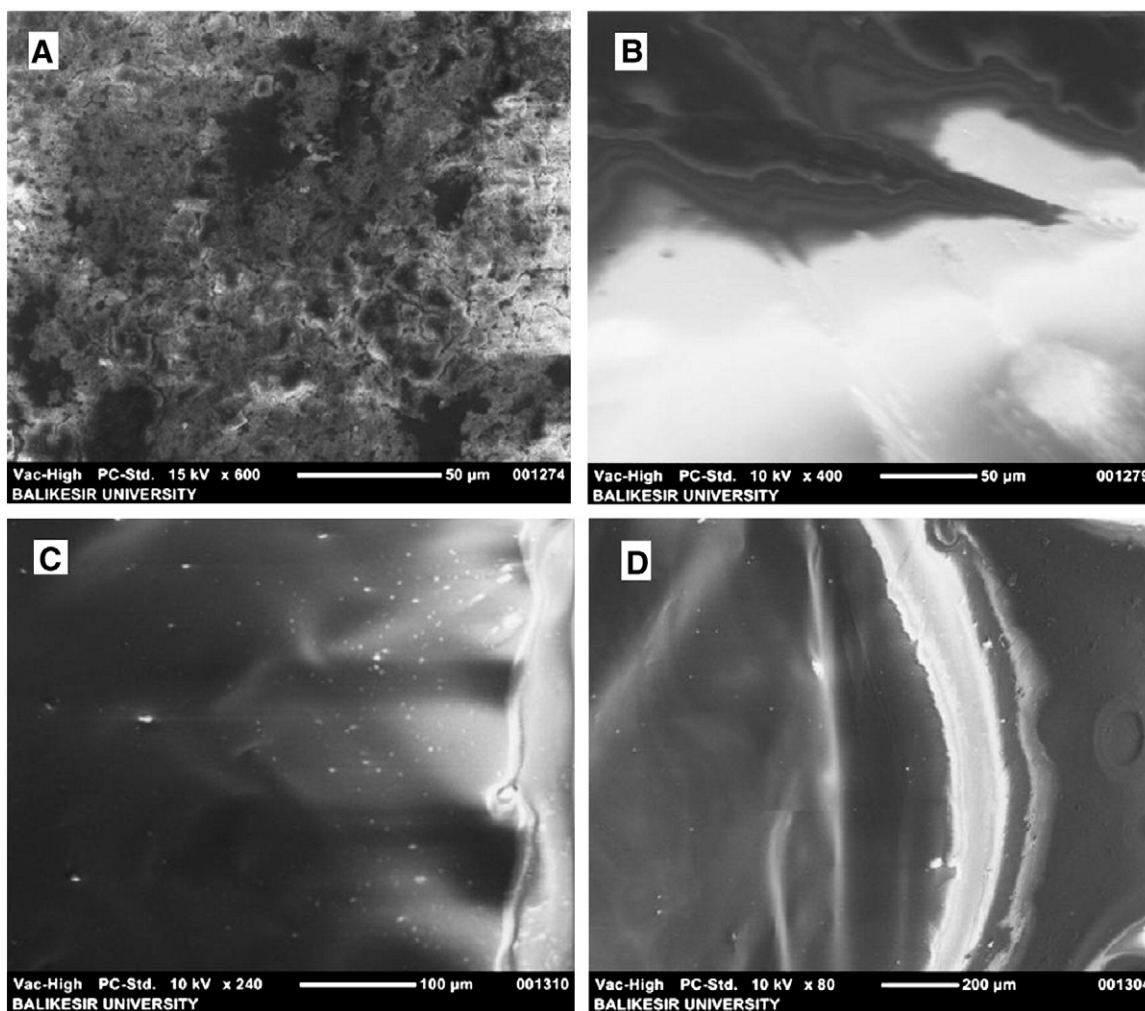


Fig. 1. SEM images of CMC-G (A); CMC-G-SOD (B); CMC-G-TiO₂ (C); CMC-G-TiO₂-SOD biosensor (D) at 10 kV.

SOD on the G-CMC film was performed without any mediators. The current density values of the CMC-G-Pt electrode was seen to slightly increase in the presence of SOD compared with relation to absence of SOD (Fig. 2A, b and c). In addition, xanthine oxidase alone did not cause any electrochemical response at the Pt electrode and no redox peaks were obtained at the bare platinum electrode, only small charge currents were observed at the platinum surfaces in PB (0.05 M, pH 7.4). An obvious increase in reduction and oxidation peaks which can be ascribed to the electron transfer of the SOD was observed with the CMC-G-SOD electrode (Fig. 2Aa). The result indicates that the SOD is effectively immobilized on the CMC-G and provides the necessary conduction pathways. The results suggest that the electron transfer of the SOD can be well-promoted on the G-CMC Pt electrode and the SOD-based biosensors show a bifunctional electrocatalytic activity toward O₂²⁻.

The formal potentials ($E^0 = (E_{p,a} + E_{p,c})/2$) of SOD where $E_{p,a}$ and $E_{p,c}$ are the anodic and cathodic peak potentials respectively, confined on G-CMC Pt surfaces are estimated to be 200 mV vs Ag/AgCl. These values are mostly within a range from 0.04 to 0.403 V vs normal hydrogen electrode (NHE) reported for the SODs in the literature [36–38,40,42,56,57]. The peak separation (ΔE_p , defined as $\Delta E_p = E_{p,a} - E_{p,c}$) of SOD (400 mV at 100 mV s⁻¹) and the asymmetric anodic and cathodic peak currents indicate that the electron transfer between SOD and the G-CMC Pt is quasireversible.

Cyclic voltammetry of electroactive species, Fe(CN)₆^{4-/3-}, is an available tool for evaluating the kinetic barrier of the interface [58,59]. Fig. 2B shows the CV graphs of Fe(CN)₆^{4-/3-} at CMC-G-SOD, and

CMC-G-TiO₂-SOD, electrodes in 0.05 M pH 7.4 PB and CMC-G-SOD electrode, CMC-G-TiO₂-SOD electrode in 0.05 M pH 7.4 PB containing xanthine (0.5 mM) and XOD (0.7 U). The formal potential of SOD confined on CMC-G-SOD, and CMC-G-TiO₂-SOD surfaces are estimated to be 207 mV and 200 mV vs Ag/AgCl. The peak separation of SOD (221.4 mV for CMC-G-TiO₂-SOD and 293 mV for CMC-G-SOD at 100 mV s⁻¹) and the asymmetric anodic and cathodic peak currents indicate that the electron transfer between SOD and CMC-G-TiO₂ is quasireversible [26,36,37]. After modifying the electrode with CMC-G, the peak belongs to Fe(CN)₆^{4-/3-} redox prob disappears. CMC-G film layer acts as the inert electron transfer blocking layer, and thus prevents the diffusion of Fe(CN)₆^{4-/3-} to the electrode surface. On the contrary, when TiO₂ nanoparticles were seeded to the electrode surface, the voltammetric peak of Fe(CN)₆^{4-/3-} was increased. This is originated from high surface area of nanoparticles which increases and promotes the electron transfer rate [38,60]. The nanoparticles tend to act like nanoscale electrodes thus promoting the electron transfer between the analyte and the electrode surface. Immobilization of SOD decreased the current response. The results indicated that the immobilized SOD in CMC-G-TiO₂ could greatly promote the electron transfer between SOD and the electrode.

Evonik Degussa P-25 TiO₂ nanoparticles were used as comparison material in the development of biosensor to make a comparison with synthesized TiO₂. For this purpose CMC-G-Degussa-SOD electrode was developed and used to investigate the electron transfer between SOD and electrode surface. Fig. 2C shows the CV graphs of Fe(CN)₆^{4-/3-} at CMC-G-SOD, CMC-G-Degussa-SOD electrodes in 0.05 M pH 7.4 PB

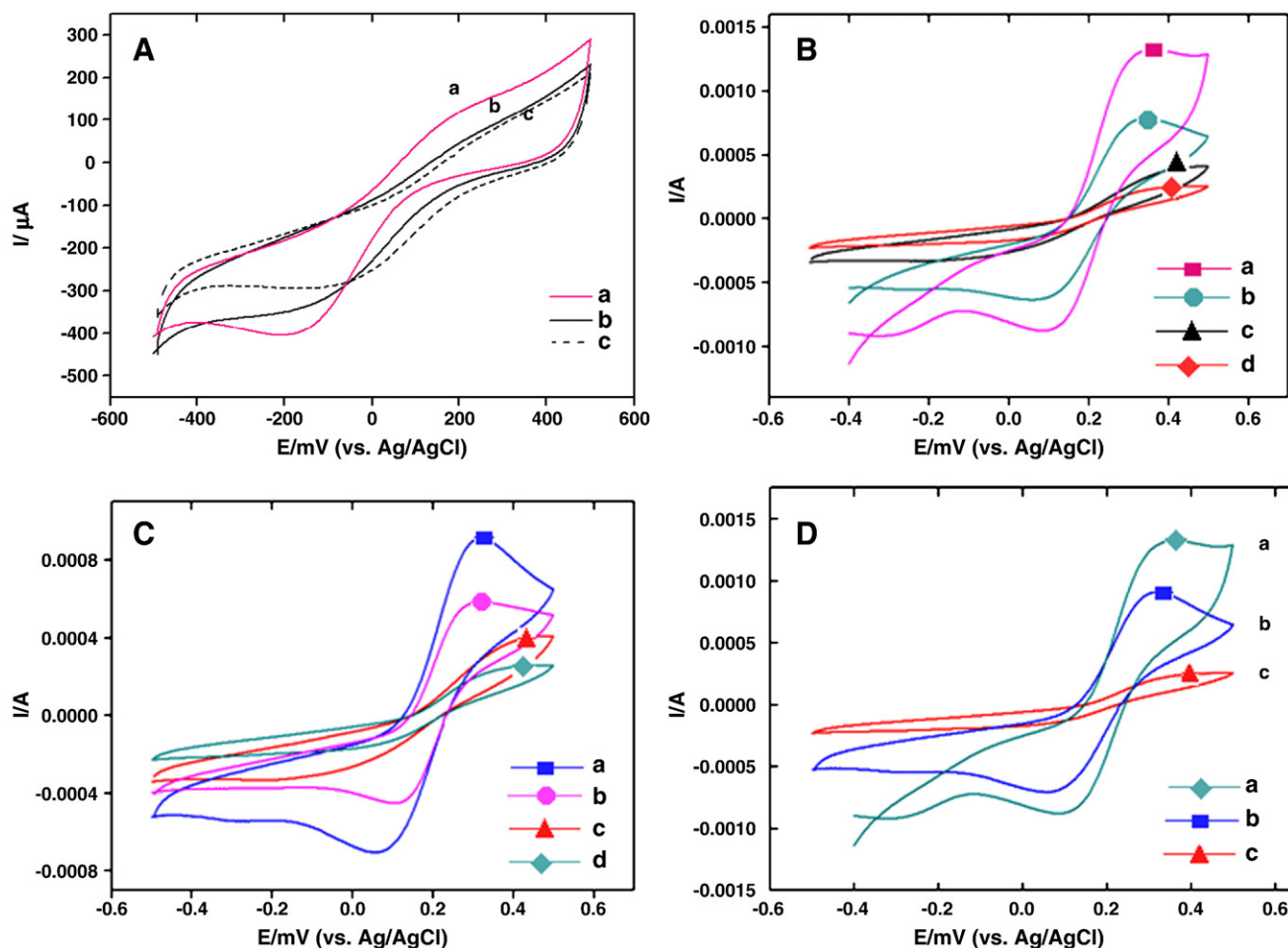


Fig. 2. (A) Cyclic voltammograms of CMC-G-SOD biosensor (a); CMC-G electrodes in the presence of SOD (b); and CMC-G-SOD in the absence of SOD (c) without mediator in 0.05 M pH 7.4 PB containing xanthine (0.5 mM) and XOD (0.7 U). Scan rate 100 mV s^{-1} . (B) Cyclic voltammograms of CMC-G-SOD (d); CMC-G-TiO₂-SOD electrodes (b); in 0.05 M pH 7.4 PB and CMC-G-SOD (c); CMC-G-TiO₂-SOD electrodes (a) in 0.05 M pH 7.4 PB containing xanthine (0.5 mM) and XOD (0.7 U). Solution composition: 0.1 M KCl, 5 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆], pH 7.4. Scan rates 100 mV s^{-1} . (C) Cyclic voltammograms of CMC-G-SOD (d); CMC-G-Degussa-SOD electrodes (b); in 0.05 M PB (pH 7.4) and CMC-G-SOD (c); CMC-G-Degussa-SOD electrodes (a) in 0.05 M pH 7.4 PB containing xanthine (0.5 mM) and XOD (0.7 U). Solution composition: 0.1 M KCl, 5 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆], pH 7.4. Scan rates 100 mV s^{-1} . (D) Comparison of cyclic voltammograms; CMC-G-TiO₂-SOD (a), CMC-G-Degussa-SOD (b), CMC-G-SOD (c) biosensors. Solution composition: 0.1 M KCl, 5 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆], pH 7.4. Scan rates 100 mV s^{-1} .

and CMC-G-SOD, CMC-G-Degussa-SOD electrodes in 0.05 M pH 7.4 PB containing xanthine (0.5 mM) and XOD (0.7 U). The formal potential confined on CMC-G-Degussa-SOD surface is estimated to be 210 mV vs Ag/AgCl, respectively. The peak separation of SOD (215 mV at 100 mV s^{-1}) and the asymmetric anodic and cathodic peak currents indicate that the electron transfer between SOD and CMC-G-Degussa is also quasireversible [26,36,37]. Degussa in CMC-G was also increased the voltammetric peak of $\text{Fe}(\text{CN})_6^{4-/3-}$ but far extent much lower than CMC-G-TiO₂-SOD.

Fig. 2D presents the the response comparison of CMC-G-SOD, CMC-G-Degussa-SOD, and CMC-G-TiO₂-SOD biosensors. The CMC-G-TiO₂-SOD biosensor was increased the current response 10.8 times compare to previously developed CMC-G-SOD biosensor without using $\text{Fe}(\text{CN})_6^{4-/3-}$. When the response of CMC-G-SOD, CMC-G-Degussa-SOD, and CMC-G-TiO₂-SOD biosensors compared the response of CMC-G-TiO₂-SOD biosensor was about a 2.1 and 3.6-fold more than CMC-G-Degussa-SOD, CMC-G-SOD biosensor with $\text{Fe}(\text{CN})_6^{4-/3-}$ respectively.

CVs of the CMC-G-SOD electrode without $\text{Fe}(\text{CN})_6^{4-/3-}$ and CMC-G-SOD electrode with $\text{Fe}(\text{CN})_6^{4-/3-}$ and CMC-G-TiO₂-SOD with $\text{Fe}(\text{CN})_6^{4-/3-}$ at different scan rates were also obtained (Fig. 3). The peak current was enhanced with the increasing of the scan rate. Both anodic and cathodic peak current was proportional to the scan rate from 20 to 1000 mV s^{-1} . Linear regression equations were

expressed for CMC-G-SOD electrode without $\text{Fe}(\text{CN})_6^{4-/3-}$ as $I_{pa}/\mu\text{A} = (20.18) + (1.17) v/\text{mV s}^{-1}$ with the slope of $(1.18) \mu\text{A (V s}^{-1})^{-1}$ for anodic peak and $I_{pc}/\mu\text{A} = -(218) - (1.72) v/\text{mV s}^{-1}$ with the slope of $-(1.72) \mu\text{A (V s}^{-1})^{-1}$ for cathodic peak, respectively. Linear regression equations were expressed for CMC-G-SOD with $\text{Fe}(\text{CN})_6^{4-/3-}$ as $I_{pa}/\mu\text{A} = (32.5) + (0.99) v/\text{mV s}^{-1}$ with the slope of $(0.99) \mu\text{A (V s}^{-1})^{-1}$ for anodic peak and $I_{pc}/\mu\text{A} = -(46.3) - (0.38) v/\text{mV s}^{-1}$ with the slope of $-(0.38) \mu\text{A (V s}^{-1})^{-1}$ for cathodic peak, respectively. Linear regression equations were expressed for CMC-G-TiO₂-SOD as $I_{pa}/\mu\text{A} = (673) + (6.03) v/\text{mV s}^{-1}$ with the slope of $(6.03) \mu\text{A (V s}^{-1})^{-1}$ for anodic peak and $I_{pc}/\mu\text{A} = -(449) - (3.6) v/\text{mV s}^{-1}$ with the slope of $-(3.6) \mu\text{A (V s}^{-1})^{-1}$ for cathodic peak, respectively. The result for all electrodes indicated that the electrode reaction involved with a surface-controlled quasi-reversible electrochemical process.

The electron-transfer coefficient (α_s) and electron-transfer rate constant (k_s) could be determined based on Laviron theory [61]:

$$E_{pc} = E'^o + \frac{RT}{\alpha_s n F} - \frac{RT}{\alpha_s n F} \ln v \quad (4)$$

$$E_{pa} = E'^o + \frac{RT}{(1-\alpha_s) n F} - \frac{RT}{(1-\alpha_s) n F} \ln v \quad (5)$$

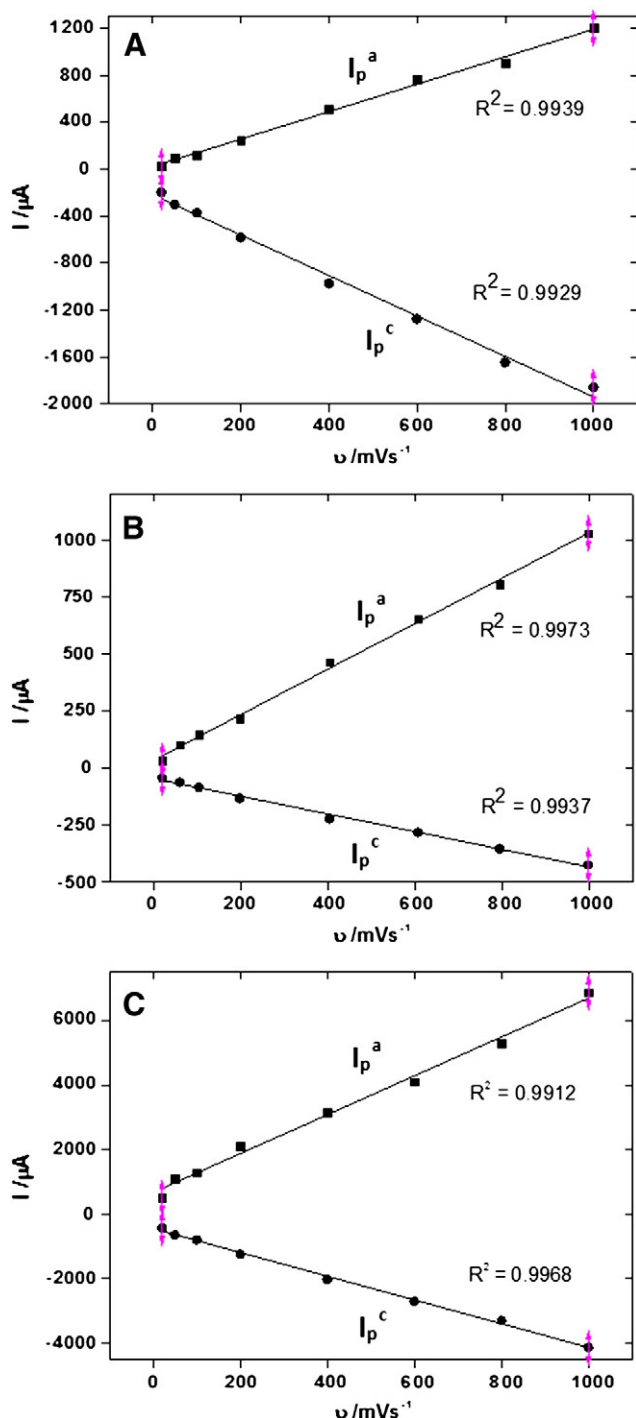


Fig. 3. Dependence of anodic peak currents and cathodic peak currents on scan rate ν for cyclic voltammograms obtained at (a) CMC-G-SOD without $\text{Fe}(\text{CN})_6^{4-/3-}$. Linear regression equations were expressed for CMC-G-SOD electrode without $\text{Fe}(\text{CN})_6^{4-/3-}$ as $I_{p^a}/\mu\text{A} = (20.18) + (1.17) \nu/\text{mV s}^{-1}$ with the slope of $(1.18) \mu\text{A} (\text{V s}^{-1})^{-1}$ for anodic peak and $I_{p^c}/\mu\text{A} = -(218) - (1.72) \nu/\text{mV s}^{-1}$ with the slope of $-(1.72) \mu\text{A} (\text{V s}^{-1})^{-1}$ for cathodic peak, respectively. (b) CMC-G-SOD with $\text{Fe}(\text{CN})_6^{4-/3-}$. Linear regression equations were expressed for CMC-G-SOD with $\text{Fe}(\text{CN})_6^{4-/3-}$ as $I_{p^a}/\mu\text{A} = (32.5) + (0.99) \nu/\text{mV s}^{-1}$ with the slope of $(0.99) \mu\text{A} (\text{V s}^{-1})^{-1}$ for anodic peak and $I_{p^c}/\mu\text{A} = -(46.3) - (0.38) \nu/\text{mV s}^{-1}$ with the slope of $-(0.38) \mu\text{A} (\text{V s}^{-1})^{-1}$ for cathodic peak, respectively. (c) CMC-G-TiO₂-SOD in 0.05 M pH 7.4 PB. Linear regression equations were expressed for CMC-G-TiO₂-SOD as $I_{p^a}/\mu\text{A} = (673) + (6.03) \nu/\text{mV s}^{-1}$ with the slope of $(6.03) \mu\text{A} (\text{V s}^{-1})^{-1}$ for anodic peak and $I_{p^c}/\mu\text{A} = -(449) - (3.6) \nu/\text{mV s}^{-1}$ with the slope of $-(3.6) \mu\text{A} (\text{V s}^{-1})^{-1}$ for cathodic peak, respectively.

where n is the electron transfer number, R is the gas constant ($R = 8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T is the temperature in Kelvin ($T = 298 \text{ K}$) and F is the Faraday constant ($F = 96493 \text{ C mol}^{-1}$). Fig. 4 shows the

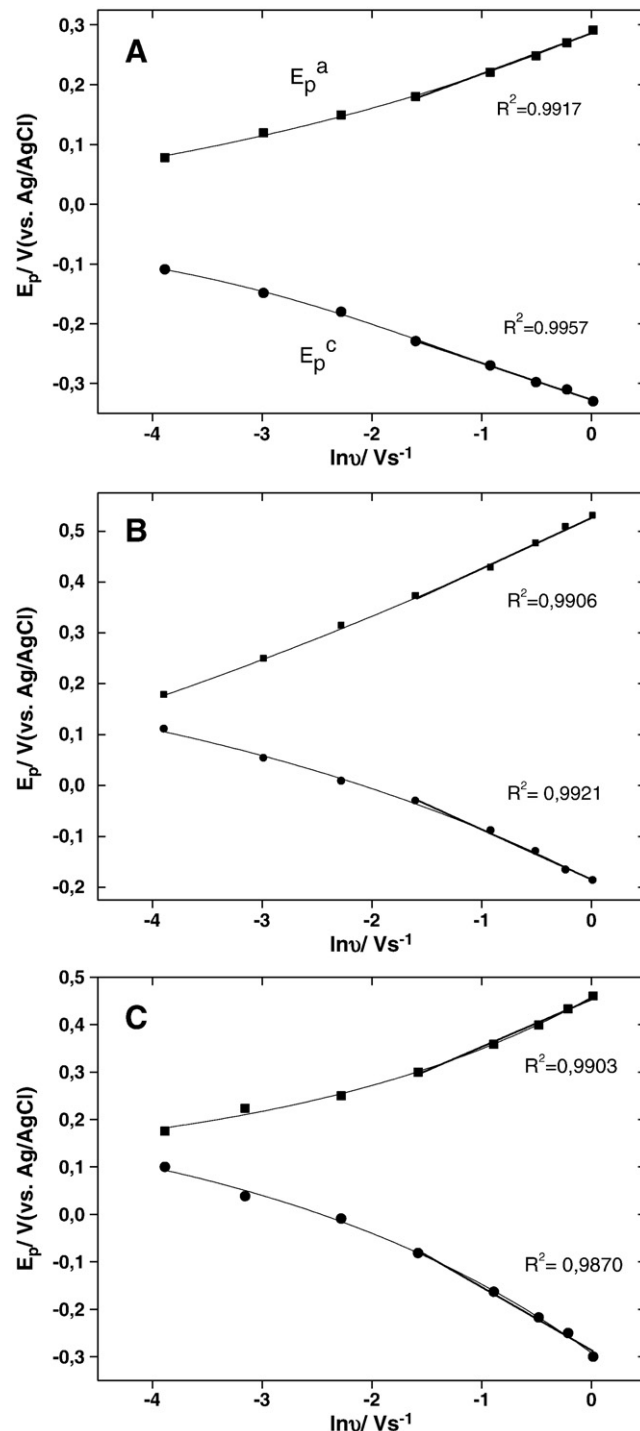


Fig. 4. Plots of the peak potential (E_{p^a} , E_{p^c}) versus the natural logarithm of scan rate ($\ln \nu$) for (a) CMC-G-SOD without $\text{Fe}(\text{CN})_6^{4-/3-}$. Linear regression equations for CMC-G-SOD without $\text{Fe}(\text{CN})_6^{4-/3-}$ were expressed as $E_{p^a}/\text{V} = (0.28 \pm 0.014) + (0.0679 \pm 0.003) \ln \nu/\text{V s}^{-1}$ with the slope of $(0.0679 \pm 0.003) \text{ V} (\text{V s}^{-1})^{-1}$ for anodic peak and $E_{p^c}/\text{V} = -(0.326 \pm 0.016) - (0.061 \pm 0.003) \ln \nu/\text{V s}^{-1}$ with the slope of $-(0.061 \pm 0.003) \text{ V} (\text{V s}^{-1})^{-1}$ for cathodic peak, (b) CMC-G-SOD with $\text{Fe}(\text{CN})_6^{4-/3-}$. Linear regression equations for CMC-G-SOD with $\text{Fe}(\text{CN})_6^{4-/3-}$ were expressed as $E_{p^a}/\text{V} = (0.525 \pm 0.026) + (0.099 \pm 0.005) \ln \nu/\text{V s}^{-1}$ with the slope of $(0.099 \pm 0.005) \text{ V} (\text{V s}^{-1})^{-1}$ for anodic peak and $E_{p^c}/\text{V} = -(0.124 \pm 0.0062) - (0.098 \pm 0.005) \ln \nu/\text{V s}^{-1}$ with the slope of $-(0.098 \pm 0.005) \text{ V} (\text{V s}^{-1})^{-1}$ for cathodic peak, (c) CMC-G-TiO₂-SOD in 0.05 M pH 7.4 PB, linear regression equations for CMC-G-TiO₂-SOD were expressed as $E_{p^a}/\text{V} = (0.4529 \pm 0.002) + (0.1 \pm 0.005) \ln \nu/\text{V s}^{-1}$ with the slope of $(0.1 \pm 0.005) \text{ V} (\text{V s}^{-1})^{-1}$ for anodic peak and $E_{p^c}/\text{V} = -(0.2856 \pm 0.014) - (0.1327 \pm 0.007) \ln \nu/\text{V s}^{-1}$ with the slope of $-(0.1327 \pm 0.007) \text{ V} (\text{V s}^{-1})^{-1}$ for cathodic peak respectively.

plots of the peak potential (E_p , E_{pc}) versus the natural logarithm of scan rate ($\ln v$) for CMC-G-SOD without $\text{Fe}(\text{CN})_6^{4-/3-}$ (a), CMC-G-SOD with $\text{Fe}(\text{CN})_6^{4-/3-}$ (b) CMC-G-TiO₂-SOD (c). The plots of E_p versus $\ln v$ for all electrodes yielded two straight lines with slopes of $RT/(1-\alpha)nF$ and $-RT/\alpha nF$ for the anodic and cathodic peak, respectively.

According to Laviron's procedure, from the potential scan rate dependence of the anodic and cathodic peak potentials, the relevant kinetic parameters of the electrodes were estimated. When $n\Delta E_p > 200$ mV, the electron transfer rate k_s could be estimated with the Laviron's equation [60]:

$$k_s = \frac{\alpha n F v}{RT} \quad (6)$$

Therefore, the anodic (k_{sa}) and cathodic (k_{sc}) electron transfer rate constants and the anodic (α_a) and cathodic (α_c) transfer coefficients were evaluated: $k_{sa} = 6.15 \pm 0.31 \text{ s}^{-1}$, $\alpha_a = 0.79$, and $k_{sc} = 1.48 \pm 0.07 \text{ s}^{-1}$, $\alpha_c = 0.19$ for CMC-G-SOD without $\text{Fe}(\text{CN})_6^{4-/3-}$, $k_{sa} = 6.77 \pm 0.34 \text{ s}^{-1}$, $\alpha_a = 0.87$, and $k_{sc} = 1 \pm 0.05 \text{ s}^{-1}$, $\alpha_c = 0.13$ for CMC-G-SOD with $\text{Fe}(\text{CN})_6^{4-/3-}$, $k_{sa} = 6.85 \pm 0.34 \text{ s}^{-1}$, $\alpha_a = 0.88$, and $k_{sc} = 0.76 \pm 0.04 \text{ s}^{-1}$, $\alpha_c = 0.1$ CMC-G-TiO₂-SOD. The electron transfer rate between SOD and the Pt electrode was remarkably enhanced by immobilizing SOD in CMC-G film on Pt electrode surface. The fast electron transfer of electrodes might be attributed to the protection of SOD conformation at the electrode surface and TiO₂ nanoparticles. When TiO₂ nanoparticles were seeded to the electrode surface, k_{sa} was increased. This is originated from high surface area of nanoparticles which increases and promotes the electron transfer rate [38,60]. The nanoparticles tend to act like nanoscale electrodes thus promoting the electron transfer between the analyte and the electrode surface. The support system enhanced the immobilization of SOD and promoted the electron transfer of SOD minimizing its fouling effect.

3.2.2. Electrochemical impedance spectroscopy

EIS is an effective technique for probing the features of surface modified electrodes. The impedance curve include a semicircle part and a linear part. The semicircle diameter at higher frequencies corresponds to the charge transfer resistance (R_{ct}), and the linear part at lower frequencies corresponds to the diffusion process.

Nyquist plot was obtained and used to determined relative change in surface-charge resistance. Interfacial R_{ct} in Nyquist plot of impedance was obtained from real (Z') and imaginary ($-Z''$) impedance at different frequencies using the following equations [62]. for a parallel RC circuit.

$$Z(\omega) = Z' + jZ'' = R_s + \frac{R_p}{(1 + j\omega C_d)} \quad (7)$$

$$Z' = R_s + \frac{R_p}{(1 + \omega^2 R_p^2 C_d^2)} \quad (8)$$

$$-Z'' = \frac{\omega R_p^2 C_d}{1 + \omega^2 R_p^2 C_d^2} \quad (9)$$

where, R_s was the electrolyte solution resistance and R_p was polarization resistance. R_p obtained at zero potential was described as surface-charge resistance (R_{ct}). The frequency associated with maximum Z'' and R_{ct} were used to calculate C_d using the following equation.

$$R_{ct} C_d = \frac{1}{2\pi f_{\max}} \quad (10)$$

Warburg resistance (Z_w) was also obtained in Nyquist plot and an equivalent circuit to describe the electrical response at electrode

labeled in Fig. 5. Warburg impedance was expressed by an intercept of straight line having a slope of unity and can be derived from the following equation.

$$Z_w(\omega) = W_{\text{int}} + \frac{R_p \lambda}{\sqrt{2\omega}} (1-j) \quad (11)$$

$$W_{\text{int}} = R_s + R_p - R_p^2 \omega^2 C_d \quad (12)$$

Where $\lambda = (k_f/\sqrt{D_0} + k_b/\sqrt{D_R})$, k_f and k_b were forward and backward electron-transfer rate constants, D_0 and D_R were the diffusion coefficient of oxidant and reductant.

The immobilization of SOD molecules on the CMC-G-Pt, CMC-G-TiO₂, CMC-G-Dequassa electrodes is further validated by the measurement of the open-circuit potential (E_{oc}) of CMC-G-SOD, CMC-G-TiO₂-SOD, CMC-G-Dequassa-SOD electrode in PB (0.05 M, pH 7.4). Fig. 5 shows the AC impedance spectra of $\text{Fe}(\text{CN})_6^{4-/3-}$ at CMC-G electrode, CMC-G-SOD electrode, CMC-G-TiO₂-SOD, CMC-G-Dequassa-SOD electrode obtained in 0.05 M pH 7.4 PB. Also AC impedance spectra of $\text{Fe}(\text{CN})_6^{4-/3-}$ at CMC-G-TiO₂-SOD, CMC-G-Dequassa-SOD electrode obtained in 0.05 M pH 7.4 PB containing xanthine (0.5 mM) and XOD (0.7 U). When the electrode is covered with CMC-G, R_{ct} of CMC-G electrode shows a slight increase compare to the bare Pt electrode. This implies that CMC-G are successfully assembled on the Pt surface and has some conducting properties. With the incorporation of nanoparticles onto the CMC-G Pt surface, R_{ct} decreases dramatically due to the large surface area and superior magnetic properties of TiO₂, accelerating the electron transfer rate [63,64]. The R_{ct} for CMC-G-TiO₂ electrode is lower than CMC-G-Dequassa electrode due to the large surface area of TiO₂ compared to Dequassa. Subsequently, when the SOD was immobilized on the surface of CMC-G-TiO₂ electrode, the EIS showed a large increase in circle diameter, suggesting that the SOD formed an external barrier, preventing any surface reaction. Therefore, from changes of R_{ct} , immobilization of SOD on the electrode surface was confirmed. Further increase in R_{ct} after addition of xanthine (0.5 mM) and XOD (0.7 U) indicates the formation of a reaction [37].

3.2.3. The dependence of formal potentials of CMC-G-SOD on pH

The dependence of formal potentials (E°) of SOD on solution pH for CMC-G-SOD and CMC-G-TiO₂-SOD is plotted in Fig. 6. As shown, the

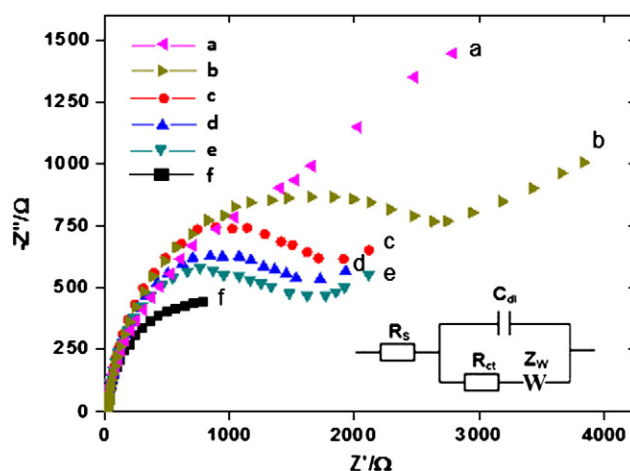


Fig. 5. Nyquist diagrams of: CMC-G-TiO₂-SOD (a) and CMC-G-Dequassa-SOD (b) in 0.05 M PB (pH 7.4) containing xanthine (0.5 mM) and XOD (0.7 U). CMC-G-SOD (c); CMC-G-Dequassa-SOD (d); CMC-G-TiO₂-SOD (e); CMC-G. (f) Solution composition: 0.1 M KCl, 5 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆], pH 7.4. The frequency range 0.01 Hz to 100 kHz at 220 mV open circuit potential, (E_{oc}), scan rates 100 mV s⁻¹.

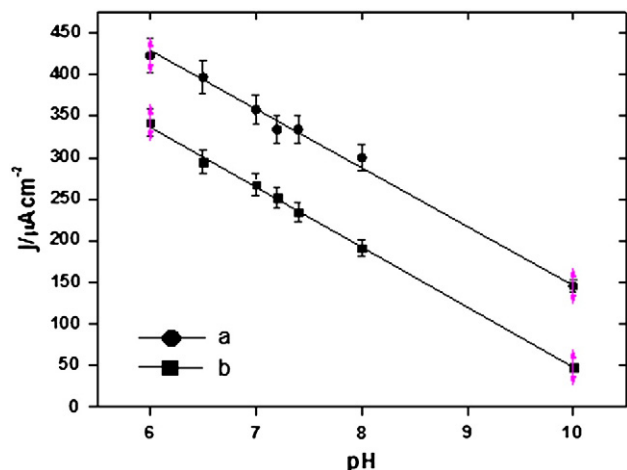


Fig. 6. Plots of the formal potentials of (a) CMC-G-SOD, (b) CMC-G-SOD-TiO₂ at Pt electrode vs solution pH.

formal potential of SOD decreases linearly with increasing solution pH. The slope of CMC-G-SOD and CMC-G-TiO₂-SOD was ~ -72.8 mV/pH and -69.5 mV/pH with correlation coefficient 0.998 and 0.993 respectively indicating one proton and one electron are involved in the electrode reaction of SOD, which is similar to the previously proposed scheme for the enzymatic catalytic mechanism of the Cu/Zn-SOD [40].

3.2.4. Determination of O₂^{•−} with CMC-G-TiO₂-SOD biosensor

The interaction of immobilized SOD with O₂^{•−} was evaluated using EIS in 5 mM Fe(CN)₆^{4−/3−} solution containing 0.1 M KCl (pH 7.4). The variation of the relative impedance, $R_{ct}(i) - R_{ct}(0)/R_{ct}(0)$, was used as response. $R_{ct}(0)$ is the charge transfer resistance when SOD is immobilized on the electrode surface while $R_{ct}(i)$ is the value of the charge transfer resistance after the dismutation reaction of the O₂^{•−} with release of oxygen and hydrogen peroxide catalyzed by SOD. Calibration curves of CMC-G-TiO₂-SOD biosensor with different xanthine concentrations: 0.976 μM–2 mM and 0.0015–0.48 μM are shown in Fig. 7A and B respectively.

The amperometric response of the CMC-G-TiO₂-SOD biosensor resulted from O₂^{•−} was investigated in the stirred buffer solution. A simple and efficient method was used for generation of O₂^{•−} by oxidation of xanthine to uric acid in the presence of xanthine oxidase at an applied potential of 650 mV. The measurement solution should be operated at a constant and high stirring speed (1000 rpm) to acquire reproducible results. A peak-like response was observed. If only a certain amount is added, a superoxide “burst” will occur which rapidly decreases to baseline level because of the dismutation reaction [39,54]. This suggests that the measurement system responded to actual concentration changes in solution. With ascending xanthine concentration, the amperometric response of the CMC-G-TiO₂-SOD biosensor increases. These results showed that the CMC-G-TiO₂-SOD biosensor has excellent responses to O₂^{•−}. Superoxide dismutase processes its biological activity after immobilized on platinum surfaces, which also enable the electron transfer of SOD itself. This formed a strong basis for the development of a SOD biosensor for O₂^{•−} because SOD catalyzes the dismutation of O₂^{•−} to O₂ and H₂O₂ via a cyclic oxidation–reduction electron transfer. The effect of xanthine concentration on the response time of the CMC-G-TiO₂-SOD biosensor was analyzed (data was not shown). The response time of the biosensor was 1.8 s for the lowest xanthine concentrations (0.0015–0.48 μM) and increased with increasing xanthine concentration. The detection limit was 1.5 nM. The developed biosensor exhibits high analytical performance with a wide linear concentration range and 1.8 s response time.

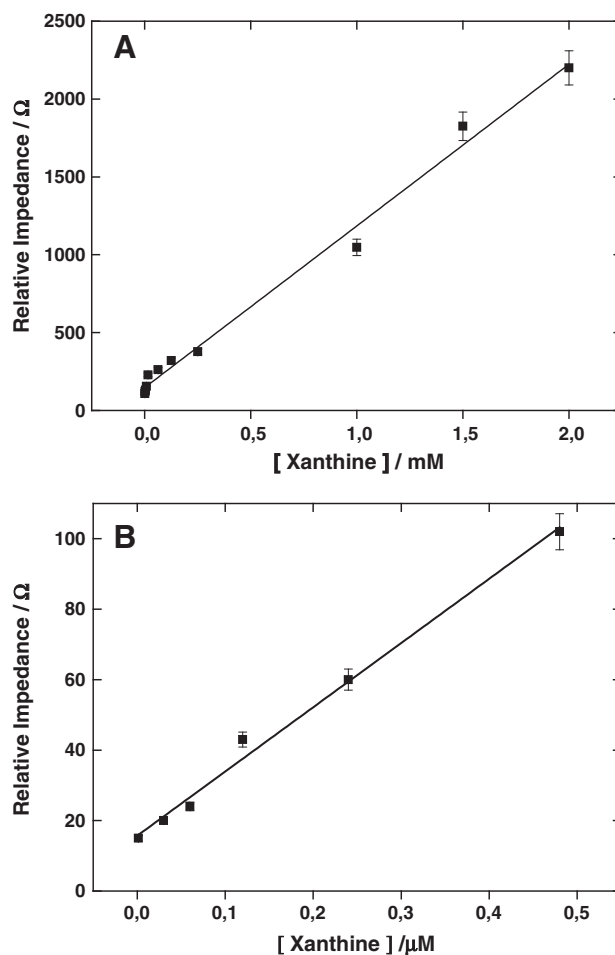


Fig. 7. (A) Calibration graph of the CMC-G-TiO₂-SOD biosensor for 0.976 μM–2 mM concentration range. (B) Calibration graph of the CMC-G-TiO₂-SOD biosensor for 0.0015–0.48 μM concentration range.

3.2.5. Test on healthy and diseased tissue

The biosensor response was tested on healthy and cancerous brain tissue. Different signals were obtained from the biosensor depending on whether the tissue was healthy or cancerous. The response was proportional to the concentration of superoxide radical present in the tissue. Cancerous brain tissue contains a large amount of superoxide radical compare with healthy tissue (Fig. 8). This is probably because

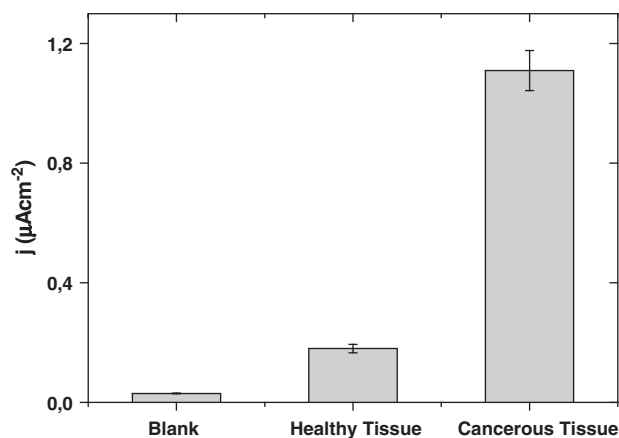


Fig. 8. Variation of the signal from CMC-G-TiO₂-SOD biosensor after addition of homogenized healthy and cancerous brain tissue to phosphate buffer (0.05 mM, pH 7.4). Data are average results from three biosensors.

a smaller quantity of scavengers or endogenous SOD is present in the cancerous tissue [39,54].

4. Conclusion

The development of highly sensitive electrochemical CMC–G–TiO₂–SOD biosensor for the determination of O₂^{•−} was performed by immobilization of SOD within a CMC–G–TiO₂ on a Pt electrode surface. The CMC–G support system provided a biocompatible microenvironment for SOD and a necessary pathway of electron transfer between SOD and the Pt surface. The electron transfer was greatly promoted after introducing TiO₂ to the modified electrode. TiO₂ nanoparticles tend to act like nanoscale electrodes. The response of the CMC–G–TiO₂–SOD biosensor was proportional to O₂^{•−} concentration with a correlation coefficient of 0.991 and the detection limit was 1.5 nM. The developed biosensor exhibits high analytical performance with a wide linear range (1.5 nM to 2 mM), high sensitivity and fast response time (1.8 s). This approach would provide a new strategy for further study on the development of biosensors.

Acknowledgements

This work financially supported by the Scientific and Technological Research Council of Turkey (no. 108T131).

References

- [1] G. Rotilio, R.C. Bray, E.M. Fielden, A pulse radiolysis study of superoxide dismutase, *Biochim. Biophys. Acta* 268 (1972) 605.
- [2] D. Klug, J. Rabani, I. Fridovich, A direct demonstration of the catalytic action of superoxide dismutase through the use of pulse radiolysis, *J. Biol. Chem.* 247 (15) (1972) 4839.
- [3] G.D. Lawrence, D.T. Sawyer, Potentiometric titrations and oxidation-reduction potentials of manganese and copper-zinc superoxide dismutases, *Biochemistry* 18 (14) (1979) 3045.
- [4] F. Auchere, F. Rusnak, Superoxide scavenging by neelaredoxin: dismutation and reduction activities in anaerobes, *J. Biol. Inorg. Chem.* 7 (2002) 667.
- [5] R.T. Dean, S. Fu, R. Stocker, M.J. Davies, Biochemistry and pathology of radical-mediated protein oxidation, *Biochem. J.* 324 (1997) 18.
- [6] B. Halliwell, J.M.C. Gutteridge, Oxygen toxicity, oxygen radicals, transition metals and disease, *Biochem. J.* 219 (1984) 14.
- [7] F. Van Lente, Free radicals, *Anal. Chem.* 65 (1993) 377R.
- [8] B. Halliwell, J.M.C. Gutteridge, Biological origin of free radicals, and mechanisms of antioxidant protection, *Arch. Biochem. Biophys.* 246 (1986) 514.
- [9] J.G. Scandalios, Oxygen stress and superoxide dismutases, *Plant Physiol.* 101 (1993).
- [10] F.A. Carey, R.J. Sundberg, *Advanced Organic Chemistry*, second ed. Plenum, New York, 1984.
- [11] K. Prasad, J. Kalra, B. Bhardwaj, Increased chemiluminescence of polymorphonuclear leucocytes in dogs with volume overload heart failure, *J. Exp. Pathol.* 70 (1989) 463.
- [12] B. Halliwell, J.M.C. Gutteridge, Formation of thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals, *FEBS Lett.* 128 (1981) 347.
- [13] I. Fridovich, Superoxide dismutase biosensors for superoxide radical analysis, *J. Biol. Chem.* 245 (1970) 4035.
- [14] J.L. Zweier, J.H. Flaherty, M.L. Weisfeldt, Direct measurement of free radical generation following reperfusion of ischemic myocardium, *Proc. Natl. Acad. Sci.* 84 (1987) 1047.
- [15] Y. Ohara, T.E. Peterson, D.G.J. Harrison, Hypercholesterolemia increases endothelial superoxide anion production, *Clin. Invest.* 91 (1993) 2551.
- [16] J. Vasquez-Vivar, N. Hogg, K.A. Pritchard, B. Kalyanaram, Superoxide anion formation from lucigenin: an electron spin resonance spin-trapping study, *FEBS Lett.* 403 (1997) 130.
- [17] H. Zhang, J. Joseph, J. Vasquez-Vivar, H. Karoui, C. Nsanzumuhire, P. Martasek, P. Tordo, B. Kalyanaram, Detection of superoxide anion using an isotopically labeled nitron spin trap: potential biological applications, *FEBS Lett.* 473 (2000) 62.
- [18] T. Ohyashiki, M. Nunomura, T. Katoh, Detection of superoxide anion radical in phospholipid liposomal membrane by fluorescence quenching method using 1,3-diphenylisobenzofuran, *Biochim. Biophys. Acta* 1421 (1999) 139.
- [19] D. Li, B. Shlyahovsky, J. Elbaz, I. Willner, Amplified analysis of low-molecular-weight substrates or proteins by the self-assembly of DNAzyme-aptamer conjugates, *J. Am. Chem. Soc.* 129 (2007) 5804.
- [20] I. Willner, M. Zayats, Electronic aptamer-based sensors, *Angew. Chem. Int. Ed.* 46 (2007) 6418.
- [21] H. Bayraktar, C. You, V.M. Rotello, M.J. Knapp, Facial control of nanoparticle binding to cytochrome C, *J. Am. Chem. Soc.* 129 (2007) 2733.
- [22] S. Srivastava, B. Samanta, B.J. Jordan, R. Hong, Q. Xiao, M.T. Tuominen, V.M. Rotello, Integrated magnetic bionanocomposites through nanoparticle-mediated assembly of ferritin, *J. Am. Chem. Soc.* 129 (2007) 11780.
- [23] J.J. Gren, E. Chiu, E.S. Leshchiner, J. Shi, R. Langer, D.G. Anderson, Electrostatic ligand coatings of nanoparticles enable ligand-specific gene delivery to human primary cells, *Nano Lett.* 7 (2007) 879.
- [24] T. Oshaka, Y. Shintane, F. Matsumoto, T. Okajima, K. Tokuda, Mediated electron transfer of polyethylene oxide-modified superoxide dismutase by methyl viologen, *Bioelectrochem. Bioenerg.* 37 (1995) 73.
- [25] K. Tammeveski, T.T. Tenno, A.A. Mashirin, E.W. Hillhouse, P. Manning, C.J. McNeil, Superoxide electrode based on covalently immobilized cytochrome c: modelling studies, *Free Radic. Res. Commun.* 25 (1998) 973.
- [26] M.K. Beissenhirtz, F.W. Scheller, M.S. Viezzoli, F. Lisdat, Engineered superoxide dismutase monomers for superoxide biosensor applications, *Anal. Chem.* 78 (2006) 935.
- [27] K. Tanaka, Y. Muto, Amperometric determination of superoxide anions generated from phytoplankton *Chlorella antiqua*, *Bioelectrochem. Bioenerg.* 29 (1992) 143.
- [28] J.M. McCord, I. Fridovich, Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein), *J. Biol. Chem.* 244 (1969) 6055.
- [29] I. Fridovich, in: L.W. Oberley (Ed.), *Superoxide Dismutase*, vol. 1, CRC Press, Boca Raton, FL, 1982, (Chapter 3).
- [30] X. Yu, L. Wang, J.J. Di, Deposition of high density gold nanoparticles on indium/tin oxide electrode for fabrication of biosensors, *Nanosci. Nanotechnol.* 11 (12) (2011) 11088.
- [31] X.R. Li, B. Wang, J.J. Xu, H.Y. Chen, *In vitro* detection of superoxide anions released from cancer cells based on potassium-doped carbon nanotubes-ionic liquid composite gel, *Nanoscale* 3 (12) (2011) 5033.
- [32] Y. Luo, G. Hu, A. Zhu, B. Kong, Z. Wang, C. Liu, Y. Tian, Biosensors as innovative tools for the detection of food borne pathogens, *Biosens. Bioelectron.* 15 (29(1)) (2011) 194.
- [33] P. Santhosh, K.M. Manesh, S.H. Lee, S. Uthayakumar, A.I. Gopalan, K.P. Lee, Sensitive electrochemical detection of superoxide anion using gold nanoparticles distributed poly(methyl methacrylate)-polyaniline core-shell electrospun composite electrode, *Analyst* 21 (136(8)) (2011) 1561.
- [34] Y. Wang, Y. Wu, J. Wang, J. Di, Disposable superoxide anion biosensor based on superoxide dismutase entrapped in silica sol-gel matrix at gold nanoparticles modified ITO electrode, *Bioproc. Biosyst. Eng.* 32 (4) (2009) 536.
- [35] X.J. Chen, A.C. West, D.M. Crokep, S. Banta, Detection of the superoxide radical anion using various alkanethiol monolayers and immobilized cytochrome c, *Anal. Chem.* 15 (80(24)) (2008) 9629.
- [36] H. Liu, Y. Tian, P. Xia, Pyramidal, rodlike, spherical gold nanostructures for direct electron transfer of copper, zinc-superoxide dismutase: application to superoxide anion biosensors, *Langmuir* 17 (24(12)) (2008) 6366.
- [37] Z. Deng, Q. Rui, X. Yin, H. Liu, Y. Tian, *In vivo* detection of superoxide anion in bean sprout based on ZnO nanodisks with facilitated activity for direct electron transfer of superoxide dismutase, *Anal. Chem.* 1 (80(15)) (2008) 5846.
- [38] J. Di, S. Peng, C. Shen, Y. Gao, Y. Tu, One-step method embedding superoxide dismutase and gold nanoparticles in silica sol-gel network in the presence of cysteine for construction of third-generation biosensor, *Biosens. Bioelectron.* 30 (23(1)) (2007) 94.
- [39] E. Emregul, Development of a new biosensor for superoxide radicals, *Anal. Bioanal. Chem.* 383 (6) (2005) 954.
- [40] B. Ge, F.W. Scheller, F. Lisdat, Electrochemistry of immobilized CuZnSOD and FeSOD and their interaction with superoxide radicals, *Biosens. Bioelectron.* 18 (2003) 302.
- [41] J. Di, S. Bi, M. Zhang, Third-generation superoxide anion sensor based on superoxide dismutase directly immobilized by sol-gel thin film on gold electrode, *Biosens. Bioelectron.* 19 (2004) 1486.
- [42] G. Moschopoulos, S. Kintzios, Application of "membrane-engineering" to bioelectric recognition cell sensors for the ultra-sensitive detection of superoxide radical: a novel biosensor principle, *Anal. Chim. Acta* 573-574 (2007) 90.
- [43] E. Barsoukov, J.R. MacDonald, *Impedance Spectroscopy: Theory, Experiment and Application*, 2nd ed. Wiley Interscience, 2005.
- [44] R.E. Ionescu, N. Jaffrezic-Renault, L. Bouffier, C. Gondran, S. Cosnier, D.G. Pinacho, M.P. Marco, F.J. Sánchez-Baeza, T. Healy, C. Martelet, Impedimetric immunosensor for the specific label free detection of ciprofloxacin antibiotic, *Biosens. Bioelectron.* 30 (23(4)) (2007) 555.
- [45] G. Tsekenis, G.-Z. Garifallou, F. Davis, P.A. Millner, T.D. Gibson, S.P.J. Higson, Detection of fluoroquinolone antibiotics in milk via a labelless immunoassay based upon an alternating current impedance protocol, *Anal. Chem.* 80 (2008) 2062.
- [46] A.G. Mantzila, V. Maipa, M.I. Prodromidis, Development of a faradic impedimetric immunosensor for the detection of *Salmonella typhimurium* in milk, *Anal. Chem.* 80 (2008) 1175.
- [47] E. Tully, S.P. Higson, R. O'Kennedy, The development of a 'labelless' immunosensor for the detection of *Listeria monocytogenes* cell surface protein, internalin B, *Biosens. Bioelectron.* 23 (2008) 912.
- [48] Q. Xie, C. Xiang, Y. Yuan, Y. Zhang, L. Nie, S. Yao, A novel dual-impedance-analysis EQCM system—investigation of bovine serum albumin adsorption on gold and platinum electrode surfaces, *J. Colloid Interface Sci.* 262 (2003) 107.
- [49] X. Luo, A. Morrin, A.J. Killard, M.R. Smyth, Enhancement of a conducting polymer-based biosensor using carbon nanotube-doped polyaniline, *Electroanalysis* 18 (4) (2006) 319–326.
- [50] H.-E. Wang, Facile synthesis and electrochemical of porous and dense TiO₂ nanospheres for lithium-ion battery applications, *J. Power Sources* 196 (2011) 6394–6399.
- [51] Y. Wang, T. Chen, Q.J. Mu, Electrochemical performance of W-doped anatase TiO₂ nanoparticles as an electrode material for lithium-ion batteries, *Mater. Chem.* 21 (2011) 6006–6013.

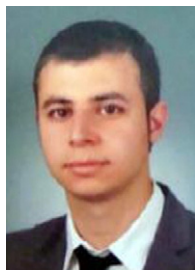
- [52] S.-J. Bao, C.-M. Li, J.-F. Zang, X.-Q. Cui, Y. Qiao, J. Guo, New nanostructured TiO₂ for direct electrochemistry and glucose sensor applications, *Adv. Funct. Mater.* 18 (2008) 591–599.
- [53] Y.P. Luo, H.Q. Liu, Q. Rui, Y. Tian, Detection of extracellular H₂O₂ released from human liver cancer cells based on TiO₂ nanoneedles with enhanced electron transfer of cytochrome c, *Anal. Chem.* 81 (2009) 3035–3041.
- [54] H. Tang, F. Yan, Q.D. Tai, H.L.W. Chan, The improvement of glucose bioelectrocatalytic properties of platinum electrodes modified with electrospun TiO₂ nanofibers, *Biosens. Bioelectron.* 25 (2010) 1646–1651.
- [55] O. Kocabay, E. Emregul, S. Aras, K.C. Emregul, Carboxymethylcellulose–gelatin–superoxidase dismutase electrode for amperometric superoxide radical sensing, *Bioproc. Biosyst. Eng.* 35 (2012) 923.
- [56] X. Wu, X. Meng, Z. Wang, Z. Zhang, Study on the direct electron transfer process of superoxide dismutase, *Chem. Lett.* 12 (1999) 1272.
- [57] X. Wu, X. Meng, Z. Wang, Z. Zhang, The electrochemical and spectroelectrochemical behaviors of SOD at cysteine modified gold electrode, *Bioelectrochem. Bioenerg.* 48 (1999) 231.
- [58] C.-C. Wu, C.-H. Lin, W.-S. Wang, Development of an enrofloxacin immunosensor based on label-free electrochemical impedance spectroscopy, *Talanta* 79 (2009) 66.
- [59] B. Rezaei, N. Majidi, H. Rahmani, T. Khayamian, Electrochemical impedimetric immunosensor for insulin like growth factor-1 using specific monoclonal antibody–nanogold modified electrode, *Biosens. Bioelectron.* 26 (2011) 213.
- [60] K.-J. Huang, D.-J. Niu, W.-X. Xie, W. Wang, A disposable electrochemical immunosensor for carcinoembryonic antigen based on nano-Au/multi-walled carbon nanotubes–chitosans nanocomposite film modified glassy carbon electrode, *Anal. Chim. Acta* 659 (2010) 102–108.
- [61] E. Laviron, General expression of the linear potential sweep voltammogram in the case of diffusionless electrochemical systems, *J. Electroanal. Chem.* 101 (1979) 19–28.
- [62] A.J. Bard, L.R. Faulkner, *Electrochemical Methods: Fundamentals and Applications*, (Hardcover).
- [63] P. Norouzi, V.K. Gupta, F. Faridbod, M. Pirali-Hamedani, B. Larijani, M.R. Ganjali, Carcinoembryonic antigen admittance biosensor based on Au and ZnO nanoparticles using FFT admittance voltammetry, *Anal. Chem.* 83 (2011) 1564–1570.
- [64] S. Saha, S.K. Arya, S.P. Singh, K. Sreenivas, B.D. Malhotra, V. Gupta, Zinc oxide–potassium ferricyanide composite thin film matrix for biosensing applications, *Anal. Chim. Acta* 653 (2009) 212–216.



Emel Emregul received her PhD in 1999 from Ankara University. Then she has been in the USA as a visiting scientist and joined the Research Group of Prof Dr Victor C YANG in the University of Michigan (Ann Arbor – USA) from 2001 to 2003. She has authored around 13 papers. She is interested in the synthesis of polymer, synthesis of nanoparticles, biosensors, immunosensors, bioreactors and controlled release. She is now Assoc. Professor of Chemistry at the Science Faculty, Department of Chemistry Biochemistry Section, University of Ankara.



Ozge Kocabay graduated from Ankara University from the Department of Chemistry. She is interested in biosensors, immunosensors, polymer and nanoparticles. She is a PhD student under the supervision of Assoc. Prof. Emel EMREGUL at the Science Faculty, Department of Chemistry Biochemistry Section, University of Ankara.



Burak Derkus graduated from Ankara University from the Department of Chemistry and Chemical Engineering. He is interested in biosensors, immunosensors, synthesis of polymer and nanoparticles. He is an MS student under the supervision of Assoc. Prof. Emel EMREGUL at the Science Faculty, Department of Chemistry Biochemistry Section, University of Ankara.



Tugrul Yumak received a BS degree from the Ankara University Faculty of Science in June 2008. He is currently on a doctorate course and is a research assistant in the Ankara University Faculty of Science under the supervision of Prof. Dr. Ali Sinağ. His research interests include the conversion of biomass and synthesis of nano sized catalysts for biomass conversion and photocatalytic applications in Prof. Dr. Sinağ's Renewable Energy and Nanotechnology Research Group.



Kaan Cebesoy Emregul received his PhD in April 1998 from Ankara University. Then he was granted NATO Scholarship Norwegian Technical Institute, Trondheim Norway and joined the Research Group of Prof Dr Kemal Nisacioglu in Norwegian Technical Institute, Trondheim Norway (1995–1996). He has authored around 30 papers. He is interested in Electrochemistry Voltammetric techniques, Electrochemical Impedance Spectroscopy, corrosion and metallurgy inhibition of metals, design of organic inhibitors, TGA-DSC analysis, nanotechnology and nanochemistry, biosensors and application in the diagnose of disease. He is now Professor of Chemistry at the Science Faculty, Department of Chemistry, University of Ankara.



Ali Sinağ received his PhD in April 2001 from Ankara University. Then he was granted DAAD scholarship and joined the Research Group of Prof Dr Ing Rainer Reimert in Karlsruhe Technical University (Karlsruhe – Germany) from May 2001 to September 2002. From October 2003 to April 2005, he was granted Karlsruhe Research Center Scholarship joining the Research Group of Prof Dr hab Andrea Kruse. He has authored around 40 papers, held 1 European and German Patent. He is interested in the synthesis of carbonaceous materials by hydrothermal carbonization processes from biomass, hydrothermal synthesis of nanoparticles and photocatalyst. He is now Professor of Chemistry at the Science Faculty, Department of Chemistry, University of Ankara.



Kamran Polat works at Ankara University, where he has been on the Faculty of Science, Department of Chemistry since 1988. He received a PhD in Organic Chemistry at Ankara University in 1997. He continues research in Ankara University as Assoc. Professor since 2004. His research has dealt with various aspects of practical organic and electrochemistry, particularly industrial organic synthesis, organic electroorganic synthesis, electrokinetic and electrode reaction mechanism and spectroscopy of organic compounds. Professor Polat currently runs a research group of four master students.