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ORIGINAL PAPER

Carboxymethylcellulose–gelatin–superoxidase dismutase electrode for amperometric superoxide radical sensing

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Abstract A novel, highly sensitive superoxide dismutase biosensor for the direct and simultaneous determination of superoxide radicals was developed by immobilization of superoxide dismutase within carboxymethylcellulose–gelatin on a Pt electrode surface. The parameters affecting the performance of the biosensor were investigated. The response of the CMC–G–SOD biosensor was proportional to $O_2^{\cdot-}$ concentration and the detection limit was 1.25×10^{-3} mM with a correlation coefficient of 0.9994. The developed biosensor exhibited high analytical performance with wider linear range, high sensitivity and low response time. The biosensor retained 89.8% of its sensitivity after use for 80 days. The support system enhanced the immobilization of superoxide dismutase and promoted the electron transfer of superoxide dismutase minimizing its fouling effect. The biosensor was quite effective not only in detecting $O_2^{\cdot-}$, but also in determining the antioxidant properties of acetylsalicylic acid-based drugs and the anti-radical activity of healthy and cancerous human brain tissues.

Keywords Carboxymethylcellulose–gelatin · Superoxide dismutase · Biosensor · Superoxide anions · Scavengers

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Introduction

Oxygen radicals have attracted considerable attention due to their harmful interaction with biological molecules and their involvement in signaling pathways. Superoxide radicals, ($O_2^{\cdot-}$), the primary species of the reactive oxygen species (ROS), produced in biological systems, play a central role in physiological processes [1–3]. They can be generated at a rate that is matched by the capacity of tissue to catabolize them under normal metabolic conditions. [4]. When its level exceeds the body's natural ability to deal with the potentially cytotoxic species, various pathological situations such as cancer, stroke, neurodegeneration, and aging may result [5–9]. In plants, $O_2^{\cdot-}$ is commonly generated under illumination of chloroplasts by the occasional transfer of an electron from an excited Chl molecule or photosystem I component, under conditions of high NADPH/NADP ratios, to molecular O_2 [10]. In most aerobic organisms different environmental complications, like hyperoxia, pathogens, ozone, temperature fluctuations, and other stresses are known to stimulate $O_2^{\cdot-}$ formation [10]. Therefore, in order to understand the role of $O_2^{\cdot-}$ in pathology and physiology and the relationship between $O_2^{\cdot-}$ and environmental stresses, the quantitative determination of $O_2^{\cdot-}$ in a variety of in vitro and in vivo models is essential. A major challenge of the quantification of superoxide radicals in biological models is of its short duration due to its spontaneous dismutation to oxygen and hydrogen peroxide at low concentration [11, 12]. Determination of free radicals is usually performed by ESR [13], chemiluminescence [14], or by certain semi-quantitative colorimetric tests [15, 16]. These indirect methods are ex situ detection techniques with low sensitivity and poor selectivity. However, among the analytical systems, electrochemical techniques have proved to be powerful tools

with the advantages of simplicity, portability, cost, fast response, high selectivity, and real-time measurements [17–23].

The enzymatic and non-enzymatic modified electrodes are used for the determination of $O_2^{\cdot-}$. Superoxide dismutases (SODs), which protect the organism against the toxic effects are ubiquitous metalloenzymes in oxygen-tolerant organisms, catalyzing the dismutation of $O_2^{\cdot-}$ to O_2 and H_2O_2 via a cyclic oxidation–reduction electron-transfer mechanism [24–26]. Superoxide dismutase, a selective scavenger of $O_2^{\cdot-}$has been the best approach for the electrochemical detection of $O_2^{\cdot-}$compared to cyt c [27, 28]. Therefore, SOD, a specific enzyme for $O_2^{\cdot-}$ dismutation, offers great potential for the sensitive and selective quantification of $O_2^{\cdot-}$ in electrochemical biosensors. Mostly, copper, zinc–superoxide dismutase-immobilized electrodes have paved an elegant way to detect $O_2^{\cdot-}$.

The objective of this study was to develop a superoxide dismutase biosensor for determination of $O_2^{\cdot-}$. Superoxide dismutase was immobilized on a gelatin–carboxymethylcellulose (G–CMC) support system by cross-linking with glutaraldehyde. On the other hand G–CMC is a new support for the development of SOD electrodes. The aim was to obtain a strong and durable structures for G–CMC to produce long-lifetime biosensors in the biosensor structure. For this purpose different conditions were examined to optimize the immobilization procedure. Crosslinker concentration, enzyme concentration, composition of the support system, and lifetime were among the factors studied. The scavenging properties of aspirin, and aspirin containing vitamin C was also investigated using the carboymethylcelulose–gelatin–superoxide dismutase (CMC–G–SOD) biosensor. The biosensor response was tested on healthy and cancerous brain tissue.

Materials and methods

Materials

Superoxide dismutase (EC. 1.15.1.1, 4,733 U mg^{-1}, from bovine erythrocytes), xanthine oxidase (XOD; EC 1.1.3.22, 0.3U mg^{-1}, from milk), xanthine (2,6-dihydroxypurine) sodium salt, gelatin, CMC, and chemicals used for preparation of buffers and cross-linking agents were purchased from Sigma (St Louis, MO, USA). Current was measured with Gamry Instrument using Framework Version 5.50 software Aspirin (500 mg acetylsalicylic acid per tablet) and aspirin containing vitamin C (400 mg acetylsalicylic acid and 240 mg ascorbic acid per tablet) were purchased from a pharmacy.

Electrolysis cell and electrodes

A three-electrode electrochemical cell comprising working, counter, and reference electrodes was used for all experiments. The working (area 0.5 cm^2) and counter electrodes were platinum and the reference electrode was Ag/AgCl, introduced into the cell via Luggin capillary.The surface of the working electrode was polished using 0.05 μm alumina slurry prior to each experiment.

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). Superoxide dismutase (4,733 U, 1 mg) and glutaraldehyde (0.005 M) were added to the support system solutions to obtain a final volume of 50 μL at 32 °C. The platinum foil electrode was covered with a total of 50 μL immobilization gel, 25 μL on each surface. The CMC–G–SOD electrodes were left at room temperature for 48 h. Enzyme leakage tests were performed for all enzyme 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–SOD biosensors obtained.

Principle of the method

The biosensor used to determine the $O_2^{\cdot-}$ was obtained by coupling an amperometric electrode for hydrogen peroxide with the superoxide dismutase enzyme immobilized on CMC–G support system.

The capacity of the SOD biosensor was determined as follows: the $O_2^{\cdot-}$ is produced in aqueous solution by oxidation of xanthine to uric acid in the presence of xanthine oxidase:

$$\text{Xanthine} + H_2O + O_2 \xrightarrow{\text{XOD}} \text{Uricacid} + 2H^+ + O_2^{\cdot-} \quad (1)$$

Superoxide dismutase immobilized on the Pt electrode catalyzes the dismutation reaction of the $O_2^{\cdot-}$ with release of oxygen and hydrogen peroxide according to Eq. 2.

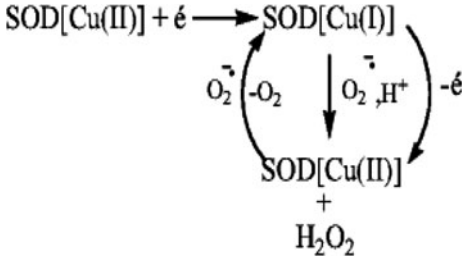
$$2H^+ + O_2^{\cdot-} \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad (2)$$

The H_2O_2 can be detected at the electrode surface in accordance with Eq. 3.

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e' \quad (3)$$

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 $O_2^{\cdot-}$ in solution.

The reaction scheme on the electrode surface is as follows:



Measurement of procedure

All 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 1,000 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 calibration plot was obtained as a function of increasing xanthine concentration. All experiments were repeated five times to ensure reproducibility in this work.

Tests on scavenger molecules

The effect of molecules regarded as scavengers of the superoxide radical was determined in vitro using a CMC–G–SOD biosensor. The response of the CMC–G–SOD biosensor to the superoxide radical both in the presence and absence of scavenger molecules such as aspirin, and aspirin containing vitamin C was determined. The selected scavenger molecule (12.5 gL^{-1}). obtained by dissolving and homogenizing the weighed sample in phosphate buffer (0.05 M, pH 7.4) was added to the cell containing phosphate buffer (0.05 M, pH 7.4) and XOD (0.7 U) with magnetic stirring. After the biosensor response became constant, different concentrations of xanthine (1.25×10^{-3}–2 mM) were added to the solution in the cell. The decrease of current density due to the superoxide-scavenging activity of the tested sample was recorded. A new calibration plot for the scavenger molecules was obtained using the method described in the measurement procedure. The scavenging properties of the molecule considered were evaluated from the percentage ratio of the slope values of the calibration plot, both in the presence and absence of the scavenger compounds considered.

Tests on healthy and diseased tissues

The biosensor response was investigated on healthy and cancerous brain tissue, meingioma (grade I, WHO 2,000). 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 μL) was added and the biosensor response was recorded. The tissues were stored at −20 °C before use.

Results and discussion

Enzyme leakage tests were performed by washing of each electrode in phosphate buffer (0.05 M, pH 7.4) for 1 h at 25 °C. All the CMC–G–SOD biosensors immobilized onto carrier systems, prepared using different ratios of CMC–G (0–0.25 w/w) with cross-linker glutaraldehyde (0.0025–0.01 M), were analyzed. As expected, enzyme leakage decreased with increasing cross-linker concentration and CMC–G ratio. Maximum leakage occurred at CMC–G ratio 0.053 and glutaraldehyde concentration 0.0025 M. Further increase of CMC–G ratio resulted in a decreased leakage to a level below the value obtained for pure G. A possible interpretation of the results is that SOD is more strongly bound with the increase of CMC–G ratio and this binding is the controlling factor, whereas when CMC–G is below 0.05, enzyme release increases with increasing CMC–G ratio due to the effect of swelling of the support material [29, 30].

The effect of CMC/G ratios

The best biosensor response was investigated by changing the composition of the support system. For this purpose the effects of CMC/G ratios (0.05–0.25 w/w) on CMC–G–SOD biosensor response were determined with 0.005 mol L^{-1} glutaraldehyde and 2,366 U SOD concentration. Maximum response was obtained for a support composition CMC–G 0.100 (w/w). Low CMC–G ratio increases leakage due to increased pore size. At higher levels the response increases due to an increase in the amount of enzyme bound. Finally further increase causes decrease of biosensor response, probably due to excess enzyme binding, leading to inactivation [29]. The best results have been obtained for CMC–G ratio 0.100 (Fig. 1).

The effect of glutaraldehyde concentration

In this work cost, physiological inertness, and structural suitability in the development of biosensors to be used in clinical applications were the criteria for selecting G–CMC as carrier material and glutaraldehyde as cross-linker. The aim was to obtain mechanically strong and durable structures for G–CMC, so the biosensor could be used for long periods of time. Glutaraldehyde was used as cross-linker

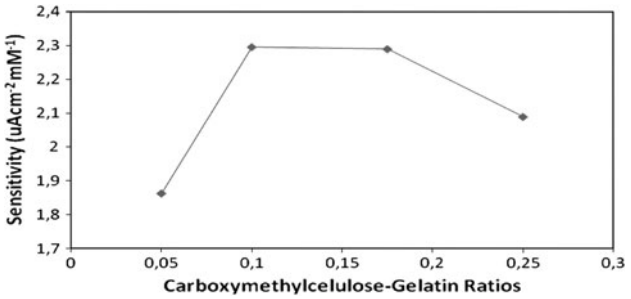


Fig. 1 Effect of carboxymethylcellulose–gelatin ratios on the sensitivity of the CMC–G–SOD biosensor. Conditions for preparation of the CMC–G–SOD biosensor: 0.005 mM glutaraldehyde, 1.25×10^{-3}–2 mM xanthine

for superoxide dismutase immobilization on to G–CMC and its effect on the response of G–SOD biosensor was studied. Diverse concentrations of cross-linkers (0.0025–0.01 mol L^{-1}) were used to immobilize SOD (4,733 U) onto carrier systems. The responses of CMC–G–SOD biosensors prepared using different concentrations of glutaraldehyde are shown in Fig. 2. Decreasing the glutaraldehyde concentration reduced the response of immobilized SOD, due to deactivation of the enzyme molecules and formation of tight gel because of the excess cross-linker. The diffusion of xanthine and superoxide radical in and out of the CMC–G–SOD film was decelerated. The diffusion limitation reduces the response of enzyme more drastically at higher cross-linker concentrations. The decrease in the flexibility of the polymer chain that occurs on cross-linking, resulting in reduced electron-transfer efficiency, may cause the lower current density [30, 31]. Response time increased with ascending cross-linker concentration. In the 0–0.005 mM cross-linker concentration range, the

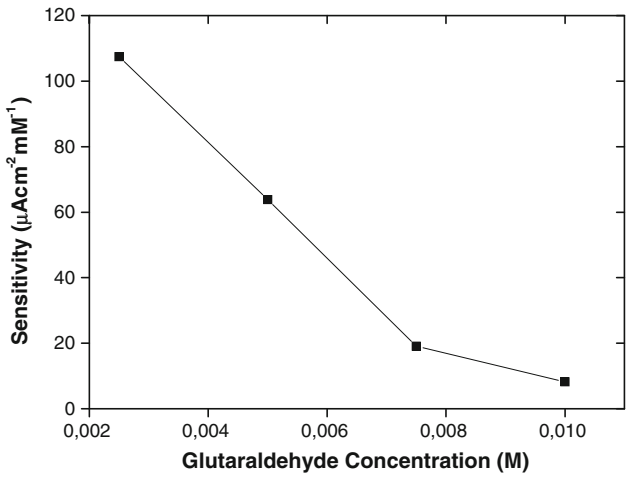


Fig. 2 Effect of glutaraldehyde concentration on the sensitivity of the CMC–G–SOD biosensor. Conditions for preparation of the CMC–G–SOD biosensor: 0.005 mM glutaraldehyde, 1.25×10^{-3}–2 mM xanthine

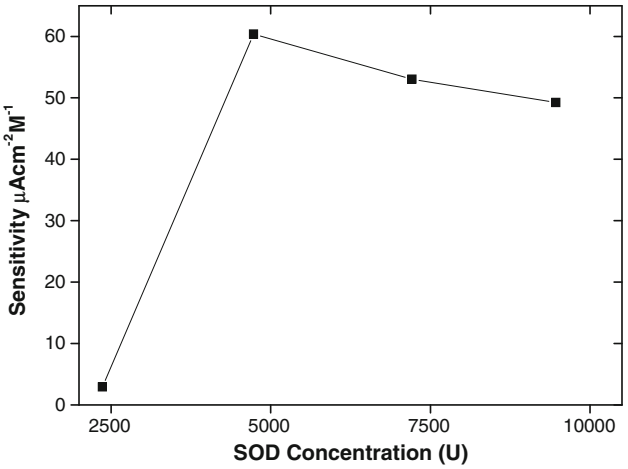


Fig. 3 Effect of enzyme concentration on the sensitivity of the CMC–G–SOD biosensor. Conditions for preparation of the CMC–G–SOD biosensor: 0.005 mM glutaraldehyde, 1.25×10^{-3}–2 mM xanthine

immobilized SOD was not mechanically strong and enzyme leakage was high.

The effect of enzyme concentration

The effects of enzyme concentrations (2,366–9,466 U) on CMC–G–SOD biosensor response were investigated. The enzyme concentration and response time correlation was also determined.The relationship between enzyme concentration and biosensor response was linear up to 4,733 U then decreased (Fig. 3) and the response time decreases with increasing enzyme concentration (Data were not shown). These behaviors can be explained by the possibility of increased enzyme–enzyme cross-linking. Also, with increased loading the enzyme becomes oversaturated within the matrix pore, which restricts the product and substrate diffusion [31, 32]. The optimum SOD concentration was found to be 4,733 U. This enzyme concentration giving the highest response is lower than for the biosensors developed by Di et al., Campanella et al., Pastor et al., and Fujita et al. [33–36], making it more cost efficient.The CMC–G–SOD biosensor was prepared using 4,733 U enzyme for further studies.

Amperometric response of CMC–G–SOD biosensor to $O_2^{\cdot-}$

The amperometric response of the CMC–G–SOD biosensor resulted from superoxide anion was investigated in the stirred buffer solution.

A simple and efficient method was utilized for generation of $O_2^{\cdot-}$ by oxidation of xanthine to uric acid in the presence of xanthine oxidase at an applied potential of

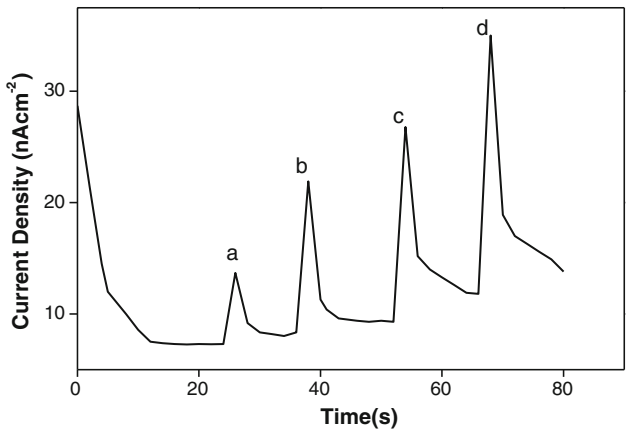


Fig. 4 Amperometric response of CMC–G–SOD biosensor for different xanthine concentrations at 650 mV. The xanthine concentrations were: *a* 1.25 μM, *b* 2 μM, *c* 2.5 μM, *d* 3 μM

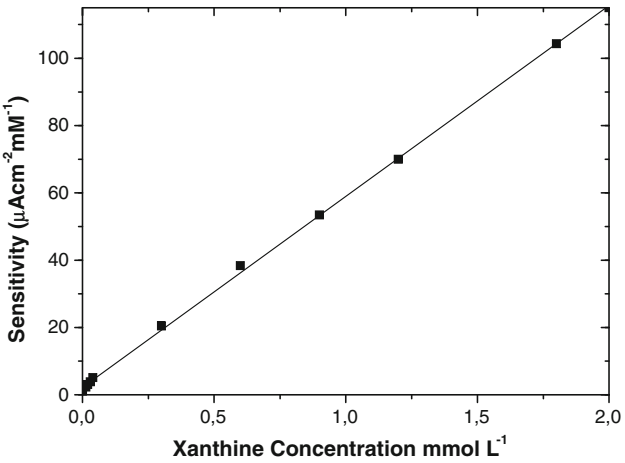


Fig. 5 Calibration graph for the CMC–G–SOD biosensor

650 mV. The measurement solution should be operated at a constant and high stirring speed (1,000 rpm) to acquire reproducible results. A peak-like response was observed, which is in agreement with a report by other worker [28, 33, 36–38]. If only a certain amount is added, a superoxide "burst" will occur which rapidly decreases to baseline level because of the dismutation reaction. This suggests that the measurement system responded to actual concentration changes in solution (Fig. 4). A calibration plot as a function of increasing xanthine concentration was obtained for the CMC–G–SOD biosensor (Fig. 5). With ascending xanthine concentration, the amperometric response of the CMC–G–SOD biosensor increases. These results showed that the CMC–G–SOD biosensor has excellent responses to superoxide anion. 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 $O_2^{\cdot-}$ because SOD catalyzes the dismutation of $O_2^{\cdot-}$ to O_2 and H_2O_2 via a cyclic oxidation–reduction electron transfer.

Table 1 shows the main analytical data and the working conditions for the CMC–G–SOD biosensor. The linear range of the CMC–G–SOD biosensor was between 1.25×10^{-3} and 2. The response of CMC–G–SOD biosensor was proportional to $O_2^{\cdot-}$ concentration and the detection limit was 1.25×10^{-3} mM at a signal-to-noise ratio of 3. The effect of xanthine concentration on the response time of the CMC–G–SOD biosensor was analyzed. The response time of the biosensor was 2 s for the lowest xanthine concentrations (1.25×10^{-3}–1 mM) and increased with increasing xanthine concentration. The enzyme is uniformly distributed throughout the film, and for the lowest xanthine concentrations the reaction occurs predominantly on the surface of the film. Reaction on the surface of the film and diffusion occur simultaneously at higher concentrations, resulting in a delay of the response time [29–31]. The response time of the biosensor was 2 s. As H_2O_2 is a metabolite in the degradation of $O_2^{\cdot-}$ and a product of the enzyme reaction of endogenous oxidases such as monoamine oxidase and L-amino acid oxidase in biological systems, the specificity of the biosensor against H_2O_2 is of great importance in its application for the determination of $O_2^{\cdot-}$ in biological system. No detectable current response to 0.5 mM H_2O_2 was observed in the CMC–G–SOD biosensor. When a high concentration of H_2O_2 were added, a step-like current response was observed, proving its selectivity towards $O_2^{\cdot-}$ measurement. The present CMC–G–SOD electrode exhibited excellent analytical performance with wider a linear range, high sensitivity, and low response time compared to other studies [38–41].

Lifetime of CMC–G–SOD electrode

The CMC–G–SOD biosensors were prepared using 4,733 U enzyme. The reproducibility of the amperometric

Table 1 Working conditions and analytical data

	CMC–G–SOD biosensor
Equation of calibration plot (y = current density (μA cm^{-2}); x = xanthine concentration (mM))	$y = (56.75 \pm 0.4)x + (2.19 \pm 0.4)$
Correlation coefficient	0.9994
Linear range (mM)	1.25×10^{-3}–2
Minimum detection limit (μM)	0.125
Response time (s)	2
Xanthine concentration (mM)	1.25×10^{-3}–2
Time of analysis (min)	10
Lifetime (days)	80

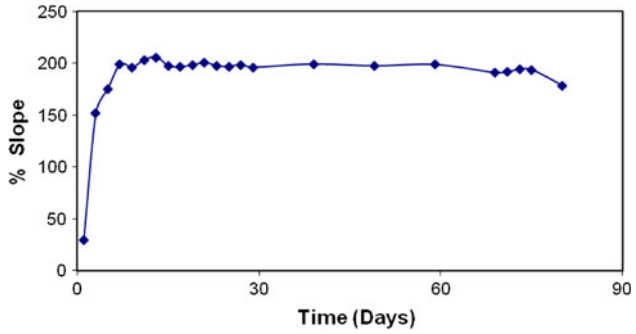


Fig. 6 Lifetime of the CMC–G–SOD biosensor: conditions for the preparation of G–SOD biosensor: 0.005 mM glutaraldehyde, 1.25×10^{-3}–2 mM xanthine

response for the CMC–G–SOD biosensor was examined. Re-use experiments were carried out for a period of 3 month under previously specified conditions (Fig. 6). The electrodes were washed in phosphate buffer (0.05 M, pH 7.5) after each use and stored in the same buffer at 4 °C. The CMC–G–SOD biosensor retained its activity for 3 months. A decrease in the response of the biosensor was observed after 80 days but it still retained 89.8% of its activity. The decrease observed in the CMC–G–SOD response is mainly the result of enzyme deactivation. The CMC–G–SOD biosensor is stable enough for clinical applications and efficiently retains enzyme activity. It has the longest lifetime of biosensors developed using SOD, with the advantage of high current responses at relatively low enzyme concentrations [33, 34, 42]. Good long-term stability can be attributed to the mild immobilization procedure and a beneficial environment for preventing enzyme leakage. In addition, the hydroxyl groups in gelatin tend to form hydrogen bonds within the enzyme molecules, which prevents leakage. Immobilization of SOD on gelatin provides a biocompatible microenvironment around the enzyme, maintaining its biological activity to a large extent.

Tests on scavenger molecules

The biosensor was used to asses the scavenging properties of acetylsalicylic acid-based drugs in vitro. The response of the biosensor to superoxide radical in the absence and presence of the scavenger molecules was determined. Aspirin and aspirin containing vitamin C were used to show the scavenger capacity to free radicals. The antioxidant properties of the scavenger molecules considered were evaluated from the percentage ratio of the slope values of the calibration plot both in the absence and presence of each scavenger molecule in solution. The results are presented in Fig. 7. Addition of an aspirin and aspirin containing vitamin C sample resulted in a decrease of the

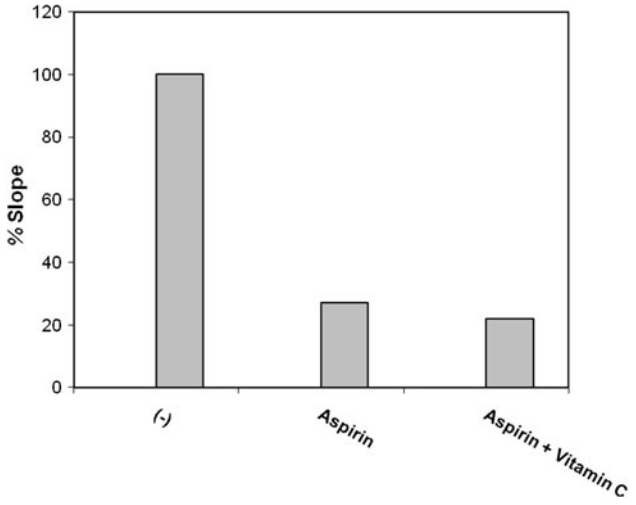


Fig. 7 Evaluation of scavenger properties of acetylsalicylic acid-based drugs using the CMC–G–SOD biosensor. The sensitivity of the CMC–G–SOD biosensor to superoxide radical in the absence (−) and presence of of acetylsalicylic acid-based drugs (12.5 gL^{-1})

signal strength as the antioxidant species reacted with the superoxide radical, thus reducing its concentration in the solution. There was a consequent decrease in the amount of H_2O_2 released and in the amperometric signal [30, 35, 37]. The developed CMC–G–SOD biosensor can be used for in vitro determination of the antioxidant properties of acetylsalicylic acid-based drugs.

Tests on healthy and cancerous tissues

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

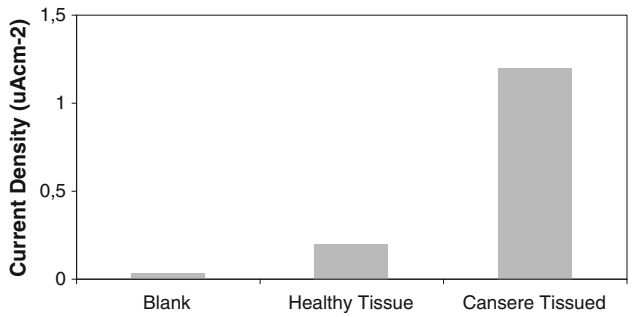


Fig. 8 Variation of the signal from CMC–G–SOD biosensors after addition of homogenized healthy brain tissue and cancerous brain tissue to phosphate buffer (0.05 mM, pH 7.4). Data are average results from three biosensors

probably because a smaller quantity of scavengers or endogenous SOD is present in the cancerous tissue [30].

Conclusion

In this study, SOD was immobilized on G–CMC for the first time although superoxide dismutase has been immobilized with different carriers in several studies [30, 31, 34, 35]. Direct electron transfer to SOD in the G–CMC film was performed without any mediators. Superoxide dismutase retained its activity after cross-linking with G–CMC. The biosensor sensitivity was 89.8% in the end of 80 days. Reusability is one major advantage of this CMC–G–SOD biosensor. It is one of the longest-lasting biosensors among those yet developed with the advantage of high biosensor response at low enzyme concentrations [25, 26, 28, 42, 43]. Immobilization of SOD on to G–CMC provides a biocompatible microenvironment around the enzyme and stabilizes enzyme activity very efficiently. This support composition is very efficient for retaining the enzyme activity and preventing the enzyme leakage out of the support system. The support system enhanced the immobilization of SOD and promoted the electron transfer of SOD minimizing its fouling effect. The response of the CMC–G–SOD biosensor was proportional to $O_2^{\cdot-}$ concentration and the detection limit was 1.25×10^{-3} mM at a signal-to-noise ratio of 3. The response time of the developed biosensor was 2 s. The present biosensor showed excellent analytical performance with wider linear range, lower response time longerstability and beter reproducibility.

Superoxide radical is produced in living systems from molecular oxygen, as a reduced intermediate, either by autooxidation processes or by enzymes involved during normal metabolism [44]. It can also appear under uncontrolled conditions, however, causing reversible or irreversible damage to biomolecules and contributing to several pathological processes including aging, atherosclerosis, hypertension, diabetes, ischemic-reperfusion injury, neurodegenerative disorders, and cancer [45–50]. This radical is quite reactive and is easily attacked by other active biomolecules and scavenged by enzymes and antioxidant agents. When the stability and sensitivity of the biosensor had been demonstrated, several applications were explored to test its performance. The response of the biosensor was tested on healthy and cancerous tissue. The response was proportional to the concentration of superoxide radical present in the tissue. Also the scavenging properties of acetylsalicylic acid-based drugs was assessed in vitro using the biosensor.

Depending on the size and thickness, CMC–G–SOD biosensors can be useful for detecting superoxide radicals in blood plasma, urine, whole cells, and tissue. The construction of a CMC–G–SOD biosensor is advantageous because of its easy and quick construction, its selectivity, and its lifetime.

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