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Development of Gelatin-Alginate-TiO_2-SOD Biosensor for the Detection of Superoxide Radicals

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Abstract: In this work, a biosensor that uses gelatin and alginate hydrogels in addition to titanium dioxide (TiO_2) nanoparticles (NPs) as a sensor matrix was developed to detect superoxide radicals ($O_2^{\bullet-}$), which play a role in carcinogenesis when present in excess levels. Parameters affecting the performance of the biosensor such as the amount of gelatin-alginate ratio, amount of TiO_2 NPs, the concentration of SOD enzymes, and glutaraldehyde (GA) cross-linker were investigated. Chronoamperometry was used as an electrochemical technique for the development of biosensor as well as characterization steps. The developed biosensor exhibited two linear ranges between 0.0009 mM – 0.125 mM and 0.25 mM – 2 mM, which was utilized as calibration curves. The detection limit of the biosensor was found 0.9 µM, which was at the appropriate level for the detection of $O_2^{\bullet-}$ in tumor samples. Finally, the constructed biosensor showed significant analytical performance, such as low detection limit, reusability, and reproducibility.

Keywords: Hydrogel, nanoparticle, biosensor, superoxide dismutase, cancer.

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INTRODUCTION

Reactive oxygen species (ROS) are a class of highly reactive reduced oxygen molecules and include various structures such as superoxide anions, hydroxyl radicals, and hydrogen peroxide (H_2O_2). Superoxide radicals, the primary species of ROPs, are present in low concentrations under normal physiological conditions, and they are converted to H_2O_2 and oxygen (O_2) as a result of dismutation with superoxide dismutase (SOD) enzymes (1).

Superoxide anions are often considered to be toxic since they are converted to other radicals in biological systems such as alkoxyl radicals ($RO^{\bullet}$), hydroxyl radicals ($HO^{\bullet}$), and peroxynitrite radicals ($ONOO^-$). Increased superoxide anions result in mutagenesis and consequently, cell death (2-4). Besides, the concentration of superoxide may increase significantly in traumatic brain injuries (5,6). Superoxide radicals can also emerge as a consequence of gradual neurodegenerative damages such as aging (7), cancer (8), and Parkinson's (9). Taken all together, developing sensitive platforms and tools towards detecting superoxide anions is essential.

So far, different sensing strategies have been followed for constructing biosensor systems aiming for the detection of superoxide anions. Fluorescence (10) and electrochemical (11) techniques are the most widely preferred ones. The biosensors developed by the modification of electrodes with hydrogels, without utilizing NPs, resulted in an insufficient detection limit (12, 13), while other designs containing nano-structures require multi-component recognition material (14, 15) or complex fabrication processes (16), which reduces the feasibility of the developed system. Moreover, a big part of the superoxide biosensors focusses on biosensor design but validating the system with biological samples such as blood or tissue (12, 14). Therefore, we need simple, low-cost, and sensitive biosensing tools for the determination of the level of superoxide anions in

biological samples, which might give information about the development of cancer.

Though enzymes catalyze biochemical reactions with unique celerity, they lack electrical conductivity due to their protein-nature, and therefore coupling enzymes with metal NPs might be an excellent strategy to enhance electron transfer to the electrode. By now, several types of NPs - such as Ag (17), Au (18), ZnO (19), and TiO_2 (20) NPs - have been used to develop biosensors. TiO_2 NPs have attracted a critical interest in their advantages, having a large surface area, high uniformity, and biocompatibility such that TiO_2 NPs have been used as a component of the sunscreen-technology as well as they are well accepted in the food industry. Researchers demonstrated a direct electron transfer between biomolecules and electrodes modified with TiO_2 NPs (21, 22). The improved catalytic activity of the developed biosensors has been attributed to their smaller sizes, enabling a larger surface area for biomolecule immobilization and a faster electron transfer from biomolecule to an electrode surface. All these superior properties make TiO_2 NPs a suitable material for electrode design.

In this work, we focused on creating SOD-based biosensor for fast, specific, easy, and sensitive detection of superoxide anions. To this end, we combined gelatin and alginate biopolymers as a carrier system and utilized from TiO_2 NPs to construct a nanocomposite material that brings various advantages such as high surface area, which enabled us to increase SOD immobilization efficiency, and semi-conductivity. SOD enzymes were bonded onto this nanocomposite material, which was coated onto platinum (Pt) electrode, via crosslinking with glutaraldehyde (GA) reagent. The developed gelatin-alginate-TiO_2-SOD biosensor was then tested for detecting superoxide radicals in cancer tissues.

EXPERIMENTAL SECTION

Preparation of gelatin-alginate-TiO_2-SOD biosensors

Gelatin-alginate-TiO_2-SOD electrodes were prepared following the previously published protocols (12, 23) -with small modifications-. After the gelatin was dissolved at 50 °C, different amounts of alginate and titanium dioxide (obtained from Sigma-Aldrich, with diameter <25 nm, in anatase form) were added and homogenized by vortexing. SOD enzymes dissolved in phosphate buffer were added to different units and re-vortexed for homogenization. Finally, the cross-linker glutaraldehyde was added to obtain stiff immobilization gel. The gels (25 µL) were modified on each surface of the Pt working electrode – square plate having 1 cm^2 surface area- with a total of 50 µL of gel, and were allowed to dry for 6 hours. Un-bonded enzymes were washed away with PBS for 3 times. Each step of electrode modification included coating the electrode with gelatin-alginate, gelatin-alginate-SOD, gelatin-alginate-TiO_2, and gelatin-alginate-TiO_2-SOD were characterized with electrochemical impedance spectroscopy (EIS) technique. Pt square plate electrode with 1 cm^2 surface area was used as a counter electrode, and Ag/AgCl with saturated KCl was used as a reference electrode, which was introduced into the cell via a Luggin capillary. All electrochemical measurements were performed with Gamry Potentiostat-Galvanostat with Framework Version 5.50.

Optimization of gelatin-alginate-TiO_2-SOD biosensors

Firstly, different gelatin-alginate ratios (0.25, 0.5, 1, 2) were tried to optimize the immobilization conditions. TiO_2, SOD enzyme, and glutaraldehyde concentrations were kept constant in the immobilization gel. To optimize the cross-linking process, different electrodes were prepared by using different glutaraldehyde concentrations (0,001, 0,002, 0,004, 0,01, and 0,016 M). Also, to obtain an efficient electrochemical performance from the developed biosensors, amperometric responses were recorded for electrodes prepared with varying enzyme concentrations that change between 25 and 5000 U, which provided an optimal enzyme concentration. For TiO_2 NPs optimization, different amount of TiO_2 NPs (0.0001 - 0.0005 g) were tested by keeping the gelatin-alginate ratio (1 w/w), SOD concentration (100 U), and glutaraldehyde cross-linker concentration (0.004 M) constant. Finally, the level of xanthine, triggering the dismutation reaction, was optimized (0.0009-2 mM).

Reproducibility and reusability of biosensor

Amperometric measurements were recorded for 6 replica electrodes to examine the reproducibility of the gelatin-alginate-TiO_2-SOD biosensor, and consistency of the amperometric responses was examined. On the other hand, for reusability study of the developed biosensor, amperometric measurements were obtained every day, up to 10 days, against xanthine (2 mM) at +0.65 V. After each use, the electrodes were washed with phosphate buffer (0.05 mM, pH 7.4) and stored in the buffer at +4 °C until further use. Reproducibility and reusability of the biosensor were determined by interpreting the amperometric responses.

Obtaining the calibration graphs

A series of gelatin-alginate-TiO_2-SOD biosensors were prepared, and amperometric measurements were recorded for varying xanthine concentrations at +0.65 V. The obtained current densities were plotted against xanthine concentration to obtain calibration curves and equations. The sensing limit and linear ranges were determined from the obtained graphs. During the calibration study, the electrodes were stored in the phosphate buffer (pH 7.4) at +4 °C until use.

Clinical application of gelatin-alginate-TiO_2-SOD biosensor

Cancerous meningioma (grade I) tissue was obtained from Ankara University, Department of Neurosurgery. Biosensor response was tested on healthy and cancerous brain tissues to demonstrate the usability of the developed biosensor in biological applications. After stabilizing the gelatin-alginate-TiO_2-SOD biosensors in the electrochemical cell containing phosphate buffer (0.05 M, pH 7.4), homogenized solutions containing healthy or cancerous tissue were added, and the changes in amperometric signal were determined.

RESULTS and DISCUSSION

Characterization of gelatin-alginate-TiO_2-SOD electrodes

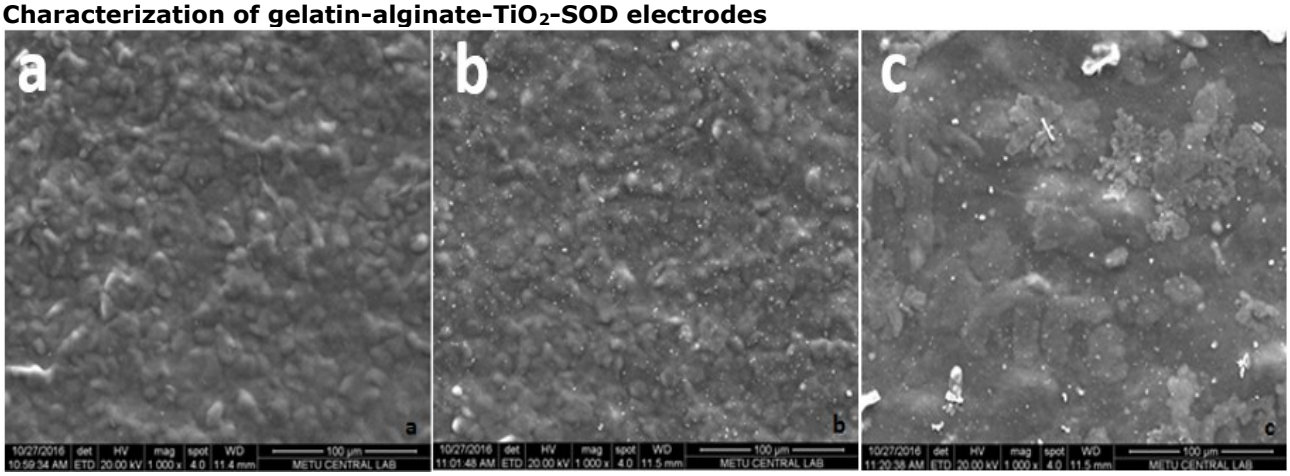

Figure 1. SEM images of gelatin-alginate (a), gelatin-alginate-TiO_2 (b), and gelatin-alginate-TiO_2-SOD (c) electrodes. Scale bars 100 µm.

The design of surface topography is one of the critical parameters for building a successful biosensor. SEM images were obtained in comparison with gelatin-alginate and gelatin-alginate-TiO_2 modified electrodes to examine the surface properties of the developed gelatin-alginate-TiO_2 electrode. It was seen in the micrograph that the gelatin-alginate biopolymer mixture covered the electrode surface properly (Figure 1a) and evaluated as a proper matrix in this regard. When TiO_2 NPs were included in the biopolymer mixture, microstructures were turned in to lower-scale microstructures (Figure 1b) due to surface enhancing property of nanoparticles, and resulted in a suitable nanocomposite material for biosensor design. Some nanoparticle aggregates were visible on this micrograph. When SOD enzymes included via cross-linking to the system, besides some little salt crystals, SOD enzymes characterized by leaf-shaped appearance seemed efficiently immobilized onto the surface (Figure 1c). Hence, it can be concluded that the gelatin-alginate-TiO_2-SOD electrode was successfully prepared, and in terms of surface properties, it seems suitable for biosensing applications.

Electrode modifications were also characterized by an electrochemical technique, namely electroimpedance spectroscopy (EIS). The Nyquist plots of the bare electrode, gelatin-alginate electrode, gelatin-alginate-SOD electrode, gelatin-alginate-TiO_2 electrode, and gelatin-alginate-TiO_2-SOD electrode were obtained for a Randles circuit model (Figure 2). Each EIS spectrum consists of a semicircle and a linear portion, which correspond to a charge transfer and diffusion process, respectively. The diameter of the semicircle is related to the charge-transfer resistance (R_{ct}) at the interface of the electrode surface and solution and can be used to evaluate the processes taking place on the electrode surface. Thus, in our strategy, change in R_{ct} values helps us to understand the success of modification steps. Unless conducting or nano-materials used, R_{ct} increases after electrode modifications due to the decreased electron transfer. While the charge transfer resistance of the bare electrode was nearly 25Ω (Figure 2a), this value increased to 56Ω when the Pt electrode was modified with gelatin-alginate composite, which shows the success of the modification (Figure 2b). SOD immobilization into gelatin-alginate composite hydrogel was further improved the R_{ct} from 56 Ω to 68 Ω (Figure 2c), indicating an efficient immobilization of SOD enzymes into the gelatin-alginate composite structure. Incorporation of TiO_2 NPs decreased R_{ct} of gelatin-alginate, 56 Ω, to 48 Ω (Figure 2d) as a result of both enhanced surface area owing to nano-size of the particles and their semi-conductive property. When SOD enzymes were immobilized into gelatin-alginate-TiO_2 nanocomposite structure, R_{ct} was increased from 48Ω to 66Ω (Figure 2e), which showed the success of the SOD immobilization. This increase in R_{ct} after SOD immobilization into gelatin-alginate-TiO_2 (about 18 Ω) was higher than that of SOD immobilization into gelatin-alginate (about 12 Ω), indicating a more efficient SOD immobilization in gelatin-alginate-TiO_2.

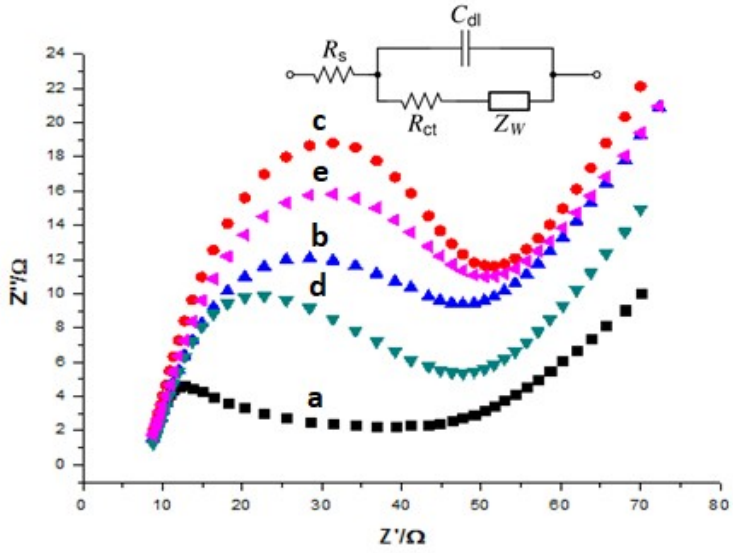

Figure 2. Nyquist Plot showing the change in charge transfer resistance after each modification step: (a) Bare electrode, (b) gelatin-alginate coated electrode, (c) gelatin-alginate-SOD coated electrode, (d) gelatin-alginate-TiO_2 modified electrode, and (e) gelatin-alginate-TiO_2-SOD electrode

Lastly, we obtained the Fourier-Transform Infrared (FTIR) spectra of gelatin-alginate-SOD and gelatin-alginate-TiO_2-SOD for further evaluate the effect of TiO_2 NPs on enzyme immobilization. Both spectra indicated the characteristic peaks of alginate and gelatin biopolymers (Figure 3). The peak near 1028 cm^{-1} was of C-O stretching vibration of pyranose rings constituting the alginate, while the peaks at 1530 and 1637 cm^{-1} related to amide I and II born of gelatin (24). Both spectra exhibited the peaks related to alginate and gelatin, which showed the success of composite preparation. Due to the chemically inert nature of TiO_2 NPs, no specific peak related to TiO_2 NPs was observed. When the spectra of gelatin-alginate-SOD and gelatin-alginate-TiO_2-SOD compared, a slight increase in amide I and II bands in the spectrum of gelatin-alginate- TiO_2-SOD can be seen clearly. This slight increase in amide I and II bands in gelatin-alginate- TiO_2-SOD can be attributed to a higher enzyme immobilization via amide bond formation between gelatin (NH_2-) and enzyme –via glutaraldehyde crosslinker –. This finding consolidated our previous findings and led us to conclude that including the TiO_2 NPs into the system improved enzyme immobilization.

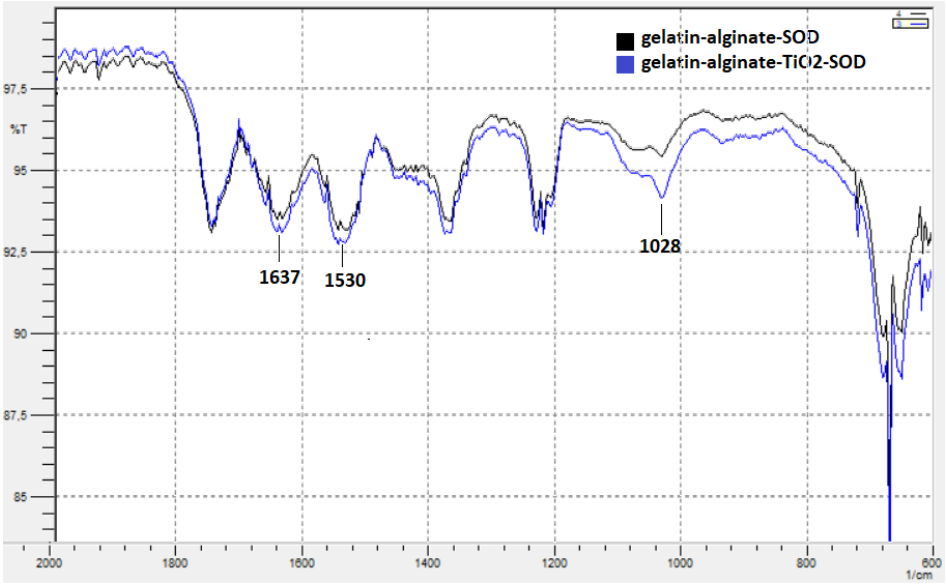

Figure 3. FTIR Spectra of gelatin-alginate-SOD (black) and gelatin-alginate-TiO_2-SOD (blue)

Optimization of gelatin-alginate-TiO_2-SOD biosensor

There are many parameters, namely biopolymer, crosslinker, nanostructure, and bio-recognizing element concentrations which affect biosensor performance, and need to be optimized before further experiments. To this end, we tested different ratios of gelation: alginate biopolymers (0.25-2 w/w) as well as different concentrations of GA cross-linker (0.001-0.016 M), SOD enzyme (25-5000 U), and TiO_2 NPs (0.0001-0.0005 g).

For the optimization of gelatin: alginate ratio, we prepared different electrodes with varying concentrations of gelatin: alginate ratio (0.25-2 w/w) by keeping the GA (0.01 M), SOD (100 U), and TiO_2 (0.0003 g) constant. The optimal gelatin: alginate ratio was found to be 1 (w/w) at which the most efficient amperometric response was recorded (Figure 4a). At higher ratios, as a result of the change in the pore structure/size and thus the reduced electron transfer, a decrease in sensitivity was observed. On the other hand, at the lower ratios (0.25-0.5 w/w), similar reduced amperometric responses were observed unlikely due to mechanical instability. For an efficient enzyme immobilization, different GA cross-linker concentrations (0.001-0.016 M) were tested by keeping the gelatin: alginate ratio (1 w/w), SOD (100 U), and TiO_2 (0.0003 g) constant. 0.004 M GA was found to be the optimal concentration (Figure 4b). The increase in GA concentrations led to more efficient enzyme immobilization, thus a more intense amperometric current as a result of larger electrochemical reactions. However, when we further increased the GA concentration, a decrease in amperometric response was observed mainly due to enzyme inactivation and reduced electron diffusion through electrode surface due to smaller pore size (Figure 4b) (23). The concentration of bio-recognizing elements, SOD enzymes, in this case, is another critical parameter for the electrochemical performance of enzymatic biosensors. Amongst different SOD concentrations (25-5000 U), the optimal value was determined as 100 U (Figure 4c) by keeping the gelatin: alginate ratio (1 w/w), GA (0.004 M), and TiO_2 (0.0003 g) constant. In lower SOD concentrations, lower current density was observed as a consequence of inadequate enzyme concentrations. In contrast, when we increased the SOD concentration over the optimal value, at this time, decreased current density was obtained due to enzyme-enzyme cross-linking in addition to over-saturation of pores with an excessive amount of enzymes that result in not-efficient diffusion of products and electrons through electrode surface (23). Finally, we optimized the amount of TiO_2 NPs, which were used to increase SOD immobilization and electron transfer rate by increasing the surface area. By keeping the gelatin: alginate ratio (1 w/w), GA cross-linker (0.004 M), and SOD (100 U) concentrations constant, we tested different amounts of TiO_2 NPs (0.0001 - 0.0005 g) and found the optimal value to be 0.0004 g (Figure 4d). Low amounts of nanoparticles were not able to increase the surface area as much as necessary, while too high amounts of nanoparticles completely covered the polymer surface, increased resistance, and inhibited electron transfer.

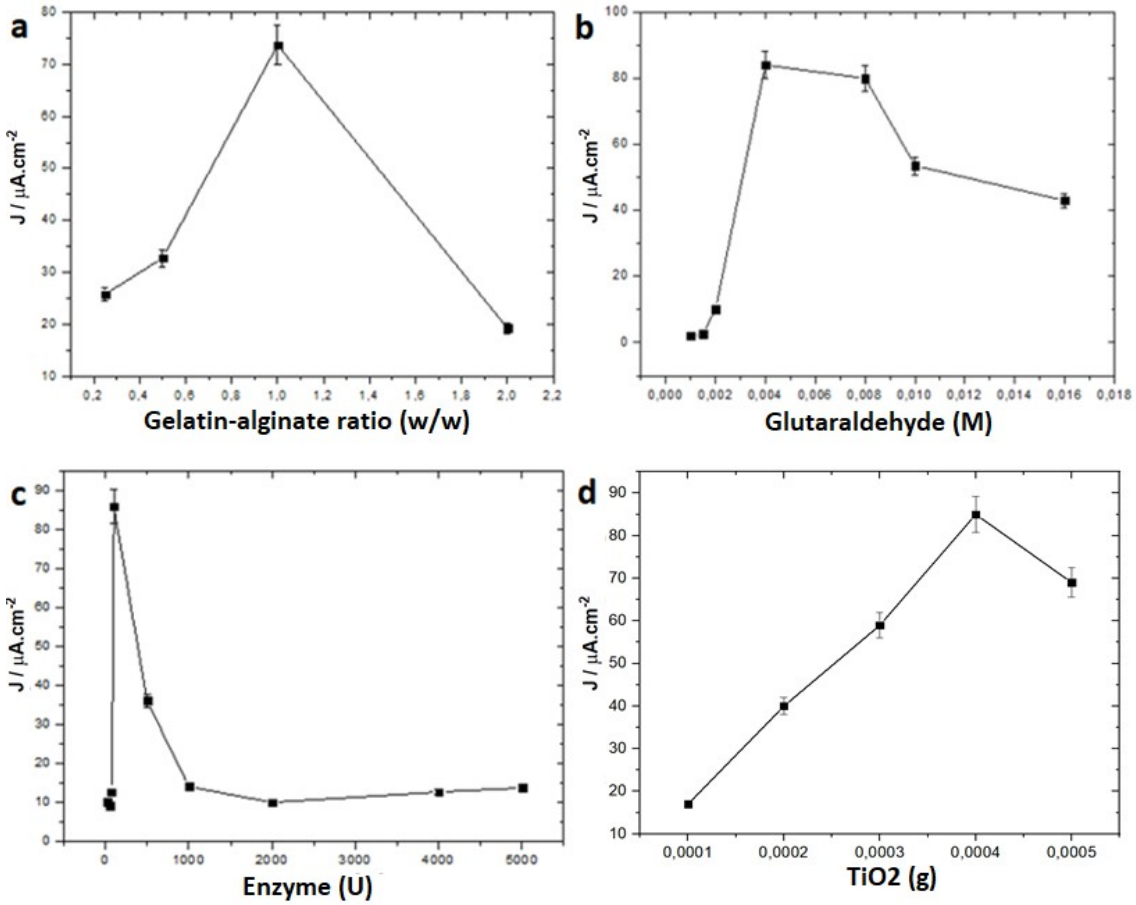

Figure 4. Effect of gelatin: alginate ratio (a), GA concentration (b), SOD concentration (c), and amount of TiO_2 (d) on the amperometric response of biosensor.

Reproducibility and reusability of the developed biosensor

Reproducibility and reusability are two parameters that are important in the practical use and commercialization of biosensors. To investigate the reproducibility and reusability potentials of the developed biosensor, we prepared 6 and 3 replicas, respectively, of a gelatin-alginate-TiO_2-SOD biosensor. For reproducibility study, after the 6 replica electrodes were stored at 4 °C for 1 day, amperometric measurements were performed by adding 2 mM xanthine into the electrochemical cell. Each measurement was performed triplicate. For the reusability study, amperometric measurements were recorded in the presence of 2 mM xanthine up to 10 days. After each use, electrodes were washed with phosphate buffer (pH 7.4) and stored in the refrigerator at 4 °C.

The mean value and standard deviation for amperometric measurements with 6 replica electrodes in the context of reproducibility study were found to be 67.5 μA.cm^{-2} and 5.8%, respectively (Figure 5a). This finding showed that gelatin-alginate-TiO_2-SOD electrodes are reproducible with trust. On the other hand, the amperometric response of the gelatin-alginate-TiO_2-SOD electrodes decreased to % 61.6 of its initial performance (Figure 5b). This finding suggests that the developed biosensor has short-term reusability, up to 7 times, after which the response go beyond 70%. One reason why the reusability of the gelatin-alginate-TiO_2-SOD electrode was not found better might be the distortive effect of NPs, which lead to detachment of nanocomposite film from the surface.

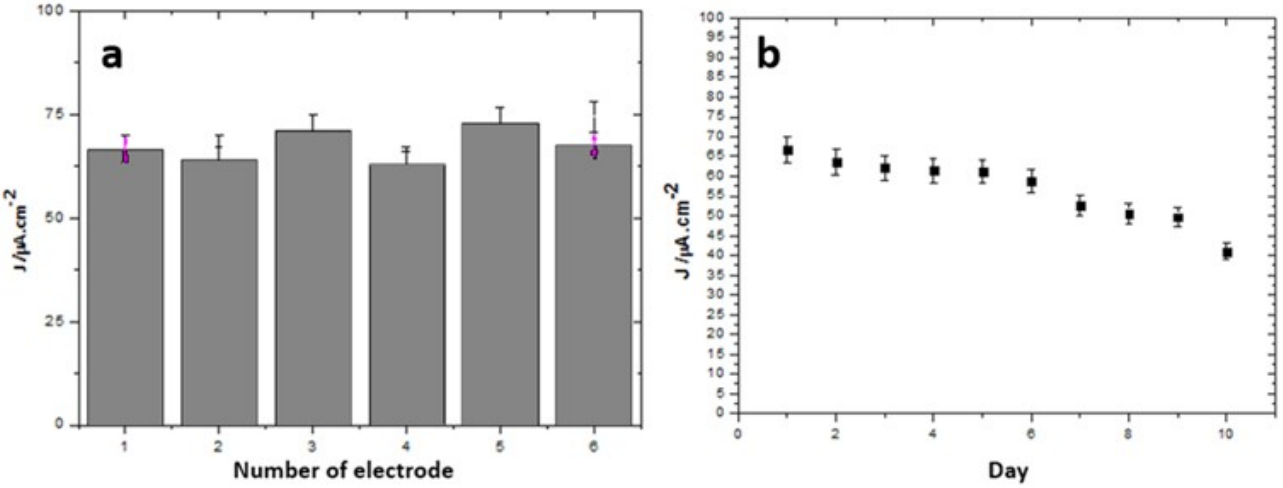

Figure 5. Reproducibility (a) and reusability (b) of gelatin-alginate-TiO_2-SOD electrodes.

Calibration study for gelatin-alginate-TiO_2-SOD electrode

Calibration study that defines the lower/higher measurement limits of a biosensor is another key step in biosensor design. To obtain calibration graph for gelatin-alginate-TiO_2-SOD electrode, we tested a broad range of xanthine concentration varying between 0.0009-2 mM. The amperometric current densities obtained within this range showed two linear graphs for 0.0250-2 mM (Figure 6a) and 0.0009-0.0125 mM (Figure 6b). Linear regression equations were expressed for gelatin-alginate-TiO_2-SOD electrode as [J, mM] = (-2.5) + (32.8) x [Xanthine, mM] for high xanthine concentrations (Figure 6a) and [J, mM] = (0.13) + (22.8) x [Xanthine, mM] for low xanthine concentrations (Figure 6b).

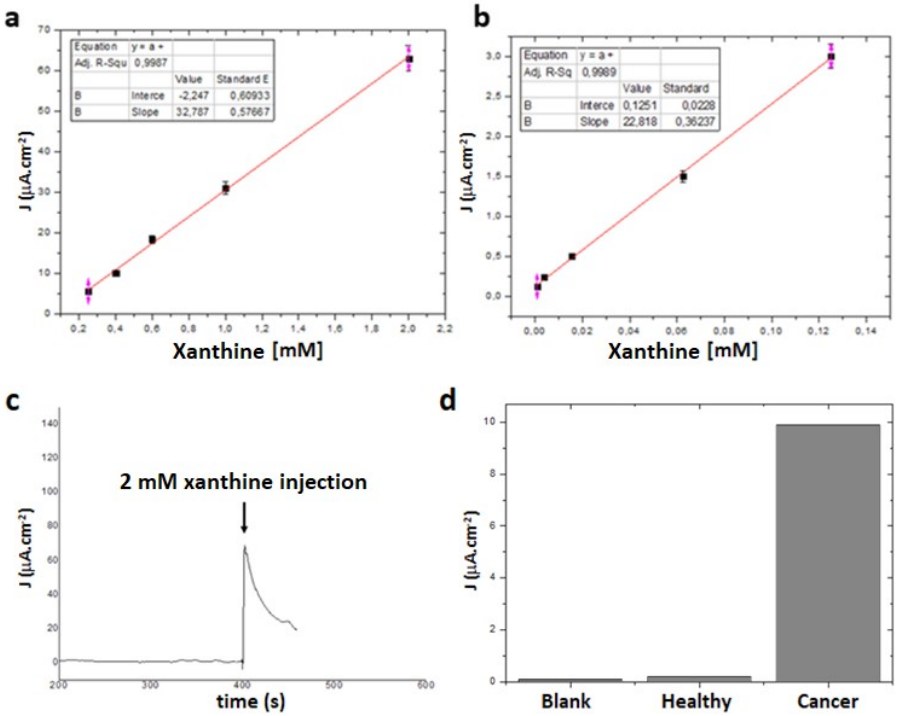

Figure 6. Calibration graphs of gelatin-alginate-TiO_2-SOD biosensor for high-range (a) and low-range (b) xanthine concentrations. (c) The amperometric response of gelatin-alginate-TiO_2-SOD biosensor after 2 mM xanthine injection. (d) Clinical application of gelatin-alginate-TiO_2-SOD biosensor.

The response of the electrode was proportional to the concentration of $O_2^{\bullet-}$ and the coefficient of determination (R^2) was found 0.9987 and 0.9989 for high concentration range and low concentration range, respectively. The detection limit for the low linear range was determined as 0.9 µM. The amperometric response of gelatin-alginate-TiO_2-SOD biosensor after injection of 2mM xanthin was demonstrated in Figure 6c.

Clinical application of gelatin-alginate-TiO_2-SOD biosensor

The ultimate goal of biosensor studies is to apply the developed systems in human clinics to contribute to human health by providing easy-to-use, practical, and low-cost diagnostic tools. To examine the potential of the developed biosensor to be applied in clinics, we tested the electrodes in healthy and cancer tissues to detect superoxide radicals. The gelatin-alginate-TiO_2-SOD biosensor exhibited nearly 10-times higher amperometric response to cancer tissue homogenate compared to healthy tissue (Figure 6d). This result confirmed our anticipation of the higher level of ROS in cancer cells and tissues compared to healthy ones (25, 26). Also, various groups utilized SOD-based biosensors for detecting superoxide radicals in cancer cells (27) and tissues (13, 28), and reported higher levels of superoxide radicals in the cancer samples. For instance, Campanella et al. (2000) has reported an approximately 15 times more intense amperometric response for cancerous renal tissue compared to healthy tissue (13). These reports support and consolidate our findings.

Various published works have lower or higher LOD and linear range compared to our design (Table 1). Our design exhibited a detection limit suitable to be used in superoxide detection; besides, it showed good performance when applied in real samples (cancer tissue). Consequently, the findings we obtained enabled us to conclude that the developed biosensor can be applied in cancer clinics after a comprehensive work, including repetitive biological trials.

Table 1. Comparison of gelatin-alginate-TiO_2-SOD sensors sensing with electrochemical (amperometry) with literature.

Electrode	LOD	Linear Range	Application	Reference
Gelatin-SOD	10.0 μM	0.02-2 mM	Cancer tissue	12
GC-PEDOT-CNT-SOD	1.0 μM	20-3000 μM	Wine/Juice	15
PtPd-PDA-RGO-SOD	2.0 μM	0.016-0.24 mM	-	29
GC-MPA-MnSOD	91.1 μM	135.2-1160 μM	-	30
Au-Fc-PEG-SOD	0.001 μM	5-100 μM	Cancer cells	16
Gelatin-Alginate-TiO_2-SOD	0.9 μM	0.0009-2 mM	Cancer tissue	This work

GC: Glassy Carbon, PEDOT: Poly(3,4-ethylenedioxythiophene), CNT: Carbon Nanotube, PDA: Polydopamine, RGO: Reduced Graphene Oxide, MPA: 3-mercaptopropionic acid, PEG: Polyethylene glycol, Fc: Ferrocene.

CONCLUSION

Superoxide radicals take place in many biological processes such as cancer, and their detection might be crucial in particularly cancer cases. In this work, it was aimed to develop a sensitive biosensor which uses gelatin-alginate-TiO_2 nanocomposite as an electrode material, and SOD enzyme as a biorecognizing element. After optimization and calibration studies, the developed sensor found suitable for detecting $O_2^{\bullet-}$ in clinical samples. The developed biosensor might be a promising candidate to be applied in cancer clinics.

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