Research Article

Angélica Domínguez-Aragón, Rocio B. Dominguez, María del Rosario Peralta-Pérez, and Erasto Armando Zaragoza-Contreras*

Catalase biosensor based on the PANi/cMWCNT support for peroxide sensing

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Abstract: Polymeric-based composites can contribute to enhancing the detection, stability, and performance of enzymatic biosensors, due to their high structural stability, conductivity, and biocompatibility. This work presents the fabrication of a nanocomposite of polyaniline (PANi)/gold nanoparticles (AuNP)/carboxylated multiwalled carbon nanotubes (cMWCNT) as functional support for covalently linked catalase (CAT) enzyme. PANi was electropolymerized on a screen-printed carbon electrode (SPCE) and decorated with AuNP to improve charge transfer properties. CAT was bonded through amide formation using the carboxylic groups of cMWCNT, resulting in PANi/AuNP/cMWCNT/CAT biosensor. The structural and electroactive characteristics of the nanocomposite were studied by SEM, FT-IR, and cyclic voltammetry. The optimal performance was achieved after CAT immobilization over PANi/AuNP/cMWCNT/nanocomposite, showing improved analytical features such as a fast amperometric response of 1.28 s, a wide detection range from 0.01 to 6.8 mM, a correlation coefficient (R^2) of 0.9921, a low detection limit of 2.34 μ M, and an average recovery rate

of 99.6% when evaluated in milk samples. Additionally, the bioelectrode showed excellent selectivity and retained bioactivity after 30 days of storage. Such remarkable performance proved the synergistic effects of both the high surface area of the cMWCNT and AuNP and the inherent PANi electroactivity, yielding direct electron transfer from CAT.

Keywords: biosensor, catalase, hydrogen peroxide, nanocomposite, polyaniline

1 Introduction

Enzyme-based electrochemical biosensors have been the subject of extensive research due to their promising applications in food safety, clinical diagnosis, health-care, and environmental monitoring (1). Compared with typical analytical techniques, biosensors offer advantages such as portability, low cost, and facility of use; however, they can suffer from poor stability and limited sensitivity that might affect the analytical performance (2). Commonly, enzymatic biosensors rely on carbon electrodes modified with high-performance nanomaterials like metal nanoparticles, graphene, or conductive polymers that allow enzyme linking and facilitates electron transfer between the enzyme redox center and the electrode. It is expected that the nanostructured support enhances the kinetics and chemical stability of the enzyme (3,4). The design of polymeric-based composites as enzymatic supports contributes to enhancing the detection, stability, and performance of biosensors because of their unique properties at the nanoscale such as high structural stability, conductivity, and biocompatibility (5). In this sense, polyaniline (PANi) has been incorporated in enzymatic biosensors due to its attractive features such as low cost, facile synthesis, conductivity, environmental stability, and biocompatibility, functioning as the transducer, mediator for electron transfer, or immobilization matrix (6). PANi, also, is an effective electroactive platform for enzymatic biosensor design due to its tunable redox properties, which facilitate the charge

* **Corresponding author: Erasto Armando Zaragoza-Contreras**, Departamento de Ingeniería y Química de Materiales, Centro de Investigación en Materiales Avanzados, S.C., Miguel de Cervantes No. 120, CP 31136, Chihuahua, Chih., Mexico, e-mail: armando.zaragoza@cimav.edu.mx, tel: +52-614-439-4811, fax: +52-614-439-1130

Angélica Domínguez-Aragón: Departamento de Ingeniería y Química de Materiales, Centro de Investigación en Materiales Avanzados, S.C., Miguel de Cervantes No. 120, CP 31136, Chihuahua, Chih., Mexico

Rocio B. Dominguez: Departamento de Ingeniería y Química de Materiales, CONACyT-Centro de Investigación en Materiales Avanzados, S.C., Miguel de Cervantes 120, CP 31136, Chihuahua, Chih., Mexico

María del Rosario Peralta-Pérez: Facultad de Ciencias Químicas, Universidad Autónoma de Chihuahua, Campus Universitario #2, Circuito Universitario, CP 31125, Chihuahua, Chih., Mexico
ORCID: E. Armando Zaragoza-Contreras 0000-0003-3010-556X

transfer process from the redox enzyme's catalytic site to the polymeric structure (7). Furthermore, PANi is capable of immobilizing enzymes through different methods such as physical adsorption, covalent linking, or entrapping (8), resulting in an ideal component for developing electrochemical bioelectrodes.

Although the electrochemical properties of PANi are suitable for electrochemical detection, incorporation of electroactive entities into PANi, such as gold nanoparticles and carbon nanotubes, has been shown to allow synergistic effects on improved surface area and conductivity, improving the electrochemical performance of the platform with a faster response (7,9). Thus, to extend the PANi characteristics and tune its performance, composites based on PANi and AuNP have been studied, considering that the high surface to volume ratio of AuNP is highly beneficial for loading biomolecules (10). Besides, AuNP improves the electron transfer rate between the active centers of the immobilized enzyme and the electrode, resulting in enhanced performance (11). In addition, the combination of PANi with carbon-based components such as MWCNT provides advantages to the sensing systems such as chemical stability, enhanced mechanical properties, extensive surface area, and high conductivity (12).

Additionally, MWCNTs can act as an immobilization nano-matrix for covalent coupling through the induction of carboxylic acid groups and the reaction with the free amino groups on the enzyme (13). These modifications facilitate charge transfer and conductivity of the sensing platform (3,14), stabilizing the PANi and favoring retention of its inherent electrochemical activity, which provides more active nucleation sites, contributing, in conjunction, to improve biosensor performance (12).

Heme enzymes, including peroxidase, catalase, cytochrome c, hemoglobin, and myoglobin are involved in several fundamental biological processes. The redox chemistry of the heme iron as an active site and the biochemical diversity has led to the application as biological recognition elements in electrochemical biosensors for H_2O_2 and phenolic compounds (15).

Catalase, from the bovine liver (CAT, EC 1.11.1.6), is an essential oxidoreductase enzyme (16), with four equal subunits of 60 kDa molecular weight and a heme prosthetic group at its active site with metallic iron $Fe(III)$ (17). CAT is present in almost all aerobically respiring organisms, protecting cells from the toxic effects of hydrogen peroxide (H_2O_2) by decomposing it into oxygen and water without the formation of free radicals (18). According to Lonča and Fraaije (19), catalases are robust enzymes with unusually high catalytic rates and among the highest enzymatic rates. Therefore, CAT is used in diverse industrial applications as an effective H_2O_2 removal tool.

The fast, reliable, and accurate determination of H_2O_2 is of great interest given its substantial role in food, pharmaceutical, clinical, industrial, and environmental analyses; additionally, H_2O_2 is a biomarker of some cells and tumors (20). H_2O_2 , also, is widely used as an antiseptic agent for preserving milk in the dairy industry. It inhibits microorganism growth and milk spoilage during the storage period. The use of H_2O_2 must not exceed 0.05% of the milk weight and is approved in the USA by the food and drug administration (FDA) for this function (21). The excess of residual H_2O_2 is harmful to humans; Hence, determining H_2O_2 in food and biological systems is very important in health and product pre-treatment (22). Therefore, novel devices and configurations for sensitive H_2O_2 detection are of significant attention like $BiVO_4$ /fluorine-doped tin oxide (23) and graphene oxide-poly(amidoamine) dendrimer- Fe^{2+} /GCE (24). Also, some authors have designed H_2O_2 biosensors using CAT as the bioreceptor, linking the CAT with supports like chitosan, single-walled carbon nanotubes, and polyethyleneimine (18). Furthermore, CAT has excellent stability and could be an excellent choice for biosensor design, where shelf stability is important (25).

Because of the interesting properties of CAT as a biocatalyst and the efficient operation of PANi-cMWCNT-based immobilization supports, the development of an electroactive platform capable of both enzyme immobilization, and measure biological interaction between CAT and H_2O_2 by the electrochemical method is proposed. This work reports the construction of an amperometric bioelectrode departing from a screen-printed carbon electrode modified with electropolymerized PANi, which was subsequently optimized with AuNP and carboxylated MWCNT (cMWCNT). The PANi/AuNP/cMWCNT nanocomposite was applied as functional support for covalent immobilization of CAT used as a bioreceptor and exploited for quantifying H_2O_2 in PBS and milk samples. The assembled nanocomposite showed high retention of enzyme activity, resulting in improved analytical parameters as accuracy, sensitivity, and low limit of detection (LOD), as well as favorable long-term stability, which evidenced the beneficial synergistic effects of the assembled nanocomposite in the biosensor performance.

2 Materials and methods

2.1 Materials and reagents

Aniline, *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), chloroauric acid ($HAuCl_4$),

and CAT from bovine liver (EC 1.11.1.6, activity 5,000 units per mg) were obtained from Sigma-Aldrich. Sulfuric acid (H_2SO_4), nitric acid (HNO_3), and hydrogen peroxide solution (30 wt% aqueous) were provided from Acros Organics. Phosphate buffer solution (PBS) (0.01 M, pH 7.4) was prepared from sodium chloride (NaCl), potassium chloride (KCl), and disodium hydrogen phosphate (Na_2HPO_4), and potassium dihydrogen phosphate (KH_2PO_4) was used as the supporting electrolyte. Whole-fat milk was bought from a local supermarket and analyzed without prior treatment, and the screen-printed carbon electrodes (SPCE) were obtained from Metrohm.

2.2 Characterization

Cyclic voltammetry (CV) and amperometry measurements were measured using an EmStat3+blue potentiostat, whereas electrochemical impedance spectroscopy (EIS) was performed using a Solartron 1260A potentiostat/galvanostat. Fourier-transform infrared spectroscopy (FTIR) was registered using Perkin-Elmer GX-FTIR equipment, and the morphology was analyzed with a JEOL 300-S scanning electron microscope (SEM). FTIR and SEM characterized each stage of electrode modification and enzyme immobilization.

2.3 Assembling of PANi/AuNP/cMWCNT

MWCNTs were obtained according to a previous procedure and subsequently carboxylated by acid treatment (26). Briefly, 50 mg of MWCNT was added in a solution of 10 mL of H_2SO_4 and HNO_3 in a 3:1 volume ratio; the mixture was sonicated for 1 h to obtain a fine dispersion and then refluxed at 90°C for 1 h. The resulting carboxylated multiwalled carbon nanotubes (cMWCNTs) were filtered and washed until neutral pH was achieved and dried at 60°C for 12 h; finally, FTIR was used to verify carboxylation.

A solution of aniline (0.25 M) in H_2SO_4 (0.25 M) was used for electropolymerization onto the SPCE by CV, applying ten cycles at a potential from -0.5 to 1.0 V at a rate of 50 mV s^{-1} . The resulting SPCE/PAni was immersed in a mixture of HAuCl_4 (1 mM) in H_2SO_4 (0.25 M) to perform AuNP electrodeposition. The conditions were three cycles at a potential from -0.5 to 1 V and a rate of 20 mV s^{-1} , obtaining the SPCE/PAni/AuNP. Then, $4 \mu\text{L}$ of cMWCNT (1 mg mL^{-1}) were dropped cast on SPCE/PAni/AuNP and dried at room temperature. Finally, the modified surface was rinsed with distilled water to remove loose

material, obtaining the SPCE/PAni/AuNP/cMWCNT functional support.

2.4 Catalase immobilization

CAT was covalently immobilized onto SPCE/PAni/AuNP/cMWCNT, using the EDC/NHS chemistry. First, the COOH groups of the cMWCNT were activated by casting $4 \mu\text{L}$ of a solution of EDC (20 mM) and NHS (20 mM) in PBS (0.01 M) over the electrode surface at room temperature. Finally, $5 \mu\text{L}$ of CAT (5 mg mL^{-1}) were deposited on the modified electrode and allowed to dry at room temperature. The electrode was washed with PBS to remove unbound enzymes, obtaining the SPCE/PAni/AuNP/cMWCNT/CAT biosensor. Figure 1 illustrates the fabrication stages, which were characterized by FTIR, SEM, CV, and EIS.

2.5 Electrochemical measurements

The quantitative detection of H_2O_2 was performed using PBS (0.01 M) as the supporting electrolyte (pH 7.4) at 25°C. Before measurements, the PBS was deoxygenated by bubbling nitrogen for 10 min to protect the solution from oxygen. CV was evaluated with concentrations from 0.1 to 4 mM, using a potential from -1 to 1 V and a scan rate of 20 mV s^{-1} . The amperometric response of the modified electrode toward H_2O_2 was determined by applying a constant potential of -0.4 V in 25 mL of PBS at 0.01 M. During the measurements, the solution was kept under continuous magnetic stirring. After reaching a steady-state, a given amount of H_2O_2 solution was added to the PBS electrolyte and the change in current was recorded. Measurements were conducted by triplicate under the same conditions.

Finally, the proposed biosensor was used to determine H_2O_2 in spiked samples of whole milk purchased at a local supermarket. The milk samples were used as they came from the packing. Known concentrations of H_2O_2 were spiked using the whole milk as a matrix. The determination of H_2O_2 in the milk samples was carried out by amperometry.

3 Results and discussion

3.1 Morphology

SEM allowed corroborating the surface modifications at each stage of biosensor construction. Figure 2a portrays

Figure 1: Scheme of step by step preparation of the SPCE/PAni/AuNP/cMWCNT/CAT biosensor.

the electropolymerized PAni, showing fibrillar morphology with a diameter of approximately 200 nm and a few micrometers in length. Similar morphology of polyaniline was attributed to the electropolymerization process that promotes fiber formation (27). Figure 2b illustrates the second stage of modification with the PAni surface decorated by

AuNP. The particles are embedded in the surface of the PAni fibers, showing good dispersion and spherical morphology of around 50 nm (inset). The third stage of fabrication includes the cMWCNT attached to the PAni/AuNP (Figure 2c), creating a porous network structure that is highly convenient for catalase immobilization. Figure 2d

Figure 2: SEM images of (a) SPCE/PAni, (b) SPCE/PAni/AuNP, (c) SPCE/PAni/AuNP/cMWCNT, and (d) SPCE/PAni/AuNP/cMWCNT/CAT.

shows CAT immobilization on SPCE/PAni/AuNP/cMWCNT, with the enzyme forming a thin layer over the cMWCNT as seen in the inset. The change in morphology revealed a successful enzyme immobilization onto the support, in agreement with previous observations for CAT binding (28).

3.2 Functional group characterization

The reaction of the strong acids with the unstable carbon atoms of the MWCNT generated COOH groups (29). The spectrum of the cMWCNT (Figure A1 in Appendix) shows absorption bands at 3,298 and 1,727 cm^{-1} , which correspond, respectively, to the stretching vibrations of the O–H and C=O bonds of the carboxyl group. The band at 1,627 cm^{-1} corresponds to the skeletal vibrations of the C=C bond of cMWCNT (30). Thus, the presence of these bands confirmed carbon nanotube's carboxylation, which has been reported for equivalent treatments (29).

Figure 3a shows the spectrum of PAni/AuNP/cMWCNT showed the bands at 3,298, 1,727, and 1,627 cm^{-1} , as described for the cMWCNT. Besides, a pair of bands at 1,542 and 1,463 cm^{-1} , attributed, respectively, to the stretching vibrations of the C=C bond of the quinoid ring and the benzenoid ring, confirmed the PAni electropolymerization (31). The band at 1,370 cm^{-1} was related to the C–N stretching vibration in the neighborhood of a quinoid ring (32), suggesting that the π -bonded surface of the carbon nanotubes might interact actively with the conjugated structure of the PAni, primarily through the quinoid rings (33).

Figure 3: FTIR spectra of (a) SPCE/PAni/AuNP/cMWCNT, (b) SPCE/PAni/AuNP/cMWCNT/CAT.

Figure 3b illustrates the spectrum of PAni/AuNP/cMWCNT/CAT. As seen, peaks are present at 1,640 and 1,572 cm^{-1} , these bands correspond on amide I and amide II bands, respectively (34). These peaks are located close to the amides bands of catalase (see Figure A2). According to Zuccarello *et al.* (15), the alterations of the relative intensities, positions, and shapes of the amide I and amide II bands of the enzyme indicate structural changes upon immobilization; Thus, these bands might be attributed to the formation of an amide bond between the NH_2 groups of CAT and the COOH groups of cMWCNT. In addition, the band at 1,727 cm^{-1} practically disappeared, and the band at 3,298 cm^{-1} showed a considerable intensity decrease, which was also associated with the interaction between COOH and NH_2 groups, confirming the covalent link of CAT and cMWCNT.

Similar behavior was reported by Chawla *et al.* (35) and Rawal *et al.* (36), where they correlated the covalent immobilization of the enzyme laccase on a modified electrode containing cMWCNT through the disappearance of the COOH band and the appearance of the amide bands.

3.3 Electrochemical characterization

The voltammogram of PAni, Figure 4a, showed the typical oxidation peaks due to the transition from leucoemeraldine to emeraldine and from emeraldine to pernigraniline (37), and only one cathodic peak was observed. This behavior may be attributed to the neutral pH and the saline phosphate buffer used as electrolyte support. According to Mažeikien *et al.* (38), the redox activity depends not only on the pH but also on the composition of the electrolyte solution. They found that the redox properties of sulfonated polyaniline were reduced in sodium salt solutions due to the exchange of hydrated sodium cations between the electrolyte solution and the polymer film during the electrochemical process, producing a less effective transition between the oxidation states of PAni. However, the electropolymerized PAni used in this work showed the ability to undergo an electrochemical redox process even in neutral pH, which is suitable for this application.

After the AuNP electrodeposition, the current intensity of the anodic peaks increased, suggesting the increase of the active surface of PAni and the formation of a more effective surface area as a charge transfer mediator (39). For PAni/AuNP/cMWCNT, the high surface area of cMWCNT and their tubular structure generated a more energetically favorable pathway for the charge transfer, improving the inherent PAni electroactivity (3).

Figure 4: (a) Cyclic voltammetry and (b) electrochemical impedance spectroscopy of (i) SPCE/PAni, (ii) SPCE/PAni/AuNP, (iii) SPCE/PAni/AuNP/cMWCNT, and (iv) SPCE/PAni/AuNP/cMWCNT/CAT biosensor.

Finally, the biosensor showed a small decrease in the electroactive area, indicating a slower electron transfer at the electrode surface by the immobilized enzyme. This confirmed the enzyme binding over the PAni/AuNP/cMWCNT functional support while retaining the electroactivity of the system. Similar behavior displayed the insulating property of the protein layer (40).

Figure 4b illustrates the charge transfer at the electrode interface determined by EIS. The semicircle diameter at the high-frequency region represents the resistance charge transfer (R_{ct}), caused by electrochemical reactions in contact with the interface between the electrode and the electrolyte, and the linear part of the low-frequency region corresponds to the diffusion process (41). Figure 4b shows the Nyquist plots of the electrodes at each modification stage. As seen, the R_{ct} of PAni was more extensive than that of PAni/AuNP and PAni/AuNP/cMWCNT,

suggesting that the electrode modified by AuNP and cMWCNT decreased the R_{ct} and promoted the electron transfer between the electrolyte and the modified electrode. These results agreed with CV; consequently, the cMWCNT not only played the role of the functional nano-matrix for the enzyme immobilization but also improved the electroactivity and charge transfer capacity in the nanocomposite. The R_{ct} of the biosensor increased in comparison with the previous stage due to the non-conductive nature of CAT. Another study attributed a similar behavior because the electrode surface suffers from blockage by the poorly enzyme conductivity (42).

3.4 Direct electron transference of catalase

Figure 5a displays the CV of CAT immobilized on PAni/AuNP/cMWCNT in PBS 0.01 M solution at a scan rate of 20 mV s^{-1} . PAni/AuNP/cMWCNT/CAT exhibited a highly defined cathodic peak at -0.52 V and an anodic peak of lower intensity not well defined at -0.26 V , suggesting that the intrinsic electroactivity of PAni overlapped it. These redox peaks were attributed to the heme group $\text{Fe}^{\text{III/IV}}$ from the CAT enzyme (43). To corroborate the direct electron transfer from CAT to PAni/AuNP/cMWCNT, CAT was immobilized on the nanocomposite without PAni to verify the overlap of the anodic peak. The AuNP/cMWCNT/CAT revealed a cathodic peak, at -0.4 V and a well-defined anodic peak at -0.22 V (Figure 5a, inset), corroborating the overlapping. The cathodic peak intensity was considerably more prominent than the anodic peak in both voltammograms, attributed to the conformational changes of CAT due to the immobilization process (44). This behavior was comparable with recent observations with catalase, where the presence of the redox peaks of the heme group from catalase was taken as evidence of the direct electron transfer (45).

Thus, the nanocomposite properties, such as high surface area, improved electroactivity, conductivity, and functional sites for covalent linking, lead to good electron transfer communication between the center of CAT with the modified PAni/AuNP/cMWCNT electrode.

3.5 Electrocatalytic behavior of CAT-modified electrode toward H_2O_2

The catalytic activity of the nanocomposite for each step of modification toward H_2O_2 was evidenced by CV. Figure A3

Figure 5: (a) Cyclic voltammetry of (i) SPCE/PAni/AuNP/cMWCNT/CAT and (ii) SPCE/AuNP/cMWCNT/CAT in PBS 0.01 M at 20 mV s^{-1} . (b) Cyclic voltammetry for increasing concentrations of H_2O_2 in PBS 0.01 M, recorded using the SPCE/PAni/AuNP/cMWCNT/CAT biosensor at a scan rate of 20 mV s^{-1} .

shows the voltammograms of each electrode modification in the presence of 2 mM of H_2O_2 . The modifications without CAT presented a broad reduction peak around -0.75 V , which is attributed to reducing O_2 generated by the applied potential in the modified electrode at the H_2O_2 solution (46). While PAni/AuNP/cMWCNT/CAT exhibited higher current intensity due to a cathodic peak at -0.4 V , such response is a probe of the enhanced catalytic activity from the anchored enzyme. Thus, incorporating the CAT in the nanocomposite led to an improved response towards H_2O_2 with less potential needed for the electron transfer process. Figure 5b displays that the cathodic peak at -0.49 V manifested a proportional increase in current as the H_2O_2 concentration raised. The CAT mechanism to H_2O_2 was proposed by Alfonso-Prieto *et al.* (47). According to this mechanism, CAT catalyzes the decomposition of H_2O_2 into water and oxygen ($2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$). Equations 1 and 2 represent the enzymatic reaction of CAT to H_2O_2 .

The active species responsible for oxidation and/or oxygenation reactions is a high-valent iron intermediate, oxoferryl porphyrin cation radical ($\text{Por}^{\cdot+}\text{-Fe}^{\text{IV}}=\text{O}$, Cpd I) obtained by the reaction with H_2O_2 . Once Cpd I is formed (Eq. 1), it immediately reacts with a second molecule of

H_2O_2 , reducing Cpd I to regenerate CAT ($\text{Por-Fe}^{\text{III}}$) with water and oxygen as by-products (Eq. 2). The reaction involves a two-electron redox process (48); therefore, the linked CAT over the support provided a fast direct electron transfer reaction between the heme group of CAT and the modified electrode surface and, consequently, an electrocatalytic activity toward H_2O_2 .

It is worth saying that all components of the composite play a very important role in the electrochemical platform. The electrochemical characterization demonstrated that both cMWCNTs and AuNP helped to increase the active surface of PAni, improving the inherent PAni electroactivity. But also the incorporation of PAni on the nanocomposite is essential since it helps to maintain the direct electron transfer from the CAT (Figure A4). The nanocomposite without PAni presented a definition loss of the cathodic peak of CAT as the concentration of H_2O_2 increased. Whereas, with the presence of PAni, the cathodic peak of CAT remained defined. This behavior could be attributed to the biocompatible properties of PAni, which contribute to an optimal environment for enzyme performance. Hence, besides working as a transducer surface, the PAni could anchor some enzymes by physical adsorption or covalent linkage (6). Thus, a synergistic effect was formed in conjunction with the cMWCNTs and AuNP protecting the native structure of CAT and maintaining the accessibility of the catalytic sites and, therefore, facilitated the direct transfer of the CAT center to the electrode surface.

3.6 Amperometric determination of H_2O_2

According to the response observed in CV, the biosensor was evaluated for the detection of H_2O_2 by amperometry at -0.4 V. Figure A5 shows the amperometric response toward H_2O_2 at every stage of the nanocomposite assembly. After the modification of the SPCE with PANi and PANi/AuNP, a small current was recorded at the selected potential. However, PANi/AuNP/cMWCNT nanocomposite showed a clear improvement due to the electrocatalytic activity of cMWCNT toward H_2O_2 . This step improved the electron transfer rate enabling the H_2O_2 reduction through electron transfer, which is the principle of non-enzymatic sensors (49). Indeed, some non-enzymatic amperometric sensors for H_2O_2 detection have been developed (50–52). Although these kinds of sensors show good electrochemical response and have the advantage of avoiding enzymatic susceptibility, they usually need a higher potential for H_2O_2 reduction.

With the immobilization of CAT, it was evident that the biosensor exhibited the best amperometric response for H_2O_2 detection. Furthermore, the incorporation of CAT into the nanocomposite led to an improved response toward H_2O_2 with less working potential needed for the electron transfer process. The electrode modified with PANi/AuNP/cMWCNT exhibits an enhanced catalytic activity toward H_2O_2 with the CAT loading, corroborating the results obtained by CV. The direct electron transfer between the heme group of CAT and the electrode surface significantly improved the device sensitivity because of the effective covalent linkage of CAT and the synergistic

effects of the PANi/AuNP/cMWCNT support. Hence, CAT immobilization was critical in the biosensor assembly to enhance the amperometric response.

The biosensor capacity was evaluated by amperometry with successive additions of H_2O_2 at -0.4 V under optimized conditions. Figure 6a and b illustrates the i versus t curve and the calculated calibration curve ($n = 3$), respectively. The current as a function of H_2O_2 concentration showed a linear response of i to the increasing level of H_2O_2 , with $R^2 = 0.9921$. The biosensor exhibited a linear range from 0.01 to 6.8 mM, a sensitivity of $58.8 \mu\text{A mM}^{-1} \text{cm}^{-2}$, and a LOD of $2.34 \mu\text{M}$ ($\text{LOD} = 3.3 \cdot \sigma/S$, where σ and S are the standard deviation and sensitivity, respectively). Notably, the device showed a fast amperometric response after only 1.28 s.

Table 1 reports a comparison of electrocatalytic values of some electrochemical (bio)sensors for H_2O_2 . The comparative data showed that the biosensor properties are comparable or even better concerning previously reported catalase biosensors, especially considering LOD. For instance, the biosensor based on CAT/Fe@G-MWCNTs showed a similar linear range, but higher LOD (45). In another report, CAT immobilized onto an inorganic hybrid microflower HMFs ($\text{Cu}_3(\text{PO}_4)_2$)-modified GCE exhibited a wide linear concentration range but higher LOD (53). Similarly, the CAT/PNR_{Ethaline}/Fe₂O₃NP/GCE biosensor showed a higher LOD and smaller linear range (54). It is worth saying that the notable performance of the PANi/AuNP/cMWCNT/CAT biosensor expressed the synergistic effects of both the high surface area of the cMWCNT and AuNP and the PANi inherent electroactivity.

Figure 6: (a) Chronoamperometry response for successive H_2O_2 additions recorder using SPCE/PANi/AuNP/cMWCNT/CAT in PBS 0.01 M at -0.4 V. (b) Calibration curve.

Table 1: Comparison of electroanalytical parameters of electrochemical (bio)sensors for H₂O₂ detection

Electrode	LOD (μM)	Linear range (mM)	Sensitivity	k_m (mM)	Reference
CAT/Fe@G-MWCNTs	28.2	0.1–7	0.059 μA mM ⁻¹ cm ⁻²	17.9	(45)
PEI/CAT/MWCNT-NF/GCE	1	0.01–5	30 μA mM ⁻¹	3	(55)
CAT-BOX/GAD/MWCNTs/GCE	54.4	0.05–0.99	21.34 μA mM ⁻¹	2.5	(56)
CAT/MWCNT/Nafion/thionine	8.7	0.01–0.1	6051.0 μA mM ⁻¹ cm ⁻²	—	(57)
CAT-HMFs/GCE	50	1–10	3.471 μA mM ⁻¹	—	(53)
Cat/PNR _{Ethaline} /Fe ₂ O ₃ NP/GCE	4.3	0.01–0.150	25.1 μA mM ⁻¹ cm ⁻²	0.13	(54)
BiVO ₄ /FTO	8.5	0.05–1.5	—	—	(23)
GO-PAMAM-Fe ²⁺ /GCE	0.18	0.0005–2	2.71 μA μM ⁻¹ cm ⁻²	—	(24)
HRP-NpAc-IL/MWCNT/GCE	2.7	0.01–2.07	55.98 μA mM ⁻¹ cm ⁻²	—	(58)
HRP/AgNPs/PANI/G/GCE	30	0.25–2.25	7.46 μA mM ⁻¹	5.03	(59)
CAT/cMWCNT/AuNP/PAni/SPCE	2.34	0.01–6.8	58.83 μA mM ⁻¹ cm ⁻²	0.808	This work

3.7 Kinetic study

When the concentration of H₂O₂ increases, a response plateau appears (Figure A6(a)), which is characteristic of the typical Michaelis–Menten kinetic behavior (60). To study the enzyme-substrate kinetics, the apparent Michaelis–Menten constant (k_m) was calculated from the linear dependence observed in Figure A6(b), using the electrochemical version of the Lineweaver–Burk, Eq. 3 (40):

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{k_m}{I_{max}} \cdot \frac{1}{C} \quad (3)$$

where I_{ss} is the steady-state current after the addition of H₂O₂, I_{max} is the maximum current measured under saturated conditions, and C is the bulk concentration of the substrate. The analysis of the reciprocals of the steady-state

current ($1/I_{ss}$) versus H₂O₂ concentration ($1/C$) yields a k_m of 0.808 mM. The low value of k_m indicated, by comparing with other configurations with catalase (Table 1), that the CAT immobilized onto PANi/AuNP/cMWCNT retains bioactivity and high affinity toward H₂O₂.

3.8 Sensor stability and specificity

Common interfering substances, such as glucose, ascorbic acid, KCl, and NaCl, were tested for confirming sensor selectivity. Figure 7a shows no significant responses for the evaluated species but a repeatable current response after H₂O₂ injection, confirming high biosensor selectivity toward the analyte. The biosensor was kept at 4°C and assessed for 30 days to evaluate stability (Figure 7b). After

Figure 7: (a) Selectivity and (b) stability of the SPCE/PAni/AuNP/cMWCNT/CAT biosensor.

storage, the biosensor retained almost 85% of the initial current. Such favorable long-term stability expressed the stable enzyme bonding with the cMWCNT at the electrode surface and the favorable biocompatible environment provided by the assembled nanocomposite.

3.9 Determination of H_2O_2 in real samples

H_2O_2 is widely used as an antiseptic agent for preserving milk in the dairy industry. For practical purposes, the standard addition method was used to determine H_2O_2 concentrations in commercial milk (whole fat milk) at PANi/AuNP/cMWCNT/CAT by amperometric method. The standard addition method (regularly denoted as “spiking” the sample) is commonly used to determine the analyte concentration in complex samples containing other components that could interfere with the analyte signal, causing inaccuracy determination. The idea is to spike the sample and monitor the amperometric response change, assuming the response is only due to a change in analyte concentration (61). The analysis was carried out by spiking a known concentration of H_2O_2 in the milk samples without prior treatment and recording their recoveries. Before analysis, a blank was verified without the addition of H_2O_2 in the milk sample, which presented no signal. For every sample, three individual measurements were performed.

Table 2 shows the excellent recovery rates obtained, in the range of 92–104.8%. Besides, the H_2O_2 concentrations used, 0.2, 0.5, and 0.7 mM correspond to 0.00068%, 0.0017%, and 0.0023%, respectively; The use of H_2O_2 is approved in the USA by the FDA for treating milk, and it cannot exceed 0.05% of the milk weight (21). Therefore, in terms of detecting peroxide in milk for quality control, concentrations smaller than the minimum required were achieved.

It should be noted that in this experiment, undiluted milk samples were used; the milk samples were used as they came from the packaging. This presents an advantage

since the peroxide was detected in milk without a previous dilution step. Other similar biosensors have been studied to detect peroxide in milk samples; however, they diluted the milk in PBS to perform the detection (62–64). These results evidenced the biosensor suitability for the analysis of H_2O_2 in real samples, confirming its practical application for food quality control.

4 Conclusion

Electrochemical characterization demonstrated that both cMWCNT and AuNP facilitated the increase in the active surface of PANi, and PANi helped maintain direct electron transfer from CAT. The assembly of a PANi/AuNP/cMWCNT platform manifested a synergistic effect as functional support for catalase immobilization allowed retaining the catalytic activity and facilitated the direct electron transfer between the CAT and the modified electrode. The biosensor exhibited notable electrocatalytic activity toward H_2O_2 with high sensitivity, low LOD, wide linear range, excellent recovery with real milk samples, and favorable long-term stability. Such performance evidenced the catalytic activity of CAT due to the effective covalent linkage on the support. Therefore, the electrode, modified by the PANi/AuNP/cMWCNT nanocomposite, is functional as support for covalent immobilization of enzymes, with high performance, sensitivity, and selectivity.

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Table 2: Detection of hydrogen peroxide spiked in real milk samples

Sample	Spiked (mM)	Found (mM)	Recovery (%)	RSD ^a (%)
Milk	0.2	0.184	92	1.51
	0.5	0.524	104.8	5.30
	0.7	0.715	102.1	6.03

^a Triplicates were performed.

relationships that could have appeared to influence the work reported in this paper.

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Appendix

Figure A1: FTIR spectra of (a) MWCNT and (b) cMWCNT.

Figure A2: FTIR spectra of catalase.

Figure A3: Cyclic voltammetry of (i) SPCE/PAni, (ii) SPCE/PAni/AuNP, (iii) SPCE/PAni/AuNP/cMWCNT, and (iv) SPCE/PAni/AuNP/cMWCNT/CAT biosensor in PBS 0.01 M in presence of 2 mM of H_2O_2 at 20 mV s^{-1} .

Figure A4: Cyclic voltammetry of (i) SPCE/PAni/AuNP/cMWCNT/CAT and (ii) SPCE/AuNP/cMWCNT/CAT in PBS 0.01 M in presence of H_2O_2 2 mM at 20 mV s^{-1} .

Figure A5: Amperometric response towards H_2O_2 addition on (i) SPCE/PAni, (ii) SPCE/PAni/AuNP, (iii) SPCE/PAni/AuNP/cMWCNT, and (iv) SPCE/PAni/AuNP/cMWCNT/CAT biosensor.

Figure A6: (a) Amperometric response of the SPCE/PAni/AuNP/cMWCNT/CAT biosensor towards successive addition of H_2O_2 and (b) Lineweaver–Burk plot of $1/I_{ss}$ versus $1/C$ H_2O_2 for the determination of k_m for the SPCE/PAni/AuNP/cMWCNT/CAT biosensor.