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Detection of Food-Grade Hydrogen Peroxide by HRP-Biocomposite Modified Biosensors

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ABSTRACT - A new amperometric biosensor has been developed for the detection of hydrogen peroxide (H_2O_2). The sensor was fabricated through the one-step deposition of a biocomposite layer onto a glassy carbon electrode at neutral pH. The biocomposite, as a H_2O_2 sensing element, was prepared by the electrochemical deposition of a homogeneous mixture of graphene oxide, aniline, and horseradish peroxidase. The experimental results clearly demonstrated of that the sensor possessed high electrocatalytic activity and responded to H_2O_2 with a stable and rapid manners. Scanning electron microscopy, cyclic voltammetry, and amperometry were performed to optimize the characteristics of the sensor and to evaluate its sensing chemistry. The sensor exhibited a linear response to H_2O_2 in the range of 10 to 500 μM concentrations, and its detection limit was calculated to be 1.3 μM. The proposed sensing-chemistry strategy and the sensor format were simple, cost-effective, and feasible for analysis of "food-grade H_2O_2" in food samples.

Key words : Food-Grade Hydrogen Peroxide, Biosensor, Amperometry, Enzyme Immobilization

Hydrogen peroxide (H_2O_2) is widely used in food industry for sterilization of equipment related to mixing, transporting, bottling and packing. During sterilization, H_2O_2 may become incorporated into the surface of bottles and packages and thereafter an additional process is required to decompose or remove the residual H_2O_2[1]. H_2O_2 is a substance composed of two hydrogen atoms and two oxygen atoms and its structure makes it highly reactive, and it can be easily broken down in aqueous solution. The breakdown of H_2O_2 leads to the formation of water and oxygen. Many different grades of H_2O_2 are available for different purposes. Among them, the only grade acceptable for home or personal use is "food-grade" H_2O_2, which consists of a 35% solution of H_2O_2 in water and is intended for the preparation or storage of food[2]. However, food-grade H_2O_2 is toxic and can adversely affect human health. The consumption of food-grade H_2O_2 has been reported to lead to gastrointestinal irritation or ulceration[3-6]. In 2006, the U.S. Food and Drug Administration (FDA) issued a warning for the intravenous administration of H_2O_2. In 2009, the Korea Ministry of Food and Drug Safety (MFDS) also announced regulations for H_2O_2 as a food additive, and these regulations were strengthened in 2016 after the detection of H_2O_2 in imported food[7]. Consequently, the ability to detect rapidly H_2O_2 in food is an important objective. In the present study, therefore, a new electrochemical H_2O_2 biosensor format is introduced for the "in-field" analysis of food samples.

Amperometric biosensors have been intensively developed for the detection of H_2O_2 using a variety of enzyme-immobilized electrodes, and the development of these biosensors using redox enzymes as bioactive materials has evoked substantial interest in recent years[8,9]. The direct electrical communication between the redox center of an enzyme and an electrode interface is considered to be an enzyme-catalyzed electrode processes. To establish an effective electrochemical transformation, the immobilization of biological sensing molecules, such as enzymes and proteins, to an electrode surface is important from a practical perspective. The electrochemical polymerization of aniline (ANI) has attracted significant interest and has been widely applied to the development of electrochemical sensor systems[10,11]. Polyaniline (PANI) is commonly produced by the chemical or electrochemical polymerization of ANI under strongly acidic conditions[12] and frequently requires toxic catalysts or oxidants that produce undesirable byproducts[13]. These low-pH aqueous conditions destabilize the biological sensing elements during fabrication. To overcome these drawbacks, nanomaterials have been widely introduced into electrochemical sensor fabrication processes[14].

As "next-generation materials," graphene-based nanomaterials have shown great promise for many potential applications including nanoelectronics and biosensors. Graphene is a remarkable two-dimensional sp^2-hybridized carbon nano-

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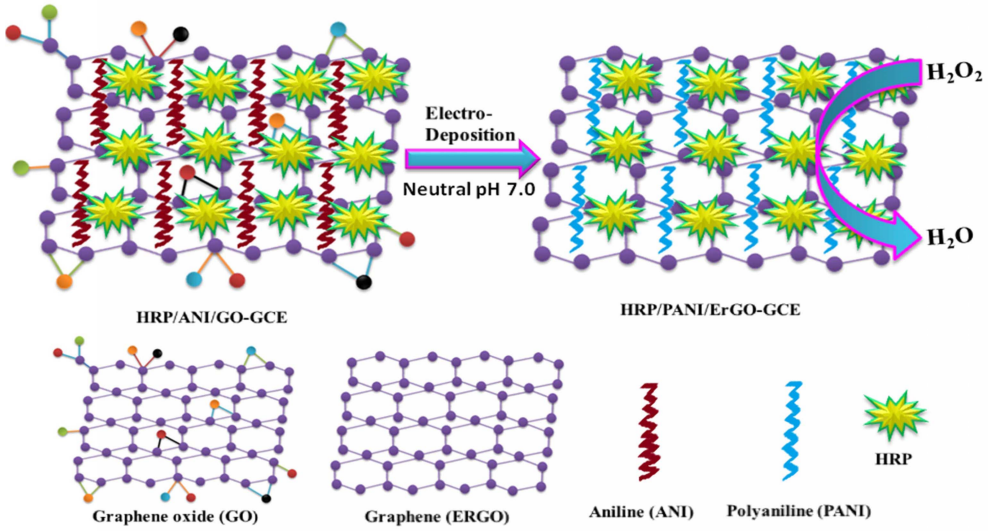

Fig. 1. Schematic illustration showing the construction of the electrochemical H_2O_2 biosensor.

material that has a large surface area, high electrical conductivity, flexibility, chemical inertness, and special charge-transport properties, which makes it unique to other sensor materials[15-17]. Techniques for the preparation of graphene materials include chemical, thermal, and electrochemical methods[18]. Among these techniques, the electrochemical method offers several advantages such as low cost, environmental friendliness, efficiency, and the lack of hazardous chemicals and reagents[19]. Graphene-based nanocomposites have been developed and used as materials for the effective immobilization of enzymes onto electrode surfaces. Chemically or electrochemically reduced graphene oxide (CrGO or ErGO) has been employed as an enzyme immobilization matrix for the detection of H_2O_2[20-23]. In addition, horseradish-peroxidase-modified (HRP-modified) graphene sensors have been developed due to their high electrochemical performance characteristics for the detection of H_2O_2[24,25]. In most previous reports, however, immobilized HRP-graphene systems were prepared using multi-step procedures. To the best of my knowledge, the present study is a pioneering investigation into a new approach for the one-step electrochemical preparation of HRP/PANI/ErGO biocomposites at neutral pH. This simple one-step process is a "green" fabrication technique for a H_2O_2 sensor that can be applied to the amperometric detection of food-grade H_2O_2 in food samples. The green one-step sensor fabrication process and the associated sensing chemistry are illustrated in Fig. 1.

Materials and Methods

Materials and Chemicals

Graphite, ANI, HRP, ascorbic acid (AA), uric acid (UA), glucose (Glu), H_2SO_4, Na_2HPO_4, NaH_2PO_4, H_2O_2 and Quantofix peroxides test sticks (Macherey-Nagel) were purchased from Sigma Aldrich (St. Louis, MO, USA). Graphene oxide (GO) was prepare from graphite by using a modification of Hummer's method[26,27]. All reagents were of analytical grade and used without further purification. All aqueous solutions were prepared using deionized water (Milli-Q water-purifying system, 18 MΩ/cm, Milford, MA, USA). As an operating buffer, phosphate solution (0.1 M PBS, pH 7.0) was prepared using standard stock solutions of Na_2HPO_4 and NaH_2PO_4.

Electrochemical Instrumentation

Cyclic voltammetry (CV) and amperometry were performed using a conventional three-electrode cell system with a modified glassy carbon working electrode (GCE, 3 mm diameter), a Ag/AgCl reference electrode, and a platinum-wire auxiliary electrode. All electrochemical experiments were performed on an electrochemical workstation (Compactstat, Ivium Technology, USA). Scanning electron microscopy (SEM) was performed using an FE-SEM instrument (Hitachi: S-4200) operating at 15 kV and 150 W.

Preparation of the HRP/ANI/GO Biocomposite

A 10 mg of GO was first dispersed in 9.9 mL of 1 M H_2SO_4, after which a 0.1 mL aliquot of ANI was added to give a brown dispersion. The dispersion was stirred for 30 min at room temperature, after which it was centrifuged and rinsed with water to remove the loosely adsorbed ANI. The ANI/GO composite obtained in this manner was dispersed in a 10 mL aliquot of distilled water to form a 1 g/L suspension. A 2 mL aliquot of the 1 g/L ANI/GO-composite dispersion was stirred with 2 mL of a 3.5 g/L HRP solution

in PBS for 3 min. The mixture was then sonicated for 10 min and allowed to stand at 4°C overnight. The mixture was subsequently centrifuged at 10000 rpm for 10 min to give the black composite, which was added to 2 mL of water and re-dispersed by gentle shaking for 10 min. This centrifugation and re-dispersion process was repeated three times to remove the unbound HRP. The HRP/ANI/GO biocomposite prepared in this manner was dispersed in 1 mL of water and stored in the dark at 5°C.

Electrochemical Construction of HRP/PANI/ErGO-GCE

A glassy carbon electrode (GCE, 3 mm diameter) was polished using 0.3 μm alumina slurries and sonicated in 50% ethanol for 10 min. After sonication, the GCE was rinsed thoroughly with distilled water and dried under N_2 at ambient temperature. After drying, 8 μL of the HRP/ANI/GO biocomposite dispersion was dropped onto the surface of the GCE, after which it was dried at ambient temperature. Cyclic voltammetric sweeping (from −1.4 to +0.8 V *vs*. Ag/AgCl) was carried out in PBS at a scan rate of 50 mV/sec up to 15 cycles to produce HRP/PANI/ErGO-GCE. After electrochemical polymerization, the modified HRP/PANI/ErGO-GCE was removed from the electrochemical cell and rinsed gently with PBS. PANI/ErGO-GCE without HRP-enzyme modification was also prepared using the same procedure. The modified electrodes were stored in PBS at 4°C until use.

Amperometric H_2O_2 Detection

The modified sensor was inserted into a 2 mL disposable electrochemical cell and connected to the electrochemical workstation. A 900 μL aliquot of PBS was added to the cell and the sensor was polarized at a potential of 0.0 V *vs.* Ag/AgCl. After achieving a stable baseline response to PBS, 100 μL of the H_2O_2 solution was added to the cell and the amperometric current response was recoded as a function of time. Measurements were repeated in order to construct a H_2O_2 calibration curve, and interference studies with AA, UA, and Glu samples prepared in PBS were also conducted.

Results and Discussion

Electrochemical Synthesis of HRP/PANI/ErGO

The HRP/PANI/ErGO biocomposite was deposited onto a GCE using the simple one-step electrochemical deposition of the HRP/ANI/ErGO, as described above. The potential sweep applied following the deposition of HRP/ANI/ErGO resulted in the reduction of ANI/GO. During potential cycling, ANI was polymerized to PANI and the oxygen-containing epoxide, carboxyl, and hydroxyl functional groups present on the surface of the GO were also reduced at the same time. Representative cyclic voltammograms depicting the electrochemical reduction of HRP/ANI/GO are shown in Fig. 2. In the first cycle, a large and broad cathodic peak appeared at −1.15 V with an onset potential of −0.88 V. In subsequent cycles, the cathodic peak gradually decreased in intensity, eventually stabilizing after 15 cycles. This result confirmed that GO was reduced quickly and irreversibly, and that the polymerization of ANI was successful[22]. As can be seen in the Inset in Fig. 2, ANI/GO-GCE prepared without HRP also showed similar deposition peaks of PANI/ErGO. These CV results, therefore, also confirm that the HRP enzyme, bound to ANI/GO either physically or covalently, did not affect the characteristics of the electrochemical deposition process.

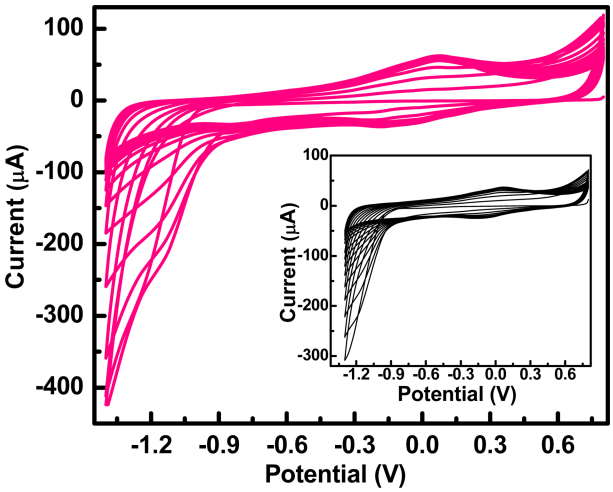

Fig. 2. Cyclic voltammogram of HRP/ANI/GO-GCE and ANI/GO-GCE (inset) in N_2-saturated 0.1 M PBS (pH 7.0) at a scan rate of 50 $mV \cdot s^{-1}$.

Morphological and Structural Characterization

Fig. 3 displays SEM images of the modified GCE after the formation of each component layer, namely GO, ErGO, and ErGO/PANI. After deposition of GO (Fig. 3A), multiple layers of GO are observed that exhibit crumbled wrinkle-like structures similar to those reported previously[22,27]. The ErGO film is observed to have a flat sheet-like structure (Fig. 3B), which is attributed to improved electrical conductivity and a significant increase in surface area following GO reduction. Compared to ErGO, ErGO/PANI exhibited a wave-like skin layer with a folded sheet-like structure (Fig. 3C) due to π-π interactions and/or hydrogen bonding between GO and ANI[22,28]. These results indicate that the electrochemical deposition of ErGO/PANI composite layer was successful.

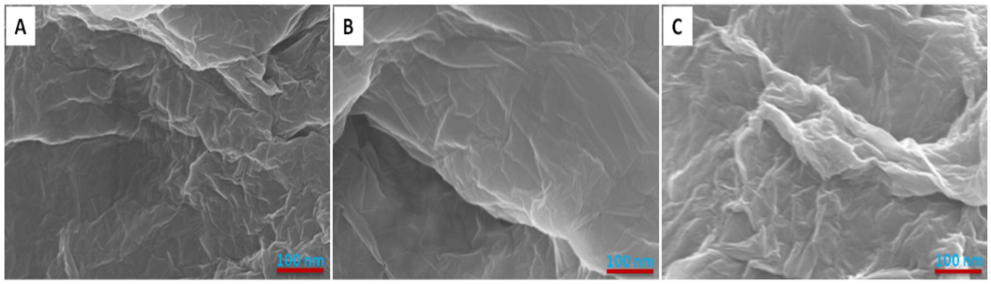

Fig. 3. FE-SEM images of (A) GO, (B) ErGO, and (C) ErGO/PANI.

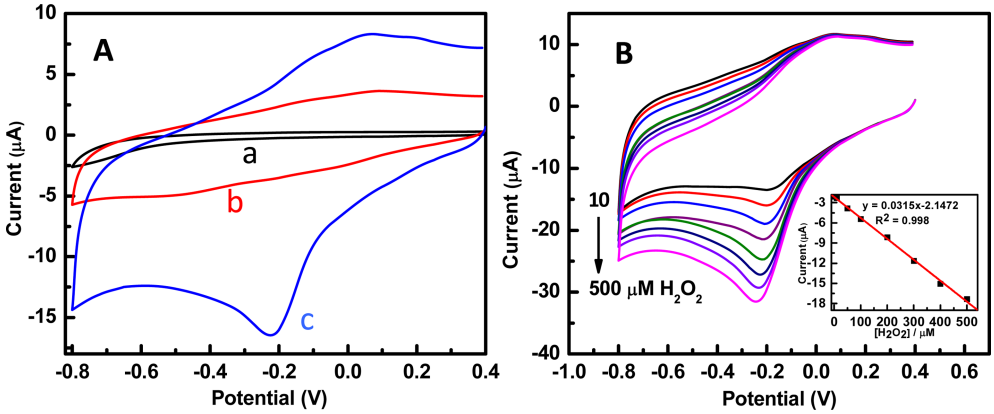

Fig. 4. (A) Cyclic voltammograms of (a) bare GCE, (b) PANI/ErGO-GCE, and (c) HRP/PANI/ErGO-GCE in 0.1 M PBS (pH 7.0) at a scan rate of 50 mV/sec. (B) Cyclic voltammograms of HRP/PANI/ErGO-GCE in H_2O_2 at concentrations of 10-500 μM at a scan rate of 50 mV/sec, (inset: cathodic peak currents at −0.22 V *vs.* Ag/AgCl).

Cyclic Voltammetry of HRP/PANI/ErGO-GCE

To investigate each deposition step during the formation of the biocomposite, CV experiments were repeated with (a) bare GCE, (b) PANI/ErGO-GCE, and (c) HRP/PANI/ErGO-GCE in N_2-saturated PBS at a scan rate of 50 mV/sec (Fig. 4A). HRP is an electrochemically active enzyme and has the heme as active site, and the heme-iron oxidation state is HRP-Fe(III) in the ground state. The native HRP-Fe(III) can be directly reduced at the electrode surface to HRP-Fe(II) by 1 e-transformation[29]. As shown in (a) and (b) in Fig. 4A, no detectable current peaks were observed for the PANI/ErGO-GCE and bare GCE. The biocomposite-modified electrode, HRP/PANI/ErGO-GCE, exhibits a prominent cathodic current peak at −0.22 V, which clearly indicates that the immobilized HRP facilitates the active electrochemical conversion between HRP-Fe(III) and HRP-Fe(II)[30]. The HRP/PANI/ErGO-GCE also exhibits enhanced electrocatalytic activity toward the reduction of H_2O_2, as shown in Fig. 4B. Upon addition of H_2O_2, the cathodic peak current (i_{pc}) was observed to increase significantly, and exhibited a linear relationship with H_2O_2 concentration in the 10-500 μM range. The mechanism of the electrocatalytic reaction of HRP with H_2O_2 is well known[30]. The equation of best fit from linear-regression analysis (inset, Fig. 4B) was determined to be: I_{pc} (μA) = 0.032 C_{H2O2} (μM) − 2.147 (R^2 = 0.998).

Sensor Characteristics

The effect of applied potential and the importance of pH on the electrochemical behavior of H_2O_2 in the constructed biocomposite sensor were investigated. A series of amperometric experiments were performed as described in the "Methods" section. These experiments were carried out with 100 μM H_2O_2 at different applied potentials that ranged from −0.3 to 0.2 V. Amperometric experiments were repeated with H_2O_2 in PBS (pH 5.0-9.0). The optimum current response was observed at 0.0 V and at pH 7.0. Therefore, these values were selected as the optimum operating conditions for use in subsequent experiments. Fig. 5A clearly reveals that (a) only HRP/PANI/ErGO-GCE exhibits a rapid change in amperometric response compared with those of (b) PANI/ErGO-GCE and (c) bare GCE, which indicates that the electrochemical deposition of the biocomposite effectively improves the electrocatalytic activity toward H_2O_2. To construct a calibration curve for H_2O_2 on the HRP/PANI/ErGO-GCE, H_2O_2 samples were prepared in PBS at concentrations that ranged from blank to 1000 μM. Fig. 5B

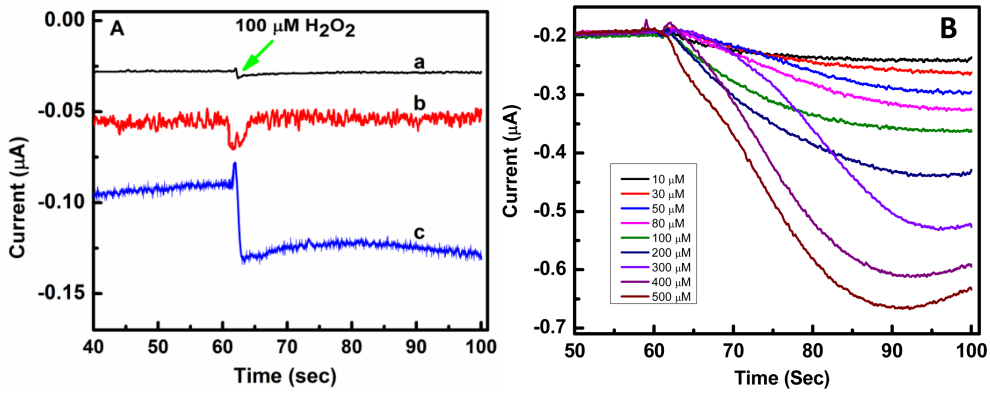

Fig. 5. (A) Amperometric responses to 100 μM H_2O_2 in 0.1 M PBS (pH 7.0) at 0.0 V of (a) bare GCE, (b) PANI/ErGO-GCE, and (c) HRP/PANI/ErGO-GCE. (B) Represent amperometric responses at various concentrations of H_2O_2 using HRP/PANI/ErGO-GCE.

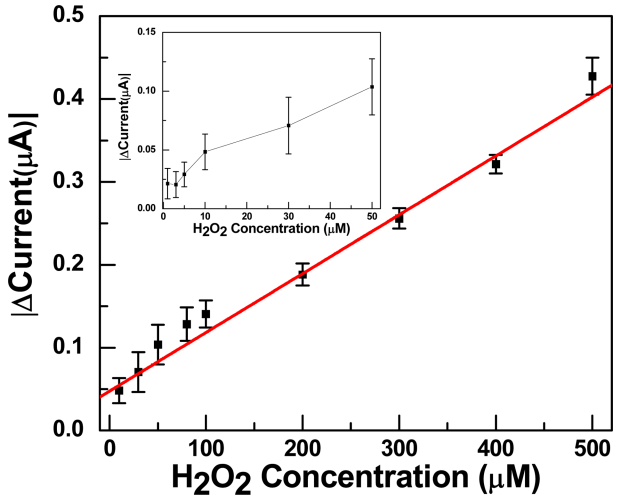

Fig. 6. Calibration curves for H_2O_2 using HRP/PANI/ErGO-GCE in 0.1 M PBS (pH 7.0).

displays the represent results of amperometric experiments involving H_2O_2 using HRP/PANI/ErGO-GCE under the optimized experimental conditions. The current responses obtained reached maximum values in less than 30 s and each current response was measured 30 s after the addition of H_2O_2 samples.

As shown in Fig. 6, the H_2O_2 calibration curve was constructed and the error bars shown on the curve represent standard deviations over five measurements for each point. In the curve obtained using the HRP/PANI/ErGO-GCE, the equation of the regression line was calculated to be I_{pc} (μA) = 0.0718 C_{H2O2} (μM) – 0.0474 (R^2 = 0.996). Responses to H_2O_2 could be detected at H_2O_2 concentration as low as 3.0 μM (inset in Fig. 6) and showed good linearity up to H_2O_2 concentration as high as 500 μM. The detection limit of the sensor was taken as six times of the standard deviation of the current change due to the addition of a blank solution. Using the obtained calibration curve, the sensor sensitivity was calculated to be 0.8 μA·mM^{-1}, and the detection limit was found to be 1.3 μM (S/N = 3).

In order to investigate the performance characteristics of the sensor, the data from the HRP/PANI/ErGO-GCE system were compared with those from other relevant reports, and are summarized in Table 1. Although the present system shows a relatively narrow linear range, it operates at zero potential (0.0 V *vs*. Ag/AgCl) and exhibits a slightly lower detection limit than the other methods listed. At a neutral operating potential and pH, most interfering signals from food contaminants are minimized or eliminated. The amperometric selectivity for the detection of H_2O_2 is often compromised by endogenous compounds such as ascorbic acid (AA), uric acid (UA), and glucose (Glu), which provide significant challenges for the detection of "food grade" H_2O_2 in conventional or functional food samples. Therefore, the H_2O_2-sensing ability of the HRP/PANI/ErGO-GCE was investigated in the presence of these interfering species. Fig. 7 reveals that the addition of 100 μM UA or Glu resulted in negligible interference, and the sensor responded only slightly to 100 μM AA, which is ascribed to the consumption of H_2O_2 during the oxidation of ascorbic acid. The sensor also operated efficiently and in a repeatable manner as evidenced by the relative standard deviation (RSD) of 3.44% (n = 5).

To demonstrate the applicability of the developed biosensor for the determination of food-grade H_2O_2, three different food samples were obtained from a local store-chain and were investigated. The food samples, two dried cuttlefish processed foods and a fishcake (product name not shown), were crushed and homogenized. one gram of each sample was mixed with 5 mL of the operating PBS by stirring by a magnetic stirrer for 20 min. The mixture was centrifuged at 3,000 rpm for 5 min and then 100 μL of each sample were used for amperometric measurement as described in the "Methods" section. After repeatable amperometric measurements under the optimized operating conditions, there were

Table 1. Comparison of the analytical performance of some recently reported H_2O_2 biosensors

Electrode	linear range (μM)	Detection limit (μM)	Operating Potential (V *vs.* Ag/AgCl)	Ref.
Au/GS/HRP/CS	5 to 5130	1.7	−0.3	30
GCE/ErGO/HRP	9 to 195	-	−0.28	23
SPCE/CS-NF/Fe_3O_4-Au-HRP	20 to 2500	12.0	−0.3	28
GCE/ND/NS (HRP)	1000 to 45000	59.0	−0.2	32
GCE/ZnO/GNPs/NF/HRP	15 to 1100	9.0	−0.3	33
GCE/HRP/RTIL/GNPs-TNTs/NF	5 to 1000	2.1	−0.4	34
Au/PolyAuNPs/HRP	5 to 1100	1.5	0.0	35
GCE/ErGO/PANI/HRP	10 to 500	1.3	0.0	This study

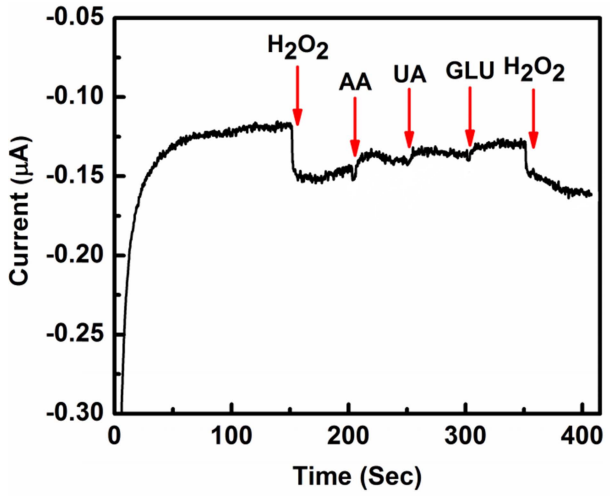

Fig. 7. Amperometric response of the HRP/PANI/ErGO-GCE sensor with successive additions of 50 μM H_2O_2, 100 μM AA, UA, and GLU, and 50 μM H_2O_2 in 0.1 M PBS (pH 7.0) at an applied potential of 0.0 V (vs. Ag/AgCl).

no detectable current responses to H_2O_2 from the food samples after 1:10 dilution with PBS. To confirm this results, a standard *in field* H_2O_2 test was performed with the samples prepared by using Quantofix peroxides test sticks. These ready-to-go test sticks are intended only for direct measurement of distinct H_2O_2 concentrations of 1, 3, 10, 30 and 100 $mg \cdot L^{-1}$ (ca. 0.32, 0.96, 3.2, 9.6 and 32 μM in pure water at 25°C, respectively)[31]. The results from the standard test showed all three food samples contained less than 3 $mg \cdot L^{-1}$ (0.96 μM). A number of existing food-related uses currently exist in FDA's food additive regulations and any residual H_2O_2 must be removed by appropriate physical and chemical means during the processing of food where it has been used (21CFR184.1366), however, potential exposure to humans from this use, if any, should be negligible due to the high reactivity of hydrogen peroxide. Three different food samples were then randomly spiked by the addition of the H_2O_2 standard to obtain final concentrations of 10, 30 and 100 $mg \cdot L^{-1}$, and amperometric measurements and the Quantofix strip tests were carried out. The recovery rates were obtained 71%, 81% and 88%, respectively, for these concentrations and the results from the strip tests confirmed the concentration levels by indicating the accurate color change regions.

In conclusion, a new redox-enzyme-modified PANI/ErGO-GCE electrochemical H_2O_2 biosensor system is introduced. HRP was immobilized by a one-step electrochemical deposition process without any additional cross-linking molecules; this deposition also improves electrocatalytic H_2O_2-sensing ability. The new biosensor design provides a promising electrochemical platform for the determination of H_2O_2. The advantageous features of the new biosensor provide a potent method for the monitoring of H_2O_2 in food samples.

Acknowledgements

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국문요약

본 연구의 목적은 식품 제조 중 표백 및 살균에 사용되는 과산화수소(H_2O_2)의 잔류 농도 검출에 활용 될 수 있는 유리탄소전극 기반의 바이오센서의 개발이다. 미국 FDA 및 국내 식품의학안전처 등 식품 용 과산화수소(food grade H_2O_2)는 국내외적으로 35% H_2O_2 수용액으로 규정한다. 연구에서 개발한 바이오센서는 감응 물질로 사용된 horseradish peroxidase를 graphene oxide와 aniline과 함께 biocomposite를 형성시킨 후 중성 pH에서 본 연구에서 새롭게 개발된 전기화학적 증착법을 수행하여 개발되었다. 센서구조 및 특성 평가를 위하여 SEM, 순환 전압 전류법 등을 수행하였으며 본 연구에서 개발된 바이오센서는 10-500 μM 농도의 H_2O_2에 대하여 직선상의 농도 의존적인 반응을 나타내었으며 최저 검출 한계는 0.12 μM 으로 산출되었다. 본 연구에서 개발된 센서의 전략적 가치는 향후 오징어포,

건어물 등 널리 유통되는 식품 중에 함유된 식품용 H_2O_2 미량을 현장에서 쉽게 분석 할 수 있어서 비용-효과적 측면에서 그 가치가 우수하다는 것을 제시한다.

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