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Measuring hydrogen peroxide due to water radiolysis using a modified horseradish peroxidase based biosensor as an alternative dosimetry method

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ABSTRACT

H_2O_2 generated during water radiolysis was measured electrochemically as an alternative dosimetry method. A biosensor was fabricated by immobilising modified horseradish peroxidase (HRP) on a glassy carbon electrode (GCE) followed by evaluation of its analytical parameters. Anthraquinone 2-carboxylic acid was used to modify HRP. To assess sensor performance, phosphate buffer solutions were irradiated with 0.510 Gy of gamma ray emitted from ^{60}Co. The results showed that this sensor can detect low quantities of hydrogen peroxide in water radiolysis. Sensitivity, detection limit and linear range of the biosensor were 260 nA/Gy, 0.392 Gy and 0.5–5 Gy, respectively. Long term stability studies showed that sensor responses were stable for at least a month. The cathodic peak current, as biosensor response, subsequently decreased to 20% of its initial value.

1. Introduction

There are a range of techniques, methods and devices that have been developed for radiation detection and dosimetry. An appropriate dosimeter should have properties such as coverage of a range of radiation energies, online responses, high resolution, efficiency, adequate count rate and acceptable cost. Ionising radiation is absorbed by an appropriate substance. Generated pulses, because of these interactions, are proportional to the total deposited energy of the incident radiation [1,2]. Aqueous solutions are typically used as the detection medium. Different chemical reactions occur during water radiolysis, and various types of free radicals are produced which may then act as either oxidising or reducing agents. The free hydroxyl radical ($OH^{\bullet}$) is one of the most important products of water radiolysis. From water radiolysis hydrogen peroxide is produced by a combination of two OH radicals [3–5]. H_2O_2 as an electroactive species could thus provide a measure of water radiolysis and therefore have application in dosimetry. In this study, a biosensor was made using modified HRP to detect H_2O_2. The characteristics of this sensor were determined in both low concentrations of hydrogen peroxide and irradiated buffer phosphate solutions.

There are many methods to measure H_2O_2, such as spectrophotometry [6], fluorimetry [7], electrochemistry [8], chemiluminescence [9], electron spin resonance (ESR) [10] and colorimetry [11]. In this study spectrophotometry and electrochemical methods were employed. Cobalt 60 was used as a source of ionising radiation. Co-60 is a beta-emitter and the energy of the excited Ni-60 generated is emitted as gamma radiation. This radioisotope is widely used for diagnosis, radiotherapy and sterilisation. It is also used in engineering for a range of applications, e.g., the checking of welding seams to detect flaws, a labelling gauge and in food and agricultural product irradiation.

2. Materials and methods

2.1. Chemicals

Hydroquinone, anilinium sulphate, ammonium molybdate, 4-aminoantipyrine 98% (4-AAP), 1-(3-dimethylaminopropyl) -and 3-ethyl carbodiimide hydrochloride 98% (DEC) were obtained from Merck Chemical Co. 3,3″-Bis [bis (carboxymethyl) aminoethyl] cresol sulfone phtrane sodium salt (xylenol orange tetrasodium), ammonium ferrous sulphate hexahydrate, sulphuric acid, 3,5-di-tert-4-butylhydroxytoluene (BHT), H_2O_2 30% (w/w) and anthraquinone 2-carboxylic acid 98% (AQ) were obtained from Aldrich, USA. Horseradish peroxidase (HRP; EC 1.11.1.7), sodium 4-(2-hydroxyethyl) -1-piperazine ethansulfonate

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(Na-HEPES) and, superfine Sephadex G-25 were obtained from Sigma in St. Louis, MO.

2.2. Horseradish peroxidase modification

To modify horseradish peroxidase, anthraquinone 2-carboxylic acid (AQ) was covalently attached to HRP. To achieve this, 20 mg AQ was dissolved in 3 mL of a Na-HEPES solution (0.15 M, pH 7.2), followed by ultrasonication for 5 min at room temperature (Elmasonic, S30H, Germany). After that, 25 mg of DEC was added to the turbid solution. Then 6 mg of enzyme was added into AQ-Na-HEPES-DEC solution. This mixture was stirred with a magnetic stirrer (Velp, U.S.A.) at 4 °C. After 22 h, this solution was centrifuged and supernatant liquid was used for further purification. The modified enzyme (AQ-HRP) was divided from un-reacted AQ molecules using a column (2 cm diameter and 15 cm height) by gel filtration. The column was filled with superfine Sephadex G-25 and equilibrated with potassium phosphate buffer (0.2 M, pH 7.0) [12]. After gel filtration, the concentration of modified HRP (AQ-HRP) was determined by the Bradford method [13].

2.3. Production of the AQ-HRP/GCE

Modified HRP (AQ-HRP) was immobilised on a glassy carbon electrode (GCE) surface. The following steps were taken in GCE preparation. First, the GCE surface was mechanically polished using Alumina powder, 10 and 0.3 μm diameter. Second, to remove alumina pieces and other contaminants from the electrode surface, the GCE was ultrasonicated for 5 min in water and ethanol. Third, to activate the electrode surface, the GCE was electrochemically treated between −1 to 0.5 V (vs Ag/AgCl) at a 100 mV/s scan rate in 0.2 M sulphuric acid for 10 min. Fourth, the electrode was immersed in 0.2 M potassium phosphate buffer (pH 7.0) at + 1.70 V (vs Ag/AgCl) for 10 min. Finally, to immobilise AQ-HRP on the GCE surface, the electrode was immersed in the oxygen free AQ-HRP solution for one hour. Cyclic voltammetry was carried out using 0–0.7 V (vs Ag/AgCl) at a 100 mV/s scan rate during the immobilisation process. Now the modified GCE was ready for the measurement of H_2O_2. The biosensor is subsequently specified as AQ-HRP/GCE. The effectiveness of this method for H_2O_2 measurement was electrochemically evaluated. Cyclic voltammetry and chronoamperometry was carried out using a potentiostat/galvanostat (DropSens, Spain and Autolab, Holland). A three electrode system which consisted of a platinum rod, Ag/AgCl and the AQ-HRP/GCE was used, respectively, as auxiliary, reference and working electrodes.

2.4. Spectrophotometric measurement

Hydroquinone–aniline catalysed by molybdate as well as by ferrous oxidation–xylenol orange (FOX-2 method), were used for spectrophotometric measurements of H_2O_2 [14,15]. For the first method, 0.25 M hydroquinone, 0.125 M anilinium sulphate and ammonium molybdate (0.5% w/v) were individually prepared in doubly distilled water. 2.4 mL of hydroquinone, 1.6 mL of anilinium sulphate and 0.25 mL of ammonium molybdate were mixed in blank and spectrophotometer cuvettes test (CECIL, CE 2501 Model). The instrument was adjusted to zero at 550 nm. For measurement at each absorbed dose, 0.75 mL of unirradiated and irradiated potassium phosphate buffer (0.2 M, pH 7) was added to blank and test cuvettes, respectively. The absorbance was measured after 10 min.

To measure H_2O_2 concentration using the FOX-2 method, 1 mM xylenol orange and 2.5 mM ammonium ferrous sulphate were prepared in 250 mM H_2SO_4. This solution is defined as working solution I; working solution II (4.4 mM BHT) was prepared in methanol. 0.2 mL of working solution I and 1.8 mL of working solution II were mixed in the blank and into a test cuvettes. The final concentration of ammonium ferrous sulphate, xylenol orange, and H_2SO_4 in each cuvette was 250 μM, 100 μM and 25 mM, respectively. To measure the H_2O_2 generated, 3 mL of irradiated potassium phosphate buffer (0.2 M, pH 7) was added to test cuvette. After 10 min, absorbance was monitored at 590 nm. Appropriate correction coefficients and dilution factors were considered in both methods to measure precise amounts of H_2O_2.

2.5. Gamma irradiation

12 samples containing 15 mL of 0.2 M potassium phosphate buffer were prepared at pH 7 (25 °C). Samples received irradiation at the following doses using a Picker-V9-Co-60 unit (USA). Zero (as reference dose), 0.1, 0.2, 0.3, 0.4, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8 and 10 Gy. The dose rate of the gamma source was 0.8 Gy/min, therefore, the appropriate absorbed dose was adjusted by changing exposure time for each sample. Irradiation was performed at the Secondary Standard Dosimetry Laboratory (S.S.D.L.) in the Iran Atomic Energy Organisation (I.A.E.O.).

3. Results and discussion

3.1. Effect of HRP modification on the AQ-HRP/GCE performance

To discover the roles of native and modified HRP on biosensor performance, the electrochemical behaviour of the HRP/GCE and the AQ-HRP/GCE was evaluated in H_2O_2 at a constant concentration of AQ (0.2 mg/mL) in phosphate buffer. Cyclic voltammetry was carried out to determine biosensor response. Fig. 1 (cyclic voltammograms A, B and C) represents the responses of a bare GCE electrode (in the absence of HRP) and native (HRP/GCE) and modified HRP (AQ-HRP/GCE) electrodes, respectively. Voltammogram A shows that cathodic and anodic peak current is not produced in the absence of HRP, therefore it indicates that the surface of the GCE is free of any confounding materials. Sharp anodic and cathodic peak currents in voltammograms B and C provide evidence of excellent native HRP and AQ-HRP immobilisation on GCE surface. However, anodic and cathodic currents due to AQ-HRP/GCE immobilisation are considerably greater than those due to HRP/GCE immobilisation. So, use of AQ-HRP as biosensing agent leads to a significant improvement in AQ-HRP/GCE response. It appears that

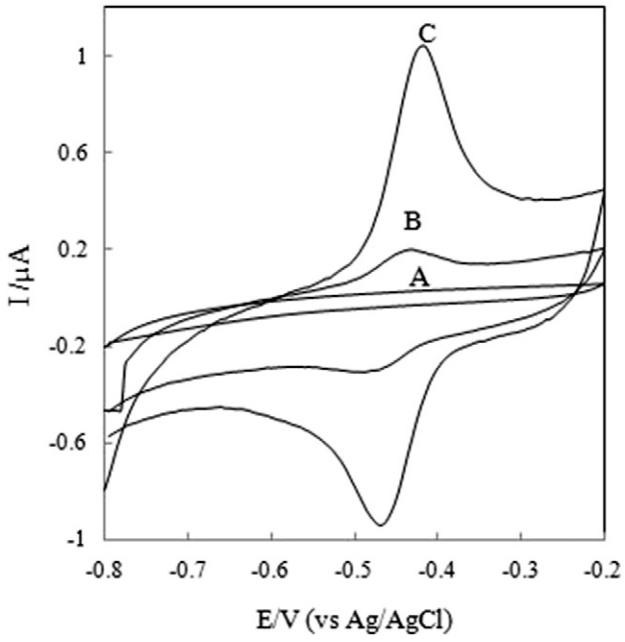
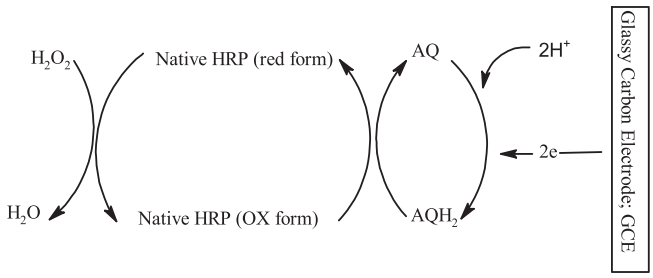


Fig. 1. Voltammograms A, B and C represent the biosensor responses. Voltammogram A is related to bare glassy carbon electrode. Voltammograms B and C are related to biosensor responses after immobilisation of native HRP (HRP/GC) and modified HRP (AQ-HRP/GCE) on glassy carbon electrode surface, respectively. The cyclic voltammetry experiments were carried out in N_2 saturated 0.2 M buffer phosphate solution at pH 7, 25 °C in 200 nM H_2O_2 solution at 50 mV/s scan rate.

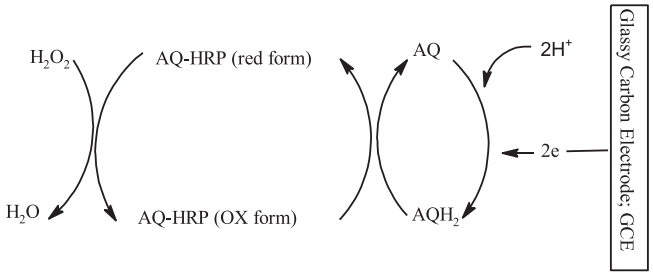


Scheme 1. The schematic represents a redox reaction of anthraquinone 2-carboxylic acid in H_2O_2. Native (unmodified) HRP is immobilised on GCE surface.

AQ also has a major role in this improvement. The mechanisms for the HRP/GCE and AQ-HRP/GCE responses are presented in Schemes 1 and 2. The redox reactions of AQ in solution and in H_2O_2, for these schemes, are observed at the surface of the HRP/GCE and AQ-HRP/GCE. Since the AQ mediatory effect on electron shuttling of HRP has been confirmed [12,16], it appears that for the AQ-HRP/GCE, electron transfer between the HRP heme prosthetic group, and the GCE is facilitated through HRP modification. Due to the low concentration of the H_2O_2 present during water radiolysis, improvement of the AQ-HRP/GCE function is extremely necessary for the appropriate performance of the biosensor, particularly, when the biosensor is used at low doses of radiation.

3.2. AQ-HRP/GCE sensitivity

AQ-HRP/GCE sensitivity was determined at low concentrations of H_2O_2 (in the range 25 nM to 200 nM) and a constant concentration of AQ (0.2 mg/mL) in phosphate buffer. Fig. 2 shows cyclic voltammograms and cathodic peak currents for the AQ-HRP/GCE at different H_2O_2 concentrations. As shown in Fig. 2A, cathodic and anodic peak currents are increased with an H_2O_2 increase, therefore, the biosensor is capable of determining H_2O_2 at low concentrations. Nevertheless, at each H_2O_2 concentration, the anodic peak current is less than the cathodic peak current. For instance, the cathodic and anodic peak currents at 175 nM were, respectively, 0.96 μA and −0.87 μA. This result is in accordance with previous results [17,18]. Moreover, as illustrated in Fig. 2B, cathodic peak currents increase with different slopes. A small slope (0.996 nA/nM) is observed in the range of 25 nM to 100 nM, whereas, the AQ-HRP/GCE responses linearly increase with a large slope (3.972 nA/nM) at a concentration greater than 100 nM. Different slopes in the two ranges of H_2O_2 concentrations can be attributed to AQ-HRP/GCE sensitivity; these data therefore indicate that the fabricated biosensor is operationally more sensitive at a concentration range greater than 100 nM. An H_2O_2 concentration between 20 nM at 0.5 Gy and 110 nM at 10 Gy is generally expected during water radiolysis in real conditions, to which our results are well-matched, assuming these expected values. These values were obtained according to the H_2O_2-G-value, details are given in Section 3.5. Additionally, the influence of ascorbic acid and uric acid on biosensor performance was studied as two major biological



Scheme 2. The schematic represents a redox reaction of anthraquinone 2-carboxylic acid in H_2O_2. AQ-HRP (modified HRP) is immobilised on GCE surface.

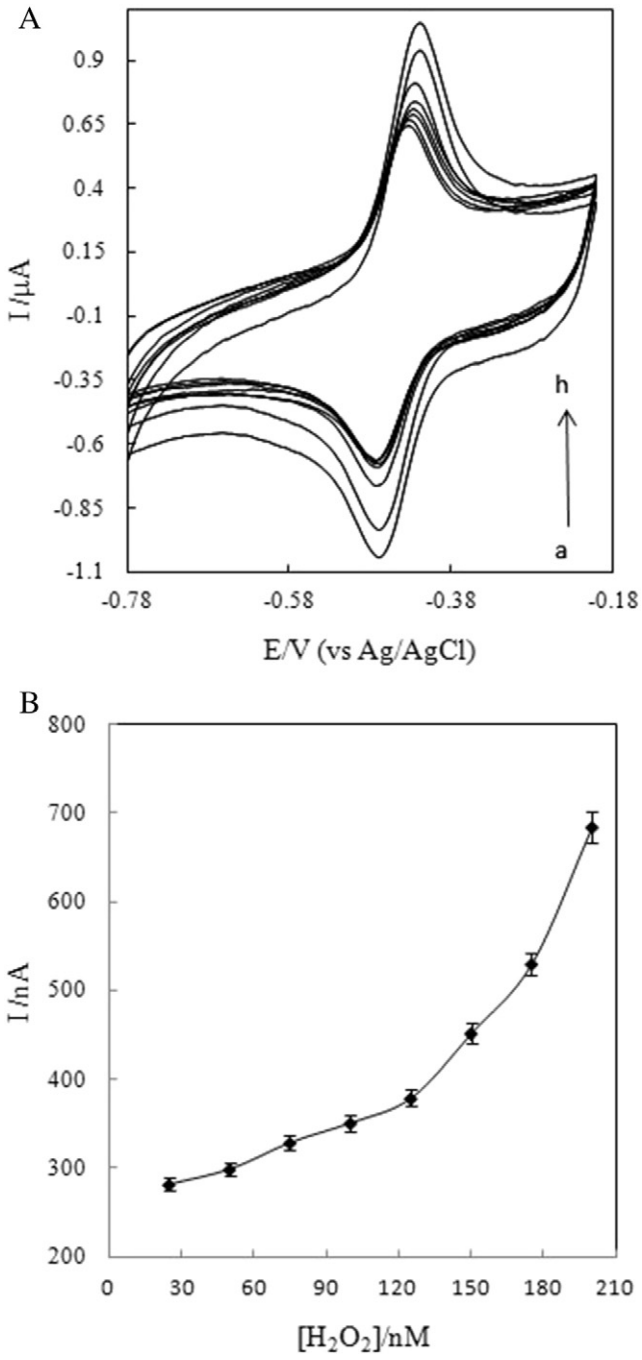


Fig. 2. A. The AQ-HRP/GCE voltammograms at different H_2O_2 concentration and 0.2 mg/mL AQ. The H_2O_2 concentrations from a to h are 25, 50, 75, 100, 125, 150, 175 and 200 nM, respectively. Cyclic voltammetry experiments were carried out in N_2 saturated 0.2 M buffer phosphate at pH 7, 25 °C and 50 mV/S scan rate. B. Voltammograms changes against H_2O_2 concentration raises. Each experiment was repeated three times and means ± standard errors are reported.

interferences. Since the redox activities of these biological compounds occur at positive potentials [19–21], they would not affect on AQ-HRP/GCE responses. The interference effect was also investigated for other potentially co-existing electroactive components in biological systems such as dopamine, catechol and acetaminophen. The results showed that highly sensitive detection of H_2O_2 using AQ and the AQ-HRP/GCE system was possible and that it was also resistant to the effects of interfering components. The findings show promise that the biosensor could measure water radiolysis through H_2O_2 production.

3.3. Electrochemical measurement of H_2O_2 during water radiolysis

In the first stage, the biosensor was used as a working electrode in a cyclic voltammetry experiment. Fig. 3 represents cyclic voltammograms between 0.3 and 0.7 V as biosensor responses at low concentrations of H_2O_2 (in the absence of AQ in the experimental solution). As shown in Fig. 3, the AQ-HRP/GCE successfully detected a low concentration of H_2O_2 (less than 1200 nM). The cathodic peak current increases with increasing H_2O_2 concentration in the absence of AQ in solution, whereas, the anodic peak current is decreased. These results are in line with nearly all enzymatic HRP biosensors. In the next stage, the H_2O_2 generated during water radiolysis at different absorbed doses was electrochemically determined. Cyclic voltammograms and choronoamprograms were considered as the biosensor responses. These were immediately recorded using a galvanostat/potentiostat by a three electrode system for each absorbed dose. In the chronoamperometry experiments, electrical currents were measured at 0.425 V (vs Ag/AgCl) after 60 s; these results were in agreement with cyclic voltammetry. Fig. 4 represents cathodic peak current changes versus absorbed doses. The AQ-HRP/GCE response at a very low range of absorbed dose (less than 1 Gy) is illustrated in the inset of Fig. 4. The principal findings are as follows: First, the AQ-HRP/GCE is fully capable of measuring low concentrations of H_2O_2 produced at low doses of ionising radiation. Second, since H_2O_2 concentration is directly correlated with absorbed dose, the current increase is proportional to the absorbed dose of irradiated phosphate buffer solution. This result is consistent with other radiochemistry studies [5–9]. Third, in Fig. 4 the AQ-HRP/GC responses are linear from 0.5 to 5 Gy, however, the slopes of the lines at very low (less than 0.5 Gy) and higher doses (from 0.5 Gy to 5 Gy) are dissimilar. These lines crossed each other at one point (X = 0.392 Gy and Y = 109 nA), therefore, 0.392 Gy is determined as the biosensor detection limit [22]. Fourth, Fig. 4 shows the AQ-HRP/GCE saturated region, which is another important point. Based on Fig. 4, saturated regions occur above 5 Gy and that, the AQ-HRP/GCE is insensitive to absorbed radiation dose more than 5 Gy. The majority of absorbed doses using in medical are fortunately in the range 0.5 Gy to 5 Gy, so this biosensor could be used for medical diagnostic purposes and in radiotherapy. Fifth, the slope of the line in the linear range (0.5–5 Gy) can be considered as the range of AQ-HRP/GCE radiation sensitivity. From Fig. 4, the AQ-HRP/GC sensitivity is 260 nA/Gy. Finally, Figs. 2B and 4 represent the AQ-HRP/GCE responses in un-irradiated and irradiated samples of buffer solutions; these appear dissimilar because saturation is not observed in Fig. 2B. This inconsistency may be due to the destructive effects of other products of AQ-HRP catalysis. For instance, reactive oxygen species (ROS), hydrated electrons (e^-_{aq}) and hydrogen free radicals ($H^•$) are produced by the hydroxyl radical (as precursors of H_2O_2) during water radiolysis [3,23]. Consequently, AQ-HRP response may decrease due to the existence of other toxic radiation products.

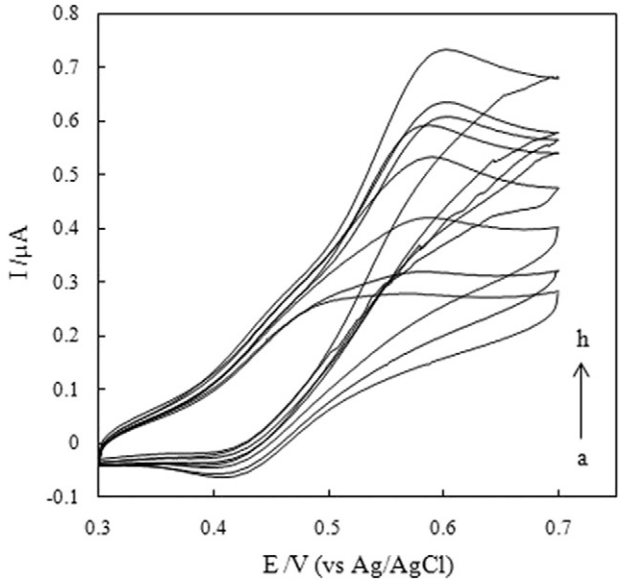


Fig. 3. The AQ-HRP/GCE voltammograms at different H_2O_2 concentration. The H_2O_2 concentrations from a to h are attributed to 70, 190, 375, 700, 900, 1000, 1070, and 1300 nM, respectively. Cyclic voltammetry experiments were carried out in N_2 saturated 0.2 M buffer phosphate at pH 7, 25 °C and 50 mV/S scan rate.

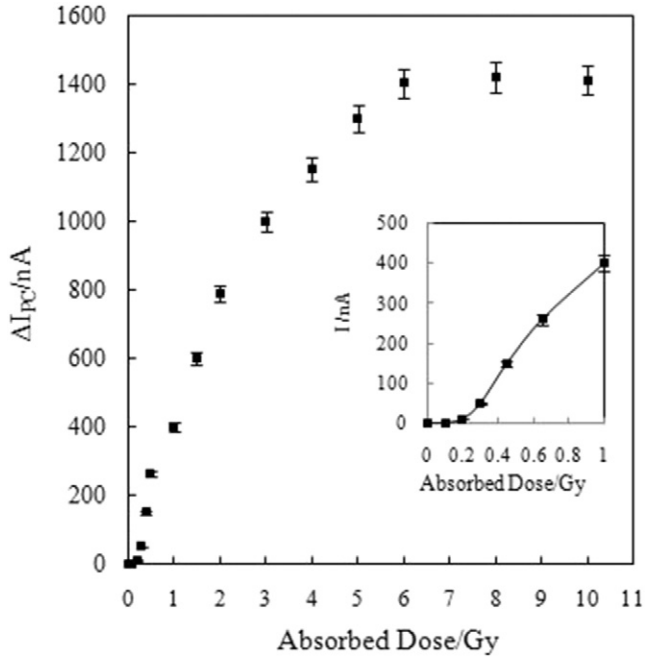


Fig. 4. Cathodic peak currents changes versus absorbed doses. The inset shows the AQ-HRP/GCE responses at less than 1 Gy of absorbed doses. The cathodic peak currents from irradiated 0.2 M buffer phosphate solution at pH7, 25 °C were separately obtained by cyclic voltammetry and chronoamperometry. Each experiment was repeated three times and means ± standard errors are reported.

3.4. Reversibility study

Sensor responses were measured at different scan rates to assess the reversibility of the AQ-HRP/GCE response. For this purpose, cyclic voltammetry was used. Since 2.5 Gy is in the middle of the linear range of the AQ-HRP/GCE response, this value was selected for the reversibility study. Fig. 5 represents the cyclic voltammograms at different scan rates as well as cathodic and anodic peak current changes against scan rates. As shown in Fig. 5 (inset), both cathodic and anodic currents are linearly increased with scan rate; therefore, the redox reactions on the AQ-HRP/GCE surface are reversible, according to the Randles–Sevcik equation [24]. Moreover, Fig. 5 indicates that the locations of the peak potentials in the cyclic voltammograms are independent of the scan rates, and based on Fig. 5 (inset), peak current ratios are approximately 1 at each scan rate. This clearly indicates that redox reactions, particularly, the catalytic activity of AQ-HRP on the AQ-HRP/GCE surface is fully reversible.

3.5. H_2O_2 measurement

The quantitation of H_2O_2 was undertaken for each absorbed dose by spectrophotometry. H_2O_2 concentrations were also calculated based on the G-value definition. This information assists better understanding of the AQ-HRP/GCE performance and its characteristics. The G-value is defined as the number of free radical species produced during water radiolysis per 100 eV radiation [2,25]. The G-value has been reported as 0.7 or 13 nM/Gy for H_2O_2 when aqueous medium, such as phosphate buffer

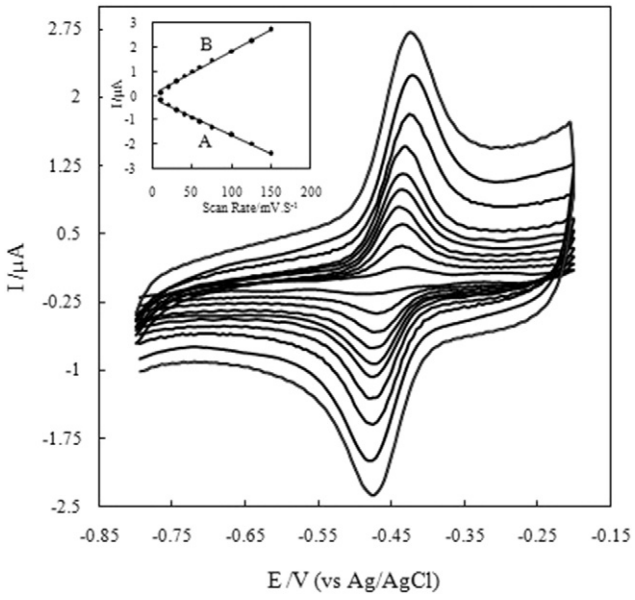


Fig. 5. Scan rate effect on cyclic voltammograms as the AQ-HRP/GCE responses. The cyclic voltammetry experiments at different scan rates were carried out in irradiated 0.2 M buffer phosphate solution. The absorbed dose of samples was 2 Gy and the scan rates from a to j are attributed to 10, 20, 30, 40, 50, 60, 75, 100, 125 and 150 mV/S, respectively. Inset indicates scan rate effect on the anodic (A) and cathodic (B) peak currents.

solution, is irradiated with gamma rays [2,4]. As a result, this G-value was used to calculate generated H_2O_2 during water radiolysis at each absorbed dose. Fig. 6 demonstrates H_2O_2 concentration changes against absorbed doses. Generally, there is a good agreement between the results by the three methods; also, H_2O_2 alterations against radiation absorbed dose at low (0 to 1 Gy), middle (1 Gy to 5 Gy) and high (5 Gy to 10 Gy) doses are similar to each other, additional important points for consideration. H_2O_2 concentration slope changes which we obtained by calculation, especially at low doses (less than 1 Gy), were greater than slope changes observed with spectrophotometric H_2O_2 measurement. This difference may be due to a lag in the H_2O_2 monitoring reaction; considering the short H_2O_2 life span, some generated H_2O_2 could be spontaneously transformed into water molecules during spectrophotometric measurements. Therefore, a delay in the reporter reaction should be considered in describing the AQ-HRP/GCE dose response curve (i.e., calibration curve), particularly when it is used for very low doses. Finally, the FOX-2 method appears more sensitive than the hydroquinone–aniline system catalysed by the molybdate method, indicated by the greater change of slope.

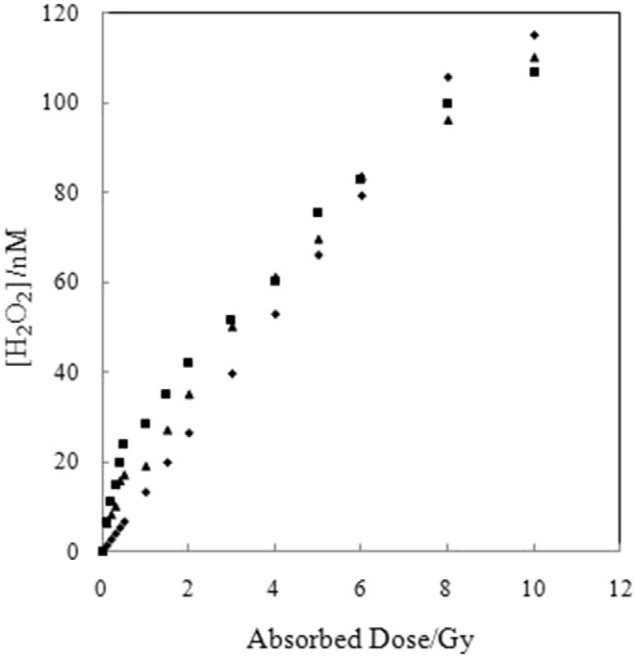


Fig. 6. Spectrophotometric measurements and measured H_2O_2 during water radiolysis at different absorbed doses. H_2O_2 concentration using hydroquinone–aniline system catalysed by molybdate method (♦), ferrous oxidation–xylenol orange (FOX-2 method) (▲) and calculated values (■) are presented in this figure.

3.6. Long term stability study

Long term stability, an important characteristic of biosensors, was defined here for the AQ-HRP/GCE. To evaluate this, cathodic peak currents were measured in irradiated phosphate buffer (0.2 M at pH7, 25 °C, 50 mV/s scan rate) at an absorbed dose of 2 Gy. This value was selected because it is at the midpoint of the linear region of the AQ-HRP/GC response. Fig. 7 shows cyclic voltammograms that were obtained every four days. It can be seen that up to day 34, any reduction in biosensor response is insignificant; however, cathodic peak currents are considerably decreased later. As shown in Fig. 7, the AQ-HRP/GCE responses are decreased and the cathodic peak current is reduced to 20% of its initial value. These results also show that a correction coefficient may be needed when the AQ-HRP/GCE is used as an individual dosimeter in normal practice or in radiological accident assessment after one month. It can be concluded that making small changes either in the immobilisation procedure or in HRP modification can improve the long term stability of this sensor.

4. Conclusion

Measurement of generated H_2O_2 using electrochemical biosensor in aqueous solutions via ionisation with ionising radiation is an alternative method for radiation dosimetry, as examined in this study. These electrochemical biosensors in comparison with conventional dosimetry procedures have several operational advantages, including being inexpensive, practical, reproducible, with appropriate sensitivity, having

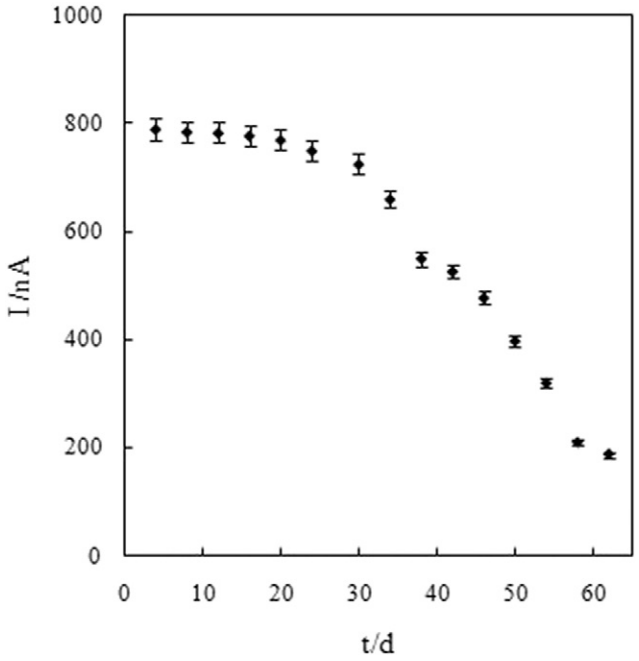


Fig. 7. The AQ-HRP/GCE long term stability. The cathodic peak current was assessed in irradiated 0.2 M buffer phosphate solution at pH7, 25 °C and 50 mV/s scan rate. Absorbed dose was 2 Gy and cyclic voltammetry experiment was carried out at each four days.

an acceptable detection limit and a linear response at clinically desired dose ranges.

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