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Electrochemical evaluation of total antioxidant capacities in fruit juice based on the guanine/graphene nanoribbon/glassy carbon electrode

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ABSTRACT

Based on electro-immobilization of guanine on graphene nanoribbon (GNR) modified glassy carbon (GC) electrode, a new electrochemical DNA biosensor was developed for the evaluation of total antioxidant capacities (TAC) in fruit juices. The biosensor relies on the guanine damage that is induced by hydroxyl radical (· OH) generated by Fenton-type reaction. Ascorbic acid (AA), which has the ability to scavenge the · OH and to protect the guanine immobilized on the electrode surface, was used as the standard antioxidant to evaluate the TAC in fruit juice. Under optimized conditions, the proposed biosensor has excellent analytical performance for antioxidant capacity assessment: wide linear range (0.1 to 4 mg L^{-1}), high sensitivity (4.16 μA/mg L^{-1}) and low detection limit (0.05 mg L^{-1}). Compared with the other electrochemical sensors developed previously, the proposed electrode demonstrates the improved detection limit of about 5 times to one order of magnitude for antioxidant capacity assessment. Additionally, the biosensor was successfully applied to the determination of the TAC in fruit juices.

1. Introduction

Reactive oxygen species (ROS), such as hydroxyl radicals (· OH), superoxide anions ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), are highly unstable molecules. In living organisms, ROS are produced by normal metabolism or exogenous stressors (pollution, sunlight exposure, cigarette smoking, excessive alcohol consumption, etc.) [1–3]. At high ROS concentration or an overproduction state, ROS can cause oxidative stress which can induce damage of lipids, proteins or DNA, impeding normal cell functioning [4,5] and leading to numerous human diseases, as well as to the aging process [6,7]. To protect the cells and organ systems of the body against ROS, most living organisms have evolved a highly sophisticated and complex endogenous and exogenous antioxidant protection system [8]. In the endogenous system, superoxide dismutase, glutathione peroxidase, and catalase are the main enzymes involved in the removal of ROS. In the exogenous system, small molecules such as ascorbic acid (AA), α-tocopherol, glutathione, carotenoids, flavonoids, and other antioxidants act as radical scavengers. Since the exogenous antioxidants are naturally present in fresh fruits and vegetables, increasing intake of dietary antioxidant may help to maintain an adequate antioxidant status [9]. Therefore, the determination of total antioxidant capacities (TAC) in natural products is necessary and receives much attention [10,11].

Among the ROS, hydroxyl radical (· OH) is one of the most reactive species. In biological systems, · OH is produced by the reaction between Iron (Fe^{2+}) and H_2O_2, the so-called Fenton reaction. Based on the · OH-induced DNA damage theory, several electrochemical DNA-based biosensors have been developed for the evaluation of TAC in biological and food samples. These biosensors were based on the immobilized double-stranded DNA [12–15], single-stranded DNA [16,17] and purine bases [18–21] as oxidation targets and a Fenton-type reaction was used for · OH generation. However, the performance of these electrochemical DNA sensors for TAC evaluation, such as the linear response range and the detection limit, still needs to be improved further.

On the other hand, it was reported that the immobilized amount of DNA strands directly influences the analytical performance of the electrochemical DNA sensors and high surface area of the sensing materials will result in high-immobilized amount of DNA strands [22,23]. Moreover, the sensing materials with good electrocatalytic properties are beneficial to the sensitive analysis of the electroactive species. Therefore, to improve the performance of the electrochemical biosensor for TAC analysis, we need explore appropriate sensing material with high surface area and good electrocatalytic properties.

Recently, retaining the feature of a high length–diameter ratio and representing a particular kind of graphene-related material,

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graphene nanoribbon (GNR) prepared from multi-walled carbon nanotubes (MWCNTs) has received wide attention, from experimental to theoretical studies [24–26]. In addition, their large surface area, superior electronic conductivity and edge structure can offer a plenty of space for chemical modification, which would result in good electrocatalytic properties [27–29] and application in biosensors [30–35]. In this work, GNR was produced by longitudinally unzipped MWCNTs and used to construct the guanine electrochemical biosensor for the first time to determine the TAC in commercial fruit juices. Based on the guanine damage which is induced by ·OH generated by Fenton-type reaction and AA as the standard antioxidant, the analytical performance of the proposed biosensor for antioxidant capacity assessment has been investigated.

2. Experimental

2.1. Chemicals

Guanine was purchased from Bio. Basic Inc., Canada. MWCNTs (diameter 20–60 nm, length 5–15 μm) were purchased from Shenzhen Nanotech Port Ltd, China and used without any purification. AA was acquired from Sinopharm Chemical Reagent Co., Ltd, China. All other chemicals were of analytical grade and used as received.

Guanine stock solution (1 g L^{-1}) was prepared by dissolving a definite amount of guanine in NaOH (0.1 mol L^{-1}) and diluting in phosphate buffered solution (PBS, 50 mM, pH 7.4), and then stored at 4 °C. The working standard solutions were prepared daily by diluting the previous stock solution with PBS (50 mM, pH 4.92) just before use. AA standard solution was of 0.5 g L^{-1}, and prepared daily and immediately before measurements by dissolving an amount of the solid AA in water until the desired concentration. Fe^{2+}–EDTA solution was prepared daily by adding the appropriate amount of $FeSO_4 \cdot 7\,H_2O$ to EDTA solution (Fe^{2+}/EDTA 1:3 mol mol^{-1}) in ultrapure water, giving a slightly green and clear solution. Fenton reaction for hydroxyl radical generation was carried out in a mixture of H_2O_2 and Fe^{2+}/EDTA (Fe^{2+}–EDTA/H_2O_2 1:6 mol mol^{-1}). In the Fenton reaction, the concentration of Fe^{2+} is equal to that of ·OH [36]. All solutions used throughout were prepared with ultra pure water obtained from a Millipore system (resistivity >18 MΩ cm).

2.2. Apparatus

The morphology of GNR was characterized by scanning electron microscopy (SEM, JSM 6700F, JEOL, Japan). All electrochemical measurements were performed on a CHI 660B Electrochemical Workstation (Chenhua Instrument Company of Shanghai, China). A conventional three-electrode cell was used with a glassy carbon (GC, with a diameter of 3 mm) electrode as the working electrode, a platinum wire as the counter electrode and a saturated calomel electrode (SCE) as the reference electrode. All the potentials in this paper were referred to SCE. Except the specific statement, the electrochemical measurements were carried out in PBS (50 mM, pH 4.92) at room temperature (25 ± 2 °C).

2.3. Preparation of GNR

The GNR was prepared by longitudinal unzipping of MWCNTs according to literatures [37,38] with some modification. First, MWCNTs (150 mg) were treated with concentrated H_2SO_4 (98%, 36 mL) for 1 h. H_3PO_4 (85%, 4 mL) was then added, and the mixture was allowed to stir 15 min before the addition of $KMnO_4$ (750 mg). The mixture was heated at 65 °C for 2 h, cooled down to room temperature, and then poured into ice water (100 mL) containing H_2O_2 (5 mL, 30 wt.%). The resulting light-brown colored precipitate was collected on a 200 nm pore size PTFE membrane, washed 2 times with HCl (20 vol%, 6 mL each) and resuspended in H_2O (60 mL) by stirring for 2 h. Then HCl (30 vol%, 60 mL) was added to coagulate the product, which was then collected on the PTFE membrane, washed 2 times with HCl (20 vol%, 6 mL each), and dispersed in ethanol (40 mL) for 2 h with stirring. Then the product was again coagulated by the addition of anhydrous diethyl ether (60 mL), filtered over the PTFE membrane, washed 2 times with anhydrous diethyl ether (10 mL each), and dried in vacuum.

2.4. Fabrication of the guanine/GNR/GC electrode

The obtained GNR (1.5 mg) was dispersed in PBS (3.0 mL, 50 mM, pH 7.4) and ultrasonicated for 0.5 h to obtain the homogeneous GNR suspension (0.5 mg mL^{-1}). Prior to use, the GC electrode was polished carefully to a mirror-like plane with 0.5 and 0.05 μm alumina powder, rinsed with ultra-pure water, and subsequently sonicated in acetone and ultra-pure water, respectively. Then, the GNR suspension (6 μL, 0.5 mg mL^{-1}) was dropped on the surface of GC electrode. After drying at room temperature, the GNR/GC electrode was obtained.

For preparation of guanine/GNR/GC electrode, the GNR/GC electrode was immersed in PBS (50 mM, pH 4.92) containing guanine (20 mg L^{-1}) and applied an accumulation potential of 0.4 V for an accumulation time of 270 s. The electrode was then rinsed with ultra pure water and denoted as guanine/GNR/GC electrode. For comparison, guanine/GC electrode was also prepared according to the above procedure.

2.5. Assays for guanine damage by ·OH and its protection by AA

Guanine damage was carried out by immersing the guanine/GNR/GC electrode in a freshly prepared Fenton solution (Fe^{2+}–EDTA/H_2O_2 1:6 mol mol^{-1}). After a definite incubation time, the guanine/GNR/GC electrode was rinsed with water and immediately immersed in PBS (50 mM, pH 4.92) to carry out the square wave voltammetry (SWV, frequency 15 Hz, step potential 4 mV and amplitude 0.025 V) experiment. The electro-oxidation peak current of the remaining unoxidized guanine was obtained and used as the detection signal.

For the study of the protection effect of antioxidant AA, the guanine/GNR/GC electrode was immersed in a freshly prepared Fenton solution in the presence of AA. And then the electro-oxidation peak current of the remaining unoxidized guanine was measured according to the above procedure.

2.6. TAC measurement in commercial fruit juices

Three kinds of fruit juices produced by Uni-president company were obtained from local supermarket. The pure fruit juice content of these juices is 10%, according to the package label. Another group of three kinds of fruit juices was homemade and obtained by squeezing the corresponding fruits obtained from local supermarket. Prior to measurement, all fruit juices were centrifuged at 10,000 rpm for 5 min, filtered and the filtrate was stored in the dark at 4 °C. For TAC assay, the fruit juices were diluted in the freshly prepared Fe^{2+} solution (2 mM) to a final volume of 5 mL. The guanine/GNR/GC electrode was immersed in the solution and H_2O_2 (60 μL, 1 M) was added for 180 s. And then the electro-oxidation peak current of the remaining unoxidized guanine was measured according to the above procedure. Measurements were made at least three times and the new prepared guanine/GNR/GC electrode was used for every TAC assay.

3. Results and discussion

3.1. Characterization of GNR

The morphology of the as-prepared GNR and MWCNTs was characterized by SEM and the corresponding results are shown in Fig. 1. From Fig. 1, it is noted that the width of the GNR (Fig. 1B) is 60–150 nm and much larger than the diameter of MWCNTs (about 20–60 nm, Fig. 1A). This confirms that the MWCNTs have been opened by treatment with H_2SO_4 and $KMnO_4$, and the GNR was obtained.

3.2. Electro-oxidation of the immobilized guanine on the GNR/GC electrode

$Fe(CN)_6^{3-/4-}$, as an electrochemical probe, is usually used to evaluate the electrochemical properties of the electrode. Fig. 2 shows cyclic voltammograms obtained at the bare GC and GNR/GC electrodes in 5 mM $Fe(CN)_6^{3-/4-}$(1:1)+0.1 M KCl solution. It is noted that the difference in potential between the anodic and cathodic peaks (ΔE_p) is 81 mV for the GNR/GC electrode and 110 mV for the bare GC electrode. As ΔE_p is a function of the electron transfer rate, the lower ΔE_p, the higher electron transfer rate. The electron transfer rate constant k^0 for the ferricyanide system decreases with the increase of the value of ΔE_p [39,40]. Furthermore, the redox peak currents at the GNR/GC electrode are larger than that at the bare GC electrode. This may result from the good electronic conductivity and large specific surface area of GNR. The smaller value of ΔE_p and the higher redox peak currents indicate that the GNR/GC electrode has better electrochemical properties than the bare GC electrode.

Fig. 3 shows the SWV results of guanine/GC electrode (a) and guanine/GNR/GC electrode (b) in PBS (50 mM, pH 4.92). It can be observed that a well-defined oxidation peak appears at about 0.80 V on guanine/GC electrode and 0.85 V on guanine/GNR/GC electrode, indicating electrochemical oxidation of guanine immobilized on the electrodes [41,42]. However, the oxidation peak current of guanine on the guanine/GNR/GC electrode is 34.5 μA and about 3.5 times higher than that on the guanine/GC electrode (9.9 μA). This should results from the high surface area and good electrocatalytic properties of GNR, which will effectively increase the loading of guanine and promote electrochemical oxidation of guanine.

For electrochemical DNA biosensors, the immobilized amount of DNA strands directly influences the performance of the DNA electrochemical sensors. Therefore, the loading of guanine on the GNR/GC electrode by electro-immobilization is important to the biosensor's performance. In order to obtain the highest oxidation peak current of guanine, we investigated the effects of the accumulation time and the concentration of guanine on the oxidation peak current of guanine/GNR/GC electrode and the corresponding results are shown in Fig. 4. From Fig. 4A, it can be observed that the oxidation peak current of guanine/GNR/GC electrode increases with the increase of accumulation time and the maximum appears at 270 s. Thus, this time (270 s) is selected as the optimum accumulation time. On the other hand, as shown in Fig. 4B, it is noted that the oxidation peak current of guanine/GNR/GC electrode increases with the increase of the concentration of guanine and reaches to the maximum at 20 mg L^{-1}. Therefore, this optimum concentration of guanine (20 mg L^{-1}) is used throughout in this study unless specified statement.

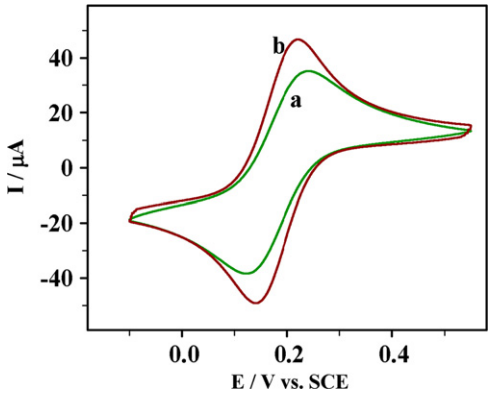

Fig. 2. Cyclic voltammograms obtained at the bare GC (a) and GNR/GC (b) electrodes in 5 mM $Fe(CN)_6^{3-/4-}$ (1:1)+0.1 M KCl aqueous solution. Scan rate: 50 mV s^{-1}.

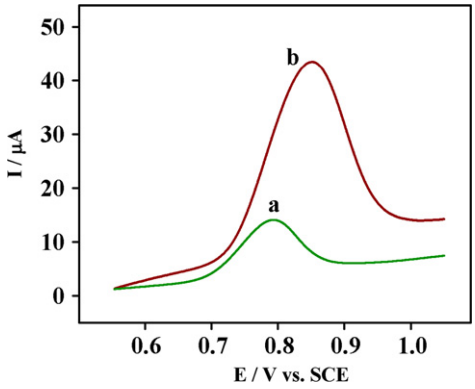

Fig. 3. Square wave voltammograms obtained at the guanine/GC (a) and guanine/GNR/GC electrode (b) in 50 mM PBS (pH 4.92).

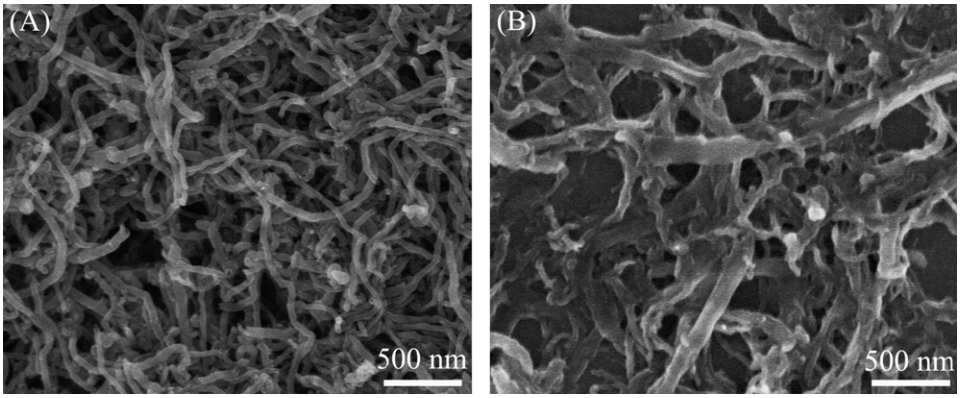

Fig. 1. SEM images of MWCNTs (A) and GNR (B).

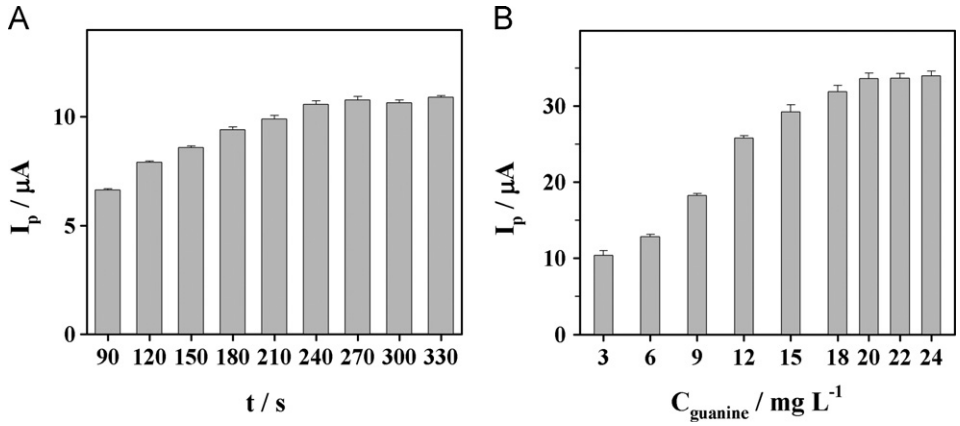

Fig. 4. The effects of the accumulation time (A) and the concentration of guanine (B) on the oxidation peak current (I_P) of the guanine/GNR/GC electrode in 50 mM PBS (pH 4.92).

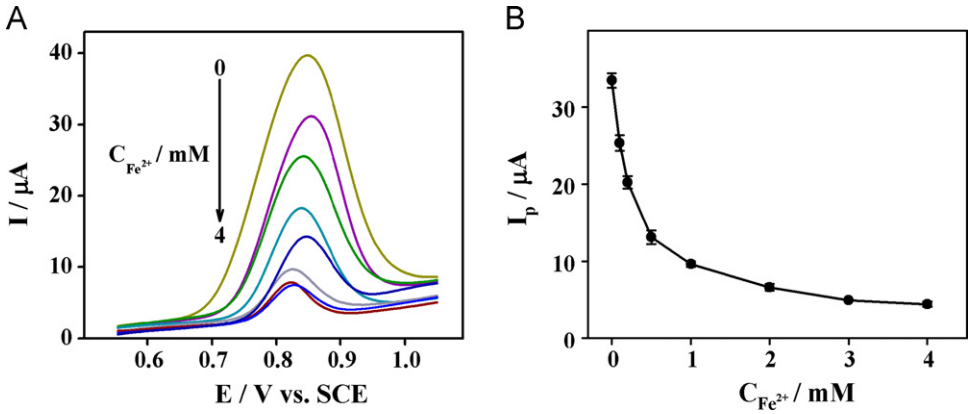

Fig. 5. (A) The oxidation peak current (I_P) of the guanine/GNR/GC electrode after incubation for 180 s in Fenton solution (Fe^{2+}/H_2O_2 1:6 mol mol^{-1}) with different concentration of Fe^{2+}: 0, 0.1, 0.3, 0.5, 0.8, 1.0, 1.5, 2.0 mM. (B) The relationship between I_P and the concentration of Fe^{2+}.

3.3. Assays for guanine damage by ·OH and its protection by AA

In living systems, hydroxyl radicals are usually generated from the metal ion-dependent breakdown of hydrogen peroxide. The Fenton-type system is important because it has been implicated as an important mediator of oxidative damage in vivo. At the present work, Fe^{2+}–EDTA was used as an inorganic reagent for mediating ·OH production from H_2O_2. The damage level of DNA was evaluated as a function of the variation of the concentration of ·OH (Fe^{2+}) keeping the constant molar ratio (1:3:6) of Fe^{2+}:EDTA:H_2O_2. In this Fenton reaction, the concentration of Fe^{2+} is equal to that of ·OH and the damage degree of guanine depends on the ·OH concentration.

In order to evaluate effectively the protection ability of the antioxidant in low concentration, the appropriate concentration of ·OH should be selected to ensure the maximum damage degree of guanine on the guanine/GNR/GC electrode. Fig. 5 shows the effect of the concentration of Fe^{2+} (·OH) on the electro-oxidation peak current (I_P) of the remaining unoxidized guanine on the electrode. It is noted that the value of I_P decreases sharply with the increase of the ·OH (Fe^{2+}) concentration and no obvious decrease of the I_P value is observed when the concentration of ·OH (Fe^{2+}) is higher than 2.0 mM. Therefore, 2.0 mM is selected as the optimum concentration of ·OH (Fe^{2+}) in the evaluation of the protection ability of the antioxidant and TAC in commercial fruit juices.

The influence of the incubation time of the guanine/GNR/GC electrode in Fenton solution (2 mM Fe^{2+}) was also investigated and the corresponding results are shown in Fig. 6. It is noted that the peak current of guanine decreases with the increase of the incubation time and no obvious decrease is observed after 180 s. This implies that 180 s is enough for guanine damage and used as the optimum incubation time.

In order to study the protective effect promoted by antioxidants on the deactivation of ·OH and consequently protection of the guanine bases from the oxidative damage, AA is selected as the typical antioxidant. It was reported that ·OH oxidized AA and produced dehydroascorbic acid by abstracting proton [20]. Fig. 7A shows the SWV results of the guanine/GNR/GC electrode treated in Fenton solution (2 mM Fe^{2+}) with different concentration of AA. It is noted that the I_P value increases with the increase of the concentration of AA, due to the scavenging effect of AA for ·OH. Here, the scavenging ability of AA is expressed as the value of ΔI_p ($\Delta I_P = I_{pp} - I_{pa}$, where I_{pp} is the electro-oxidation peak current of the remaining unoxidized guanine after the guanine/GNR/GC electrode was treated in Fenton solution (2 mM Fe^{2+}) with antioxidant, I_{pa} is the electro-oxidation peak current of the remaining unoxidized guanine after the guanine/GNR/GC electrode was treated in Fenton solution (2 mM Fe^{2+}) without antioxidant) and the relationship between ΔI_p and the concentration of AA is shown clearly in Fig. 7B. It can be observed that the ΔI_p value increases linearly with the increase of AA concentration from 0.1 to 4.0 mg L^{-1} and the corresponding linear function is ΔI_p (μA) = $4.1575 C_{AA} - 0.1128$ with a correlation coefficient of $R^2 = 0.9967$. The limit of detection (LOD) is calculated to be 0.05 mg L^{-1} ($S/N = 3$). The comparison of the purine-based

electrochemical DNA sensors for the evaluation of the antioxidant capacity was listed in Table 1. From Table 1, it can be seen that the guanine/GNR/GC electrode shows the improved performance, not only the linear range but also the detection limit. In particular, the detection limit of the proposed electrode is 5 or 10 times lower than that of the other purine-based electrochemical DNA sensors developed previously [18,19,21].

The reproducibility and stability of the guanine/GNR/GC electrode for determination of antioxidants was evaluated by detecting AA (2.0 mg L^{-1}) in Fenton solution. The results reveal that the modified electrode possesses a satisfying reproducibility with a relative standard deviation (RSD) of 2.6% for 10 times parallel measurements with different electrodes. After the modified electrode was stored at room temperature for 30 days, only a small decrease of the response current (about 3.7%) was observed. These results demonstrate that the electrode exhibits satisfactory reproducibility and stability, indicating that the guanine/GNR/GC electrode is suitable for the practical analysis of TAC in real samples.

3.4. Determination of TAC in commercial fruit juices

To ascertain its practical application, the guanine/GNR/GC electrode was used to analyze the TAC in commercial fruit juices and homemade fruit juices. The values of TAC in fruit juices are expressed in AA content and shown in Table 2. It is noted that all samples exhibit antioxidant activity. The order of TAC in the commercial fruit juices and homemade fruit juices are the same and as follows: orange > grape > pear. From Table 2, we also know that the TAC in the commercial juice is close to the homemade fruit juices, although the pure fruit juice content in the commercial juice are only 10%. This should result from the additives (such as vitamin C, citric acid, malic acid, etc.) added into the commercial fruit juices, which was labeled on the package label. On the other hand, to further testify the feasibility of this method, the concentration of AA in each sample of fruit juices was also determined by the standard addition method and the results are shown in Table 2. From Table 2, the recovery of AA is from 97.6 to 102.6% for commercial fruit juices and from 95.4 to 103.4% for homemade fruit juices, respectively. These results imply that the proposed electrochemical DNA sensor (the guanine/GNR/GC electrode) has great potential application in TAC detection in fruit juices.

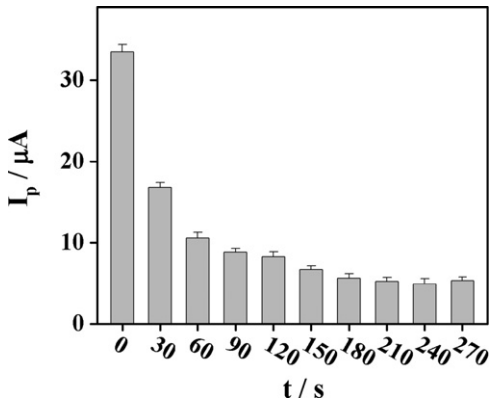

Fig. 6. The influence of the incubation time on the oxidation peak current (I_P) of the remaining unoxidized guanine on the guanine/GNR/GC electrode. Fenton solution, 2 mM Fe^{2+} + 12 mM H_2O_2.

4. Conclusion

Based on electro-immobilization of guanine on GNR modified GC electrode, a new electrochemical DNA biosensor was fabricated for the evaluation of protection ability of antioxidant and TAC in commercial fruit juices. For antioxidant assay, the proposed guanine/GNR/GC electrode shows wide linear range, high sensitivity and low detection limit, due to the large surface area and good electrochemical properties of GNR. Particularly, using AA as the standard antioxidant, the detection limit of the guanine/GNR/GC electrode is 5 or 10 times lower than that of the other purine-based electrochemical DNA sensors developed previously.

Table 1
Comparison of different purine-based electrochemical sensors for analysis of the antioxidant capacity (expressed in AA).

Electrode	Free radicals	Linear range (mgL^{-1})	Limit of detection (LOD)	Ref.
Guanine/GC	$SO_4^{\bullet -}$	0.5–4.0	0.47 m g L^{-1}	[18]
Adenine/GC		0.5–4.0	0.50 mg L^{-1}	
Guanine/GC	$O_2^{\bullet -}$	1.0–5.0	0.77 mg L^{-1}	[21]
Adenine/GC		0.5–4.0	0.50 mg L^{-1}	
Guanine/GC	·OH	0.5–2.5	0.29 mg L^{-1}	[19]
Adenine/GC		2.0–6.0	0.99 mg L^{-1}	
Guanine/GNR/GC	·OH	0.1–4.0	0.05 mg L^{-1}	This paper

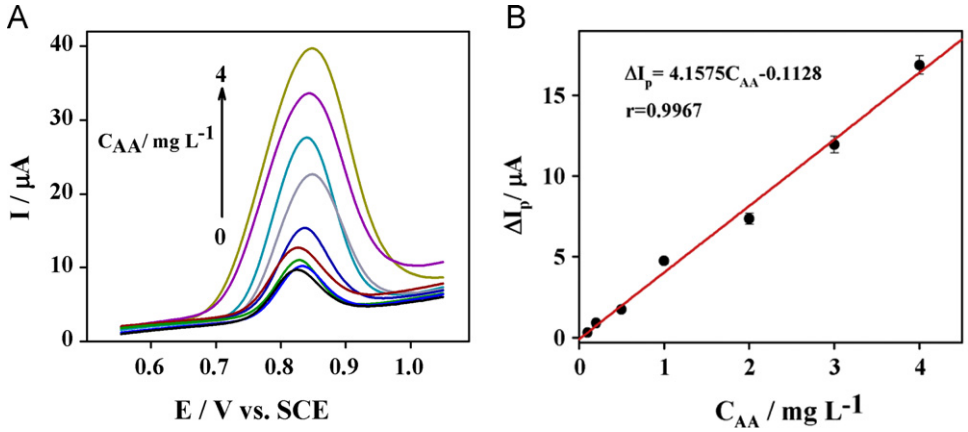

Fig. 7. (A) The oxidation peak current (I_P) of the guanine/GNR/GC electrode after incubation for 180 s in 2 mM Fe^{2+} + 12 mM H_2O_2 Fenton solution with different concentration of AA: 0, 0.1, 0.2, 0.5, 1, 2, 3, 4 mg L^{-1}) and (B) The calibration plot of ΔI_P against AA concentration.

Table 2
TAC values in fruit juices (expressed in AA) and AA recovery in the corresponding fruit juices ($n=3$).

Sample	Fruit juices (wt%)	TAC [mg L^{-1}]	AA added [mg L^{-1}]	AA found [mg L^{-1}]	AA recovery (%)
Commercial fruit juices	Orange 10	231.5	50	280.3.	97.6
	Grape 10	182.6	50	232.3	99.4
	Pear 10	113.2	50	164.5	102.6
Homemade fruit juices	Orange 100	326.8	50	374.5	95.4
	Grape 100	238.2	50	289.9	103.4
	Pear 100	69.3	50	120.2	101.8

On the other hand, the guanine/GNR/GC electrode has good reproducibility and stability, and provides a promising sensing platform for the evaluation of the TAC in commercial fruit juices.

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