Research Article

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Electrochemical antioxidant screening and evaluation based on guanine and chitosan immobilized MoS_2 nanosheet modified glassy carbon electrode (guanine/CS/MoS_2/GCE)

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Abstract: In this study, an electrochemical biosensor based on guanine and chitosan immobilized MoS_2 nanosheet modified glassy carbon electrode (guanine/CS/MoS_2/GCE) was developed and employed for antioxidant screening and antioxidant capacity evaluation. The oxidation peak current of guanine was improved and nearly tripled after modifications of chitosan and MoS_2 nanosheet. The immobilized guanine could be damaged by hydroxyl radicals generated in Fenton solution. However, in the presence of antioxidants, the guanine was protected and the oxidation peak current of guanine increased. This process mimics the mechanism of antioxidant protection in human body. The factors affecting preparation of sensor and detection of antioxidant capacity were optimized. At the optimum conditions, the guanine/CS/MoS_2/GCE showed wide linear range, low detection limit, satisfactory reproducibility and stability for detection. Ascorbic acid was used as a model antioxidant to evaluate the antioxidant capacity. A good linearity was observed with a correlation coefficient of 0.9959 in the concentrations between 0.5 and 4.0 mg L^{-1}. The antioxidant capacities of three flavonoids were also tested and the rank of antioxidant capacities was ascorbic acid (51.84%), quercetin (45.82%), fisetin (34.39%) and catechin (16.99%). Due to the rapid measurement and low cost, this sensor could provide an available sensing platform for antioxidant screening and evaluation.

Keywords: Antioxidant capacity; Chitosan; Guanine; Hydroxyl radical; MoS_2 nanosheet.

1 Introduction

There is a growing attention paid to the damage of living cells caused by oxidative stress, which is generally associated with the generation of reactive oxygen species (ROS). ROS includes oxygen radicals (O^{2-}, ·OH, $RO_2^{\cdot}$, $HO_2^{\cdot}$) and byproducts of nonradical oxidizing agents (H_2O_2, HOCl, O_3) that can be easily converted into radicals [1]. When the level of ROS increases dramatically to cause oxidative stress, it could lead to cellular damage to DNA, proteins and lipids, as well as being linked with various human diseases such as rheumatoid arthritis, cardiovascular disorders, cancer and diabetes, etc. [2]. To protect living organisms and counteract the deleterious effects of ROS, complex and sophisticated endogenous and exogenous antioxidant systems are evolved in organisms [3]. The exogenous antioxidant system can be provided by chemical antioxidants such as ascorbic acid, phenolic compounds and flavonoids, which exist in fruit, vegetables and beverages. These antioxidants are important for the protection of human health [4]. Therefore, finding antioxidants and the following evaluation of antioxidant capacity for antioxidants has significant meaning in related research, and there is a

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high demand to explore rapid and appropriate methods for quantitative determination of antioxidants in real samples.

Several traditional analytical methods have been proposed for measuring the total antioxidant capacities in various samples. Usually, these analytical methods are based on photometric, chromatographic and fluorometric techniques [5-7]. However, the radicals used in these methods ($DPPH^{\cdot}$, $ABTS^{+\cdot}$ or iron) are usually chemical reagents, which belong to exogenous ROS. And the corresponding measures of antioxidant capacities are chemical types as well. Moreover, unstable results and sophisticated procedures are other disadvantages with these methods. Therefore, the simulation of antioxidant mechanism and oxidative stress state taking place *in vivo* are helpful to explore effective and satisfactory antioxidants. However, this kind of simulation face more difficulties as well.

Recently, several ROS scavenging-type electrochemical antioxidant sensors have been developed and reported [8-10]. For this kind of biosensors, guanine is immobilized on electrodes as an oxidation target, and Fenton solutions are used for ·OH generation. However, the immobilization of guanine on electrodes suffers some drawbacks, such as inactivation, insufficient adsorption and complicated procedures. MoS_2 nanosheet is one of the graphene-like two-dimensional layered materials [11]. It has advantages like large specific surface area, unique optical properties, high electrocatalytic ability, good biocompatibility, and so on [12, 13]. Due to the large specific surface area, MoS_2 nanosheet shows a good adsorption performance and could improve the immobilization of guanine. Driven by this property, the immobilization of guanine on the surface of MoS_2 nanosheet modified glassy carbon electrode (GCE) and subsequent activation of guanine are better than those of bare GCE. What's more, good biocompatibility and nontoxicity of MoS_2 nanosheet allow its development in biochemical and biological researches.

In this work, MoS_2 nanosheet modified GCE (MoS_2/GCE) is prepared, and then guanine and chitosan are immobilized on MoS_2/GCE forming the guanine and chitosan immobilized MoS_2 nanosheet modified glassy carbon electrode (guanine/CS/MoS_2/GCE) as an electrochemical sensor for screening and evaluation of antioxidants. The working principle of this sensor is shown in **Scheme 1**. The ·OH radicals generated by Fenton solution could damage guanine and lead to the change of peak current in square wave voltammetry (SWV). In this process, MoS_2 nanosheet serves as a co-catalyst and could greatly enhance the efficiency of ·OH radicals generation [14]. Large number of ·OH radicals generated on the surface of MoS_2/GCE mimic the ROS environment and antioxidant protection mechanism *in vivo*. Meanwhile, ·OH radicals could be attracted by the glucoside bonds of chitosan. Hence, guanine could be damaged more completely under the existence of chitosan [15]. Compared with traditional methods, this sensor could directly determine the antioxidant capacity of target compound without extra processes. Therefore, this sensor could be applied in rapid detection of antioxidant capacity and screening of antioxidants from complexes.

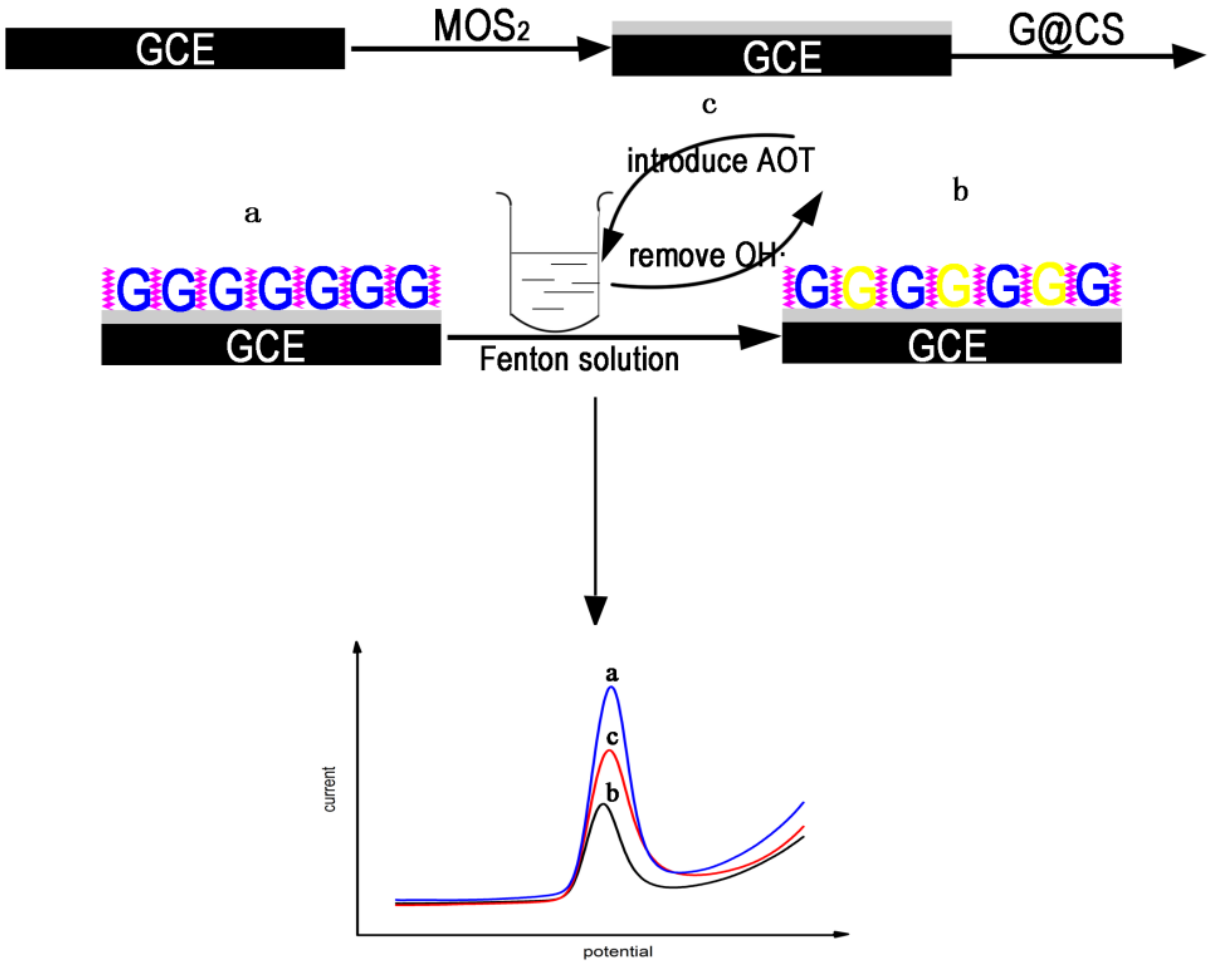
2 Materials and Methods

2.1 Chemicals and reagents

Guanine, chitosan, MoS_2 powder, ferrous sulfate and ethylenediaminetetraacetic acid (EDTA) were purchased from Merck KGaA (Darmstadt, Germany). Quercetin, fisetin and catechin were acquired from Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China). Voltammetric measurements were carried out in phosphate buffered solutions (PBS, 50 mM) as supporting electrolytes. Stock solutions of guanine (1 g L^{-1}) were prepared by dissolving a certain amount of guanine in NaOH (0.1 M). Stock solutions of flavonoids (quercetin, fisetin and catechin) were prepared by dissolving the solid in ethanol. The working standard solutions were prepared daily by diluting stock solutions with supporting electrolytes to certain concentration. Fe^{2+}-EDTA solution was prepared daily by adding definite amount of ferrous sulfate to EDTA solution (1 mM). Fenton solution for ·OH generation was carried out in a mixture of hydrogen peroxide (20 mM) and Fe^{2+}-EDTA. Ultra-pure water (18.25 MΩ·cm, 25 °C) was obtained from a Millipore system (Millipore Corporation, Milford, MA, USA). All other chemicals were of analytical grade without further purification.

2.2 Apparatus

All electrochemical measurements were performed on a CHI 660E Electrochemical Workstation (Chenhua Instrument Company of Shanghai, Shanghai, China) with a standard three electrodes system including a glassy carbon electrode (GCE, a diameter of 3 mm) as the working electrode, a platinum wire as the auxiliary electrode and an Ag/AgCl (KCl 3.0 M) electrode as the reference electrode. All the potentials obtained in this study are reported against Ag/AgCl/KCl electrode. The morphology of MoS_2



Scheme 1: The preparation process of the modified electrode and the detection method for guanine damage: SWV.

was obtained by using a scanning electron microscopy (SEM, JSM 6610Lv, JEOL, Japan) operated at 30 kV. An ultrasonic processor FS-250 (20% amplitude; Shanghai Shengxi, Shanghai, China) was used for synthesizing the MoS_2 nanosheet. All the experiments were carried out at room temperature.

2.3 Preparation of MoS2 nanosheet

The MoS_2 nanosheet was prepared by MoS_2 powder according to literature [16] with some modifications. Briefly, 300 mg of MoS_2 powders were dispersed in 100 mL of mixtures consisted of acetone and water (v:v, 89:11) to obtain MoS_2 suspension (3 mg mL^{-1}). Afterwards, the suspension was ultrasonically treated in iced water for 1 h using an ultrasonic processor FS-250 with pulsed ultrasonic irradiation mode (Power, 35 W; Pulse frequency: 20 s on and 10 s off) to attain MoS_2 nanosheet. The pulsed ultrasonic irradiation mode could reduce solvent heating and avoid degradation of produced MoS_2 nanosheet. Finally, the dispersion was centrifuged at 3000 rpm for 30 min (Beckman Coulter Allegra 64R, Brea, CA, USA). The supernatant was collected and dried in a vacuum oven for further use.

2.4 Preparation of the guanine/CS/MoS2/GCE

The MoS_2 suspension (0.5 mg mL^{-1}) was obtained by dispersing MoS_2 nanosheet in water. The MoS_2/GCE was obtained by dropping 6 μL of MoS_2 suspension on the surface of a GCE and dried at room temperature to form a MoS_2 film on the GCE. Then, chitosan solution (1.0 mg mL^{-1}, in 1% acetic acid) was added to a guanine solution (0.6 mg mL^{-1}) at a volume ratio of 1:5 (v/v), and then 6 μL

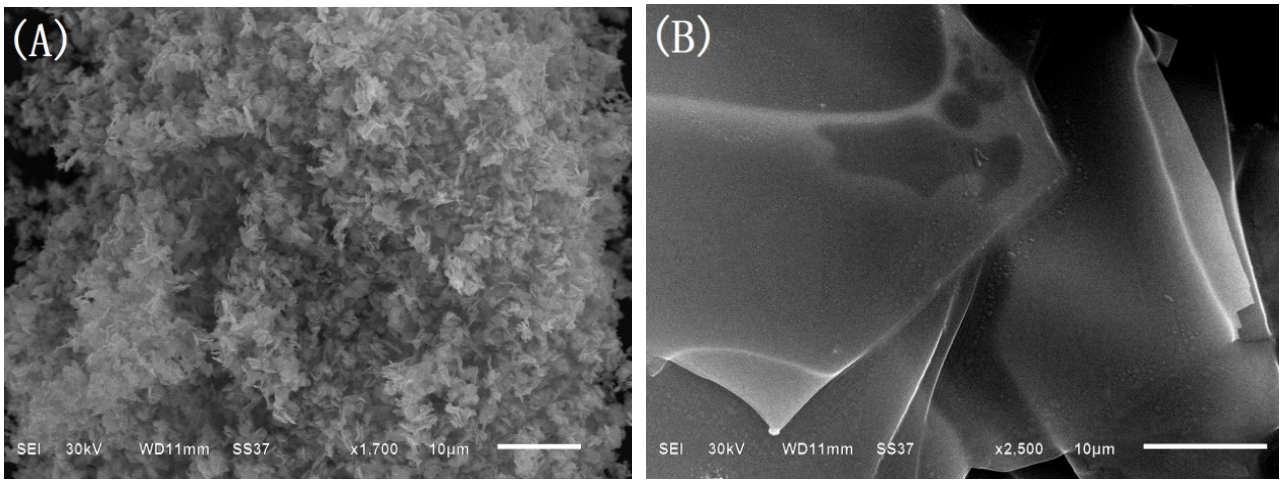


Figure 1: SEM images of MoS_2 powder (A) and MoS_2 nanosheet (B).

of the mixture added on to the surface of MoS_2/GCE and dried at room temperature. Ultimately, we obtained a guanine/CS/MoS_2/GCE.

2.5 Antioxidant capacity assays

Hydroxyl (·OH) radicals could be produced by adding hydrogen peroxide into freshly prepared Fe^{2+}-EDTA solution in PBS (50 mM, pH 3.5). Damage of guanine was performed by immersing the guanine/CS/MoS_2/GCE in Fenton solution. After a certain incubation time, the guanine/CS/MoS_2/GCE was washed with water and immersed in PBS (50 mM, pH 1.5) immediately to perform the SWV detection. The oxidation peak of guanine in SWV peaks was measured at about 1.20 V.

For antioxidant capacity assay, antioxidant (quercetin, fisetin and catechin) with a certain concentration was firstly added into freshly prepared Fenton solution. Then, the guanine/CS/MoS_2/GCE was immersed in the Fenton solution containing antioxidant. After the same procedures as mentioned before, the SWV detection of guanine/CS/MoS_2/GCE was performed as well. The oxidation peak of guanine in SWV peaks could be observed and used to calculate the antioxidant activity of sample. The antioxidant capacity of sample was calculated using the following equation:

$$\text{Antioxidant capacity } \% = (I_0 - I_s) / (I_0 - I_c) \times 100 \quad (1)$$

Where I_0 is the oxidation peak current of guanine/CS/MoS_2/GCE before damage in Fenton solution, I_c is the oxidation peak current after damage in Fenton solution without sample, and I_s is the oxidation peak current after damage in Fenton solution with sample.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and discussion

3.1 Characterization of MoS2 nanosheet

The morphologies of MoS_2 powder (**Figure 1A**) and MoS_2 nanosheet (**Figure 1B**) were characterized by scanning electron microscopy (SEM). It could be observed in **Figure 1A** that the MoS_2 powders were agglomerated by a large number of particles with 2 μm of diameter, and the particles were uniform in morphology. **Figure 1B** showed that the exfoliated MoS_2 nanosheet with a typical layered and wrinkled structure. The SEM characterization confirmed that MoS_2 nanosheet was successfully prepared from MoS_2 powders.

3.2 Electrochemical study of the guanine/CS/MoS2/GCE

Figure 2 showed the SWV results of guanine/GCE (a), guanine/CS/GCE (b) and guanine/CS/MoS_2/GCE (c) in PBS (50 mM, pH 1.5). It can be noted from the picture that an oxidation peak potential of guanine appeared at about 1.20 V. When the guanine molecules were previously mixed with chitosan and then immobilized together on the electrode, the corresponding oxidation peak current

got a clear improvement (**Figure 2b**). It indicated that chitosan could effectively adsorb guanine to the surface of electrode. Moreover, the oxidation peak current of guanine on the guanine/CS/MoS_2/GCE is 42.6 μA, which is nearly 3 times than that on the guanine/GCE (15.2 μA). This might be due to the good adsorption performance and biocompatibility of MoS_2 nanosheet.

In order to get a better understanding about the interaction between chitosan and guanine, the geometry of chitosan and guanine was optimized with the density functional theory method (B3LYP/6-31G*). The density functional theory [17] B3LYP/6-31G* basis set was employed to optimize the guanine and chitosan without any constraints via the Gaussian software [18]. The B3LYP functional includes Becke's three-parameter-exchange functional and Lee-Young-Parr correlation functional [19-21]. **Figure 3** showed the optimized structures and the electrostatic potential surface of aromatic systems of guanine (a) and chitosan (b). Electrostatic potentials were caused by the interactions of electrons or nuclei with electrons [22, 23]. The blue area corresponded to the negative charges. The red region expressed the positive charges. As shown in **Figure 3**, the N_6 and N_{11} atom in guanine had negative charges. However, the positive charges have been found in the amino unit of chitosan which was consisted well with previous experiment [24]. It indicated that chitosan and guanine could bind together well with electrostatic interaction.

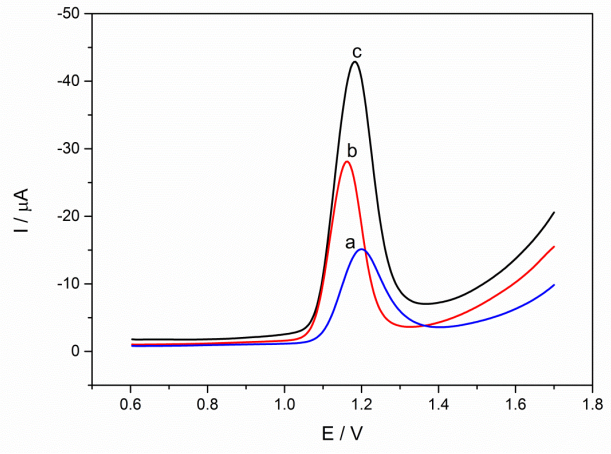


Figure 2: SWVs obtained at the guanine/GCE (a), guanine/CS/GCE (b) and guanine/CS/MoS_2/GCE (c) in PBS (50 mM, pH 1.5).

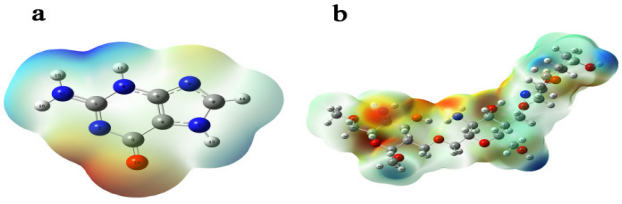


Figure 3: The optimized structure of guanine (a) and chitosan (b) and electrostatic potential surface of aromatic systems calculated at B3LYP/ 6-31G*.

3.3 Effect of pH and immobilized concentration

The effect of pH value on the electrochemical response of guanine on guanine/CS/MoS_2/GCE was studied by SWV and the results were shown in **Figure 4A**. An insert displayed a plot of guanine electro-oxidation signals with the change of pH. The oxidation peak currents decreased gradually with the increasing of pH values. The response reached the maximum value at pH 1.5. The oxidation peak potentials obeyed the equation in the range of pH from 1.5 to 4.0 (**Figure 4B**), Ep = 1.288 − 0.0696 pH (R^2 = 0.9861). The calculated slope (69.6 mV pH^{-1}) was closed to the theoretical value of 59 mV indicating that direct electrooxidation of guanine is an isoelectron and isoproton process [25, 26]. Therefore, 50 mM phosphate buffered solution with pH 1.5 was used as the supporting electrolyte in all electrochemical determinations during this research.

The amount of modifiers on the surface of electrode is one of the important factors that influence the voltammetric response of guanine. As shown in **Figure 5**, the peak currents increased gradually with the increase of guanine/CS concentrations (from 0.1 to 0.6 mg mL^{-1}) and decreased rapidly when the concentration was higher than 0.6 mg mL^{-1}. This result might indicate that the thicker film of guanine/CS blocked the electron transfer between guanine and electrode. Therefore, 0.6 mg mL^{-1} of guanine/CS suspension was chosen as the optimum concentration to modify the GCE.

3.4 Optimization of electrochemical assays

Hydroxyl radicals (·OH) are part of reactive oxygen species in living systems, which are generated from the metal ion-dependent breakdown of hydrogen peroxide. The ·OH produced by Fenton's reaction plays a major role during oxidative damage *in vivo*. EDTA is usually used as a chelating agent in Fenton solution to increase the solubility of Fe^{2+} ions. Meanwhile, MoS_2 nanosheet could enhance the efficiency of ·OH generation as a co-catalyst. Therefore, Fenton solution containing EDTA was used to

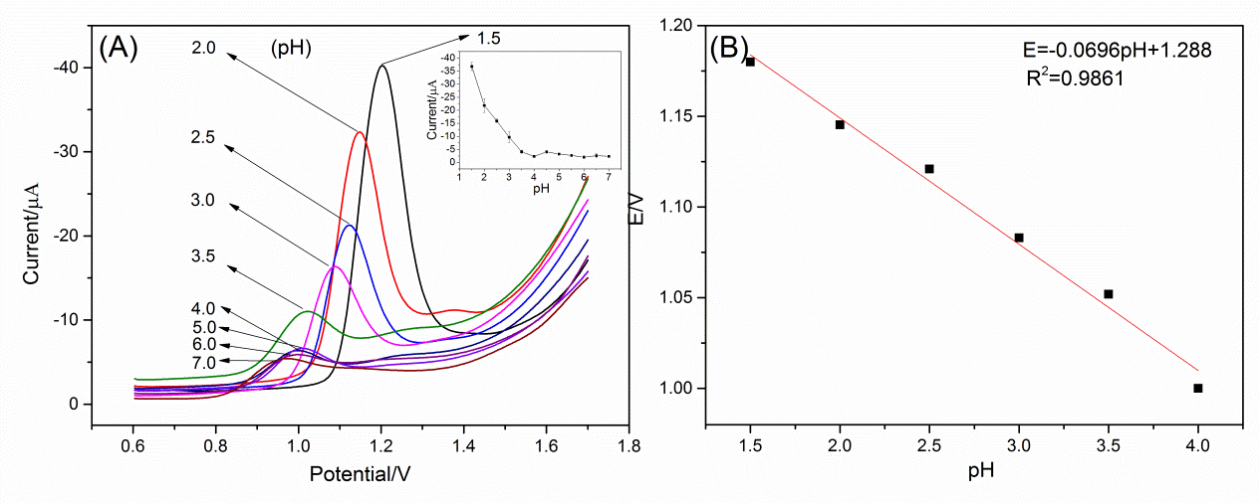


Figure 4: (A) Effect of pH on the electrochemical response of guanine on the guanine/CS/MoS_2/GCE. Insert: relationship between oxidant peak current and pH. (B) The calibration plot of potential against pH. The experimental conditions were incubated in Fenton solution (1 mM EDTA, 2.0 mM Fe^{2+} ions and 20 mM H_2O_2) for 300 s.

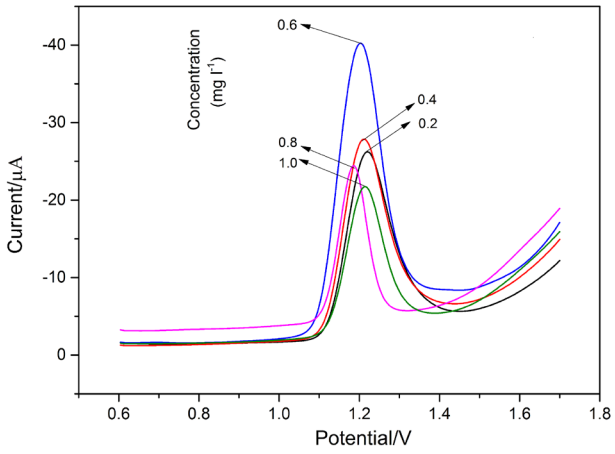


Figure 5: Effect of immobilized concentration on the electrochemical response of guanine on the guanine/CS/MoS_2/GCE. The experimental conditions were incubated in Fenton solution (1 mM EDTA, 2.0 mM Fe^{2+} ions and 20 mM H_2O_2) for 300 s.

generate ·OH for the damage of guanine on electrode in this study.

Reported studies indicated that the concentration of Fe^{2+} ions had a close relationship with the production of ·OH and affected the damage of guanine [27]. The relationship between concentration of Fe^{2+} ions and electrochemical oxidation peak current of guanine/CS/MoS_2/GCE was studied. As shown in **Figure 6**, it could be observed that the peak currents decreased with increasement of Fe^{2+} ions. When the concentration of Fe^{2+} ions is higher than about 2.0 mM, the change of peak current tended to gentle.

Incubation time could affect the damage degree of guanine. Therefore, the effect of incubation time was also investigated in this study (**Figure 7**). With the incubation time increased, the peak currents decreased gradually until the incubation time reached 300 s. It indicated that 300 s incubation was enough for guanine damage. Therefore, 2.0 mM Fe^{2+} ions and 300 s incubation time were chosen as experimental conditions for future studies.

3.5 Antioxidant capacity assays

The ·OH generated by Fenton solution could attack electrode surface and damage guanine molecules. When antioxidants were added into Fenton solution, the produced ·OH was scavenged by antioxidants and correspondingly the damage of guanine was reduced. Through the electrochemical detection, a higher oxidation peak current of guanine could be observed and the antioxidant capacity could be then calculated.

As a well-known and powerful antioxidant, ascorbic acid (AA) is regarded as an important water-soluble ROS scavenger in human body [28]. In this work, AA was selected as a model antioxidant to evaluate the antioxidant capacity performance in developed electrochemical biosensor. **Figure 8A** showed the SWV curves of guanine/CS/MoS_2/GCE incubated for 300 s in Fenton solution (1 mM EDTA, 2.0 mM Fe^{2+} ions and 20 mM H_2O_2) with different concentrations of AA. **Figure 8B** showed the plots of peak currents against AA concentrations. It was noted that the presence of AA significantly affected the oxidation peak

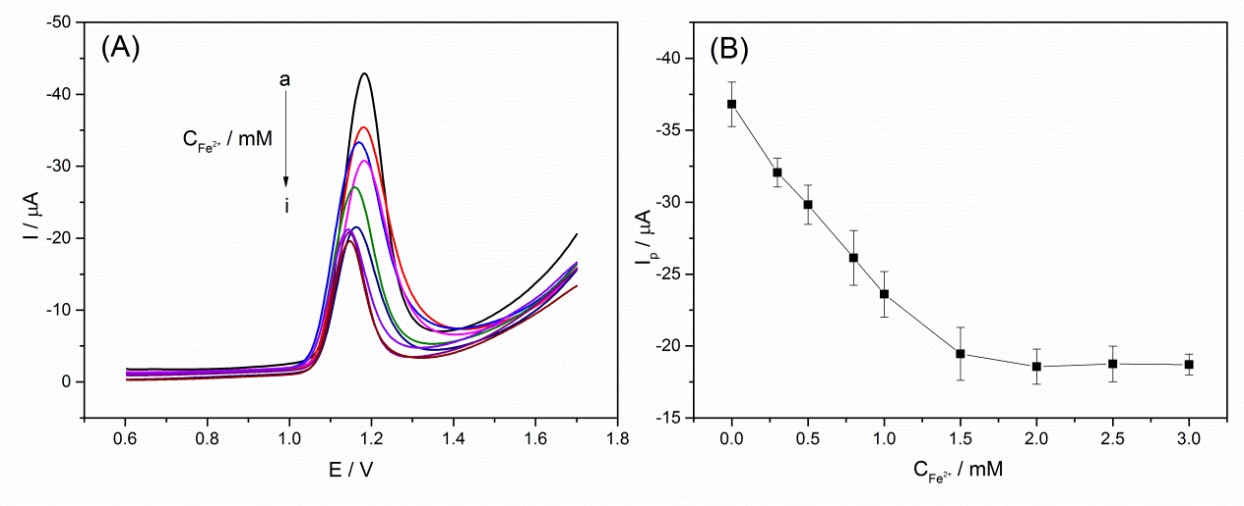


Figure 6: (A) The oxidation peak current of the guanine/CS/MoS_2/GCE after incubation for 300 s in Fenton solution (1 mM EDTA and 20 mM H_2O_2) with different concentration of Fe^{2+} ions (a-i): 0, 0.3, 0.5, 0.8, 1.0, 1.5, 2.0, 2.5 and 3.0 mM. (B) The relationship between oxidant peak current and the concentration of Fe^{2+} ions.

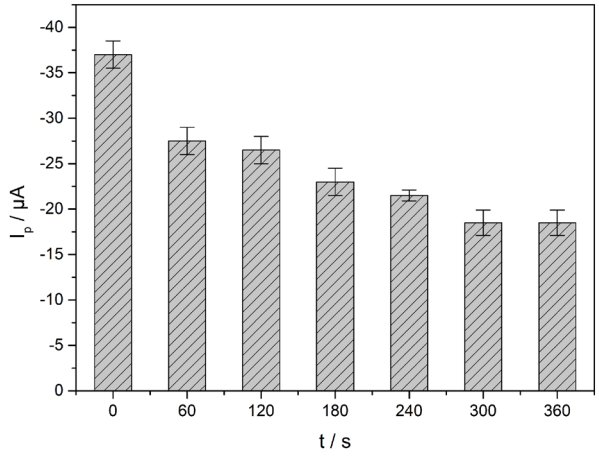


Figure 7: Effect of incubation time of the guanine/CS/MoS_2/GCE with the Fenton solution (1 mM EDTA, 2.0 mM Fe^{2+} ions and 20 mM H_2O_2).

current, and the peak current increased with increasing concentration of AA. A linear relationship of peak current and AA concentration was also observed (**Figure 8B**) when the concentration of AA was between 0.5 and 4.0 mg L^{-1}. The corresponding linear function was I_p (μA) = -4.4395 C_{AA} – 18.2693 with a correlation coefficient of R^2 = 0.9959. The detection limit was calculated to be 0.33 mg L^{-1} (S/N = 3). Based on these results, it could be considered the developed sensor showed good linear range and detection limit.

In order to confirm the abilities in antioxidant capacity detection and antioxidant screening of proposed sensor, the antioxidant capacities of several flavonoids were analyzed by guanine/CS/MoS_2/GCE. The concentration of antioxidant in this assay was set at 2.0 mg L^{-1}. **Figure 9** presented the results of antioxidant capacities for three flavonoids and AA. AA displayed the highest antioxidant capacity (51.84%), then followed by quercetin (45.82%), fisetin (34.39%) and catechin (16.99%). Three investigated flavonoids showed different antioxidant capacities, which showed this sensor could be used for evaluation of samples.

3.6 Reproducibility and stability tests

The reproducibility and stability of modified electrode were also evaluated by detecting AA (2.0 mg L^{-1}) in Fenton solution. As shown in **Figure 10**, Different electrodes were used for 10 times of parallel measurements (Black plots), and it showed a good reproducibility with the relative standard deviation is 2.9%. The same electrode was also tested for 10 times of parallel measurements (Red plots), and it showed a better reproducibility with the relative standard deviation of 1.2%. The modified electrode was stored at the room temperature (25°C) for a week. The current of electrode was reduced about 1.8%. The modified electrode displayed acceptable reproducibility and stability, indicating that the guanine/CS/MoS_2/GCE is suitable for antioxidant screening.

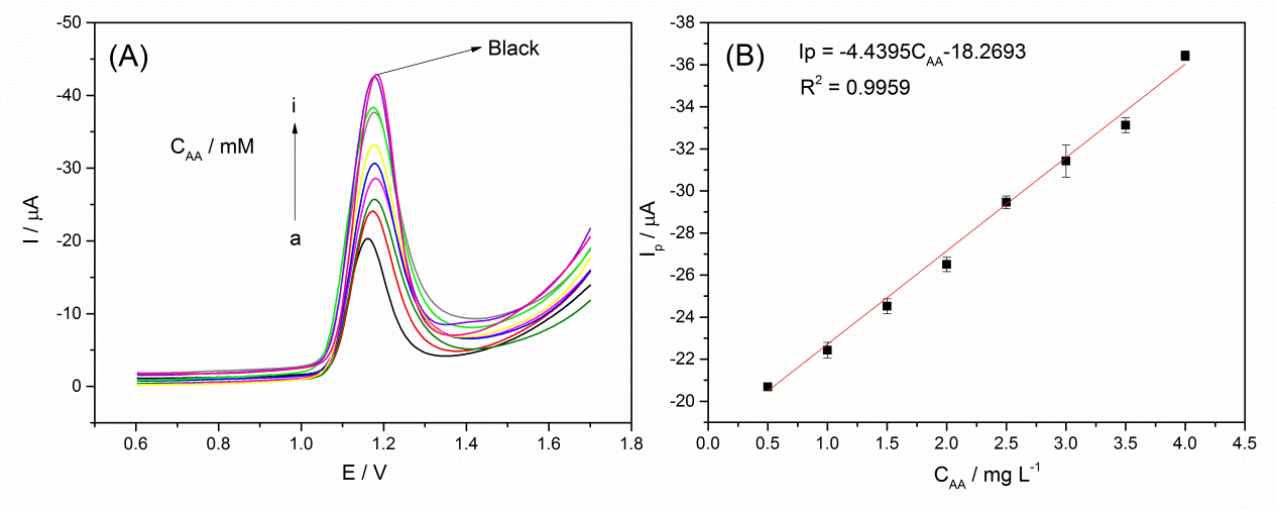


Figure 8: (A) The oxidation peak current of the guanine/CS/MoS_2/GCE after incubation for 300 s in Fenton solution (1 mM EDTA, 2.0 mM Fe^{2+} ions and 20 mM H_2O_2) with different concentration of AA (a-i): 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg L^{-1}. (B) The calibration plot of oxidant peak current against the concentration of AA.

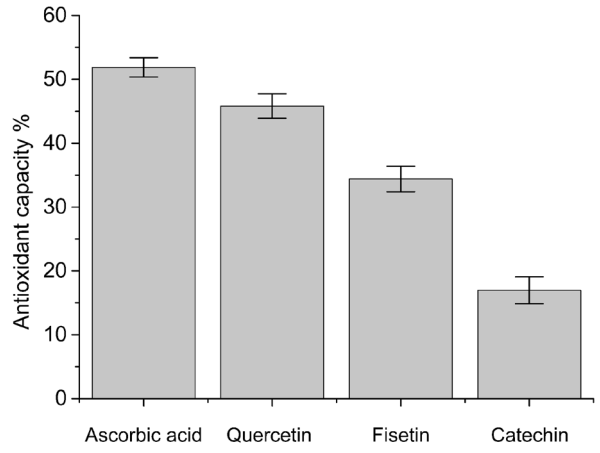


Figure 9: Antioxidant screening of several antioxidant compounds using the guanine/CS/MoS_2/GCE electrode.

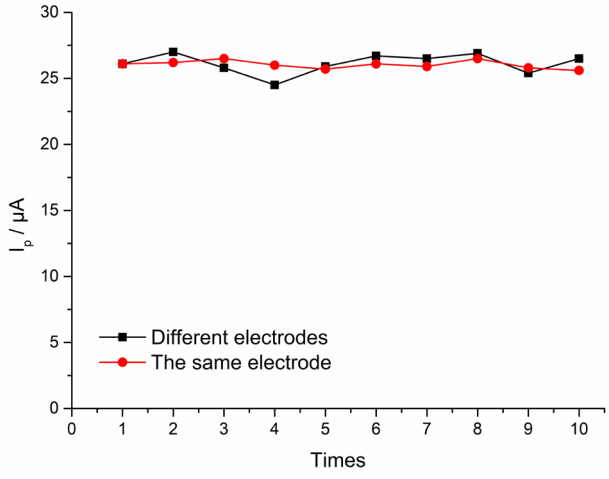


Figure 10: The reproducibility of guanine/CS/MoS_2/GCE (Black: different electrodes; Red: the same electrode) in detection of AA (2.0 mg L^{-1}). The experimental conditions were incubated in Fenton solution (1 mM EDTA, 2.0 mM Fe^{2+} ions and 20 mM H_2O_2) for 300 s.

4 Conclusions

In this study, the guanine/CS/MoS_2/GCE was assembled and investigated as an electrochemical biosensor for antioxidant screening and antioxidant capacity test. Due to the co-catalyst and adsorption effects of MoS_2 nanosheet, the detection could mimic the antioxidant protection mechanism *in vivo*. The factors affected preparation of sensor and detection of antioxidant capacity were optimized. Under the optimum conditions, the guanine/CS/MoS_2/GCE showed advantages like wide linear range, low detection limit, satisfactory reproducibility and stability. The antioxidant capacities of three flavonoids were also tested and compared with AA. As a result, the rank of antioxidant capacity was AA (51.84%), quercetin (45.82%), fisetin (34.39%) and catechin (16.99%). The proposed biosensor provided a rapid and inexpensive sensing platform for antioxidant screening and evaluation.

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Conflicts of Interest: The authors declare no conflict of interest.

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