

A graphene-based label-free voltammetric immunosensor for sensitive detection of the egg allergen ovalbumin

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A graphene-based label-free voltammetric immunosensor for the sensitive detection of the egg white allergen ovalbumin has been developed. Graphene-modified screen printed carbon electrodes have been covalently functionalized using electrochemical reduction of *in situ* generated aryl diazonium salt forming a carboxyphenyl film on the graphene surface. The blocking property of the carboxyphenyl film grafted on to the graphene electrodes using different cyclic voltammetry cycles has been characterized using differential pulse voltammetry in $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution. Then, the terminal carboxylic groups on the graphene surface were activated using EDC/NHS and used to immobilize the ovalbumin antibody and construct the immunosensor. The fabrication steps of the immunosensor have also been characterized using differential pulse voltammetry. The decrease in the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ reduction peak current after the immunochemical reaction with ovalbumin has been used for the ovalbumin detection. The developed immunosensor has been used for ovalbumin detection in the concentration range of 1 pg mL^{-1} to $0.5 \text{ }\mu\text{g mL}^{-1}$ with a detection limit of 0.83 pg mL^{-1} in PBS buffer. The food matrix effect studied with ovalbumin spiked cake extract showed a good percentage of recovery, indicating the possible applicability of the developed immunosensor in real food samples.

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1 Introduction

Food allergy has become one of the major health concerns nowadays.^{1,2} So far, there has been no effective treatment for food allergen sufferers and the only way to manage the health hazards resulting from allergens is to avoid the specific allergen containing food. Egg is one of the most common causes of food allergies in children, particularly infants under 3 years old.^{3–6} It is considered the first cause of allergies in some countries exceeding even the cows' milk allergy. The most common allergens in egg are the four chicken egg white proteins: ovalbumin, ovomucoid, ovotransferrin, and lysozyme.^{7,8} Ovalbumin is the most abundant protein in egg white comprising 58% of the whole egg white proteins; therefore it can be used as a marker for the presence of egg contamination in food products. Under the current regulations, egg should be mentioned in the ingredient list on the food product label. However, traces of egg residues can be unexpectedly present in some food products where egg is not mentioned in the ingredient list, due to

inadequate cleaning of the food processing equipment or contamination during the transfer or the storage processes, leading to serious allergic reactions for the sensitive individuals such as urticaria, angioedema, or anaphylaxis.⁶ Therefore, sensitive detection methods are highly demanded for regulatory agencies to protect the health of people with allergies especially children. Several traditional methods are used for the detection of ovalbumin including protein-based methods such as western blot,⁹ immunoperoxidase staining¹⁰ and capillary electrophoresis.^{10,11} Currently, the most commonly and widely used method for ovalbumin detection is the enzyme-linked immunosorbent assay (ELISA).^{12–18} A rolling circle amplification procedure has also been developed to enhance signals of ovalbumin immunoassay combined with fluorescent dye and a circular DNA probe allowing the sensitive and real-time detection of ovalbumin.¹⁹ Despite the relatively high sensitivity of the currently used analytical methods, they are highly laborious, time consuming, require highly trained personnel, as well as expensive. Recently, biosensors have received great interest and exhibit potential roles in various applications such as clinical diagnosis, environmental and food quality analysis.²⁰ Several optical-based biosensors have been developed for the detection of the egg white allergen ovalbumin. Anraku *et al.*²¹ have reported polymer brush modified cap-shaped gold nanoparticles as sensing elements for ovalbumin using localized-surface plasmon resonance. Recently, an optical immunochip biosensor has been reported utilizing the optical near-field

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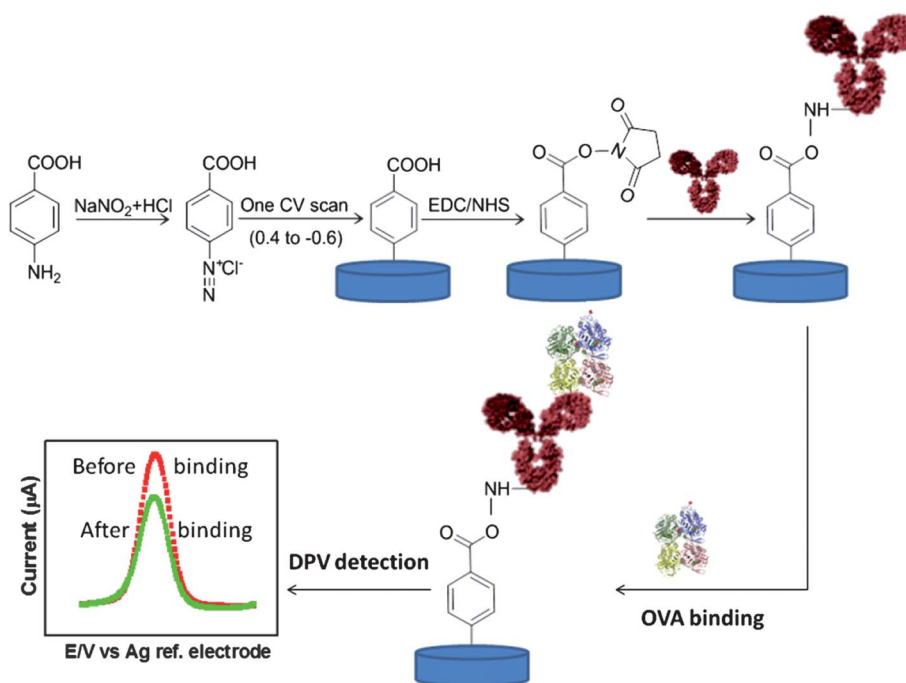
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phenomenon called resonance-enhanced absorption (REA).²² In this approach gold nanoparticles (AuNPs) have been used as signal transducers in an interferometric setup yielding colorimetric response. An optical planar waveguide array sensing platform has also been developed for the detection of multiple analytes including ovalbumin using fluorescence sandwich immunoassays.²³ The samples were run over the sensor surface functionalized with capture antibodies, and then secondary labeled fluorescent antibodies were introduced. The optical biosensing platforms are sensitive; however, they are not suitable for routine analysis due to the bulky and costly instrumentation. Furthermore, most of the assays require secondary recognition receptors and labeling with fluorescent tags which is laborious as well as costly. In recent years, electrochemical-based biosensors have been perceived to be one of the most promising analytical tools due to their low cost, simple use, fast analysis, low detection limits and capability of automation and miniaturization.²⁴ To the best of our knowledge, no electrochemical biosensor has been reported for the detection of the egg allergen ovalbumin. Therefore, a rapid, sensitive and selective electrochemical immunosensor for ovalbumin would provide a potential tool for the screening of egg allergen leading to improvement in food safety and helping in consumer protection.

Graphene, a one-atom-thick carbon nanomaterial consists of sp^2 hybridized carbon atoms forming a honeycomb two-dimensional lattice, has been widely recognized as one of the most promising materials for a variety of important applications.²⁵ Since its isolation in 2004, graphene has shown outstanding features such as high electrical and thermal conductivity, large surface area and high mechanical strength.^{26–29} Developing biosensor platforms is one of the

interesting fields that graphene appeared to play a major role in, such as electrochemical,^{30,31} electrical³² and optical biosensors.^{33,34} Graphene has been widely utilized in developing electrochemical biosensors using two main approaches: first, as an electrode material^{35–37} exploiting its large surface area, high electrical conductivity and fast electron-transfer kinetics, second, as a label-bearing nanocarrier.³⁸ Moreover, graphene has shown superior performance in electrochemical sensors over other carbon materials including carbon nanotubes.^{39,40} The functionalization of the electrode surface and the subsequent immobilization of biomolecules is one of the critical steps in developing electrochemical biosensing platforms which could affect the sensitivity and the selectivity. Graphene has been functionalized by different methods; either non-covalently through π - π stacking of pyrene derivatives³⁶ or covalently using different approaches such as the spontaneous reduction of aryl diazonium salts in organic solvents.^{41–45} Recently, an easy, fast and controlled functionalization approach of graphene modified electrodes using electrochemical reduction of diazonium salts in aqueous solution has been reported.^{46,47} In an attempt to demonstrate the versatility of the electrochemical functionalisation of graphene and its successful applicability for fabricating biosensing platforms, we report here for the first time a biosensing platform for ovalbumin detection based on carboxyphenyl modified graphene. The carboxyphenyl modified graphene was prepared through cyclic voltammetry reduction of *in situ* generated carboxyphenyl diazonium salt in aqueous solution. Then the functionalized graphene electrodes were activated by carbodiimide/succinimide (EDC/NHS) and used for the covalent immobilization of the ovalbumin antibody *via* amide bond formation. A simple label-free differential pulse voltammetric method has been employed



Scheme 1 The construction of the OVA label-free immunosensor and its working principal using DPV detection.

for the detection of ovalbumin based on the decrease of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ reduction peak current after ovalbumin binding (Scheme 1). This proposed method allowed a fast, low cost and single step electrochemical detection method of the egg allergen ovalbumin with very low limit of detection as compared with previously reported methods.

2 Experimental

2.1 Reagents and materials

Anti-ovalbumin monoclonal antibody was obtained from Abcam (Cambridge, USA). Ovalbumin from chicken egg white, 4-aminobenzoic acid, potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6$), potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), dipotassium hydrogen orthophosphate, potassium dihydrogen orthophosphate, sodium chloride, sodium nitrite, hydrochloric acid, 2-(*N*-morpholino) ethanesulfonic acid (MES), bovine serum albumin (BSA), chicken egg lysozyme, β -lactoglobulin (β -LG) and casein were purchased from Sigma (Ontario, Canada). 1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Fisher Scientific (Ontario, Canada). A phosphate buffered saline PBS solution (10 mM, pH 7.4) was used for the preparation of the antibody and the protein standard stock solutions and dilutions. A 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer solution (100 mM, pH 5.0) was used in the activation of the carboxylic groups. A Milli-Q water was used throughout the work.

2.2 Instrumentation

The electrochemical experiments were performed using an Autolab PGSTAT302N (Eco Chemie, The Netherlands) potentiostat/galvanostat connected to a personal computer and controlled by NOVA software version 1.6. Disposable graphene-modified screen printed carbon electrodes (GSPEs) were purchased from Dropsens, Inc. (Spain, ref. 110GPH). The electrodes incorporate a conventional three-electrode configuration, which consists of a carbon working electrode modified with graphene, a carbon counter electrode and a silver reference electrode. A sensor connector (Dropsens, Inc.) allows connecting the GSPE to the Autolab potentiostat.

2.3 Procedure

2.3.1 Electrochemical functionalization of the graphene electrode surface and covalent immobilization of ovalbumin antibodies. The 4-carboxyphenyl diazonium salt was prepared *in situ* by mixing 2 mM sodium nitrite solution with 2 mM 4-aminobenzoic acid in 0.5 M HCl. The mixture was stirred for 5 min at room temperature. The functionalization of the GSPE was then performed by the electrochemical reduction of the *in situ* generated diazonium cations using one cyclic voltammetry scan from +0.4 to -0.6 V at a scan rate of 100 mV s^{-1} . The electrode was then gently rinsed with Milli-Q water. The 4-carboxyphenyl (CP) film on the graphene surface was activated by incubating the electrodes in MES buffer (pH 5.0) containing; 100 mM EDC and 20 mM NHS for 60 min. After washing with MES buffer, the modified electrode surface was covered with a

50 μL droplet of anti-OVA-MAb solution ($10 \mu\text{g mL}^{-1}$ in 10 mM PBS buffer, pH 7.4) and incubated for 60 min at room temperature in a water-saturated atmosphere (Scheme 1). The removal of the unreacted antibodies was performed by gently washing the electrodes with PBS buffer (10 mM, pH 7.4). Then, the electrodes were blocked by the addition of 50 μL of blocking buffer (0.1% of BSA in PBS buffer, pH 7.4) and incubated for 30 min to deactivate the remaining active groups and block the free surface. The prepared immunosensor was then rinsed with PBS and used in the assay or kept at 4°C in PBS buffer pH 7.4 until further use.

2.3.2 Electrochemical measurements. For the ovalbumin measurements and the negative control experiments, 50 μL of either ovalbumin or the control proteins (lysozyme, casein, BSA, and β -LG) of the required concentration in PBS buffer pH 7.4 were added onto the modified electrode surface and incubated for 45 min in a water-saturated atmosphere. The immunosensor was then washed thoroughly with PBS buffer pH 7.4 prior to electrochemical measurements. All the electrochemical measurements were performed in 10 mM PBS (pH 7.0) containing the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox pair of 10 and 5 mM using cyclic voltammetry, and differential pulse voltammetry (DPV), respectively at room temperature. The decrease of the DPV current which was relative to the concentration of ovalbumin has been used to construct the calibration curve. The parameters used for the DPV measurements: modulation amplitude 25 mV; modulation time 0.05 s; interval time 0.5 s, and step potential -5 mV .

2.3.3 Spiking of egg-free cake sample extract. An egg-free cake sample (from Les Aliments Ange-Gardien, Quebec, Canada) was blended to form a homogeneous mixture. 0.1 g of the blended cake was mixed with 1 mL of PBS buffer and incubated at 60°C for 1 hour with mixing. The clear supernatant was then collected after centrifugation at 3500 rpm for 10 min. The supernatant was diluted 1 : 100 in 10 mM PBS buffer and spiked with different concentrations of ovalbumin (0.01, 0.10, 10 ng mL^{-1}). Finally, the spiked extracts were incubated for 45 min with the immunosensor at room temperature and the DPV responses were recorded as previously described.

3 Results and discussion

3.1 Electrochemical functionalization of the graphene electrode surface

The electrochemical functionalization of the graphene electrode surface was performed by the cyclic voltammetry reduction of the *in situ* generated carboxyphenyl diazonium salts from 0.4 to -0.6 V at a scan rate of 100 mV s^{-1} , leading to the formation of a carboxyphenyl film on the graphene surface. A single irreversible cathodic peak at -0.12 V vs. the Ag reference electrode was observed in the first cyclic voltammetry scan on the GSPE as shown in Fig. 1(A). This irreversible peak is resulted from the reduction of the carboxyphenyl diazonium species *via* a one electron transfer process, resulting in the elimination of a nitrogen molecule and the formation of an aryl radical which forms a covalent bond with the graphene surface.⁴⁷ The intensity of the cathodic peak then gradually diminished in the

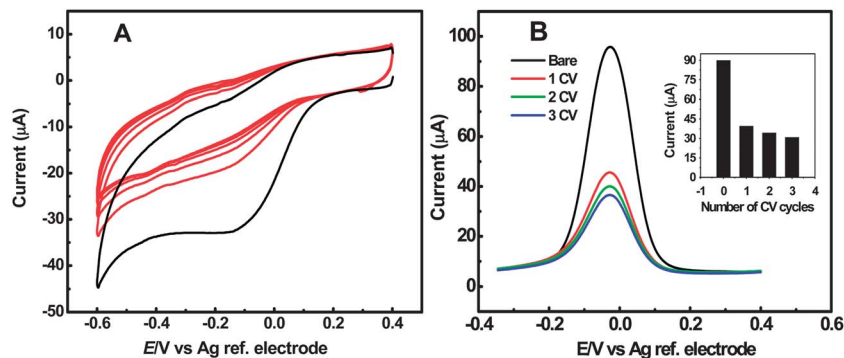


Fig. 1 (A) Successive cyclic voltammograms for the *in situ* generated 4-carboxyphenyl diazonium salt in the diazotization mixture (2 mM NaNO_2 + 2 mM 4-amino-benzoic acid in 0.5 M HCl) at a scan rate of 100 mV s^{-1} on the GSPE, the black line represents the first CV and the red lines represent the subsequent cycles, (B) DPVs of the bare GSPE and 4-CP-GSPE functionalized by diazonium salt reduction using different CV cycles in 5 mM $[\text{Fe}(\text{CN})_6]^{4-/3-}$ at 100 mV s^{-1} . Inset is the plot of the reduction peak current for 5 mM $[\text{Fe}(\text{CN})_6]^{4-/3-}$ measured at 4-CP-GSPE vs. the number of CV scanning cycles for diazonium reduction on the GSPE.

second and the third CV scans indicating the gradual decrease of electron transfer between the diazonium cations in the solution and the aryl modified graphene surface due to the formation of a denser organic layer, leading to more blocking of the surface. After the third CV cycle, the peak almost disappeared indicating the coverage of the graphene surface with a packed monolayer of carboxyphenyl groups. However, more CV cycles could lead to the continuous growth of multilayers of aryl groups on the surface as reported elsewhere.^{46–49} The electrochemical behaviour of the carboxyphenyl modified electrodes was further studied using differential pulse voltammetry in the presence of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe in order to study the blocking effect of the grafted carboxyphenyl layer. As shown in Fig. 1(B) the DPV reduction peak current of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ species decreased with increasing the number of cyclic voltammetry reduction cycles used for the carboxyphenyl grafting. This decrease in the reduction peak current of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox species indicates the blocking effect of the carboxyphenyl layer for the reduction of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ on the modified electrode surface. This effect is due to the thickness of the organic layer as well as the electrostatic repulsion between the negatively charged carboxylic groups and the $[\text{Fe}(\text{CN})_6]^{4-/3-}$ redox probe. The DPV peak currents of $[\text{Fe}(\text{CN})_6]^{4-/3-}$ were plotted *versus* the number of CV scanning cycles used for the diazonium cation reduction. A decrease in the peak current was observed with increasing the number of CV scans as can be seen in the inset of Fig. 1(B). However, the largest drop in the peak current was observed after the electrografting using one CV cycle, while a smaller difference in the peak current was observed after the subsequent cycles. These results confirm that the increase in the number of CV cycles leads to an increase in the density of the organic layer, formed on the electrode surface, which in turn results in more hindrance for the electron transfer process. Therefore, it is clear that one CV scan was enough to cover most of the surface with the carboxyphenyl film. In this work, one CV scan has been chosen for the electrografting step in further experiments to ensure that the electron transfer still takes place and to avoid the formation of multilayers which could significantly affect the biosensor sensitivity.^{48,49}

3.2 Differential pulse voltammetric characterisation of the immunosensor construction

Differential pulse voltammetry in 5 mM $[\text{Fe}(\text{CN})_6]^{4-/3-}$ solution was used to follow the modifications of the graphene electrode surface. The DPVs for all modification steps are shown in Fig. 2. A well-defined cathodic peak was observed in the case of the bare GSPE. After the electrografting of carboxyphenyl groups on the GSPE using one cyclic voltammetry scan from 0.4 to -0.6 V at a scan rate of 100 mV s^{-1} , the intensity of the $[\text{Fe}(\text{CN})_6]^{4-/3-}$ reduction peak decreased (Fig. 2(b)), due to the partial blocking of the surface as illustrated above. After the activation of the carboxylic group and the covalent antibody immobilization, the reduction peak current of $[\text{Fe}(\text{CN})_6]^{4-/3-}$ increased again (Fig. 2(c)). This increase in the current could be attributed to the neutralization of the negative charge of the terminal carboxylic groups on the surface with the positively charged antibodies. A similar behaviour was reported previously elsewhere.^{47,50} Then, after blocking of the free active sites on the antibody bound electrodes with 0.1% BSA, the reduction peak further decreased, indicating the retardation of the electron transfer kinetics between the redox probe and the graphene electrode, due to the bulky size of this protein.

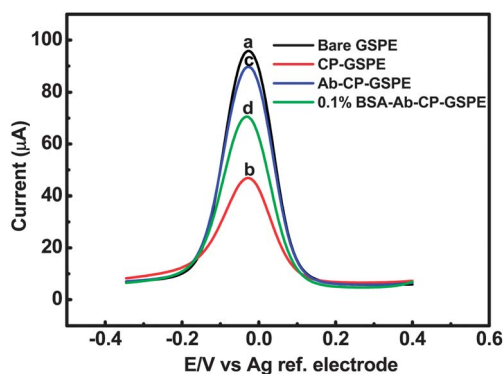


Fig. 2 DPVs of the 5 mM $[\text{Fe}(\text{CN})_6]^{4-/3-}$ probe in PBS, pH 7.4 for the bare GSPE (a), 4-carboxyphenyl/GSPE (b), Ab/4-CP/GSPE (c), and after blocking with 0.1% BSA for 30 min (d). The parameters of DPV: modulation amplitude, 25 mV; modulation time, 0.05 s; interval time 0.5 s, step potential -5 mV .

3.3 Mechanism of the electrochemical process on the proposed graphene-based immunosensor

Cyclic voltammetry was used to investigate the mechanism of the electrochemical process taking place on the immunosensor surface. Fig. 3 shows the cyclic voltammograms of the immunosensor in 10 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at different scan rates in the range of 10–200 mV s^{-1} . As seen in the inset of Fig. 3, the plot of the anodic and cathodic peak currents of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ versus the square root of the scan rate showed a linear response over the range of the studied scan rates, with a correlation coefficient of 0.99. This behaviour is characteristic of a diffusion-controlled electrochemical process on the immunosensor surface.

3.4 Differential pulse voltammetry detection of ovalbumin on the developed immunosensor surface

The differential pulse voltammetry technique was used to monitor the binding between the ovalbumin and the antibody bound electrode, by observing the change in the reduction peak current of the redox probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$ on the electrode surface. The introduction of any blockage for the redox probe to

access the graphene electrode surface, such as the introduction of a bulky protein or negatively charged groups, will retard the charge transfer and consequently reduce the current. As shown in Fig. 4(A), a decrease in the differential pulse voltammetric reduction peak current of $[\text{Fe}(\text{CN})_6]^{4-/3-}$ was observed with incubating the immunosensor with different concentrations of ovalbumin (0, 0.01, 0.1, 1.0, 10, 100 and 200 ng mL^{-1}). This decrease in the current upon binding is attributed to the bulky size of the protein sterically hinders the access of $[\text{Fe}(\text{CN})_6]^{4-/3-}$ anions to the electrode surface. The ovalbumin also has an isoelectric point equal to 4.6,⁵¹ which has a negative charge at pH 7.4 resulting in repelling the $[\text{Fe}(\text{CN})_6]^{4-/3-}$ anions.

As shown in the inset of Fig. 4(A), a good linear relationship was obtained, between the change of the cathodic peak current of the $[\text{Fe}(\text{CN})_6]^{4-/3-}$ redox probe in the DPV, and the logarithm of the ovalbumin concentrations from 1.0 pg mL^{-1} to 0.5 $\mu\text{g mL}^{-1}$. The data points in the calibration curve represent three independent measurements and the error bars show the standard deviations ranging from 2.6% to 5.0% indicating good reproducibility of the immunosensor. The linear regression equation was $(i^0 - i) (\mu\text{A}) = 9.98 + 2.81 \times \log C [\text{ng mL}^{-1}]$, $R = 0.997$, with a detection limit (LOD) of 0.83 pg mL^{-1} ($S/N = 3$). The result confirms that the proposed immunosensor exhibits a lower detection limit compared with other previously reported methods^{22,23} without the need for sophisticated amplification steps.

3.5 Selectivity of the immunosensor

It is very important to study the selectivity of the immunosensor and to verify that the drop in the current is due to the specific interaction between the ovalbumin and the surface bound antibody. To illustrate the selectivity of the ovalbumin immunosensor, the immunosensor was incubated with four non-specific food proteins: β -lactoglobulin, BSA, egg lysozyme and casein as negative controls. As can be seen in Fig. 4B, the % decrease in the current for β -lactoglobulin, BSA, lysozyme and casein was much lower than that of the ovalbumin. These results indicate that the immunosensor exhibited good selectivity for the determination of ovalbumin with a negligible effect of the non-specific proteins.

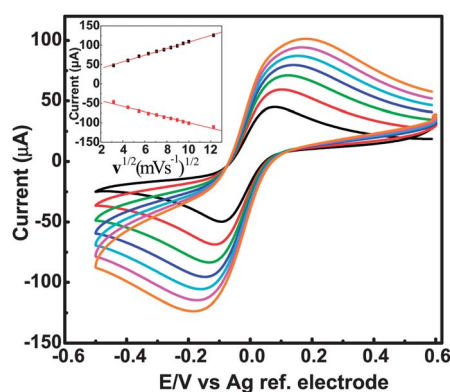


Fig. 3 Cyclic voltammograms of the modified electrode at different scan rates (from inner to outer): 10, 20, 30, 40, 50, 60, and 70 mV s^{-1} in 10 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 10 mM PBS solution (pH 7.4). The inset shows the dependence of the oxidation and reduction peak currents on the square root of scan rates.

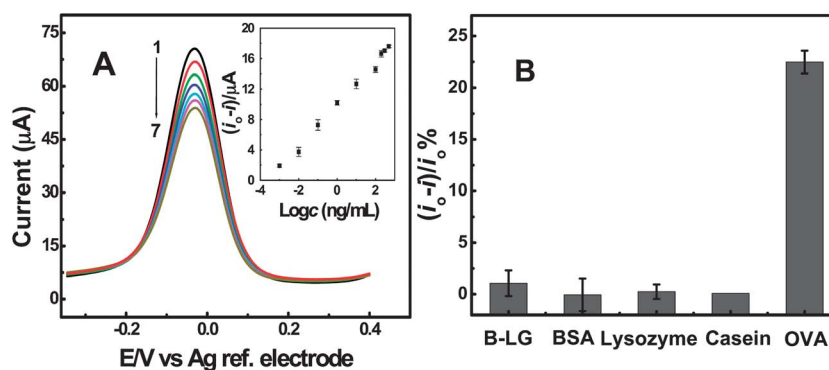


Fig. 4 (A) DPVs of the immunosensor incubated with different concentrations of ovalbumin (1–7): the concentrations of OVA are 0.00, 0.01, 0.1, 1.0, 10, 100 and 200 ng mL^{-1} in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 10 mM PBS solution (pH 7.4). The inset is the calibration curve based on the change of the DPV peak currents versus the logarithm of the concentrations, (B) comparison of the response of the immunosensor to 100 ng mL^{-1} ovalbumin, BSA, casein, lysozyme, and β -lactoglobulin.

Table 1 Results of the determination of ovalbumin in spiked cake samples extract (n = 3)

Sample	Standard concentration (ng mL ⁻¹)	Found (ng mL ⁻¹)	Recovery %	RSD ^a %
1	0.01	0.0107	107.2	4.78
2	0.1	0.1036	104.0	2.50
3	10	10.960	109.6	6.50

^a RSD%, relative standard deviation percentage.

3.6 Application of the ovalbumin immunosensor in real samples

A preliminary application has been performed in order to study the effect of the matrix on the ovalbumin detection by the developed immunosensor. Three concentrations of ovalbumin were spiked in diluted egg-free cake sample extracts and were then detected using the proposed immunosensor. As shown in Table 1, the calculated recoveries for the three samples were between 104% and 109.6%. This indicates a non-significant effect of the matrix on the immunosensor and confirms the possible applicability of the immunosensor in real food samples.

3.7 Stability of the immunosensor

The stability of the ovalbumin immunosensor was studied after storage at 4 °C for a 14-day period. The immunosensor was then used to detect 10 ng mL⁻¹ of ovalbumin. A small change in the immunosensor response after storage (less than 2%) was observed demonstrating a good stability of the immunosensor.

4 Conclusion

In conclusion, we have developed for the first time a label-free voltammetric approach for the detection of the egg allergen ovalbumin based on graphene modified screen printed carbon electrodes. An electrografting method was used for the covalent functionalization of the graphene surface, by electrochemical reduction of *in situ* generated carboxyphenyl diazonium salt in acidic aqueous solution using cyclic voltammetry. We found out that one cyclic voltammetry cycle was enough to cover most of the graphene electrode surface with the carboxyphenyl groups which was then used for the immobilization of the ovalbumin antibody. The differential pulse voltammetric immunosensor exhibits a linear relationship between the difference in the reduction peak current before and after binding and the ovalbumin concentration in the range of 1 pg mL⁻¹ to 0.5 µg mL⁻¹. It was found that the voltammetric immunosensor is highly selective for ovalbumin in the presence of β-lactoglobulin, BSA, egg lysozyme and casein. Finally, no significant matrix effect has been detected when we used spiked cake extracts. Such finding indicates possible applicability of the proposed graphene-based label-free immunosensor in real food samples.

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