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A Novel Immunosensor Based on Chitosan-Prussian Blue-Graphene Nanocomposite and Au Nanoparticle for Rapid Detection of Melamine

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In 2008, serious illnesses and deaths of babies caused by the contaminated powdered infant formula by melamine in China. To determine a rapid detection of melamine in raw milk, an immunosensor based on the chitosan-Prussian blue-graphene nanocomposite and Au nanoparticle was synthesized in this study. Several important parameters of the sensor were optimized and discussed in detail. The optimized concentration of the immobilized antigen and incubation time was 100ng/ml and 40min, respectively. The biosensor exhibits good electrocatalytic behavior to detection of melamine and the linear response range from 0.3 to 1000ng/mL with a correlation coefficient of 0.991 and the detection limit of 0.15ng/mL at the signal to-noise ratio of 3. The immunosensor also has good selectivity, stability and reproducibility. Recoveries of melamine from raw milk samples ranged from 86.0%-103.1%, which was suitable to test the melamine in raw milk samples.

Keywords: immunosenor; graphene; AuNps; melamine; raw milk

1. INTRODUCTION

In 2008, melamine-contaminated infant formula was responsible for the illnesses and deaths of so many infants in China. Since melamine was nitrogen-rich material and was misused to elevate falsely its protein content by the Kjeldahl method[1]. Melamine could cause a variety of toxic effects on animal or human being, such as nephrolithiasis, chronic kidney inflammation, bladder carcinoma and urinary stones, etc [2]. The World Health Organization adopted a new daily tolerable intake of 0.2 mg kg^{-1} body weight due to this melamine-contaminated incident in 2008. Several methods were investigated to determine the concentration of melamine in food, for instance, gas chromatography

mass spectrometry (GC-MS)[3], surface-enhanced Raman spectroscopy[4-6], high performance liquid chromatography (HPLC)[7], HPLC-GS[8] and enzyme-linked immunosorbent assay (ELISA)[9, 10]. However, methods above all required either the expensive equipment or the complicated procedures. Therefore, it is urgent to develop the rapid and accurate testing methods of melamine in raw milk to guarantee food safety.

Electrochemical immunosensors are miniaturized analytical devices to provide concentration-dependent signals based on immunological interactions using antibodies (Ab) or antigens (Ag) [11]. For low level concentration of substance in the testing sample, it is crucial to enhance the capture probability of the immobilized primary antibodies on the transducer [12]. Graphene (GR) is a monolayer of carbon atoms bonded together in a two-dimensional (2D) hexagonal lattice[13, 14], which got considerable attention to making nanocomposites due to excellent electrical conductivity and biocompatibility[14-17]. Since limited biomolecules that can be physically adsorbed or chemically conjugated onto GR[18], the use of graphene/chitosan, graphene/gold nanoparticles (AuNps), graphene/Prussian Blue PB), and have been investigated in recent years. GR can be well dispersed in chitosan(CS) due to the effective charge transfer behavior between GR and CS [19, 20]. In addition, CS was used widely in immunosensor because of the active chemical characteristics and absorbability of protein. Hence, the combination of chitosan/Prussian blue/graphene (CS-PB-GR) was suitable to enhance the immobilization of bioactive molecules and constructing biosensors[21, 22]. AuNps was also used to enhance the immobility and adsorption ability of graphene due to the excellent biocompatibility, good conductivity and strong adsorption ability [23, 24].

In this study, we successfully designed an immunosernsor based on the CS-PB-GR composite nanosheets and AuNps for electrochemical detection of melamine. The important parameter, such as the concentration of antibody, incubation time and incubation temperature was also investigated.

2. MATERIALS AND METHODS

2.1 Reagent and material

The ELISA kit of melamine was purchased from Wanger Biotechnology Co.,Ltd.(Beijing, China). Chitosan (CS), Chloroauric acid ($HAuCl_4$) were purchased from Sigma (St. Louis, MO, USA). $K_4Fe(CN)_6$, $FeCl_3$, and sodium citrate were purchased from Chemical Reagent Co. All chemicals reagents used were analytical reagents. Redistilled water was used through all experiments in this study. Phosphate buffer solutions (PBS) were prepared with 0.1 M KH_2PO_4 and 0.1 M Na_2HPO_4. 0.1 M KCl solution was used as the supporting electrolyte.

2.2 Apparatus

Electrochemical measurements were used by CHI 660D electrochemical workstation (Shanghai Chenhua Co., China). GS-PB-GR composite nanosheets and AuNps were characterized by scanning electron microscopy (SEM). All the electrochemical experiments were operated at room temperature.

2.3 Preparation of the electrochemical immunosensor

2.3.1 Preparation of CS-PB-GR nanocomposite

Firstly, PB-GR nanocomposite were synthesized based on the article of Zhong et al [22]. Under the condition of stirring, 2 mL (1 mg/mL) graphene dispersed into 5 mL aqueous solution (containing 6 mg $FeCl_3 \cdot 6H_2O$, 8 mg $K_3Fe(CN)_6$ and 37 mg KCl, and pH adjusted as 1.5 with HCl). The composite was centrifuged and washed for several times after 24 h-stirring, and dried in vacuum for 12 h at 40℃.

Secondly, 1 mL CS solution (5 mg/mL) was mixed with 3 mL PB-GR solution (1 mg/mL), and continuously stirred for 48 h. The final composite was centrifuged and washed for several times, then redissolved in distilled water.

2.3.2 Preparation of the AuNps

AuNPs were prepared by a trisodium citrate reduction method according to the literature[25]. Briefly, trisodium citrate (5 mL, 38.8 mM) was quickly mixed with boiling $HAuCl_4$ solution(50 mL, 1 mM), and the boiling procedure should be maintained under stirring in 30min till the color of the solution turn to wine-red. AuNPs solution should be cooled down to the room temperature and stored at 4℃.

2.3.3 Electrode surface cleaning

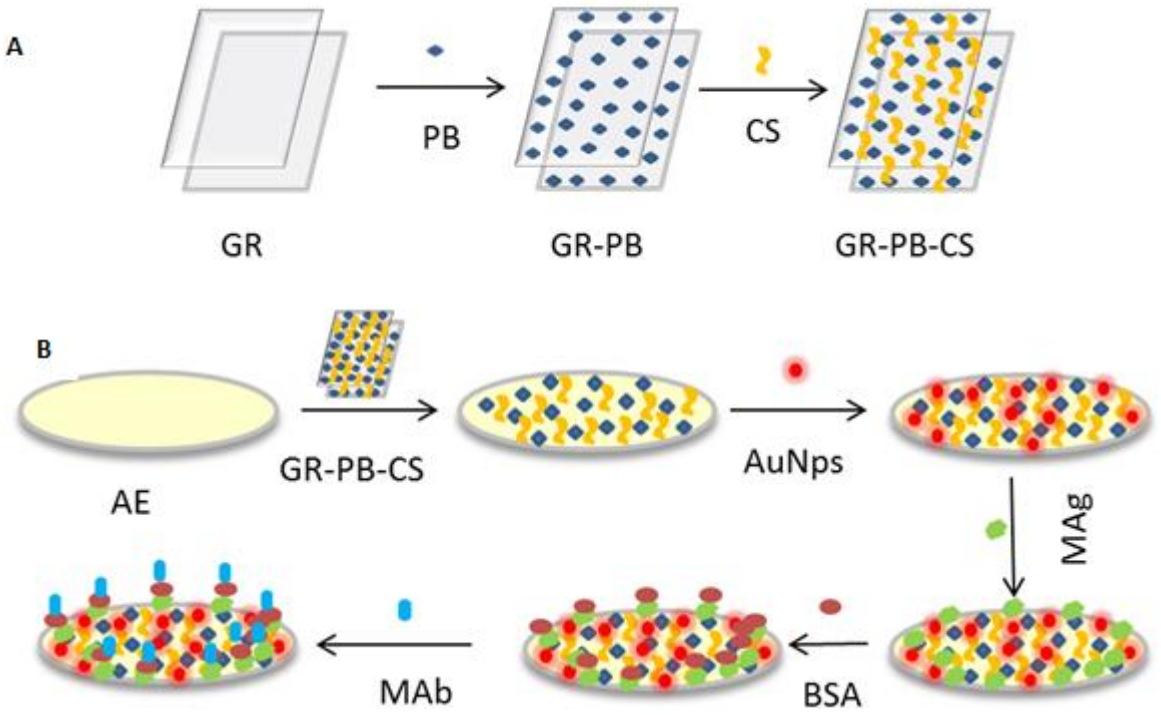
The Au electrode (AE, Ø = 4 mm) was polished with 0.3 and 0.05 μm alumina slurry and ultrasonically cleaned in ethanol and water thoroughly. Then the electrode was rinsed with distilled water and dried in air.

2.4 The fabrication of immunosensor

10 μL CS-PB-GR nanocomposite solution was dropped on the surface of cleaned AE (denoted as CS/PB/GR/AE). Then the prepared electrode was washed with distilled water to remove loosely adsorbed CS-PB-GR and allowed to dry in air at room temperature. Subsequently, AuNps was assembled onto the CS/PB/GR/AE by electrochemical deposition (denoted as AuNps /CS/PB/GR/AE). Au^{3+} was reduced to Au by chronoamperometry at a scan rate of 600s and a volume of -0.2V, and then it was flushed by redistilled water to eliminate the physically absorbed Au.

10μL of melamine antigen (MAg) (100ng/mL)was dropped onto the surface of the AuNps /CS/PB/GR/AE nanocomposite and dried in 5h. Then 10μL 0.1% BSA solution was dropped onto the modified electrode to block possible remaining active sites and avoid the non-specific adsorption (denoted as BSA/MAg/AuNPs/CS/PB/GR/AE).

The electrode should be flushed by distilled water after every procedure above finished. The constructing procedure of modified electrode was shown in Scheme 1.



Scheme 1. Schematic illustration for the preparation of the immunosensor. (A)CS-PB-GR nanocomposite; (B) illustration of the preparation process of the modified electrode.

2.5 Electrochemical measurement

The scanning electron micrographs of PB/GR and CS/PB/GR film were observed with SEM. The electrochemical characteristics of the modified electrode were investigated by CV which were performed in PBS (pH 7.2) containing 2mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ at room temperature. CV was performed over a potential range from −0.2 to 0.6 V at a scan rate of 50mV/s (vs. SCE).

2.6 Preparation and determination of real samples

5ml milk was diluted by 20ml distilled water, and stirred after adding the standard solution of melamine. Then the mixed solution was diluted to 50ml by 2mol/L H_2SO_4 and stirred completely. After 5min, the solution should be filtered and kept the supernatant to test.

3. RESULTS AND DISSCUSSION

3.1 SEM characterization of different films modified on the electrode interface

The surface morphologies of the PB-GR composite and CS-PB-GR composite were characterized by SEM (Fig. 1). As shown in Fig 1A, a lot of PB nanoparticles were deposited on the surface of GR and formed the PB-GR nanocomposite. After CS combining with composite (Fig. 1B),

wrinkled sheet-like structure coated the nanocomposite was observed, which is the typical image of CS, indicated that the CS successfully assembled into the electrode.

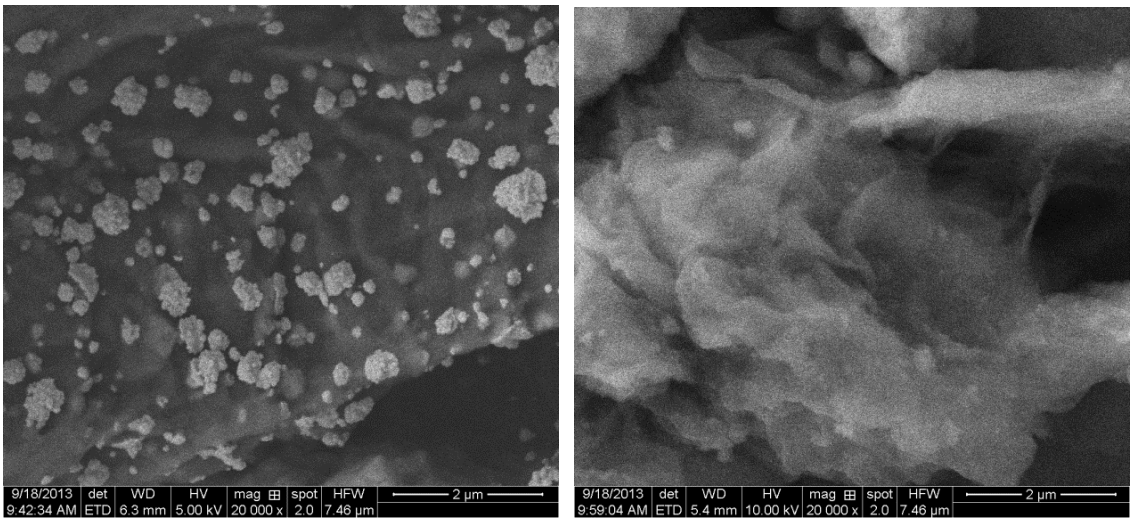


Figure 1. SEM photos of PB-GR (A), and CS-PB-GR composite (B).

3.2 Cyclic voltammetry (CV) characterization of the enzyme electrode

The assembly process of MAg /MAb/ AuNps/ CS-PB-GR multilayer films on the AE was monitored by cyclic voltammetry (CV) experiments. As shown in Fig. 2, a couple of quasi-reversible redox peak of the probes were presented in the bare AE (curve a).

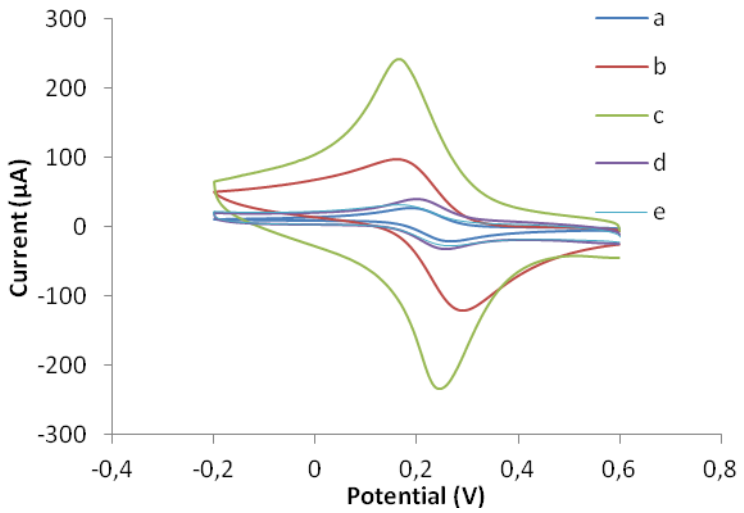


Figure 2. Cvs of the electrodes at different stages. (a)Bare AE, (b)CS-PB-GR/AE, (c)AuNps/CS-PB-GR/AE, (d)MAg/ AuNps/ CS-PB-GR/AE, (e)MAb/ MAg/ AuNps/ CS-PB-GR/AE at a scan rate of 50 mV/s and in 2.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) solution, respectively.

When the electrode was assembled with the CS-PB-GR composite sheets, an obvious increase in the amperometric response was found (curve b). After AuNps loading on the electrode (curve c), peak current increased compared with curve (b), due to the contribution of AuNps to promote the transmission of electrons. However, after MAg was modified onto the electrode, a decrease in peak current was observed (curve d). When MAg/ AuNps/ CS-PB-GR/AE electrode was immersed in the incubation solution, the redox peak was further decrease (curve e). The reason for the decrease was because the antigen in the incubation solution and antibody were insulated, which blocked the diffusion of the redox probe to the electrode.

3.3 Optimization of immunoreaction parameters

The optimized temperature of the immune reactions should be 37℃, which was used as the temperature of immune reaction in this study. Since the antibody concentration of the test kit was already optimized, the effect of antigen concentration and the incubation time on the peak current was investigated in this study.

3.3.1 Effect of the immobilized antigen concentration

The concentration of the immobilized antigen was one of the most important parameters of immunosensors, which should be optimized to achieve the best analytical performance. The response current was used as a quantitative index to evaluate the performance of the immunosensor during the optimization process[26]. The response current was observed to decrease when the concentration of the MAg increased from 10 to 100ng/ml, and then levered off (Fig.3A). This is because the antigen on the binding sites was saturated and no more increase current occurred when the concentration increased. As a result, a concentration of 100ng/ml MAg was chosen in the subsequent experiments.

3.3.2 Effect of incubation time on immunoreaction

The incubation time between the antigen and antibody was an important parameter which should also be optimized. If the incubation time is not long enough, the reaction would be no insufficiency; while if the incubation time is too long, the dissociation of the MAb complex or melamine-antibody would be caused. Response current was decreased when the incubation time increased from the 10min to 40min, and there was no obvious increase of the inhibition ratio after 40 min (Fig.3B). The explanation was that the increasing formation of insulated antibody-antigen immune complex inhibited the spread of the redox probe to the electrode in the first 40min. After 40 min, no more immune complex formed and the current remained stable. Consequently, 40min was recommended as the optimal incubation time for the detection of melamine.

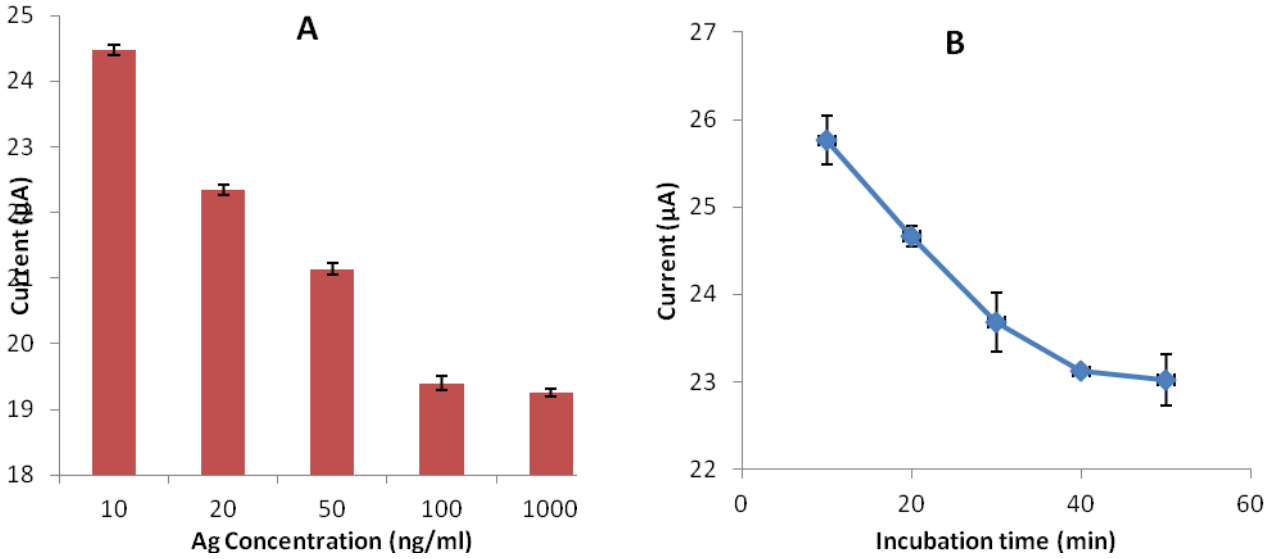


Figure 3. Optimization of experimental parameters. (A) the effect of immobilized antigen concentration; (B) the effect of the incubation time.

3.3 Amperometric responses of the biosensor to melamine

The amperometric response of the immunosensor to melamine concentration is based on the change of the reduction peak current before and after immunoreaction. The Cv response of the immunosensor was investigated under the optimized condition we addressed above. Cvs of the immunosensor for the detection and the calibration curve of melamine standard solution were shown in Fig.4, which increased as the concentration of the melamine increased.

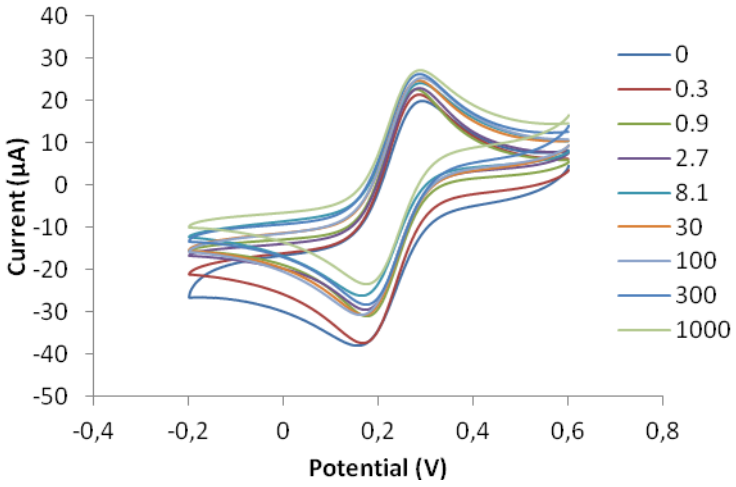


Figure 4. Cvs of the imminosensor for the detection of Cvs of the electrodes standard solution.

When the modified electrode immersed into the solution of free melamine and antibody of melamine, the melamine that immobilized in the electrode would compete react with the free melamine or antibody of melamine, resulted in the absorption of antibody on the electrode. The

composite of antibody-melamine blocked the block the electron transfer from solution diffusing to the surface of electrode. There was a linear relationship between the current and the log of the concentration, and the equation was y=1.509x+22.492, (R^2=0.991)（Fig.5）. The linear range of the melamine immunosensor was 0.3 to 1000ng/ml, and the detection limit was 0.15ng/ml.

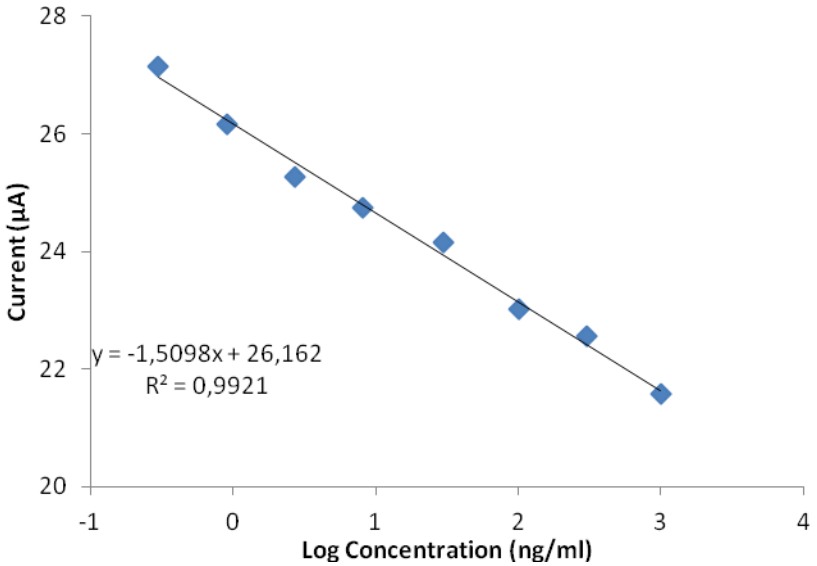


Figure 5. The calibration curve of melamine standard solution.

3.4 Selectivity, stability and reproducibility of the biosensor

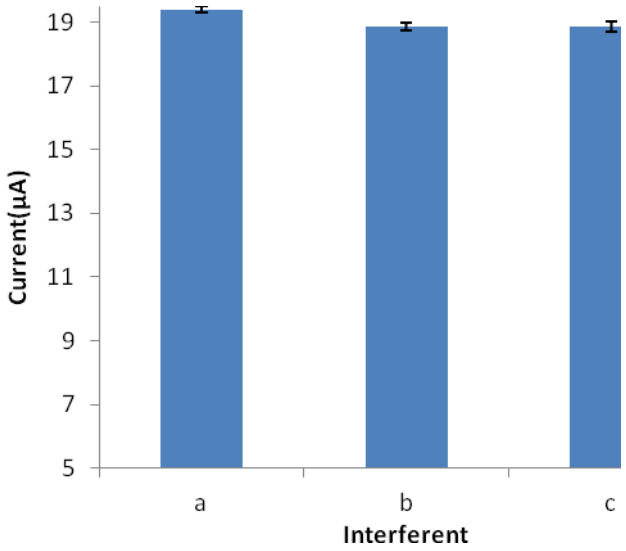


Figure 6. The peak current of the proposed immunosensor to the interference. (a) 100 ng/mL melamine; (b) 100ng/mL melamine + 100 ng/mL ammelide and; (c) 100 ng/mL melamine + 100 ng/mL ammeline.

Ammelide and ammeline were investigated as the interferences to test the selectivity of immunosenor. Addition of 10ng/ml, 20ng/ml, 100ng/ml and 1000ng/ml ammelide and ammeline to melamine solution were had almost no observable interference on the current response of melamine (Fig.6). The coefficient variation (CV) was less than 10%, which suggested the immunosensor has good anti-interferent ability.

The reproducibility and repeatability of the sensors were tested with inter-assay precision, which was evaluated by five assaying electrode prepared in the same condition. A relative standard deviation (R.S.D.) ranged from 3.8% to 5.1% in the concentration of 100 ng/mL melamine solution was tested. The concentration of 100 ng/mL melamine solution was tested by 5 successive measurements of a single sensor with a R.S.D. of 4.6%. We suggest that this immumosensor exhibited good reproducibility and repeatability.

The stability of this immunosensor was also investigated in 30 days. The modified sensor was tested in the concentration of 100 ng/mL melamine solution in every 5 days. In the first 10 days, no obvious decrease was observed. After a certain time of storage and usage, the steady ability of the modified electrode would gradually decrease[27]. In our work, after 30 days, the sensor response decreased by 18.7%, which indicated that the immunosensor have a good stability because of the biocompatibility of CS/PB/GR and AuNps.

3.5 Real sample analysis

4 milk samples collected from different supermarket were tested to estimate the analytical applicability of the immunosensor. The recovery rate ranged from 86.0% to 103.1%, with RSD values between 3.7% and 5.1% (Table 1). Compared with the other detection method, this result showed that the modified electrode was suitable to test the melamine content in milk.

Table 1. Recoveries of the proposed immunosensor in real samples.

Sample	Add (ng/mL)	Found (ng/mL)	RSD (%) (n=5)	Recovery (%)
1	1	0.86	4.5	86.0
2	20	20.13	3.7	100.6
3	200	206.2	5.1	103.1
4	400	399.2	3.8	99.8

3.6 Comparison of analytical methods

Some analytical methods of detection melamine in the raw milk or milk powder have been developed since 2008, such as sensitive methods with larger apparatus, such as HPLC, GC-MS; or less sensitive methods, such as electrochemical methods. The comparison of usual detection methods of melamine was illustrated in Table 2. The common method used the larger equipment, such as ESI-MS,

HPLC and NIR, required complicated procedure and expensive equipment, but the detection limits were relatively lower than the electrochemical methods [28-32]. Although electrochemical method to determine melamine is rapid, economical, flaw such as lower reproducibility for electrode preparation still exist [29]. To conquer this problem, our work synthesized the CS-PB-GR nanocomposite to modify the electrode. The detection limit of our work was 10^4 times lower than the larger apparatus, with a good stability and high recovery rate. The low detection limit could be caused by AuNPs functionalized GS, which could greatly decrease the detection limit of immunosensor for good conductivity and biocompatibility [33]. Graphene was a common material to enhance the electron transfer between the electrode and enzyme. Unlike carbon nanotube, graphene has the potential ability of low cost, safety and easily processing [34]. Chitosan and melamine could be held together through strong hydrogen bonds, which could enhance the interaction force between graphene and melamine [35]. Since Prussian blue (PB) is a well-known "artificial peroxidase" [36], we proposed a novel electrochemical immunosensor based on Prussian Blue (PB) as the signal for the determination of melamine.

Table 2. Comparison of analytical methods for the detection of melamine

Analytical methods		Linear range	Detection limit	Recovery	Reference
Electrospray ionization mass spectrometry (ESI–MS)		0.5–10.0 μg/mL	0.1μg/mL		[28]
HPLC HPLC-PDA		0.05-5mg/kg 0.08-10 μg/ml	35-110μg/kg 0.02 μg/mL	95-109% 92-102%	[29] [30]
GC-MS				94-102%	[31]
Near infrared spectrometry (NIR)			<1ppm	100%	[32]
AuNPs	Surface Enhanced Raman Scattering(SERS)	0.31–5.0 mg/L	0.17 mg/L	-	[37]
	UV-vis spectrometric	-	0.04μM	-	[38]
UV spectrophotometer		3-8 μg/mL	-	-	[39]
Lateral flow test strip based on colloidal selenium immunoassay		-	150 μg/kg	-	[40]
Planar waveguide fluorescence immunosensor		26.6–517.5μg/L	6.6μg/L	89.8-103.2%	[41]
Oligonucleotides/AE		3.9×10^{-8}-3.3×10^{-6} M	9.6×10^{-9} M	95%	[42]
AuNps/CS-PB-GR/AE		0.3-1000ng/ml	0.15ng/ml	86-103.1%	This work

4. CONCLUSIONS

In this work, a novel immunosensor was synthesized for rapid detection of melamine based on the GR-PB-CS nanocomposite and Au nanoparticles. Because the unique nano network structure of graphene increased the surface area of gold electrodes, the use of GR-PB-CS nanocomposite enhanced the stability of the biosensor. AuNPs on the nanocomposite film for antibody immobilization could also improve the electrochemical signal and adsorption capacity of antibody, and thus enhanced the detection sensitivity.

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