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论文题目： **An electrochemical aptasensor based on target-induced nicking site reconstruction strategy for the detection of milk allergen β-lactoglobulin**

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An electrochemical aptasensor based on target-induced nicking site reconstruction strategy for the detection of milk allergen β-lactoglobulin

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Abstract

Food allergy is an immune system reaction to a particular food, in which milk is the most common one. β-lactoglobulin (β-Lg) is the main ingredient of milk protein and the main cause of infant milk allergy. On such an occasion, the determination of β-Lg is very important and electrochemical sensor is a good alternative for this purpose since it is sensitive, selective and inexpensive. In this work, an electrochemical aptasensor was fabricated instead of an expensive immunosensor for the quantitative detection of β-Lg in dairy products. A tri-functional hairpin HP (Probe) was designed with an aptamer sequence, a nicking site and a DNA sequence. In the absence of β-Lg, the part of aptamer hybridized with the DNA sequence to form a stable stem-loop structure. While in the presence of β-Lg, the binding of aptamer with β-Lg caused the reconstruction of Probe conformation and the formation of the nicking sites. Then, the nicking enzyme was activated and the DNA sequence could be released, which bound with the end of hairpin 1-methylene blue (HP1-MB)/HP2-MB conjugation on the Au nanoparticles (AuNPs) modified electrode surface. Thus, the insulation of electrode enhanced and the current response of MB decreased, which was the quantitative basis for β-Lg detection. The proposed aptasensor exhibited a wide linear range of 0.01-100 ng mL^{-1} and a low detection limit of 5.7 pg mL^{-1}, allowing a promising alternative for cost-effective, rapid and sensitive detection of milk allergen in food quality control.

Keywords: β-lactoglobulin, electrochemical aptasensor, target-induced nicking site reconstruction, hybridization chain reaction, methylene blue.

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1. Introduction

Food allergy is a reaction of the immune system upon a particular food, which has become one of the major health concerns today [1-3]. Food allergy can cause atopic dermatitis, cough, asthma and other symptoms in the mild cases, and even endanger life in the severe cases [4]. Milk protein allergy is one of the most common types of food allergy, especially in infants. Statistically, about 2 to 3 % of infants will have allergic reaction to milk protein within one year of birth [5-7]. The main allergen protein in milk is β-lactoglobulin (β-Lg). In order to reduce the incidence of allergic reactions, food labels are required to mark the most common allergens, such as eggs, milk, peanuts, soybeans and wheat. Hypoallergenic formula milk is also available for milk allergy patients. Even so, low concentrations of β-Lg residue may cause allergic reactions during the production and packaging of products [8-10]. Therefore, it is of great significance to develop an effective detection method towards trace β-Lg in food to protect public health.

Current analytical methods for β-Lg detection include high performance liquid chromatography (HPLC) [11-13], electrochemical immunoassay (EIA) [14-15], fluorescence immunoassay (FIA) [16], surface plasma resonance (SPR) [17-18], and enzyme-linked immunoassay (ELISA) [19-21]. However, these methods also have some disadvantages, such as high cost, lack of portability, complex experimental technology, and long response time. Upon these aspects, electrochemical biosensors have obvious advantages and become a promising alternative. Therefore, the combination of biotechnology and electrochemistry could be developed into a portable and sensitive sensing tool for β-Lg detection.

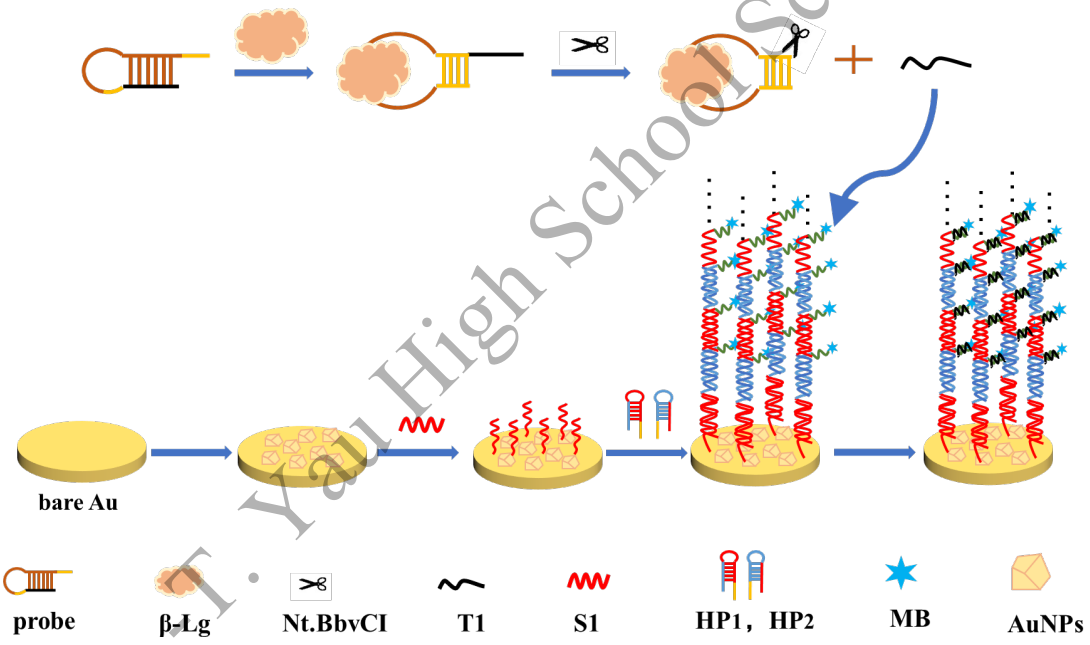
As a kind of single-stranded oligonucleotide with high recognition affinity and specificity, aptamers have been widely used in aptasensors for protein detection in recent years [22-25]. Qiao and her colleagues, for example, constructed an electrochemical sensing platform for cardiac troponin I (cTnI) based on aptamer/MoS_2

nanosheet conjugation [22]. The combination of cTnI and aptamer induced the affinity between the aptamer and MoS_2 greatly weakened and the release of the aptamers from the surface of MoS_2. Thus, the electrochemical resistance became decreased. Shui et al. designed a new sandwich-type aptasensor for the detection of the Alzheimer's disease marker tau-381 in human serum, in which both the antibody and aptamer could recognize tau-381 simultaneously to establish a sandwich-type complex [23]. Therefore, the combination of aptamers and electrochemical methods in the construction of aptasensors has the advantages of a wide detection range, low detection limit, good selectivity, portability and low cost.

In order to enhance the sensitivity and widen the linear range of electrochemical aptasensors, various signal amplification strategies have been applied in recent years, such as hybridization chain reaction (HCR) [26-27], roll ring amplification technology (RCA) [28-29], nucleic acid enzyme etc. [30-31]. In addition, the modification of electrode surface is also very important. Gold nanoparticle (AuNP) is one of the most widely used nanomaterials as an excellent scaffold for fabricating chemical and biological sensors, since it possesses some advantages such as biocompatibility, high specific surface area, excellent conductivity and feasible surface functionalization [32-33]. Simple and efficient preparation of AuNP modified electrode is considered as an important basis for the construction of electrochemical sensors. In our previous works, electrodeposition is verified as an effective and fast method, which could control the amount, morphology and distribution density of electrode surface via adjusting the working potential and the deposition charge [34-37]. Therefore, in this work an electrodeposited AuNP modified electrode was also used as the basis for fabricating a novel aptasensor for sensitive and quantitative detection of β-Lg, in which a signal amplification strategy was ingeniously designed.

Herein, a highly sensitive and homogeneous detection of β-Lg was reported by an aptamer and nicking enzyme assisted electrochemical signal amplification in a HCR system. The homogeneous reaction based on the β-Lg-triggered conformation alteration of a hairpin Probe can improve the detection accuracy with elimination of several washing and separation steps. The AuNP modified electrode promoted the HCR

structure density and the electrochemical signal from the methylene blue (MB) label. Thus a high detection selectivity and sensitivity could be reached. The detection principle is shown in Scheme 1. A special hairpin HP (Probe) was designed which is composed of three functional parts: an aptamer sequence for capturing β-Lg, a nicking site consisting two complementary sequences (site-1 and site-2), and a DNA sequence (T1) for amplification. In the absence of β-Lg, the Probe was stable with the stem-loop structure hybridized between part of aptamer and T1. In the presence of β-Lg, the aptamer can bind to β-Lg due to the high affinity between them. Thereby, the Probe conformation is reconstructed and the site-1/site-2 hybridized. And then T1 sequence was exposed, which trigged the nicking enzyme, following the release of T1.



Scheme 1. Schematic illustration of the electrochemical β-Lg aptasensor.

On the other hand, an AuNP modified Au slice electrode (AuE) was used as the basis for the immobilization of S1 via the Au-S covalent bonding. After that, HCR structure hybridized by HP1-MB and HP2-MB could be constructed, and the initial current signal (I_0) was achieved. There are two sequences in HP1-MB and HP2-MB can match with T1, respectively. Thus, the released T1 could form a large amount of T1/HP1-MB and T1/HP2-MB rigid branches in HP1-MB/HP2-MB conjugation, which brought the

electrochemical resistance of electrode increased and the current response of MB decreased. The decrease of current ($\Delta I=I_0-I$) was proportional with the β-Lg concentration, and linear relationship between ΔI and $C_{\beta\text{-Lg}}$ was considered as the basis for quantitative detection of β-Lg.

2. Experimental

2.1 Chemicals and reagents

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) are purchased from Sigma-Aldrich (St Louis, Missouri, USA). β-Lg, tris (2-carboxyethyl) phosphine hydrochloride (TCEP), 6-mercaptohexanol (MCH), the nicking endonuclease Nt.BbvCI and all oligonucleotides are synthesized from Sangon Inc. (Shanghai, China). The sequences and associated abbreviations are listed in Table 1.

Table 1. The sequences for the β-Lg sensing.

Name	Sequence(5'→3')
HP	CACACA CGGATG AACCTC AGCCGA CGATCG GACCGC AGTACC CACCCA CCAGCC CCAACA TCATGC CCATCC GTGTGT GGCTGA GGTT
S1	AGTCT AGGAT TCGGC GTGGG TTAA-$(CH_2)_6$-HS
HP1	TTAACC CACGC CGAAT CCTAG ACT CAAAGT AGTCT AGGATT CGGCGTG TTTTT*GTGTGTGCCTACTT*-$(CH_2)_6$-MB
HP2	AGTCT AGGATT CGGCGTG GGTTAA CACGC CGAAT CCTAG ACT ACTTTG TTTTT*GTGTGTGCCTACTT*-$(CH_2)_6$-MB

Note: The red and blue sequences are the complementary parts in HP1 and HP2. The italic parts are the complementary sequences with the short-chain T1 obtained in the nicking reaction.

The carboxyl group modified magnetic beads were purchased from Shannuo Biotech Co. (Tianjin, China). The amino terminated HP-Cy3 (NH_2-HP-Cy3) is the fluorescent dye Cy3 tagged HP sequence, which is used for the verification of target-induced nicking site reconstruction and nicking reaction. The immobilization buffer (IB) is

composed of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM TCEP and 0.1 M NaCl. The hybridization buffer (HB) is a 10 mM Tris-HCl buffer (pH 7.4) containing 500 mM of NaCl and 1 mM of $MgCl_2$. Phosphate buffer solution (PBS, 10 mM, pH 7.4) was prepared with Na_2HPO_4 and NaH_2PO_4, which is used for preparing β-Lg dilution, DNA stocking solutions and as washing buffer (WB). All the other chemicals are of analytical grade and used as received. The ultrapure water (≥18.2 MΩ cm^{-1}) is produced by a Youpu ultrapure water system (UPT-II-10T, Chengdu, China).

2.2 Instruments

The surface morphology of AuNPs/AuE was investigated by scanning electron microscope (SEM, Hitachi S4800, Japan). A fluorescence (FL) spectrometer (Lengguang F97XP, Shanghai, China) was used to record FL intensity. All electrochemical measurements were performed on a CHI 660D electrochemical workstation (Chenhua Instrument Co., Shanghai, China). The typical three-electrode cell system consisting of an Ag/AgCl reference electrode in saturated KCl solution, an AuNP modified Au slice working electrode (2 mm in diameter), and a Pt auxiliary electrode was used for all the electrochemical measurements. All electrochemical experiments were purged with high-purity N_2 to remove O_2.

2.3 Target-induced nicking site reconstruction and T1 release

The stocking solution of target protein β-Lg was 4 μg mL^{-1} and was diluted to appropriate concentrations. 4 μL of 100 μM HP was annealed at 95 ˚C for 5 min and slowly cooled to 30 ˚C before use. Then, a 40 μL mixture of β-Lg and HP was incubated at 37 ˚C for 2 h. After that, 10 U Nt.BbvCI was added and the nicking process was carried out at 37 ˚C for 1 h, and the T1 strands were released.

2.4 Verification of the target-induced nicking site reconstruction

The feasibility of the proposed target-induced nicking site reconstruction strategy was evaluated using fluorescence characterization. 20 mg/mL magnetic beads were centrifuged and re-dispersed in 200 μL of PBS, and then 0.5 M of 50 μL mixture of NHS and EDC was added to activate the carboxyl groups on the surface of magnetic beads. Additionally, 4 μL of 100 μM NH_2-HP-Cy3 was annealed at 95 ˚C for 5 min and slowly cooled to 30 ˚C. Incubate the NH_2-HP-Cy3 with the activated magnetic beads overnight at room temperature, and then the HP-Cy3 immobilized magnetic beads could be prepared. After the similar triggering and nicking procedures with those in section 2.3, T1-Cy3 was released via the magnetic separation. The FL intensities before and after the reaction were detected for comparison. The samples were excited at 550 nm and the emission spectra were collected at 570 nm. The excitation and emission slits used in the experiment were both 1.5 nm.

2.5 Fabrication of the aptasensor

The AuE was cleaned with acetone, ethanol, and water for 10 min, successively and dried with N_2 stream. A perforated insulative tape with the aperture of 3 mm was covered onto the AuE surface to control the geometric area of the working electrode. The AuNPs were electrodeposited on the Au slice surface via a potentiostatic electrodeposition in 12 mM $HAuCl_4$ solution containing 0.05 M $HClO_4$ under the potential of -0.1 V and the controlled charge of 0.15 C according to our previous work [37]. Afterwards, 10 μL of 2 μM SH-S1 was dropped onto the AuNPs/AuE and incubated overnight at 37 ˚C to form S1-AuNPs/AuE. The non-specific sites were passivated in 1 mM MCH for 1 h at room temperature. In addition, 10 μL mixture of 2 μM HP1 and HP2 with the same quantity was annealed at 95 ˚C for 5 min and cooled to room temperature, which were dropped onto the S1-AuNPs/AuE surface and incubated for 90 min. After that, the electrode was thoroughly rinsed, and the HCR/S1-

AuNPs/AuE was constructed.

2.6 Electrochemical measurement

The determination of β-Lg was performed by differential pulse voltammetry (DPV) method in 0.1 M of pH 7.4 PBS within the potential range of -0.45-0.05 V at a scan rate of 100 mV/s. The characterization of the aptasensor fabrication process and the parameters optimization were detected by CV, differential pulse voltammetry (DPV) or electrochemical impedance spectrum (EIS) techniques, which were measured in 0.1 M of pH 7.4 PBS containing 0.1 M KCl and 5 mM $[Fe(CN)_6]^{3-/4-}$ at a scan rate of 100 mV/s. The EIS measurements were conducted under an AC amplitude of 0.005 V and a frequency range from 100 kHz to 0.1 Hz.

2.7 Real sample analysis

Preliminary experiments for the determination of β-Lg in real samples were performed. Real samples were treated following a procedure described in previous literatures [38, 39]. Milk, soya milk and hypoallergenic formulas (HF) were purchased from local market. The milk and HF samples were manually skimmed after heating at 40 °C for 30 min. After centrifugation at 15,000 rpm for 15 min, the skimmed suspension was collected and acidified to pH 4.6 with 2 M HCl. After 20 min, the acidified suspension was centrifuged to sediment the casein, and filtered by a 0.45 μm polycarbonate membrane. The solution was neutralized by 1 M NaOH and diluted with 10 mM of pH 7.4 PBS to an appropriate dilution (between 10^5 and 10^7), which was subsequently spiked with different concentrations of β-Lg solution (0.1, 1.0 and 10 ng mL^{-1}). The soya milk sample was filtered by 0.45 μm polycarbonate membrane and diluted in 10 mM of pH 7.4 PBS with the volume ratio of 1:9. Soya milk sample was not pretreated because it has not matrix effect. The β-Lg detection in real samples were performed as described in the previous procedures, and the recovery (%) of the concentration was calculated.

3 Results and discussion

3.1 Characterization of the working electrode

The AuNPs could provide a stable and biocompatible immobilization carrier for capturing biomolecules, such as HPs, aptamers, enzymes, etc. in the construction of various biosensors. The AuNPs were electrodeposited on the AuE surface, which was used as a working electrode. The morphology of the AuNPs/AuE was characterized by SEM, as seen in Fig. 1A. Numerous angular AuNPs with the mean diameter of 120 nm were deposited onto the AuE surface, providing a rougher surface and a larger electrochemical active area. The CV curves recorded in 0.1 M of pH 7.4 PBS containing 0.1 M KCl and 5 mM $[Fe(CN)_6]^{3-/4-}$ were shown in Fig. 1B. It could be seen that a pair of well-defined redox peaks at AuNPs/AuE with a much larger peak current, compared with those at bare AuE. According to Randles-Savick equation, the electroactive surface area of AuNPs/AuE is about 5.4 times of that of bare AuE. It demonstrated that the AuNPs/AuE could provide more active sites for immobilizing S1 probe via Au-S bonding, which was helpful to form more HCR structure and enhance the sensitivity of β-Lg sensing.

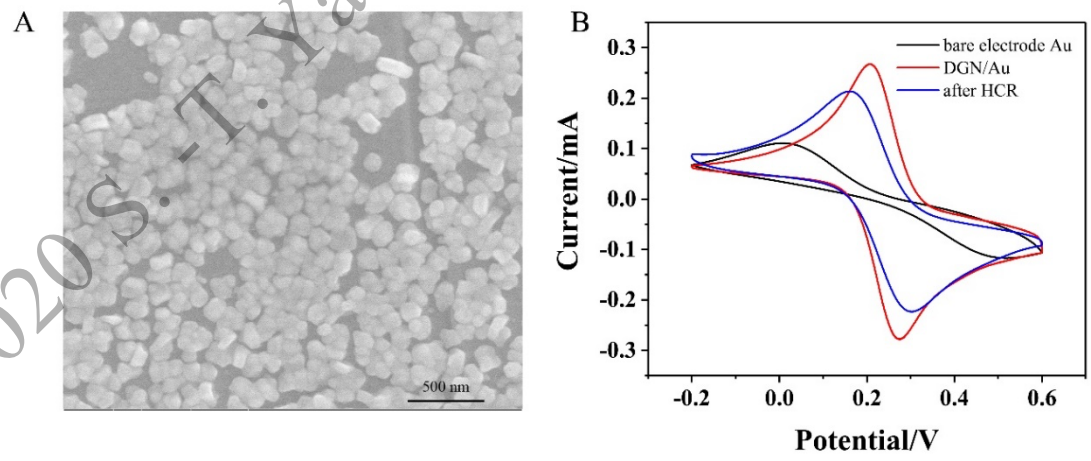


Fig. 1 (A) SEM images of AuNPs/AuE. (B) The CV comparison of AuNPs/AuE and bare AuE in 0.1 M of pH 7.4 PBS containing 0.1 M KCl and 5 mM $[Fe(CN)_6]^{3-/4-}$ at a scan rate of 100 mV/s.

3.2 Feasibility of Nt.BbvCI-assisted reconstruction strategy

The reconstruction of HP towards β-Lg is the most important procedure in the designed sensing strategy, which could further induce the Nt.BbvCI-assisted amplification process with the release of T1 [40-42]. For investigating the feasibility of this procedure, HP-Cy3 tagged magnetic beads were prepared in section 2.4. As shown in Fig. 2A, in the absence of β-Lg, the FL emission of Cy3 at the wavelength of 570 nm was recorded. In the presence of β-Lg, the recognition between the target β-Lg and aptamer, the stem structure of HP was changed to site-1/site-2, which triggered the Nt.BbvCI-aided nicking reaction. Then, the T1 tagged magnetic beads were precipitated via the magnetic attraction and the supernatant was disposed. The release of T1-labeled magnetic beads and the resulting disappearance of the FL signal at 570 nm demonstrated that the feasibility of the target-induced nicking site reconstruction and the subsequent nicking reaction.

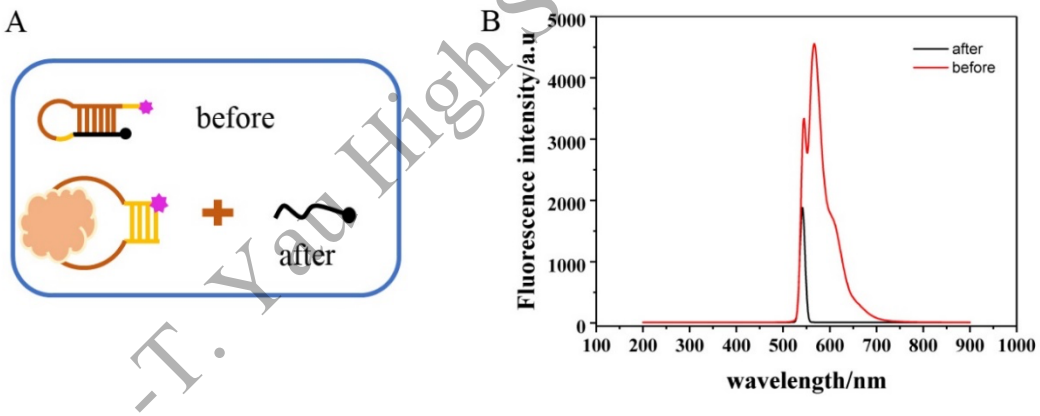


Fig. 2 (A) Schematic diagram of the target-induced nicking site reconstruction strategy. (B) FL spectra recorded in the absence (a) and presence (b) of β-Lg, respectively.

3.3 Optimization of the experimental parameters

To achieve an excellent performance of the aptasensor, the experimental parameters need to be optimized, including the incubation time of S1 and reaction time of HCR.

S1 strand is the link of electrode and HCR structure. In order to obtain the highest sensitive current response, the immobilized amount of S1 should be optimized. After

the newly prepared AuNPs/AuE was immersed in 2 μM of S1 solution for different time, EIS and CV were applied for the electrochemical characterization. From Fig. 3A and 3B, it could be seen that the electron transfer resistance (R_{et}) value reached the largest and the corresponding peak current became the lowest when the immersion time prolonged to 8 h, and the EIS and CV curves nearly kept unchanged along with the longer time. Too little amount of S1 immobilization could not cover enough surface area of electrode, while too much S1 brought high steric hindrance or even could not be immobilized too well due to the limited active sites on the electrode surface. Thus, 8 h of immobilized time was chosen for the S1 modification for the maximum coverage and minimum nonspecific adsorption. In addition, in this work, the concentration of S1 was prepared as 2 μM. If other concentrations of S1 stock solution were prepared, the immersing time should be altered accordingly.

Since the HCR is an effective procedure for signal amplification and importantly in this work, the optimal HCR structure brought the highest initial current signal, thus the HCR time should be investigated. Consolidate the immobilization time of S1 as 8 h, the concentration of HP1/HP2 mixture with the molar ratio of 1:1 as 2 μM, and the incubation temperature as room temperature, the DPV current was found rising along with the HCR time increased from 30 to 90 min. As seen from Fig. 3C and 3D, the current response decreased when the HCR time exceeded 90 min. It demonstrated that the optimal HCR time was appeared at 90 min, compromising the maximum amount of MB probe attachment and the insulation of HCR structure.

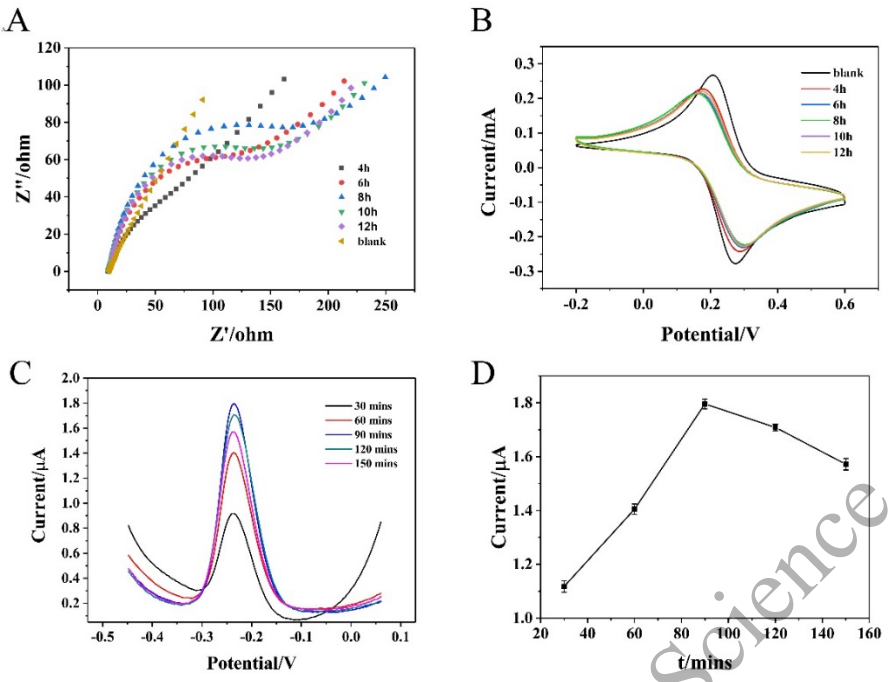


Fig. 3 (A) EIS and (B) CV curves recorded at S1-AuNPs/AuE with different incubation time in S1 solution. The electrolyte is 0.1 M of pH 7.4 PBS containing 0.1 M KCl and 5 mM $[Fe(CN)_6]^{3-/4-}$. (C) DPV curves recorded after HCR reaction with different time and (D) the relationship between HCR time and the peak current. The electrolyte is 0.1 M of pH 7.4 PBS.

3.4 Quantitation of β-Lg

To investigate the sensitivity of the proposed sensing assay, various concentrations of β-Lg were detected under the optimal conditions. As shown in Fig. 4A, the current of MB gradually decreased with the increase of β-Lg concentration from 0.01 to 100 nM. It is indicated that more β-Lg induced more released T1, which enhanced the electrochemical resistance of electrode surface. As revealed in Fig. 4B, a good linearity was obtained in 5 orders of magnitude from 0.01 nM to 100 nM, with the linear regression equation of ΔI /μA = 0.56 + 0.19 lg $C_{\beta\text{-Lg}}$ /nM (R^2 = 0.995). The detection limit was estimated as 5.7 pg mL^{-1} at three times of the standard deviation divided by the slope (LOD = 3σ/slope). The LOD is below the threshold established for proteins in cow's milk so it would be very suitable for the determination of trace concentrations in food to avoid undesired reactions in allergenic patients. In addition, the performance

of the proposed method was compared with previous electrochemical biosensors and other practical protocols, which is listed in Table 2.

The intra- and inter-assay repeatability and reproducibility of the proposed strategy were also evaluated. Here, 0.1, 1 and 10 nM of β-Lg were selected for three times of detection. The relative standard deviations (RSD) values for intra-assay were 1.5, 1.8 and 2.6%, respectively, and for inter-assay were 1.7, 2.3 and 3.5%, respectively, demonstrating a reliable, repeatable and reproducible strategy was proposed.

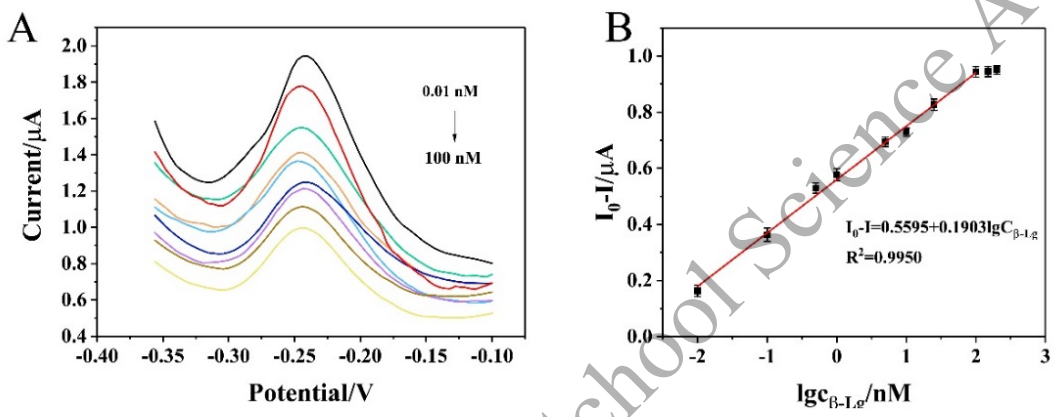


Fig. 4 (A) The DPV curves recorded in the addition of different concentration β-Lg with the concentrations of 0, 0.01, 0.1, 0.5, 1, 5, 10, 25 and 100 nM, respectively. (B) The linear relationship between *ΔI* and the logarithm of β-Lg concentration. The error bars represented the standard deviation of four measurements.

Table 2. Comparison of analytical performance of different methods for quantitative β-Lg detection.

Techniques	LOD	Linear range	Ref.
Electrochemistry	0.8 nM	2.8 nM-100 nM	[15]
Electrochemistry	0.85 pM	0.001-100 nM	[14]
ELISA	7.9 nM	62.5 nM-8 μM	[23]
SPR	5.54 nM	5 nM-4 μM	[43]
Fluorescence	37 pM	0.25-50 nM	[44]
Electrochemistry	0.057 nM	0.01-100 nM	This work

4 Conclusions

The main ingredient of milk protein of β-Lg has been accurately determined via a target-induced nicking site reconstruction and HCR amplification strategies. The AuNPs modified AuE provided more active sites for DNA probe immobilization, enhancing the sensitivity of the genosensing. MB was used as the electroactive probe for current response, which provided a quantitative basis for β-Lg detection. The method is highly sensitive and selective, and could be conceived as an effective alternative for milk quality evaluation. Furthermore, it also could be extended to other allergens detection via substituting the corresponding DNA sequences.

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