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

Qianying Qiu,^a Xiao Ni,^a Tianchen Liu,^b Zening Li,^b Xinyi An^b and Xiaojun Chen^{id} ^{*a,c}

Food allergy is an immune system reaction to a particular food, milk being 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 the electrochemical sensors are a good alternative for this purpose since they are sensitive, selective and inexpensive. In this work, an electrochemical aptasensor was fabricated for the quantitative detection of β -Lg in hypoallergenic formula (HF) milk. A tri-functional hairpin (HP) was designed, which was composed of an aptamer sequence, a nicking site and a DNA sequence (T1). In the absence of β -Lg, the aptamer part hybridized with T1 to form a stable stem-loop structure. However, in the presence of β -Lg, the capture of the aptamer sequence towards β -Lg caused the reconstruction of HP and thus the nicking sites were exposed. Then, the nicking enzyme was activated and T1 could be released, which bound with the end of the hairpin 1-methylene blue (HP1-MB)/HP2-MB conjugation on the Au nanoparticle (AuNP) modified electrode surface. Thus, the insulating property of the electrode was enhanced and the current response of MB decreased, which built the quantitative basis for β -Lg detection. In this way, 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. This aptasensor also displayed high selectivity, reproducibility and stability, and became a promising platform for β -Lg detection in real food samples.

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1. Introduction

Food allergy is a reaction of the immune system upon having a particular food, which can cause atopic dermatitis, cough, asthma and other symptoms in mild cases, and even endanger life in severe cases.^{1,2} Milk protein allergy is one of the most common types of food allergy, especially in infants. Statistically, about 2 to 3% of infants have allergic reaction to milk protein within one year of birth.^{3–5} The main allergen protein in milk is β -lactoglobulin (β -Lg). Hypoallergenic formula milk (HF) is normally available for milk allergy patients. Even so, low concentrations of β -Lg residue introduced during the production and packaging of products may cause allergic reactions.^{6,7} 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),^{8–10} electrochemical immunoassay (EIA),^{11,12} fluorescence immunoassay (FIA),¹³ surface plasma resonance (SPR),^{14,15} and enzyme-linked immunoassay (ELISA).^{16–18} However, these methods also have some disadvantages, such as high cost, lack of portability, complex experimental technology, and long response time. To address these aspects, electrochemical biosensors have become a promising alternative since they are low cost, time-effective, highly selective and sensitive, and have been shown to have the ability to detect trace levels of analyte in food safety controls.¹⁹ 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.^{20–23} Qiao and colleagues, for example, constructed an electrochemical aptasensor for cardiac troponin I (cTnI) detection, in which the high affinity between the aptamer and cTnI could effectively cause the release of the aptamer from the surface of the MoS₂ modified electrode.²¹ Shui *et al.* designed a new aptasensor for the detection of the Alzheimer's disease marker tau-

^aCollege of Chemistry and Molecular Engineering, Nanjing Tech University, Nanjing 211816, PR China. E-mail: chenxj@njtech.edu.cn

^bNanjing Foreign Language School, Nanjing 210018, PR China

^cJiangsu Key Laboratory of Molecular Biology for Skin Diseases and STIs, Nanjing, 210042, PR China

381 in human serum, based on the recognition between the aptamer and tau-381.²² Marrazza's group selected an aptamer by the SELEX process, which was further utilized for the capture and detection of β -Lg on a polymer film modified electrode.²³ In these studies, the combination of aptamers and electrochemical methods endowed aptasensors such advantages as wider detection range, lower detection limit, better selectivity and portability.

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),^{24,25} roll ring amplification (RCA) technology,^{26,27} nucleic acid enzyme strategies *etc.*^{28,29} In addition, the modification of an electrode surface with nanomaterials is also very important for improving the performance of electrochemical sensors. Gold nanoparticles (AuNP) are one of the most widely used nanomaterials as an excellent scaffold for fabricating chemical and biological sensors, since they possess some advantages such as good biocompatibility, high specific surface area, excellent conductivity and feasible surface functionalization.^{30,31} Simple and efficient preparation of an AuNP modified electrode is considered an important basis for the construction of electrochemical sensors. In our previous work, electrodeposition has been verified as an effective and fast method, which could control the amount, morphology and distribution density of AuNPs on the electrode surface *via* adjusting the working potential and the deposition charge.^{32–35} Therefore, in this work an electrodeposited AuNP modified electrode was also utilized as the basis for fabricating a novel β -Lg aptasensor.

Herein a highly sensitive and homogeneous aptasensor of β -Lg was designed with an aptamer and nicking enzyme assisted electrochemical signal amplification in an HCR system. The homogeneous reaction based on the β -Lg-triggered alteration of the conformation of a hairpin (HP) can improve the detection accuracy with the elimination of several washing and separation steps. The AuNP modified electrode enhanced the HCR structure density and the electrochemical signal from the methylene blue (MB) label. As a result, high detection selectivity and sensitivity could be reached. The detection principle is shown in Scheme 1. On the one hand, a tri-functional HP was designed, containing an aptamer sequence for capturing target β -Lg, a nicking site composed of two complementary

sequences (site-1 and site-2), and a DNA sequence (T1) for amplification. In the absence of β -Lg, the HP was stable with the stem-loop structure, in which a part of the aptamer and T1 hybridized. After incubation with β -Lg, the aptamer could bind to β -Lg due to high affinity, resulting in the reconstruction of the HP conformation. Then, the separated sequences of site-1/site-2 hybridized and T1 sequence was exposed. At this time, the nicking site could be identified by the nicking enzyme, yielding the released T1. On the other hand, the AuNP modified Au slice electrode (AuE) was used for the immobilization of S1-SH *via* the Au-S covalent bonding. After that, HCR hybridized with HP1-MB and HP2-MB could be constructed, and the initial current signal (I_0) was achieved. Two sequences in HP1-MB and HP2-MB matched 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 caused the electrochemical resistance of the electrode to increase and the current response of MB to decrease. The decrease of current ($\Delta I = I_0 - I$) was proportional with β -Lg concentration, and the linear relationship between ΔI and $C_{\beta\text{-Lg}}$ was considered the quantitative basis for β -Lg detection.

2. Experimental

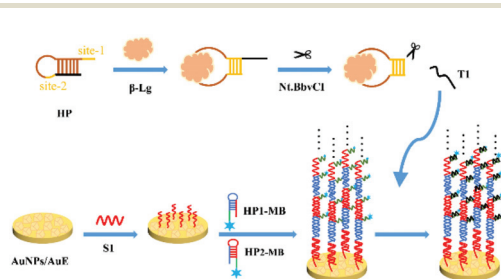
2.1 Chemicals and reagents

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were 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 were purchased from Sangon Inc. (Shanghai, China). The sequences with abbreviations are listed in Table 1. Carboxyl modified magnetic beads were purchased from Shannuo Biotech Co. (Tianjin, China). Amino terminated HP-Cy3 (NH₂-HP-Cy3) is a fluorescent dye Cy3 tagged HP sequence, which is used for verifying the target-induced nicking site reconstruction.

Table 1 The sequences for the construction of the β -Lg aptasensor

Name	Sequence(5' → 3')
HP	CACACA CGGATG AACCTC AGCCGA <u>CGATCG</u> GACCGC AGTACC CACCCA CCAGCC CCAACA TCATGC CCATCC GTGTGT GGCTGA GGTT
NH ₂ -HP-Cy3	Cy3-CACACA CGGATG AACCTC AGCCGA CGATCG GACCGC AGTACC CACCCA CCAGCC CCAACA TCATGC CCATCC GTGTGT GGCTGA GGTT-(CH ₂) ₆ -NH ₂
S1-SH	AGTCT AGGAT TCGGC GTGGG TTAA-(CH ₂) ₆ -HS
HP1-MB	TTAACC CACGC CGAAT CCTAG ACT CAAAGT AGTCT AGGATT CGGCGTG TTTTGTGTGTGCCTACTT-(CH ₂) ₆ -MB
HP2-MB	AGTCT AGGATT CGGCGTG GGTTAA CACGC CGAAT CCTAG ACT ACTTTG TTTTGTGTGTGCCTACTT-(CH ₂) ₆ -MB

Note: the aptamers corresponding to β -Lg in HP and NH₂-HP-Cy3 are marked with an underline. The red and blue sequences are the complementary parts in HP1-MB and HP2-MB, respectively. The italicized parts are the complementary sequences with the short-chain T1 obtained in the nicking reaction.



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

tion 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 NaCl and 1 mM MgCl_2 . Phosphate buffer solution (PBS, 10 mM, pH 7.4) was prepared with Na_2HPO_4 and NaH_2PO_4 , and is used for preparing β -Lg dilution and DNA stocking solutions and as the washing buffer (WB). All the other chemicals were of analytical grade and used as received. The ultrapure water ($\geq 18.2 \text{ M}\Omega \text{ cm}^{-1}$) was 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 microscopy (SEM, Hitachi S4800, Japan). A fluorescence (FL) spectrometer (Lengguang F97XP, Shanghai, China) was used to record the 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/AuE working electrode (3 mm in diameter), and a Pt auxiliary electrode was used for all the electrochemical measurements. All the 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 the target protein β -Lg was $4 \mu\text{g mL}^{-1}$ and was diluted to appropriate concentrations. $4 \mu\text{L}$ of 100 μM HP was annealed at 95°C for 5 min and slowly cooled to 30°C before use. Then, $40 \mu\text{L}$ of the 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

To verify the feasibility of the proposed target-induced nicking site reconstruction strategy for β -Lg detection, fluorescence experiments were performed. 20 mg mL^{-1} magnetic beads were centrifuged and re-dispersed in 200 μL of PBS, and then 50 μL of a 0.5 M mixture of NHS and EDC (1 : 1) was added to activate the carboxyl groups on the surface of the magnetic beads. Additionally, $4 \mu\text{L}$ of 100 μM NH_2 -HP-Cy3 was annealed at 95°C for 5 min and slowly cooled to 30°C . After the NH_2 -HP-Cy3 was incubated with the activated magnetic beads overnight at room temperature, the NH_2 -HP-Cy3 immobilized magnetic beads could be prepared. After similar triggering and nicking procedures to those in section 2.3, T1-Cy3 was released through magnetic separation. The FL intensity was detected before and after the reaction 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.

In order to prove the feasibility of nucleic acid reaction, agarose gel electrophoresis (AGE) analysis was conducted. AGE analysis was performed in $1\times$ TAE buffer at a constant voltage of 120 V for 40 min at room temperature. 2% agarose was used as the media of electrophoresis. After separation, the gel was stained with ethidium bromide (EB) and scanned using a gel imaging system (Bio-Rad, Shanghai). The 50 bp DNA ladder ranging from 50 to 500 bp (Solarbio, Beijing) was applied for the analysis of the DNA strands.

2.5 Fabrication of the aptasensor

The AuE was cleaned with acetone, ethanol, and water for 10 min, successively and dried with a N_2 stream. A perforated insulative tape with an 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* potentiostatic electrodeposition in 12 mM HAuCl_4 solution containing 0.05 M HClO_4 under a potential of -0.1 V , and the deposition charge was controlled as 0.15 C according to our previous work.³⁵ Afterwards, 10 μL of 2 μM SH-S1 was incubated on AuNPs/AuE overnight at 37°C to form S1-AuNPs/AuE. The non-specific sites were passivated by immersing the electrode in 1 mM MCH for 1 h at room temperature. In addition, 10 μL of a mixture of 2 μM HP1-MB and HP2-MB with the same concentration was annealed at 95°C for 5 min and cooled to room temperature, which was then 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 system was constructed.

2.6 Electrochemical measurement

The determination of β -Lg was performed by the differential pulse voltammetry (DPV) method in 0.1 M PBS of pH 7.4 within the potential range of -0.45 – 0.05 V at a scan rate of 100 mV s^{-1} . The characterization of the aptasensor fabrication process and the parameter optimization were detected by CV, DPV or EIS techniques, which were measured in 0.1 M PBS of pH 7.4 containing 0.1 M KCl and 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at a scan rate of 100 mV s^{-1} . 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 the previous literature.^{36,37} Hypoallergenic formula (HF) was purchased from the local market, and was 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 $0.45 \mu\text{m}$ polycarbonate membrane. The solutions were neutralized by 1 M NaOH and diluted with 10 mM PBS of pH 7.4 at the ratio of 1 : 9. HF samples were subsequently spiked with different concentrations of β -Lg solution (0.1, 1.0, 10, 50 and 100 ng mL^{-1}).

The β -Lg detection in real milk samples was 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

AuNPs can provide a stable and biocompatible immobilization carrier for capturing biomolecules, such as HPs, aptamers, enzymes, *etc.* in the construction of various biosensors. 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 a mean diameter of 120 nm were scattered on the AuE surface, providing a rougher surface and a larger electrochemically active area. The CV curves recorded in 0.1 M PBS of pH 7.4 containing 0.1 M KCl and 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ are shown in Fig. 1B. A pair of well-defined redox peaks of AuNPs/AuE with much larger peak currents compared with bare AuE could be seen. According to the Randles-Sevcik equation, the electroactive surface area of AuNPs/AuE is about 5.4 times of that of bare AuE, demonstrating that more active sites could be provided by AuNPs/AuE for immobilizing the S1-SH probe *via* Au-S bonding. It was helpful to form more HCR structures on the electrode surface and enhance the sensitivity of β -Lg detection.

3.2 Feasibility of the 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 accompanied by the release of T1.^{38–40} For investigating the feasibility of this procedure, NH_2 -HP-Cy3 tagged magnetic beads were prepared as 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 the aptamer induced the stem structure of HP to change to site-1/site-2. It triggered the Nt-BbvCI-aided nicking reaction and the T1 tagged magnetic beads were precipitated by magnetic separation. After that, the T1 tagged magnetic beads were re-dispersed and the FL signal at the 570 nm disappeared, demonstrating the feasibility of

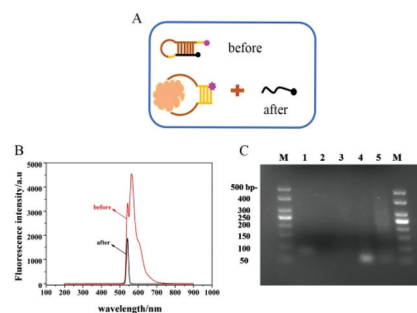


Fig. 2 (A) Schematic diagram of the target-induced nicking site reconstruction strategy. (B) FL spectra recorded before and after the β -Lg recognition, respectively. (C) The AGE analysis result. Lane 1: HP; lane 2: S1-SH; lane 3: after the nicking reaction of the HP/ β -Lg biocomposite; lane 4: HCR nanowire; lane 5: HCR/T1 composites; lane M: DNA marker.

the binding-induced nicking site reconstruction and the subsequent nicking reaction.

AGE analysis was conducted to affirm the feasibility of the nucleic acid reaction. As shown in Fig. 2C, lanes 1 and 2 corresponded to HP and S1-SH. Only an obscure band was observed in lane 1, while there were no obvious bands in lane 2 owing to the small molecular weight of S1-SH. After the nicking reaction of HP/ β -Lg biocomposite with Nt-BbvCI, a short-strand T1 was released and it was still hardly observed in lane 3. Lane 4 corresponded to the HCR nanowire and exhibited a bright band, illustrating the successful interaction among S1-SH, HP1-MB and HP2-MB. Lane 5 was the composite of HCR/T1, showing a tailing band compared with that in lane 4. This result was probably due to a variety of HCR/T1 products with high molecular weights.

3.3 Optimization of the experimental parameters

To achieve excellent performance of the aptasensor, the experimental parameters needed to be optimized, including the incubation time of S1-SH, construction time of HCR, reaction time between HP and β -Lg, and the nicking time of Nt-BbvCI catalysis and the dosage of Nt-BbvCI.

The S1-SH strand is the linkage between the electrode and HCR structure. In order to obtain the highest current response, the immobilized amount of S1-SH should be optimized. After the newly prepared AuNPs/AuE was immersed in 2 μM S1-SH solution for different times, EIS and CV were applied for electrochemical characterization. Seen from Fig. 3A and B, the largest R_{et} value and the corresponding lowest peak current were obtained at the immersion time of 8 h, and then both values nearly remained unchanged as the immersion time was prolonged further. Too little S1-SH could not cover enough of the surface of the electrode, while too much S1-SH 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 immobilization time was chosen for S1-SH modification for the maximum coverage and minimum nonspecific adsorption. In addition, in this work, the concentration of the S1-SH stock solution was prepared as 2 μM . If

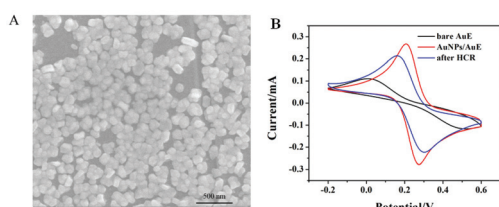


Fig. 1 (A) SEM images of AuNPs/AuE. (B) The CV comparison of AuNPs/AuE and bare AuE in 0.1 M PBS of pH 7.4 containing 0.1 M KCl and 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at a scan rate of 100 mV s^{-1} .

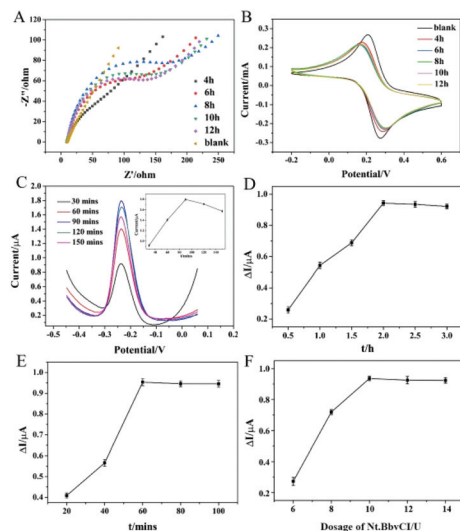


Fig. 3 (A) EIS and (B) CV curves recorded at S1-AuNPs/AuE with different incubation times in S1-SH solution. The electrolyte is 0.1 M PBS of pH 7.4 containing 0.1 M KCl and 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$. (C) DPV curves recorded at different HCR reaction times (inset is the relationship between HCR time and peak current). The relationship between ΔI and (D) capture time of HP towards β -Lg, (E) the nicking time and (F) dosage of Nt-BbvCI upon 100 ng mL^{-1} of β -Lg. The electrolyte is 0.1 M PBS of pH 7.4.

the concentrations of S1-SH changed, the immersion time should be altered accordingly.

Since HCR is an effective strategy for signal amplification and an important procedure in this work, the optimal HCR length with the highest initial current signal should be investigated. Combining the immobilization time of S1-SH as 8 h, the concentration of HP1-MB and HP2-MB mixtures with the molar ratio of 1 : 1 as $2 \mu\text{M}$, and the incubation temperature as room temperature, the DPV current was found to increase along with the HCR time increasing from 30 to 90 min. As seen in Fig. 3C, the current response decreased when the HCR time exceeded 90 min. It demonstrated that the optimal HCR time appeared at 90 min (inset), balancing the maximum amount of MB probe attachment and insulation of the HCR structure.

The HCR/S1-AuNPs/AuE surface modification has been optimized under the above-mentioned two parameters. The sensitivity of the β -Lg aptasensor is dependent on the ΔI value obtained after T1 binding. The more T1 strands hybridized with HCR, the larger the current change (ΔI) that could be induced. Since T1 is released by the target-induced reconstruction of HP and the following Nt-BbvCI-aided nicking reaction, the amount of T1 is closely correlated with the efficiency of aptamer recognition and reactivity of the nicking enzyme. The effects of capture time of HP towards β -Lg, the nicking time of Nt-BbvCI catalysis and the dosage of Nt-BbvCI on ΔI are shown in Fig. 3D–F. It could be seen that the steady capture and nicking states for 100 ng mL^{-1} of β -Lg were obtained when the time reached 2 h and 60 min, respectively. In addition, ΔI was

found to increase as the Nt-BbvCI concentration increased from 6 to 10 U, and became steady when the Nt-BbvCI concentration increased further. It demonstrated that 2 h is enough for the binding of HP towards β -Lg. The maximum activity of Nt-BbvCI in this reaction was found at 60 min with the dosage of 10 U.

3.4 Quantitation of β -Lg

Under the optimal experimental conditions, various concentrations of β -Lg could be detected quantitatively. As shown in Fig. 4A, the current of MB decreased progressively as the concentration of β -Lg increased from 0.01 to 100 ng mL^{-1} . As shown in Fig. 4B, good linearity was obtained in 5 orders of magnitude with a linear regression equation of $\Delta I (\mu\text{A}) = 0.56 + 0.19 \lg C_{\beta\text{-Lg}} (\text{ng mL}^{-1})$ ($R^2 = 0.995$). The detection limit (LOD) was estimated as 5.7 pg mL^{-1} at $\text{S/N} = 3$, which is below the threshold established for proteins in cow's milk. Therefore, the proposed aptasensor would be very suitable for trace β -Lg determination in food to avoid undesired reactions in allergic patients. In addition, the performance comparison of different detection techniques and biosensors for β -Lg determination has been listed in Table 2. The performance of our aptasensor was comparable with that of existing detection methods, illustrating the good potential of this system for practical applications. In particular, compared with the commercial ELISA kit ($\text{LOD} = 20 \mu\text{g mL}^{-1}$), the proposed electrochemical aptasensor exhibited a comparable LOD and wide linear range.

3.5 Repeatability, reproducibility, selectivity and stability

The repeatability and reproducibility of the proposed strategy are considered important for evaluating the precision of the sensor. Five replicate aptasensors were used to detect 1 ng mL^{-1} β -Lg in PBS at the same time, and the relative standard deviation (RSD) value of the five repeated measurements was calculated as 2.75%, indicating favorable repeatability of the fabricated aptasensor. Furthermore, the intra- and inter-assay were assessed to evaluate the reproducibility of the fabricated aptasensor. The intra-assay precision was evaluated by assaying three concentrations (0.1 , 1 and 10 ng mL^{-1}) of β -Lg for three times, respectively. The RSDs analyzed from the intra-assay were 1.5, 1.8 and 2.6%, respectively. However the inter-

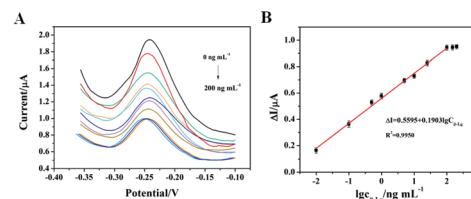


Fig. 4 (A) The DPV curves recorded at different β -Lg concentrations of 0, 0.01, 0.1, 0.5, 1, 5, 10, 25, 100, 150 and 200 ng mL^{-1} , respectively. (B) The linear relationship between ΔI and the logarithm of β -Lg concentration. The error bars represent the standard deviation of four measurements.

Table 2 Performance comparison of different techniques for quantitative β -Lg detection

Technique	LOD (ng mL ⁻¹)	Linear range (ng mL ⁻¹)	Ref.
Electrochemistry	8.5×10^{-4}	0.001–100	12
Electrochemistry	0.8	2.8–100	13
Photoelectrochemistry	3.7×10^{-3}	0.01–100	41
SPR	5.54	5–4000	42
Fluorescence	3.7×10^{-2}	0.25–50	43
Electrochemistry	5.7×10^{-3}	0.01–100	This work

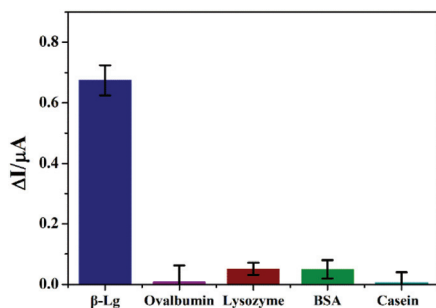
assay precision was estimated by measuring the above mentioned three concentrations with three aptasensors prepared independently under the same experimental conditions. The RSD values of the inter-assay were 1.7, 2.3 and 3.5%, respectively. The results indicated that the proposed strategy was reliable with acceptable repeatability and reproducibility.

The selectivity investigation of the aptasensor is a key factor for real-life applications. Four different non-specific proteins which may be present in milk including ovalbumin, BSA, egg lysozyme and casein were chosen for study. Fig. 5 shows a much smaller ΔI response for each interfering protein, compared with the response towards 1 ng mL⁻¹ β -Lg. The results confirmed that the aptasensor displayed good selectivity for β -Lg determination, owing to the inherent affinity between β -Lg and the corresponding aptamer.

In addition, stability is also essential for an aptasensor. Several aptasensors were prepared in the same batch and stored at 4 °C for use. Every three days, one was taken out to detect 1 ng mL⁻¹ β -Lg. After 21 days, the ΔI response could still maintain about 90.7% of the initial value, confirming the good stability of the designed electrochemical aptasensor. The possible reason might be that AuNP modification could provide an excellent micro-circumstance for biomolecule immobilization and the covalent bonding of the S1-SH probe with AuNPs/AuE enhanced the stability of the HCR structure.

3.6 Real HF sample analysis

In order to verify the potential applicability of the developed aptasensor, an HF milk sample was applied for the determination of β -Lg after a simple pre-treatment process. According

**Fig. 5** The ΔI response of the aptasensor towards 1 ng mL⁻¹ of β -Lg, ovalbumin, lysozyme, BSA and casein, respectively.**Table 3** The recovery in HF milk samples ($n = 5$)

Sample	Added (ng mL ⁻¹)	Found (ng mL ⁻¹)	Recovery (%)	RSD (%)
1	0	0.063	—	3.62
2	1	0.945	94.5	2.19
3	10	10.14	101.4	3.11
4	50	49.76	99.5	2.93
5	100	101.23	101.2	3.44

to previous literature,⁴⁴ the amount of β -Lg in HF milk is below 0.5 ppm, and the result of our detection coincided with the information. Furthermore, a decrease in the current signal was observed along with increasing the β -Lg concentration added to the HF samples. The recovery ranged from 94.5 to 101.4% with a satisfactory analytical precision, in which the RSD values were less than 4%. The results confirm the potential practicability and validity of the aptasensor in real samples (Table 3).

4. Conclusion

As one of the main ingredients of milk protein, β -Lg could be accurately determined by a target-induced nicking site reconstruction and HCR amplification strategy in this work. The AuNP modified AuE provided more active sites for DNA strand immobilization, enhancing the sensitivity of the aptasensing. MB was used as the electroactive probe for quantitative β -Lg detection. The method is highly sensitive and selective, and can be considered as an effective alternative for HF milk quality evaluation. Furthermore, it also could be extended to other allergen detection *via* substituting the corresponding aptamer sequences.

Author contributions

Qianying Qiu: investigation, data curation. Xiao Ni: methodology, materials preparation and characterization. Tianchen Liu: methodology, data curation. Zening Li: data curation. Xinyi An: real sample detection. Xiaojun Chen: funding acquisition, project administration, resources, validation, supervision.

Conflicts of interest

The authors declare no competing financial interest.

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