



A novel sandwich enzyme-linked immunosorbent assay with covalently bound monoclonal antibody and gold probe for sensitive and rapid detection of bovine β -lactoglobulin

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Abstract

Bovine milk is a recognized allergenic food source with β -lactoglobulin (BLG) as its major allergen. Reliable detection of BLG epitopes can, therefore, be a useful marker for the presence of milk in processed food products, and for potential allergenicity. At the present, enzyme-linked immunosorbent assays (ELISA) for the detection of BLG are time-consuming and generally not specific to BLG IgE epitopes. In this study, the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-activated anti-BLG IgE epitope monoclonal antibody (mAb 1G9) was covalently bound onto the KOH-treated microtiter plate surface. Using this mAb-bound plate in sandwich combination with biotinylated anti-BLG polyclonal antibody-labeled gold nanoparticles, a linear dynamic range between 31.25 and 64×10^3 ng mL⁻¹ with a limit of detection for BLG of 0.49 ng mL⁻¹ was obtained, which is 32 times wider and 16 times more sensitive than conventional sandwich ELISA (sELISA). Total recovery of BLG in spiked food samples was found, without matrix effects. Also in partially hydrolyzed infant formulas, the allergenic BLG residues were detected quantitatively. Compared with conventional and commercial BLG detection sELISAs, our sELISA is reliable, highly BLG epitope-specific, user-friendly, and time-saving and allows accurate detection of potentially allergenic residues in different types of processed foods. This improved sELISA protocol can be easily extended to detect other well-identified and characterized food allergens.

Keywords Bovine β -lactoglobulin · Sandwich ELISA · Monoclonal antibody · IgE epitope · Covalent immobilization · Gold nanoparticle

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Abbreviations

ALA	Bovine α -lactalbumin
APTES	3-Aminopropyltriethoxysilane
AuNPs	Colloidal gold nanoparticles
BLG	Bovine β -lactoglobulin
CN	Bovine casein
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
mAb	Monoclonal antibody
MES	2-(N-Morpholino)ethanesulfonic acid
MW	Molecular weight
pAb-BLG	Rabbit anti- β -lactoglobulin polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBST	PBS containing 0.1% Tween-20
sELISA	Sandwich enzyme-linked immunosorbent assays
UHT-milk	Ultra heat treated-milk

Introduction

Bovine milk is a well-accepted food in the western world due to its high contents of a wide range of nutrients. However, milk is also known as an important allergenic food especially to infants and children, with a prevalence of 2–3% in children under 1 year of age [1]. Over 80% of cows' milk allergic patients are sensitive to bovine β -lactoglobulin (BLG) [2], the major protein in milk [3]. Milk-allergic individuals can even react to trace amounts of milk [4]. Cow milk avoidance is the current treatment for these patients. However, complete avoidance of all milk-containing products is very difficult, because milk is widely used in the food industry as a highly appreciated ingredient, and current food labeling practice is still insufficient [5]. To enforce milk labeling regulations making adequate information available to milk-allergic individuals, rapid, reliable, and sensitive methods for the detection of allergenic milk residues are urgently required.

Many analytical methods have been developed to detect food allergens, based on allergen-specific DNA analysis, proteomics (HPLC and LC-MS), and ELISA technologies, with the latter most widely applied. Among the ELISA technologies, those targeting allergen epitope(s) have a dual functionality: (1) to detect the presence of the given allergen in foods and (2) to estimate the allergenic potential of the given food product [6–8]. However, to our knowledge, for the detection of BLG, an epitope-specific antibody-based immunoassay is lacking. The commercially available ELISA kits for BLG detection are unable to recognize its specific IgE epitopes. Previously, we have developed several sELISA for the detection of BLG based on IgE epitope-specific antibodies [9, 10], but these sELISAs appeared time-consuming. Thus, development of an ELISA with high epitope sensitivity and being rapid-in-use would be desirable.

Biomolecule (antibody) immobilization is a critical step in immunoassays [11]. In conventional ELISA, the specific antibody is omnidirectionally immobilized to the microtiter plate through physical adsorption (Fig. 1C-b). Recently, it was discovered that 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)-activated antibodies could be immobilized covalently on a KOH-treated microtiter plate surface (Fig. 1B), which improved binding strength and antibody orientation (Fig. 1C-a), and decreased response time [12]. In addition, colloidal gold nanoparticles (AuNPs) had the properties of a high surface-to-volume ratio, good biocompatibility, high chemical stability, and high loading capacity for antibodies (Fig. 1A), which could improve the detectability of AuNPs to specific proteins and related peptides [13, 14].

In this study, we used the anti-BLG monoclonal antibody covalently bound plate for accelerated detection time, combined with the application of biotinylated anti-BLG polyclonal antibody-labeled AuNPs as detection probe to enhance the detectability for detection of BLG. The analytical performance

of the proposed sELISA will be evaluated for its detection limit, linear detection range, accuracy, precision, specificity, and recovery. In addition, several different processed food products were tested, and the results were compared with conventional sELISA and validated with a commercial sELISA kit.

Materials and methods

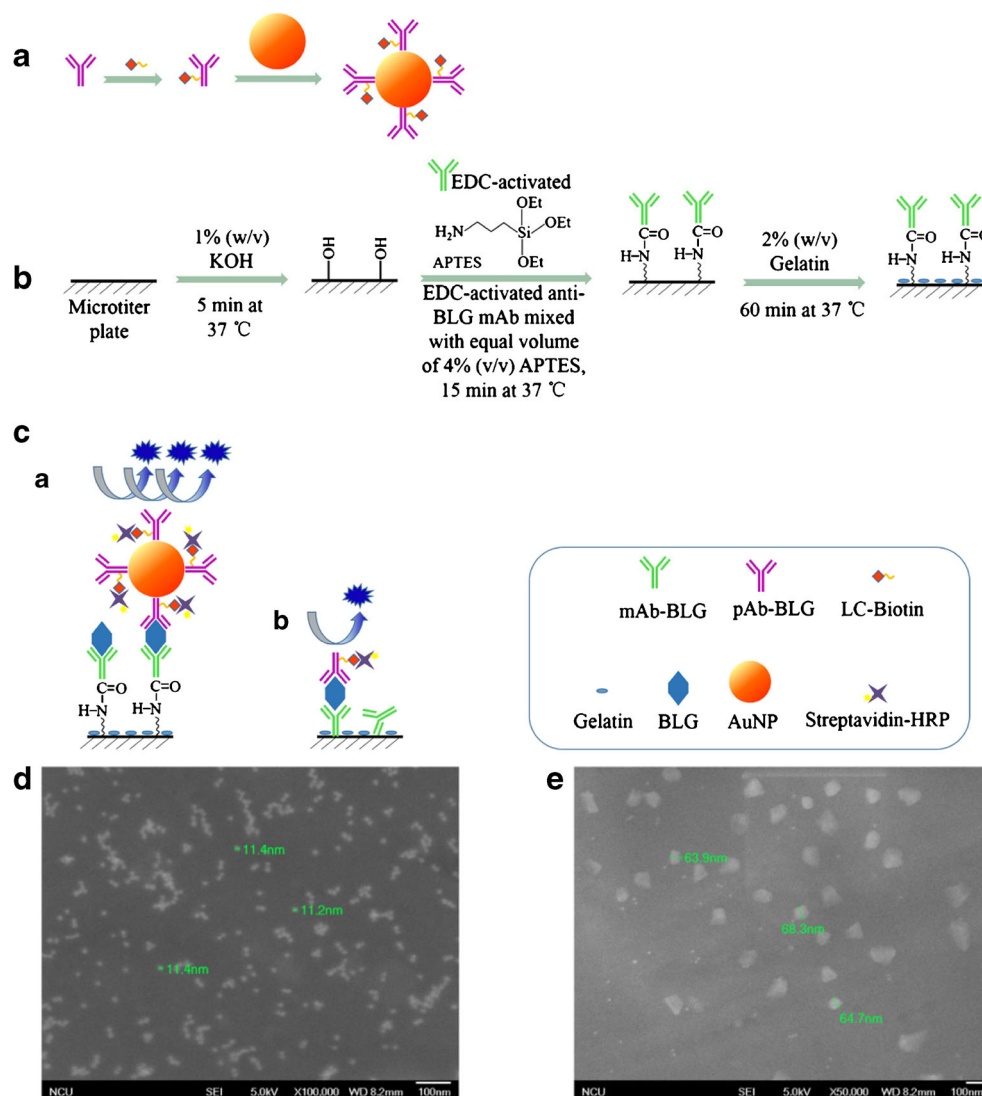
Materials

CNBr-activated Sepharose™ 4B resin and Protein G affinity column were purchased from GE Healthcare (Uppsala, Sweden). EZ-Link® Sulfo-NHS-LC-Biotinylation kit, 2-(N-morpholino)ethanesulfonic acid (MES), EDC, and 3,3',5,5'-tetramethylbenzidine (TMB) substrate kit were purchased from Thermo Scientific Pierce (Rockford, IL, USA). The 3-aminopropyltriethoxysilane (3-APTES), α -lactalbumin (ALA, purity $\geq 85\%$, electrophoresis), BLG (purity $\geq 90\%$, polyacrylamide gel electrophoresis, PAGE), α -casein (α -CN, purity $\geq 70\%$, electrophoresis), β -casein (β -CN, purity $\geq 98\%$, PAGE), κ -casein (κ -CN, purity $\geq 70\%$, PAGE), and gelatin from cold water fish were from Sigma (St. Louis, MO, USA). RADASCREEN® FAST BLG ELISA kit was purchased from R-Biopharm (Darmstadt, Germany). The 96-well polystyrene plates were purchased from Jincanhua (Shenzhen, China). Bovine serum albumin (BSA) was from Sangon Biotech (Shanghai, China), and streptavidin-HRP was purchased from Neobioscience (Shenzhen, China). The AuNPs were kindly provided by Professor Xiong from Jiangxi-OAI Joint Research Institute, Nanchang University (Nanchang, China). Egg, peanut, soybean, wheat, ultra heat treated-milk (UHT-milk), yoghurt, chocolate, cookie, and hydrolyzed infant formulas were purchased from local supermarkets. All reagents were analytical grade, and solutions were prepared using ultrapure Milli-Q water throughout the experiments.

Preparation of pAb-BLG-AuNPs probe

The rabbit anti-BLG polyclonal antibody (pAb-BLG) was prepared and purified using a Sepharose CNBr immunoaffinity column coupled with BLG as described previously [10], then purified using a Protein G affinity column before biotinylated with LC-biotin using an EZ-Link® Sulfo-NHS-LC-biotinylation kit according to the manufacturer's instructions. Briefly, 27 μL of 10 mmol L^{-1} sulfo-NHS-LC-biotin was transferred to 1 mL of 2 mg mL^{-1} purified pAb-BLG, vortexed, and allowed to stand for 1 h at room temperature. The free LC-biotin was removed by using a desalting column, and the biotinylated pAb-BLG was stored at -80°C until use.

Fig. 1 Synthesis of pAb-BLG-AuNPs probe (A), schematic principle of preparation of anti-BLG mAb-covalent bound and gelatin-preblocked plate (B), and mAb-covalent bound plate and pAb-BLG-AuNPs probe-based sELISA (C-a) and conventional sELISA (C-b) for detection of BLG. SEM images of bare AuNPs (D) and pAb-BLG-AuNPs probe (E)



The pAb-BLG-AuNPs probe was prepared according to the previously described method (Fig. 1A) [13], with slight modifications. Briefly, the pH of AuNPs solution was adjusted to 8.0 with 0.1 mol L⁻¹ K₂CO₃, and 2 mL AuNPs solution was mixed with 80 µL of 1 mg mL⁻¹ biotinylated pAb-BLG. The mixture was stirred for 45 min at room temperature, followed by blocking with 200 µL 10% BSA. After agitation for 1 h at room temperature, the probes were purified by centrifugation at 14,000×g for 30 min at 4 °C. The precipitates were resuspended in 2 mL of phosphate-buffered saline (PBS) solution (10 mmol L⁻¹, pH 7.2, containing 5% sucrose, 1% BSA, 1% PEG, and 0.25% Tween-20) and stored at 4 °C for further use.

Antibody immobilization

A mouse monoclonal antibody (1G9, IgG) against the BLG-specific peptide “EVDDEALEKFDKALKALP,” a major

human IgE linear epitope identified by Järvinen et al. [15], was prepared and found to exhibit high affinity and specificity in our previous study [9].

The microtiter polystyrene plate was treated with KOH, and the EDC-activated antibodies were immobilized to the hydroxyl-modified plate through covalent binding [12] (Fig. 1B). Briefly, the microtiter plate was treated with 200 µL 1% (w/v) KOH at 37 °C for 5 min, followed by washing five times with ultrapure water. The mAb (4.95 mL, 8 µg mL⁻¹) was activated with 50 µL of EDC (4 mg mL⁻¹ in 0.1 mol L⁻¹ MES, pH 4.7) for 10 min at 37 °C, followed by mixing with 5 mL of 4% APTES. Thereafter, 100 µL of the mixed solution was added to each well and incubated at 37 °C for 15 min. After washing with PBS containing 0.1% Tween-20 (PBST) for three times, the plate was blocked with 2% gelatin for 1 h at 37 °C. The anti-BLG mAb-bound and gelatin-preblocked plates were stored at -20 °C until use.

Developed sandwich ELISA

The anti-BLG mAb-bound and gelatin preblocked plate was washed three times with PBST, then each well was added with 100 μL of BLG (or food sample, PBST containing 2% gelatin as control), followed by incubation at 37 °C for 1.5 h. After washing again, 100 μL of pAb-BLG-AuNPs probe (diluted 1:200) was added into each well as detection antibody, and incubated at 37 °C for 1.5 h. After further washing, 100 μL of streptavidin-HRP (diluted 1:60) was added to each well and incubated at 37 °C for 1 h. After further washing with PBST for four times, 100 μL of TMB solution was added into each well, and color was developed at 37 °C in the dark for 15 min. Afterwards, 50 μL of 2 mol L^{-1} sulfuric acid was added to each well to stop color development. Finally, the optical density at 450 nm was measured using a microplate reader (Bio-Rad, USA).

Evaluation of detection limit, accuracy, precision, and specificity

The limit of detection (LOD) and quantitation (LOQ), accuracy, and precision of the developed sELISA were determined according to the Eurachem Guide [16]. LOD and LOQ were calculated according to the average of 10 blank values plus three or ten times the standard deviations (SD), respectively. The accuracy of the assay was evaluated using bias (%), which was expressed as the difference (%) between BLG content measured using the developed sELISA and the theoretical content. The precision was evaluated by determining the relative SD of repeatability (RSDr, intra-day) and reproducibility (RSDR, inter-day) at a series of BLG concentrations. Repeatability and reproducibility were evaluated by testing BLG at different concentrations in 1 day ($n = 5$) or in 5 sequential days ($n = 3$), respectively. Results were calculated as:

$$\text{RSDr (or RSDR) (\%)} = \text{SD}/\text{mean} \times 100.$$

The specificity of the developed sELISA was determined by the cross-reactivity with α -CN, β -CN, κ -CN, ALA, BSA and egg, peanut, soybean, and wheat proteins at 0.0625, 0.25, 1, and 4 $\mu\text{g mL}^{-1}$.

Application study

Whey was prepared by centrifugation (10,000 $\times g$ for 10 min at 4 °C) and isoelectric precipitation (pH 4.6) to remove the fats and caseins from UHT-milk. UHT-milk, whey and yoghurt were centrifuged (10,000 $\times g$ for 10 min at 4 °C) to remove the fats, then fortified with BLG at concentrations of 0.5 and 2.0 mg mL^{-1} for the recovery experiments. Chocolate and cookie were ground into fine powder, and 1 g powder was

extracted with 20 mL Tris-HCl buffer (20 mmol L^{-1} , 2% Tween-20, pH 8.0). After stirred overnight at 4 °C, the samples were centrifuged at 10,000 $\times g$ for 10 min at 4 °C, and the supernatant was collected. BLG content in extracts was determined and fortified with 0.5 and 2.0 mg BLG per 1 g chocolate or cookie. Recovery (%) = (detected concentration/spiked concentration) $\times$ 100. Moreover, hydrolyzed infant formulas were dissolved in PBS and centrifuged at 10,000 $\times g$ for 15 min at 4 °C to remove fats before measuring the contents of BLG and its allergenic residues. The results were compared with conventional sELISA and validated with commercial sELISA kit.

Tricine-SDS-PAGE

Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE) was carried out to analyze the molecular weight (MW) distribution of hydrolyzed formulas [17]. The sample was mixed with equal volume of loading buffer (40% sugar, 0.05 mol L^{-1} Tris, 4% SDS and 0.08% bromophenol blue, pH 6.8), followed by heating at 99 °C for 5 min. After centrifugation at 5000 $\times g$ for 1 min at room temperature, 20 μL of each sample (50 μg of protein per well) was loaded before performing electrophoresis. Electrophoretic separation was performed at 30 V for stacking gel for 1 h and 100 V for separating gels for 2.0–2.5 h, respectively. After electrophoresis, the gels were fixed in 50% methanol containing 10% acetic acid for 20 min before stained with 0.05% Coomassie Brilliant Blue R-250 for 30 min, followed by thorough rinsing in destaining solution (5% methanol and 7.5% acetic acid). The gels were imaged by GS-800TM Densitometer (Bio-Rad, USA).

Statistical analysis

Results are expressed as means $\pm$ SD. Statistical significance was determined using SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA) and differences were performed by *t* test. Statistically significant was considered at $p < 0.05$.

Results

SEM analysis of pAb-BLG-AuNPs probe

The AuNPs probe was synthesized by labeling the AuNPs with pAb-BLG. The diameter of nanoparticles increased when labeled with the antibody [13]. The morphology of the AuNPs and pAb-BLG-labeled AuNPs was visualized using a scanning electron microscope (SEM, JSM 6701F, JEOL, Japan) at a voltage of 5 kV. As shown in Fig. 1D, the average diameter of AuNPs was approximately 10 nm. After linkage of pAb-BLG to AuNPs, the average diameter of the pAb-BLG-

AuNPs was approximately 60 nm (Fig. 1E), indicating that the pAb-BLG-AuNPs probe was successfully synthesized.

Optimization of the procedure of AuNPs probe-based sELISA

The effects of physical and chemical conditions on the performance of AuNPs probe-based sELISA were determined at an incubation temperature of 37 °C. Firstly, the conditions of antibody immobilization were optimized. As shown in Fig. S1A (see the Electronic Supplementary Material (ESM)), when the plate was treated with 1% (v/v) KOH for different times, the values of the positive treatment (“P,” with 1 $\mu\text{g mL}^{-1}$ BLG) and the negative treatment (“N,” without BLG) ratios (P/N) decreased significantly when the KOH incubation time was increased from 5 to 60 min. Therefore, the plate treated with 1% (w/v) KOH for 5 min was chosen in further experiments. Afterwards, the mAb was activated with 4 mg mL^{-1} EDC from 2 to 20 min, and the values of both the positive and the negative treatment decreased at increasing incubation time, where the maximum P/N value was obtained at an incubation time of 10 min, which was significantly higher than other incubation times (Fig. 2A). The EDC-activated mAb was mixed with equal volume APTES before adding to the plate for covalent immobilization of the mAb, and the concentration of APTES was increased from 0.25% (v/v) to 4% (v/v), where the P/N value was maximum at 4% APTES, which was significantly higher than the other concentrations of APTES (Fig. 2B). Then the mAb was incubated from 15 min to 2 h, and the P/N values tended to decrease at increasing immobilization time, leading to the selection of 15 min for the optimum immobilization of mAb (Fig. 2C) in further experiments. Consequently, less than 1 h appeared to be needed for preparing a mAb-bound plate, which is much shorter compared with regular ELISA protocols which requires overnight coating of the plate [9].

The second step was to optimize the conditions for the detection of BLG based on the mAb-bound plate. The plate was blocked with 2% gelatin, and the highest P/N ratio was obtained at blocking time of 1 h (ESM Fig. S1B). Figure 2D shows that the P/N value increased significantly (from 7.23 to 9.87) at a BLG incubation time from 0.5 to 1.5 h, whereas further incubation did not significantly influence the value of P/N. Thus, 90 min was selected as the optimum BLG incubation time. As shown in Fig. 2E, the values for both the positive and negative treatments increased with increasing the incubation time of the AuNPs probe, and the maximum P/N was achieved at an incubation time of 90 min, which was significantly higher than the other incubation times. As shown in Fig. 2F, the optimum incubation time of streptavidin-HRP was found at an incubation time of 60 min. The incubation time for TMB appeared optimum after 15 min (Fig. 2G).

AuNP probe-based sELISA for detection of BLG

Under the optimized experimental conditions as determined above, BLG was detected by the AuNPs probe-based sELISA. A calibration curve was obtained with BLG at a concentration range from 0.12 to $256 \times 10^3 \text{ ng mL}^{-1}$ (Fig. 3A, red curve). The linear range of the optimized sELISA for the detection of BLG was $31.25\text{--}64 \times 10^3 \text{ ng mL}^{-1}$ ($y = 5.20728x - 5.63325$, $r^2 = 0.9983$) (Fig. 3A, red curve). This linear detection range was 32 times wider than that observed in the conventional sELISA ($62.5\text{--}4 \times 10^3 \text{ ng mL}^{-1}$) (Fig. 3A, black curve). The calculated LOD and LOQ were 0.49 and 3.9 ng mL^{-1} , respectively. The LOD achieved was 16 times lower than that found in the conventional sELISA (7.8 ng mL^{-1}) (Fig. 3A, black curve). Though the LOD achieved was the same as the 1G9-based fluorescence sELISA, the performance time was shorter than the fluorescence sELISA [9].

As shown in Table 1, the analytical figures of merit of the developed assay were compared with those of several other detection methods reported for BLG quantitation. It was shown that the LOD and linear dynamic range of our AuNPs probe-based sELISA were better than the other ELISAs.

The accuracy and precision of the AuNPs probe-based sELISA were also assessed. The average bias value of 3.13% (ESM Table S1) indicated that the developed assay had high accuracy. The intra-assay and inter-assay precisions were (RSDr) 8.07% and (RSDR) 11.79% (ESM Table S1), respectively, suggesting sufficient precision of the AuNPs probe-based sELISA.

Cross-reactivity

As shown in Fig. 3B, there was no cross-reactivity of the AuNPs probe-based sELISA with ALA, BSA, egg proteins, peanut proteins, soy proteins, or wheat proteins. Weak cross-reactivity was observed with α -CN, β -CN, and κ -CN (Fig. 3B).

Spike and recovery

To validate the applicability of the AuNPs probe-based sELISA to test BLG in real samples with different matrixes, recovery experiments were performed by spiking BLG at 0.5 and 2 mg mL^{-1} in UHT-milk, whey, and yoghurt and spiking BLG at 0.5 and 2 mg g^{-1} in chocolate and cookie, respectively. The mean recoveries of BLG from spiked UHT-milk, whey, yoghurt, chocolate, and cookie were 106.69, 108.38, 104.02, 104.87, and 103.32% (Table 2), respectively. The results of the developed sELISA are in agreement with those of the conventional sELISA and a commercial sELISA kit.

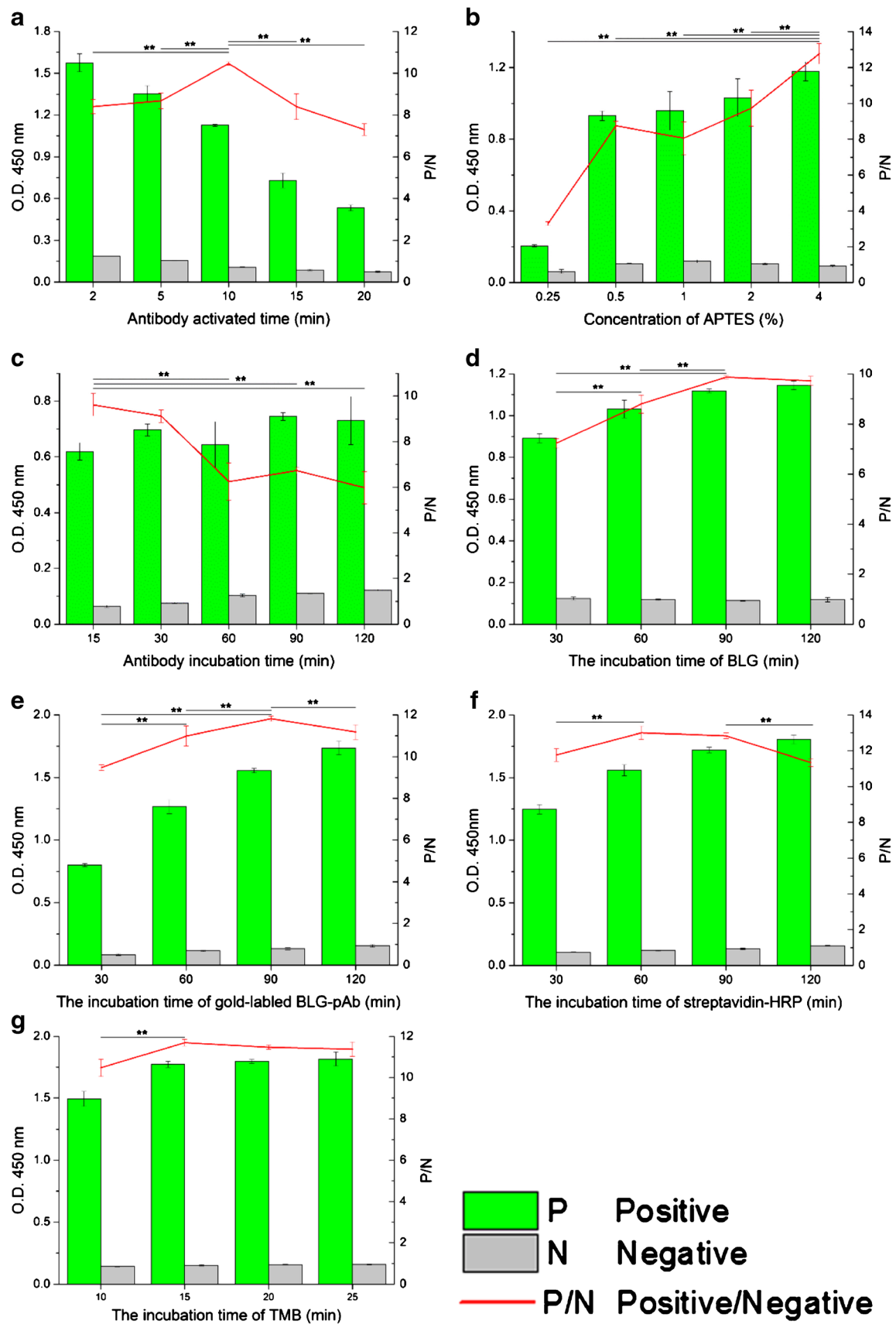


Fig. 2 Optimization of AuNPs probe-based sandwich ELISA parameters: **A** activation time of mAb, **B** concentration of APTES, **C** immobilization time of EDC-activated mAb, **D** incubation time of BLG, **E** incubation time of AuNPs probe, **F** incubation time of streptavidin-HRP, and **G** incubation time of TMB solution. Results are expressed as means $\pm$ SD ($n = 3$). Significant at $**p < 0.01$

Detection of BLG allergenic residues in hydrolyzed infant formulas

Hydrolysis may destroy epitopes of milk proteins. Therefore, hydrolyzed infant formula are good food sources to test for their cows' milk hypoallergenicity. However, due to incomplete hydrolysis, intact BLG and its allergenic peptide fragments may still be present in infant formulas and could induce allergic reactions in some milk-sensitive infants [27], suggesting that assessment of the safety or residual allergenicity of

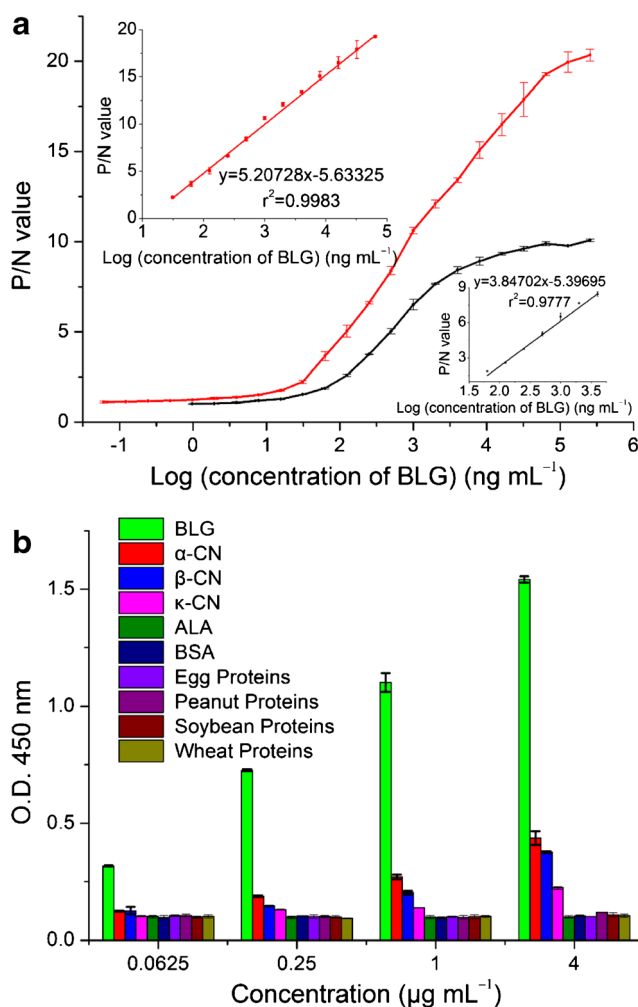


Fig. 3 Analytical performance of the developed AuNPs probe-based sELISA and conventional sELISA. **A** Calibration curve of AuNPs probe-based sELISA for detection of BLG (red curve) and calibration curve of conventional sELISA for detection of BLG (black curve). **B** Specificity of the developed AuNPs probe-based sELISA, tested with α -CN, β -CN, κ -CN, ALA, BSA, egg proteins, peanut proteins, soy proteins, and wheat proteins. Results are expressed as means $\pm$ SD ($n = 3$)

these hydrolyzed infant formulas is necessary. In this work, the contents of BLG and its allergenic residues in two extensively hydrolyzed formulas and four partially hydrolyzed formulas were measured. In the two extensively hydrolyzed formulas, BLG allergenic residues were not detected (Table 3). Among the four partially hydrolyzed formulas, the amount of BLG in “Tummy Care” was the highest with a concentration of $16,503.16 \mu\text{g g}^{-1}$ (Table 3), and the BLG content in “Precinutri (HA)” was the lowest ($50.04 \mu\text{g g}^{-1}$), approximately 300-fold lower than “Tummy Care” (Table 3). We observed concordant results between the developed sELISA and the conventional sELISA (Table 3). The BLG content in “NAN PRO” detected by the developed sELISA was similar to that of a commercial sELISA kit. However, the developed sELISA detected a BLG concentration in “NAN HA” that was higher than that detected by the sELISA kit, while the BLG concentrations in “Precinutri (HA)” and “Tummy Care” detected by the developed sELISA were much lower than that of commercial sELISA kit. This difference may be due to different antibodies used in these tests.

Tricine-SDS-PAGE is a common method for the analysis of the MW distribution of a hydrolysate [28]. The MW distribution of two extensively hydrolyzed formulas and four partially hydrolyzed formulas was analyzed by tricine-SDS-PAGE. As shown in Fig. 4, there were no distinct bands observed in the extensively hydrolyzed formulas (lanes 1 and 2), indicating that the concentrations of allergenic residues in the extensively hydrolyzed formulas were below the LOD of our sELISA (Table 3). Moreover, there were more high MW peptides/proteins found in “Tummy Care” when compared with the other three partially hydrolyzed formulas (Fig. 4), which could explain why the sELISA results showed that the BLG concentration in “Tummy Care” was the highest (Table 3). Furthermore, the image intensity of “Precinutri (HA),” “NAN PRO,” “NAN HA,” and “Tummy Care” increased successively (Fig. 4), in agreement with the results of the developed sELISA analysis (Table 3).

Discussion

Immobilized antibodies play an important role in ELISA for its sensitivity, specificity, and response time. APTES is widely used for immobilization of antibodies and enzymes on functionalized bioanalytical platforms [29]. We hereby developed a new sELISA where the plate was coated with covalently bound EDC-activated monoclonal antibody on the KOH-treated microtiter plate surface in 15 min, much shorter than the conventional ELISA (overnight). In our antibody immobilization protocol, the microtiter plate was pretreated with KOH to introduce hydroxyl groups [30], APTES can form a stable complex with the EDC-activated anti-BLG monoclonal

Table 1 Comparison of analytical methods reported for determination of BLG

Analytical methods	Recognized epitope	Linear range	LOD/sensitivity	Reference or manufacturer
AuNPs probe-based sELISA	IgE epitope	$31.25\text{--}64 \times 10^3 \text{ ng mL}^{-1}$	0.49 ng mL^{-1}	This study
sELISA	IgE epitope	$62.5\text{--}8 \times 10^3 \text{ ng mL}^{-1}$	7.9 ng mL^{-1}	This study
Fluorescence sELISA	IgE epitope	$125\text{--}4 \times 10^3 \text{ ng mL}^{-1}$	0.49 ng mL^{-1}	[9]
sELISA	IgE epitopes	$31.25\text{--}8 \times 10^3 \text{ ng mL}^{-1}$	1.96 ng mL^{-1}	[10]
sELISA	No	$5\text{--}100 \text{ ng mL}^{-1}$	Not shown	[18]
Microarray	No	$20\text{--}500 \text{ ng mL}^{-1}$	80 ng mL^{-1}	[19]
Immunochromatography	IgG epitopes	Not shown	0.2 ng mL^{-1}	[20]
Electrochemistry	No	$2.8\text{--}100 \text{ ng mL}^{-1}$	0.8 ng mL^{-1}	[21]
Electrochemistry	No	$1 \times 10^{-3}\text{--}100 \text{ ng mL}^{-1}$	$8.5 \times 10^{-4} \text{ ng mL}^{-1}$	[22]
SPR	No	$5\text{--}4 \times 10^3 \text{ ng mL}^{-1}$	5.54 ng mL^{-1}	[23]
HPLC	No	$3.6 \times 10^3\text{--}5.68 \times 10^4 \text{ ng}$	500 ng	[24]
LC-MS	Yes	$4.8 \times 10^2\text{--}3.125 \times 10^4 \text{ ng mL}^{-1}$	$2 \times 10^2 \text{ ng mL}^{-1}$	[25]
LC-MS	Yes	$1.11\text{--}1.111 \times 10^3 \text{ ng mL}^{-1}$	1.11 ng mL^{-1}	[26]
sELISA	No	$0.5\text{--}13.5 \text{ mg kg}^{-1}$	0.19 mg kg^{-1}	R-Biopharm
sELISA	No	$10\text{--}400 \text{ } \mu\text{g kg}^{-1}$	$1.5 \text{ } \mu\text{g kg}^{-1}$	Immunolab
ELISA	No	$10\text{--}400 \text{ mg kg}^{-1}$	1.5 mg kg^{-1}	Demeditec
sELISA	No	$0.1\text{--}1 \text{ mg kg}^{-1}$	Not shown	ELISA System

antibody, and the complex can be covalently bound with the hydroxyl groups on the surface of microtiter plate [12].

The combination of a monoclonal antibody covalently bound plate with a gold probe made the LOD and linear dynamic range of the developed sELISA 32 times wider and 16 times lower than the conventional sELISA. This is better than the LOD in other methods, including ELISA, microarray, electrochemistry, SPR, HPLC, and LC-MS [10, 18, 19, 21, 23–26]. Though the LOD achieved was the same as in our previously developed 1G9-based fluorescence sELISA [9], the performance time was shorter. In comparison with the electrochemical method reported by Eissa et al. [22] with its low LOD of 0.85 pg mL^{-1} , our method has the following three advantages: It (1) is sensitive to detect BLG in food samples [19], (2) is much simpler because it does not require multiple reagents and complex electrode modification protocols, and (3) is able to detect BLG IgE epitope peptide fragments.

There are only a few reported methods that can detect BLG IgE epitopes, but no commercial kits are available. Our previously reported sELISA and fluorescence sELISA could recognize IgE epitope of BLG, but were time-consuming [9, 10]. In addition, some LC-MS methods could detect epitope peptides of BLG [25, 26], but require expensive equipment, highly trained personnel and a long time for sample digestion, with a high LOD and a narrow linear dynamic range, and therefore may not be user-friendly methods for sensitive detection of BLG and its epitopes.

The developed sELISA showed weak cross-reactivity with α -CN, β -CN, and κ -CN, which is consistent with previous reports [9, 10, 31]. The reason might be the impurity of these

caseins: α -CN (purity $\geq 70\%$), β -CN (purity $\geq 98\%$), and κ -CN (purity $\geq 70\%$), with the presence of BLG in these impurities. As shown in our previous study [9], pAb-BLG was reactive with α -CN, β -CN, and κ -CN, and there were obvious bands corresponding to BLG, indicating that BLG was present in these caseins. Both cows' milk protein-sensitized mice and cows' milk-allergic patients were reported to suffer from cross-reactivity with soy proteins [32, 33], but our sELISA did not show any cross-reactivity between BLG and soy proteins (Fig. 3B). These results suggested that the developed immunoassay was highly specific to a single and specific BLG epitope but did not detect other BLG epitopes with possible homologies with allergenic soy proteins.

Regarding the hydrolyzed infant formulas tested, the results of our sELISA were similar to the conventional sELISA. However, when compared with the commercial sELISA kit, the results were only similar for the product "NAN PRO," but much higher for "NAN HA," and lower for "Precinutri (HA)" and "Tummy Care." This might be because the antibody used in the commercial kit is different from ours, and monoclonal antibody normally recognizes only one single epitope. In general, the BLG content in hydrolyzed infant formulas tested by monoclonal antibody may be lower than that tested by polyclonal antibodies. For example, Mariager et al. compared the BLG contents in hypoallergenic and ordinary infant formulas determined by monoclonal and polyclonal antibodies. The results showed that the BLG content was lower in a monoclonal antibody test than in a polyclonal antibodies test (e.g., the Cortecs kit), but the results appeared to be the reverse for BLG contents in ordinary, not

Table 2 Recoveries of BLG from spiked food samples tested by different methods

Sample	BLG Spiked (mg mL ⁻¹ , mg g ⁻¹) ^a	AuNPs probe-based sELISA (<i>n</i> = 3)			Conventional sELISA (<i>n</i> = 3)			Commercial sELISA kit (<i>n</i> = 2)		
		Detected concentration (mg mL ⁻¹ , mg g ⁻¹) ^a	Recovery (%)	CV (%)	Detected concentration (mg mL ⁻¹ , mg g ⁻¹) ^a	Recovery (%)	CV (%)	Detected concentration (mg mL ⁻¹ , mg g ⁻¹) ^a	Recovery (%)	CV (%)
UHT- milk	0	3.28 ± 0.18		5.49	3.24 ± 0.22		6.79	3.31 ± 0.13		3.93
	0.5	3.83 ± 0.18	109.96	4.70	3.76 ± 0.08	104.07	2.13	3.93 ± 0.06	124.04	1.53
Whey	2	5.35 ± 0.31	103.42	5.79	5.45 ± 0.17	110.44	3.12	5.62 ± 0.33	115.08	5.87
	0	0.73 ± 0.03		4.11	0.69 ± 0.02		2.90	0.70 ± 0.05		7.14
	0.5	1.29 ± 0.07	111.84	5.43	1.19 ± 0.04	99.87	3.36	1.26 ± 0.03	111.17	2.38
	2	2.83 ± 0.24	104.92	8.48	2.66 ± 0.16	98.65	6.02	2.90 ± 0.4	110.05	13.79
Yoghurt	0	2.43 ± 0.12		4.94	2.35 ± 0.13		5.53	2.52 ± 0.08		3.17
	0.5	2.94 ± 0.12	102.18	4.08	2.89 ± 0.08	109.23	2.77	2.97 ± 0.06	90.51	2.02
Chocolate	2	4.54 ± 0.41	105.85	9.03	4.54 ± 0.34	109.77	7.49	4.38 ± 0.14	92.79	3.20
	0	ND			ND			ND		
	0.5	0.51 ± 0.02	102.69	3.92	0.52 ± 0.03	104.15	5.77	0.60 ± 0.04	120.16	6.67
	2	2.14 ± 0.28	107.04	13.08	2.18 ± 0.24	108.76	11.01	2.12 ± 0.03	105.89	1.42
Cookie	0	5.88 ± 0.24		4.08	5.88 ± 0.21		3.57	5.98 ± 0.04		0.67
	0.5	6.37 ± 0.16	98.29	2.51	6.42 ± 0.17	108.71	2.65	6.47 ± 0.03	99.54	0.46
	2	8.05 ± 0.50	108.35	6.21	7.99 ± 0.45	105.80	5.63	8.43 ± 0.20	122.66	2.37

^aThe “mg mL⁻¹” was used for UHT-milk, whey, and yoghurt, and “mg g⁻¹” was used for chocolate and cookie

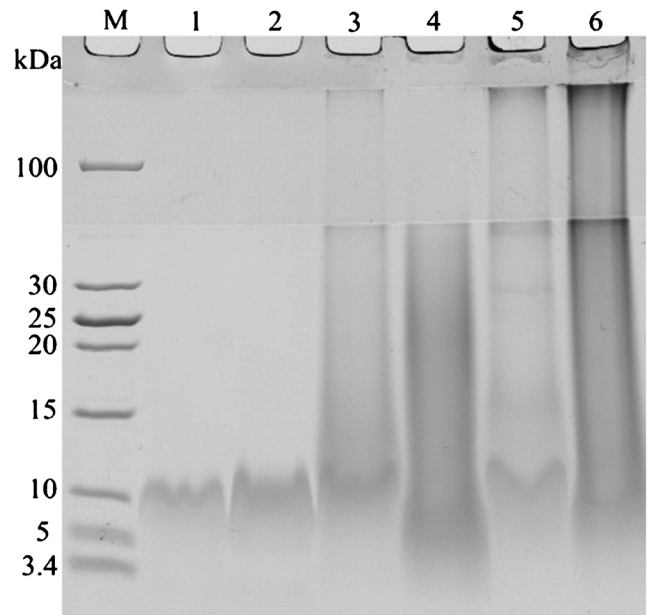
ND: Not detectable

Table 3 BLG contents in hydrolyzed infant formulas detected by different methods

Formula	BLG concentration			
	AuNPs probe-based sELISA (<i>n</i> = 3)		Conventional sELISA (<i>n</i> = 3)	
	Manufacturer	Dry weight ($\mu\text{g g}^{-1}$)	Ready-to-use formula (mg L^{-1})	Ready-to-use formula (mg L^{-1})
Product			Dry weight ($\mu\text{g g}^{-1}$)	Ready-to-use formula (mg L^{-1})
Alfaré	Nestlé	ND	ND	ND
Precinutri (Pepti)	Dumex	ND	ND	ND
NAN PRO	Nestlé	330.97 ± 22.26	48.54 ± 3.26	40.90 ± 3.04
NAN HA	Nestlé	553.94 ± 41.39	80.51 ± 6.02	77.35 ± 6.61
Precinutri (HA)	Dumex	50.04 ± 3.69	7.67 ± 0.57	9.09 ± 0.28
Tummy Care	Abbott	$16,503.16 \pm 1211.34$	2193.27 ± 160.99	$20,773.77 \pm 3047.02$
				2760.83 ± 404.95
				$40,536.93 \pm 2518.86$
				5387.36 ± 0.33

BLG allergenic residues were not detected when protein concentration of hydrolyzed infant formulas was $\leq 1.25 \text{ mg mL}^{-1}$

ND not detectable

**Fig. 4** Tricine-SDS-PAGE analysis of two extensively hydrolyzed infant formulas (lanes 1 and 2) and four partially hydrolyzed infant formulas (lanes 3 to 6). lane M: markers; lane 1: Alfaré; lane 2: Precinutri (Pepti); lane 3: NAN PRO; lane 4: NAN HA; lane 5: Precinutri (HA); lane 6: Tummy Care

hydrolyzed infant formulas [34]. This may show a limitation of our monoclonal antibody test. Nevertheless, our new method is suitable for the detection of allergenic residues in hydrolyzed infant formulas.

Conclusions

In this study, a highly sensitive and time-saving sELISA based on the mAb 1G9 with AuNPs probe covalently bound onto a microtiter plate was developed for the detection of BLG and its allergenic residues (IgE epitope) in foods, including hydrolyzed infant formulations. The mAb binding time was 15 min. The LOD was 0.49 ng mL^{-1} , which was 16 times lower than the conventional sELISA (7.9 ng mL^{-1}). The linear range for BLG detection was $31.25\text{--}64 \times 10^3 \text{ ng mL}^{-1}$, which was 32 times wider than conventional sELISA ($62.5\text{--}4 \times 10^3 \text{ ng mL}^{-1}$). The average recovery from five BLG spiked foods was 105.45%. The contents of BLG and its allergenic epitope in six hydrolyzed infant formulas were quantified. The new immunoassay had a good correlation with a conventional sELISA and a commercial sELISA kit. These results suggest that our novel AuNPs probe-based sELISA is a reliable, rapid, and user-friendly method for the detection of BLG and its IgE epitope in food samples.

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Compliance with ethical standards

Conflict of interest All academic authors declare that they have no conflict of interest. Mr. Shandong Wu declares that he is the director of the Hangzhou Zheda Dicum Biological Gene Engineering Co., Ltd., but this company was not financially involved in the present study.

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

A novel sandwich enzyme-linked immunosorbent assay with covalently bound monoclonal antibody and gold probe for sensitive and rapid detection of bovine β -lactoglobulin

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Table S1 Accuracy and precision of the sandwich AuNPs probe-based ELISA for detection of BLG

BLG (ng mL ⁻¹)	Intra-assay ^a				Inter-assay ^b		
	Mean ± SD	Bias	RSDr ^c	n	Mean ± SD	RSDR ^d	n
	(ng mL ⁻¹)	(%)	(%)		(ng mL ⁻¹)	(%)	
31.25	36.07±2.63	15.44	7.29	5	34.47±2.60	7.54	5
62.5	54.86±2.48	-12.22	4.51	5	65.85±7.09	10.77	5
125	120.58±5.83	-3.54	4.84	5	128.98±15.89	12.32	5
250	251.07±9.98	0.43	3.98	5	273.87±29.43	10.75	5
500	487.23±40.43	-2.55	8.29	5	499.60±76.57	15.33	5
1000	997.11±171.36	-0.29	17.19	5	1052.34±146.81	13.95	5
2000	2345.81±231.44	17.29	9.87	5	1934.22±287.18	14.85	5
4000	4419.21±379.09	10.48	8.58	5	4185.79±367.91	8.79	5
	Mean	3.13	8.07		Mean	11.79	

^a Assay was performed within one days (n = 5).

^b Assay was performed every day for 5 sequential days.

^c Relative standard deviation of repeatability for intra-assay precision.

^d Relative standard deviation of reproducibility for inter-assay precision.

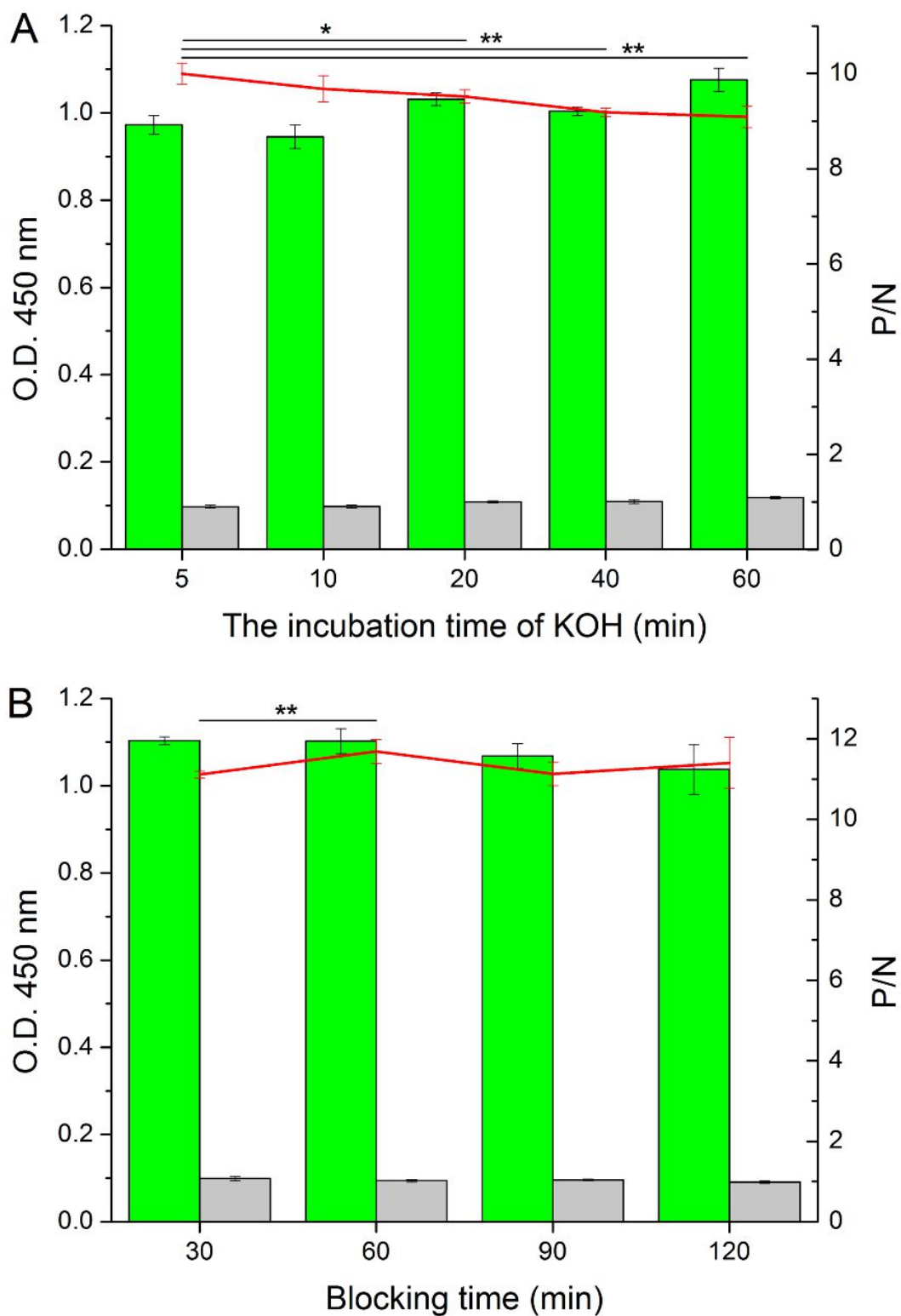


Fig. S1 Optimization of (A) incubation time of KOH and (B) blocking time of AuNPs probe-based sandwich ELISA. Results are expressed as mean $\pm$ SD (n = 3). Significant at $*p < 0.05$, and $**p < 0.01$