

Highly Sensitive Detection of Bovine β -Lactoglobulin with Wide Linear Dynamic Range Based on Platinum Nanoparticles Probe

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Supporting Information

ABSTRACT: Cow's milk allergy is one of the most frequent and severe IgE-induced food allergies for children, demanding sensitive analytical methods, and β -lactoglobulin (BLG) can be as an important biomarker for detection of milk protein. In this study, a highly sensitive sandwich enzyme-linked immunosorbent assay (sELISA) based on a specific polyclonal antibody against human IgE linear epitopes of BLG and an anti-BLG polyclonal antibody-platinum nanoparticles probe was described for detection of BLG. This sELISA exhibited an ultrawide linear range of $0.49\text{--}1.6 \times 10^4$ ng/mL, covering more than four orders of magnitude. The limit of detection was 0.12 ng/mL, which was 16-fold lower than that using traditional sELISA with the same antibodies. Furthermore, the proposed approach showed high recoveries (93.53%–111.95%) and low coefficient of variation (1.49%–12.50%) after analysis of samples fortified with BLG. The presence of allergenic BLG residues also could be detected in partially hydrolyzed infant formulas. These results, in comparison with conventional and commercial BLG detection sELISAs, highlight that this proposed sELISA could be a reliable and user-friendly tool to monitor trace amounts of BLG and its potentially allergenic residues in foods.

KEYWORDS: milk allergens, beta-lactoglobulin, platinum nanoparticle, sandwich ELISA, allergenic residues

INTRODUCTION

Food allergies have become a serious and prevalent food safety and public healthcare concern, affecting 5% of adults and 8% of children.¹ Milk is one of eight major allergenic foods, and cows' milk allergy affects 6%–7% of children and 1%–2% of adults.² Even very low amounts of milk can induce allergic reactions in milk-sensitive individuals.³ Since there is currently no effective treatment for milk allergic consumers, the only safe way is to avoid consumption of milk or milk-containing products. This requires reliable method to detect milk allergens in food products for milk labeling, aiding in the prevention of milk-sensitive patients exposure to milk allergens. Approximately 82% of cows' milk allergic patients are sensitive to bovine β -lactoglobulin (BLG),⁴ the major protein in milk.⁵ Thus, detection of BLG can be a useful marker of the presence of milk in food.

To date, a number of analytical methods have been developed to detect food allergens based on allergen-specific DNA analysis (real-time polymerase chain reaction),⁶ proteomics (such as liquid chromatography coupled mass spectrometry, LC–MS),⁷ and immunoassay (such as enzyme-linked immunosorbent assay, ELISA) technologies.^{8,9} Among them, ELISA is the most widely applied. Moreover, antibody is an important element in ELISA for testing of food allergens (such as BLG) because epitope play a key role in allergic reaction. However, conformational epitopes are unstable and

could be destroyed easily by processing,¹⁰ thus Cucu et al.¹¹ pointed out that using antibody raised against a single peptide is a new strategy in the quantification of food allergens. For example, epitope/immunotoxic peptide specific antibodies were widely used for detection of gluten,^{12,13} and IgE epitope-specific monoclonal antibody based sandwich ELISA (sELISA) was developed to detect invertebrate major allergen tropomyosin.¹⁴ Among the immunoassays, those targeting allergen epitope(s) have a dual functionality: (1) to detect the presence of the given allergen in general in foods and (2) to estimate the allergenic potential of the given food product. However, to our knowledge, an epitope-specific antibody-based immunoassay for detection of BLG is lacking. The commercially ELISA kits for BLG detection are unable to recognize its specific IgE epitopes. That yields the need to develop a reliable and sensitive method to monitor BLG and its allergenic residues in food products.

Platinum nanoparticles (PtNPs) not only possess the common properties of nanomaterials (such as high surface-to-volume ratio and high chemical stability), but also have peroxidase activity to catalyze the color reaction of peroxidase

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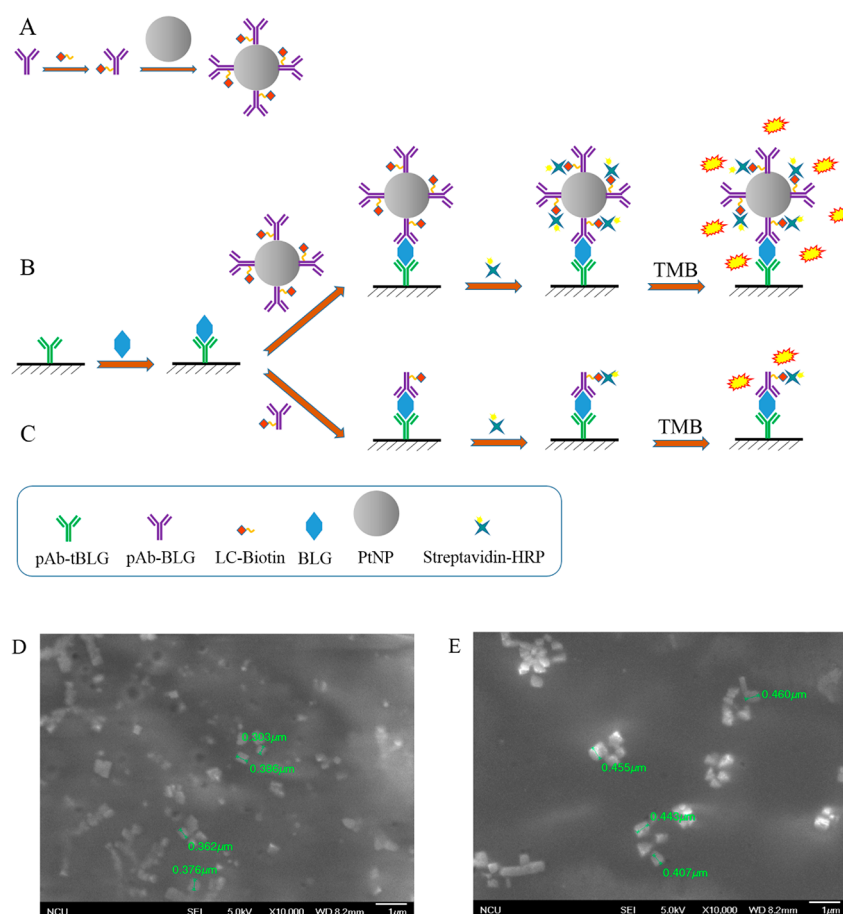


Figure 1. (A) Synthesis of pAb-BLG-PtNPs probe, (B) schematic principle of pAb-BLG-PtNPs probe based sELISA, and (C) conventional sELISA for detection of BLG. SEM images of the resuspended (D) bare PtNPs after centrifugation at $14\,087 \times g$ for 15 min and the (E) synthesized PtNPs probe.

substrate.¹⁵ At present, the PtNPs are mainly applied in microfluidics, electrochemical and colorimetric analysis to detect the targets based on their peroxidase activity.^{16–18} For example, Wang et al.¹⁹ developed a colorimetric immunoassay for detection of human chorionic gonadotropin based on the peroxidase-like activity of antibody-PtNPs probe; however, because of the higher nonspecific background signal, the limit of detection (LOD = 10 ng/mL) was about 100-fold higher than that of antibody-horseradish peroxidase (HRP) based ELISA (LOD = 0.1 ng/mL). This report indicated that the only use of the peroxidase activity of the PtNPs was not necessary to improve the sensitive. Because nanoparticles (such as AuNP and PtNP) can load several antibodies/HRP, this can improve the sensitivity.²⁰ Accordingly, we hypothesize that the method combining the high surface-to-volume ratio to carry several antibodies and peroxidase activity of the PtNPs could improve the detectability.

In this work, the antiepitope tandem of BLG polyclonal antibody (pAb-tBLG) was used as capture antibody, and the biotinylated anti-BLG polyclonal antibody (pAb-BLG) was immobilized on the surface of PtNPs as detection probe to improve detectability (Figure 1). A highly sensitive and sELISA was established for determination of BLG and its allergenic residues in food samples with ultrawide linear range. Commercial sELISA kits confirmed the reliability of the proposed immunoassay.

MATERIALS AND METHODS

Chemicals and Apparatus. CNBr-activated Sepharose 4B resin and Protein G affinity column were purchased from GE Healthcare (Uppsala, Sweden). Gelatin from cold water fish, BLG ($\geq 90\%$, PAGE), α -casein (α -CN, purity $\geq 70\%$, electrophoresis), β -CN (purity $\geq 98\%$, PAGE), and κ -CN (purity $\geq 70\%$, PAGE) were purchased from Sigma (St. Louis, USA). Bovine serum albumin (BSA) was purchased from Sangon Biotech (Shanghai, China). The 3,3',5,5'-tetramethylbenzidine (TMB) and Sulfo-NHS-LC-Biotinylation kit were obtained from Thermo Scientific Pierce (Rockford, USA). PtNPs (1 mg/mL) were obtained from Daoking (Beijing, China). HRP-streptavidin was acquired from Neobioscience (Shenzhen, China). RADASCREEN FAST β -lactoglobulin ELISA kit was purchased from R-Biopharm (Darmstadt, Germany). UHT-milk, yoghurt, chocolate, cookie, candy, and hydrolyzed infant formulas were purchased from local supermarkets. Whey was prepared by centrifugation and isoelectric precipitation to remove fat and CNs from UHT-milk.²¹ The egg proteins, peanut proteins, soybean proteins, and wheat proteins were prepared according to our previous study.²² All reagents were analytical grade, and solutions were prepared using Milli-Q water throughout the experiments.

The pAb-BLG and pAb-tBLG were produced and purified using a Sepharose CNBr immunoaffinity column coupled with BLG as described in our previous study,^{22,23} then purified using a Protein G affinity column.

The morphology of the PtNPs and pAb-BLG modified PtNPs was achieved using a field scanning electron microscope (SEM, JSM 6701F, JEOL, Japan) at a voltage of 5 kV. The optical density (OD) at 450 nm was read with a Model 680 microplate reader (Bio-Rad, USA).

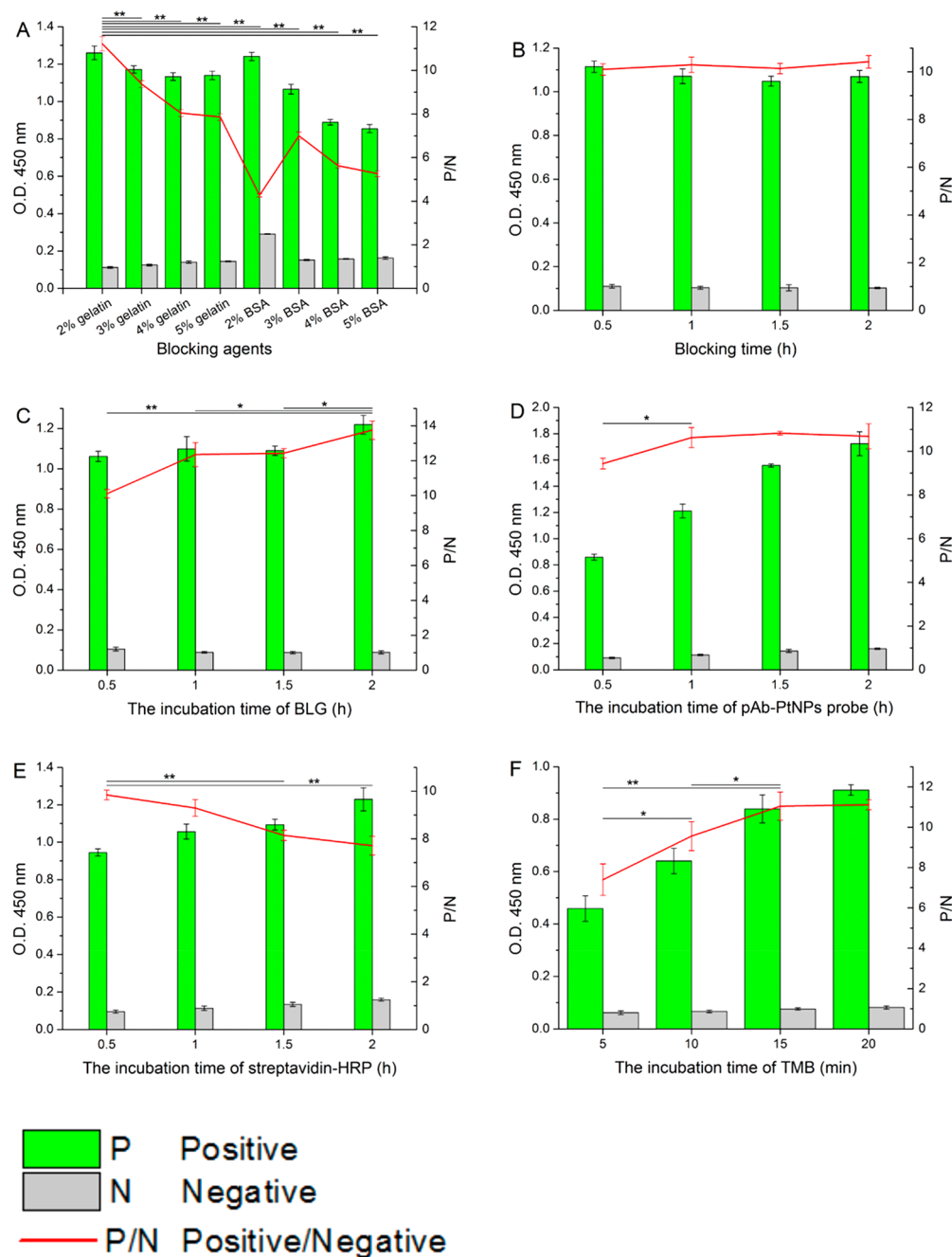


Figure 2. Optimization of PtNPs probe-based sandwich ELISA parameters: (A) blocking agents, (B) blocking time, (C) incubation time of BLG, (D) incubation time of pAb-BLG-PtNPs probe, (E) incubation time of streptavidin-HRP, (F) incubation time of TMB solution. Results are represented as mean $\pm$ SD ($n = 3$). Significant at $*p < 0.05$ and $**p < 0.01$.

Preparation of pAb-BLG-PtNPs Probe. The pAb-BLG-PtNPs probe was synthesized using biotinylated pAb-BLG label PtNPs (Figure 1A). First, the pAb-BLG was biotinylated with LC-biotin by using EZ-Link Sulfo-NHS-LC-biotinylation Kit according to the manufacturer's instructions. Briefly, 1 mL of 2 mg/mL purified pAb-BLG was biotinylated with 27 μ L of 10 mM Sulfo-NHS-LC-Biotin, vortexed, and allowed to stand for 1 h at 4 $^{\circ}$ C. The excess biotin was removed by a desalting column. Then the pAb-BLG-PtNPs probe was prepared using a previously described method with slight modification (Lin et al.²⁴). First, the PtNPs (5 mL) were centrifuged at $14\,087 \times g$ for 15 min at 4 $^{\circ}$ C, and the precipitate was resuspended in 4950 μ L of phosphate buffer saline (PBS, 10 mM, pH 6.5). Then 50 μ L of 1 mg/mL biotinylated pAb-BLG was added to PtNPs, vortexed, and allowed to stand overnight at 4 $^{\circ}$ C. The biotinylated pAb-BLG was immobilized on the surface of PtNPs via ionic interactions.²⁴

Subsequently, 500 μ L of 10% BSA was added to block the surface of PtNPs, and the unconjugated antibody was removed by centrifugation at $14\,087 \times g$ for 15 min at 4 $^{\circ}$ C. Finally, the pAb-BLG-PtNPs probe was resuspended in 5 mL of PBS solution (10 mM, pH 7.2, containing 0.1% Triton X-100, 5% sucrose, and 1% BSA).

Procedures of pAb-BLG-PtNPs Probe-Based sELISA. The 96-well microliter plates were coated with purified pAb-tBLG (3.5 μ g/mL in 50 mM carbonate buffer solution) and incubation overnight at 4 $^{\circ}$ C. The wells were washed three times with PBS containing 0.1% Tween-20 (PBST), followed by blocking with 250 μ L of 2% gelatin in PBST and incubation at 37 $^{\circ}$ C for 0.5 h. After being washed three times with PBST, 100 μ L of BLG (or food samples, PBST containing 2% of gelatin as control) was added to each well, incubated at 37 $^{\circ}$ C for 2 h, and washed again. The pAb-BLG-PtNPs probe was diluted 200 times before being added into each well as detection antibody,

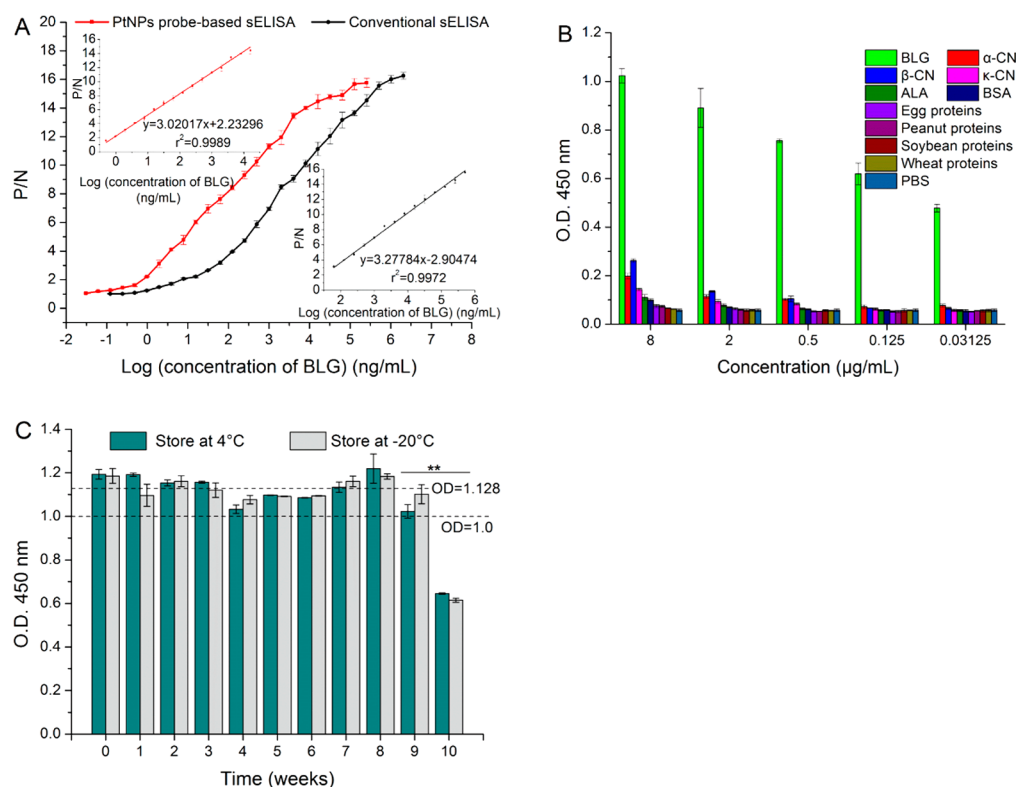


Figure 3. Analytical performance of the developed sELISA and conventional sELISA. (A) Calibration curve of PtNPs probe based sELISA (red curve) and conventional sELISA (black curve) for detection of BLG. (B) Cross-reactivity of the developed sELISA with ALA, BSA, α -CN, β -CN, κ -CN, egg proteins, peanut proteins, soy proteins, and wheat proteins. (D) Storage stability of the PtNPs probe stored at 4 °C or −20 °C with 50% glycerol. Data are represented as mean $\pm$ SD ($n = 3$). Significant at $**p < 0.01$.

followed by incubation at 37 °C for 1 h. After further washing, the streptavidin-HRP (diluted 1:60) was added to each well and incubated at 37 °C for 0.5 h. After further washing, 100 μ L of TMB solution was added to each well, and color was developed at 37 °C in the dark for 15 min. The color development reaction was stopped using 50 μ L of 2 M sulfuric acid, and the optical density at 450 nm was measured by microplate reader (Bio-Rad, USA). BLG, samples, pAb-BLG-PtNPs probe, and streptavidin-HRP were diluted with blocking buffer (2% gelatin in PBST).

Analysis of Food Samples. To investigate the applicability of the developed pAb-BLG-PtNPs probe-based sELISA, different samples were fortified with BLG for the recovery experiment, and BLG allergenic residues in hydrolyzed infant formula were tested. The results were compared with conventional sELISA and validated with commercial sELISA kit.

Fat was removed from UHT-milk, whey, and yoghurt by centrifugation at 10 000 $\times g$ for 15 min at 4 °C, then fortified with 0.5 or 2.0 mg/mL of BLG. Chocolate, cookie and candy were grounded into fine powder, and 1 g of powder was dissolved in 20 mL 20 mM Tris-HCl (pH 8.0, containing 2% Tween-20). The mixture was agitated overnight at 4 °C, followed by centrifugation at 10 000 $\times g$ for 15 min at 4 °C, the supernatant was collected. The sample was fortified with 0.5 or 2.0 mg/g of BLG. Recovery (%) = (detected concentration/spiked concentration) $\times$ 100.

The hydrolyzed infant formula was dissolved in PBS, then centrifuged at 10 000 $\times g$ for 15 min at 4 °C to remove fat before the contents of BLG and its allergenic residues were tested.

Tricine-SDS-PAGE. The molecular weight distribution of hydrolyzed infant formulas was analyzed by Tricine-SDS-PAGE.²⁵ The thickness of the gel was 1 mm, and the gel consisted of three portions: 4% acrylamide/bis(acrylamide) for stacking gel, 16.5% acrylamide/bis(acrylamide) for separating gel, and 10% acrylamide/bis(acrylamide) for spacer gel between stacking and separating gels. The hydrolyzed infant formula was dissolved in PBS with a protein

concentration of 5 mg/mL, and the sample was diluted 1:1 in loading buffer (40% sugar, 0.05 M 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 2 min at room temperature, 20 μ L of each sample (50 μ g of protein per well) was loaded before running the electrophoresis using a vertical mini gel electrophoresis (Bio-Rad, USA). Electrophoretic separations were performed at a constant voltage of 30 V until the tracking dye passed the stacking gel, and then 100 V was applied until the tracking dye reached the bottom of the gel. The gels were fixed in 50% methanol containing 10% acetic acid for 20 min and stained with 0.05% Coomassie Brilliant Blue R-250 for 30 min before being thoroughly rinsed 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), and differences were performed by t test. Statistically significant was considered at $*p < 0.05$, and $**p < 0.01$.

RESULTS AND DISCUSSION

SEM Analysis. The PtNPs probe was synthesized by coating the surface of PtNPs with biotinylated pAb-BLG. After antibody coupling, the diameter of nanoparticles will increase.²⁰ The morphology of PtNPs before and after modification with biotinylated pAb-BLG was confirmed by SEM. The average diameter of the bare PtNPs was approximately 360 nm (Figure 1D). After conjugation with biotinylated pAb-BLG, the average diameter of the composite nanoparticles was increased to approximately 440 nm (Figure 1E). The SEM test indicated that the pAb-BLG-coated PtNPs probe was successfully synthesized. In addition, 50 μ g of biotinylated pAb-BLG was coupled to 5 mL of PtNPs; thus,

Table 1. Summary of Different Analytical Methods for Detecting BLG

analytical methods	recognize epitope	linear range	LOD	reference/manufacture
PtNPs probe-based sELISA	IgE epitopes	$0.49\text{--}1.6 \times 10^4$ ng/mL	0.12 ng/mL	this paper
sELISA	IgE epitopes	$62.5\text{--}512 \times 10^3$ ng/mL	1.96 ng/mL	this paper
sELISA	IgE epitopes	$31.25\text{--}8 \times 10^3$ ng/mL	1.96 ng/mL	22
fluorescent sELISA	IgE epitope	$125\text{--}4 \times 10^3$ ng/mL	0.49 ng/mL	30
AuNPs probe-based sELISA	IgE epitope	$31.25\text{--}64 \times 10^3$ ng/mL	0.49 ng/mL	31
sELISA	no	5–100 ng/mL	not shown	32
immunochromatography	IgG epitopes	not shown	0.2 ng/mL	33
electrochemistry	no	$1 \times 10^{-3}\text{--}100$ ng/mL	0.85 pg/mL	26
electrochemistry	no	2.8–100 ng/mL	0.8 ng/mL	28
capillary electrophoresis	no	$5 \times 10^{-10}\text{--}10^{-7}$ M	5×10^{-10} M	34
microcantilever array	no	not shown	0.04 ppm	35
SPR	no	$5\text{--}4 \times 10^3$ ng/mL	5.54 ng/mL	36
HPLC	no	3.6–56.8 μ g	0.5 μ g	37
UHPLC	no	20–560 μ g/mL	7 μ g/mL	38
LC–MS	yes	0.48–31.25 μ g/mL	0.2 μ g/mL	29
sELISA	not shown	0.5–13.5 mg/kg	0.19 mg/kg	R-Biopharm
competitive ELISA	not shown	10–810 ng/mL	0.12 mg/kg	R-Biopharm
sELISA	not shown	10–400 ppb	1.5 ppb	Immunolab
ELISA	not shown	10–400 mg/kg	1.5 mg/kg	Demeditec
sELISA	not shown	0.1–1 mg/kg	Not shown	ELISA System

the concentration of biotinylated pAb-BLG immobilized onto PtNPs was about 10 μ g/mL.

Optimization of PtNPs Probe-Based sELISA Procedure. In the PtNPs probe-based sELISA, the pAb-tBLG, BLG, and pAb-BLG-PtNPs probe were used as the capture antibody, antigen, and detection probe, respectively. The concentrations of capture antibody and detection probe, the effects of physical and chemical conditions on the performance of PtNPs probe-based sELISA were determined at an incubation temperature of 37 °C.

First, the concentrations of pAb-tBLG (capture antibody) and pAb-BLG-PtNPs probe (detection probe) were optimized via checkerboard titration with serial dilutions of the two antibodies, and the minimum concentration to produce an OD_{450 nm} value around 1.0 and maximum value of positive value (P, OD of sample with BLG) to negative value (N, OD of sample without BLG) ratio (P/N) was selected. The results showed that the optimal parameters for capture antibody and detection probe were 3.5 μ g/mL and 200-fold diluted (Table S1), respectively.

Moreover, blocking plays an important role in reducing or eliminating nonspecific binding. Therefore, the blocking effects of different concentrations of gelatin and BSA in PBST were evaluated. As shown in Figure 2A, the blocking effect of gelatin was better than that of BSA, and the highest P/N value was achieved with 2% gelatin, which was significantly higher than other blocking agents. Next, the influence of blocking time from 0.5 to 2 h was tested. Different blocking time did not significantly influence the P/N value (Figure 2B). Thus, 30 min was selected as blocking time in further experiments.

In addition, the incubation times of BLG, pAb-BLG-PtNPs probe, HRP-streptavidin, and TMB were investigated. As shown in Figure 2C, the P/N value increased significantly with prolonged incubation of BLG. Therefore, 2 h was selected as the incubation time of BLG. Figure 2D showed the P/N value increased significantly at pAb-BLG-PtNPs probe incubation time from 0.5 to 1 h, whereas further incubation did not significantly influence P/N value. Thus, 1 h was selected as the best incubation time of pAb-BLG-PtNPs probe. The P/N

value declined significantly with increasing streptavidin-HRP incubation time (Figure 2E). Accordingly, 0.5 h was chosen as the optimal incubation time of streptavidin-HRP. As shown in Figure 2F, the P/N value increased significantly at TMB incubation time from 5 to 15 min, but further incubation did not significantly influence P/N value. Therefore, 15 min was chosen as optimal for the TMB.

Analytical Performance of PtNPs Probe-Based sELISA. The performance of the proposed pAb-BLG-PtNPs probe based sELISA for detection of BLG was tested under the optimized experimental conditions. The calibration curve of the developed sELISA was produced with BLG concentration range of $0.03\text{--}256 \times 10^3$ ng/mL (Figure 3A). The linear dynamic detection range was from 0.49 ng/mL up to 1.6×10^4 ng/mL with a good regression coefficient of $r^2 = 0.9989$ (Figure 3A, red curve), spanning four orders of magnitude. The limit of detection (LOD) and the limit of quantitation (LOQ) were 0.12 ng/mL and 0.49 ng/mL, respectively, which were obtained by the mean of 10 blank values plus 3- or 10-times the standard deviations, respectively. Compared with the conventional sELISA (Figure 3A, black curve), the linear detection range and LOD were improved by 4-fold and 16-times, respectively. In comparison with our previously reported sELISA based on pAb-BLG (capture antibody) and biotinylated pAb-tBLG (detection antibody),²² the linear detection range and LOD achieved were 128-times wider and 16-times lower, respectively. These results suggested that the PtNPs probe could improve the LOD and linear dynamic range, and the antibody pair could significantly affect the linear range.

As shown in Table 1, the linear dynamic range of our developed sELISA was wider than most of the other developed methods and commercial ELISA kits. The ultrawide linear dynamic range ($0.49\text{--}1.6 \times 10^4$ ng/mL) indicated that the developed sELISA was suitable for determination of BLG in food at different concentration levels. The LOD and LOQ achieved were lower than the other ELISAs (Table S2). Although the LOD obtained was not as low as the electrochemistry (0.85 pg/mL) reported by Eissa et al.,²⁶ and also higher than the sELISA (30 pg/mL) reported by

Table 2. Recoveries of BLG from Five Spiked Food Samples Tested by Different Methods^a

BLG spiked (mg/mL or mg/g)	PtNPs probe based sELISA (<i>n</i> = 3)			conventional sELISA (<i>n</i> = 3)			commercial sELISA kit (<i>n</i> = 2)		
	detected concentration (mg/mL or mg/g)	recovery (%)	CV (%)	detected concentration (mg/mL or mg/g)	recovery (%)	CV (%)	detected concentration (mg/mL or mg/g)	recovery (%)	CV (%)
UHT-Milk									
0	3.50 ± 0.37		10.57	3.40 ± 0.16		4.71	3.34 ± 0.17		5.09
0.5	4.04 ± 0.06	107.67	1.49	3.92 ± 0.04	104.05	1.02	3.86 ± 0.04*	104.03	1.04
2	5.52 ± 0.28	101.03	5.07	5.49 ± 0.24	104.55	4.37	5.50 ± 0.17	108.13	3.09
Whey									
0	0.64 ± 0.04		6.25	0.64 ± 0.06		9.38	0.75 ± 0.02*		2.67
0.5	1.15 ± 0.06	101.30	5.22	1.17 ± 0.06	105.67	5.13	1.34 ± 0.09	117.53	6.72
2	2.52 ± 0.22	93.53	8.73	2.63 ± 0.32	99.66	12.17	3.13 ± 0.07*	118.84	2.24
Yoghurt									
0	2.47 ± 0.08		3.24	2.46 ± 0.10		4.07	2.49 ± 0.09		3.61
0.5	2.99 ± 0.10	105.17	3.34	3.00 ± 0.12	108.40	4.00	3.02 ± 0.13	106.24	4.30
2	4.54 ± 0.34	103.76	7.49	4.43 ± 0.26	98.62	5.87	4.48 ± 0.27	99.26	6.03
Chocolate									
0	2.39 ± 0.13		5.44	2.36 ± 0.13		5.51	2.52 ± 0.10		3.97
0.5	2.95 ± 0.06	111.95	2.03	2.88 ± 0.10	103.55	3.47	3.02 ± 0.04	100.41	1.32
2	4.38 ± 0.24	99.33	5.48	4.46 ± 0.34	104.79	7.62	4.53 ± 0.11	100.46	2.43
Cookie									
0	6.22 ± 0.15		2.41	6.08 ± 0.35		5.76	6.10 ± 0.21		3.44
0.5	6.75 ± 0.07	106.77	1.04	6.76 ± 0.09	134.73	1.33	6.52 ± 0.04*	84.54	0.61
2	8.29 ± 0.25	103.80	3.02	8.20 ± 0.33	106.02	4.02	8.12 ± 0.03	101.03	0.37
Candy									
0	ND			ND			ND		
0.5	0.49 ± 0.05	97.79	10.20	0.51 ± 0.09	101.22	17.65	0.54 ± 0.10	108.84	18.52
2	2.00 ± 0.25	100.01	12.50	2.04 ± 0.26	101.83	12.75	2.01 ± 0.05	100.71	2.49

^aThe “mg/mL” was used for UHT-milk, whey, and yoghurt, and “mg/g” was used for chocolate, cookie, and candy. Compared to PtNPs probe based sELISA, the BLG concentration in food samples detected by conventional sELISA and commercial sELISA kit significant at **p* < 0.05. ND: Not detected.

Negrone et al.,²⁷ it should be remarked that the sensitive achieved is sufficient for detection of BLG in food samples.²⁸ Moreover, the sELISA presented was simpler than the immunosensor reported by Eissa et al.,²⁶ which needed multiple reagents and complex electrode modification protocols. Our proposed sELISA is time-saving when compared with the sELISA reported by Negrone et al.,²⁷ which required about 40 h. Furthermore, the low LOD (0.12 ng/mL) and LOQ (0.49 ng/mL) make it possible to eliminate the matrix effects of real samples with an appropriate dilution without compromising the sensitivity of the PtNPs probe-based immunoassay. Because of the pAb-tBLG could recognize seven special linear IgE epitopes of BLG,²³ making the developed sELISA could specifically detect BLG epitope peptide fragments. Though LC–MS could detect BLG epitope peptide, compared with the developed sELISA, LC–MS not only lacks sensitive and with narrower linear dynamic range, but also required expertise, expensive equipment, and need long time for sample digestion.²⁹

The mean bias value of interassay was 2.48% (Table S2), indicating good accuracy of the developed assay. The relative standard deviation of repeatability (RSD_r, interassay, *n* = 5) was in the range of 5.52–12.85% (mean = 8.68%), and relative standard deviation of reproducibility (RSD_r, intra-assay, *n* = 5) was from 7.54% to 15.33% (mean = 11.79%) (Table S2), suggesting good precision.

Selectivity of PtNPs Probe-Based sELISA. The selectivity of the developed sELISA was evaluated by testing the cross-reactivity with various nontarget food allergens at the concentration of 8, 2, 0.5, 0.125, and 0.03125 μg/mL. As

shown in Figure 3B, no cross-reactivity was observed with ALA, BSA, egg proteins, peanut proteins, soy proteins, and wheat proteins. However, the developed sELISA had cross-reactivity with α-CN, β-CN, and κ-CN (Figure 3B), which is consistent with previous reports.^{22,27,28} The reason might be due to the fact that α-CN (purity ≥70%), β-CN (purity ≥98%), and κ-CN (purity ≥70%) were impurity, and BLG was present in these caseins. As shown in our previous study,³⁰ Western blotting showed that the pAb-BLG was reactive with α-CN, β-CN, and κ-CN, and there were obvious bands that corresponded to BLG, indicating that BLG was present in these caseins. In addition, reports showed that milk proteins had cross-reactivity with soy proteins,^{39,40} but our results showed no cross-reactivity between BLG and soy proteins (Figure 3B). All these findings indicated that the developed immunoassay was highly specific.

Storage Stability of pAb-BLG-PtNPs Probe. The storage stability of the pAb-BLG-PtNPs probe was tested by running the procedure described in the Procedures of pAb-BLG-PtNPs Probe-Based sELISA section, and 1 μg/mL of BLG was used as antigen. The pAb-BLG-PtNPs were stored at 4 °C or –20 °C (containing 50% glycerol). As shown in Figure 3C, the activity of the probe under the two storage conditions had similar varying tendency. Within 9 weeks, the ODs were higher than 1.0, with an average OD of 1.128. However, the activity of pAb-BLG-PtNPs probe was declined significantly at 10th week. These results suggesting that the pAb-BLG-PtNPs probe can be used for detection of BLG within 9 weeks when stored at 4 °C or –20 °C with 50% glycerol.

Table 3. BLG Content in Hydrolyzed Infant Formulas Detected by Different Methods^a

formula		BLG concentration					
		PtNPs probe based sELISA (<i>n</i> = 3)		conventional sELISA (<i>n</i> = 3)		commercial sELISA kit (<i>n</i> = 2)	
		dry weight (μg/g)	ready-to-use formula (mg/L)	dry weight (μg/g)	ready-to-use formula (mg/L)	dry weight (μg/g)	ready-to-use formula (mg/L)
Alfaré	Nestlé	ND	ND	ND	ND	ND	ND
Precinutri (Pepti)	Dumex	ND	ND	ND	ND	ND	ND
NAN PRO	Nestlé	420.63 ± 32.58	61.69 ± 4.78	508.83 ± 50.11	74.63 ± 7.35	267.17 ± 12.75*	39.18 ± 1.87
NAN HA	Nestlé	205.32 ± 39.03	29.84 ± 5.67	271.54 ± 3.72	39.46 ± 0.54	125.46 ± 10.47	18.23 ± 1.52
Precinutri (HA)	Dumex	146.97 ± 3.04	22.53 ± 0.47	138.75 ± 5.32	21.27 ± 0.82	191.89 ± 5.93*	29.42 ± 0.91
Tummy Care	Abbott	16 764.61 ± 86.67	2228.02 ± 11.52	13 579.48 ± 1633.17	1804.71 ± 217.05	44 536.09 ± 3136.81**	5918.85 ± 41.69

^aND: Not detected. BLG allergenic residues were not detected when protein concentration of hydrolyzed infant formulas was 1.25 mg/mL. Compared to PtNPs probe based sELISA, the BLG content in hydrolyzed infant formulas detected by conventional sELISA and commercial sELISA kit significant at **p* < 0.05 and ***p* < 0.01.

Detection of BLG in Food Samples. To validate the applicability of the pAb-BLG-PtNPs probe-based immunoassay, recovery experiments were performed by spiking BLG at 0 mg/mL, 0.5 or 2 mg/mL in UHT-milk, whey, and yoghurt, and spiking 0 mg/mL, 0.5 mg/g, or 2 mg/g of BLG in chocolate, cookie, and candy, respectively. The results were compared with that of conventional sELISA and validated with commercial sELISA kit. As shown in Table 2, the detected BLG contents and recoveries by the developed sELISA were in good correlation with the results of the conventional sELISA and commercial sELISA kit, and with low variable coefficient from 1.04%–12.50%, indicating the reliability of this proposed method. Regarding to our proposed sELISA, the mean recoveries of BLG from spiked UHT-milk, whey, yoghurt, chocolate, cookie, and candy were 104.35%, 97.42%, 104.47%, 105.64%, 105.29%, and 98.90% (Table 2), respectively. All these results indicated that it was reliable to apply the developed pAb-BLG-PtNPs probe-based sELISA for detection of BLG in real samples with high recoveries.

Detection of BLG in Hydrolyzed Infant Formulas.

Cows' milk allergy is a very common adverse reaction in infants and young children,³ and hydrolyzed infant formulas are good nutritional substitutes for cows' milk sensitive children. However, it was reported that partially hydrolyzed infant formulas could induce allergic reactions in milk-sensitive infants,⁴¹ implying that safety or residual allergenicity assessment of these hydrolyzed infant formulas is necessary. In this study, BLG contents in two extensively hydrolyzed infant formulas and four partially hydrolyzed infant formulas were measured. The detected BLG content in hydrolyzed formulas by the developed sELISA was similar to those tested using conventional sELISA and commercial sELISA kit (Table 3). BLG was not detected in extensively hydrolyzed infant formulas ("Alfaré" and "Precinutri (Pepti)") by all the three methods (Table 3). Among the four kinds of partially hydrolyzed formulas, the BLG content in "Precinutri (HA)" was the lowest (146.97 μg/g) and was approximately 100-folds lower than "Tummy Care" (16 764.61 μg/g, the highest). The detected BLG contents in partially hydrolyzed formulas by the developed sELISA were similar to result of conventional sELISA (Table 3). However, the BLG contents in "NAN PRO" tested by the PtNPs probe-based sELISA were significantly higher than the tested by commercial sELISA kit, whereas the amounts of BLG in "Precinutri (HA)" and "Tummy Care" measured by the developed sELISA were

significantly lower than that of commercial sELISA kit (Table 3). These might be because pAb-tBLG specifically binded to seven IgE epitopes of BLG, resulting in BLG content measured by our developed immunoassay was strongly depend on IgE epitopes presented in the hydrolyzed formulas. These results indicated that the proposed method could be used to detect BLG and its allergenic residues in hydrolyzed infant formulas.

The molecular weight distribution of hydrolyzed infant formulas was analyzed by Tricine-SDS-PAGE. The results showed that no distinct bands existed in the extensively hydrolyzed infant formulas (Figure 4, lines 1 and 2), implying

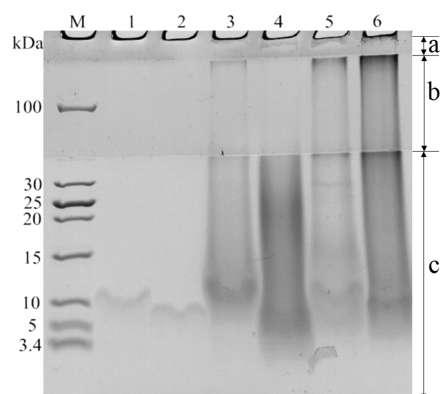


Figure 4. Tricine-SDS-PAGE analysis of two extensively hydrolyzed infant formulas (lanes 1 and 2) and four partially hydrolyzed infant formulas (lanes 3 to 6). Lane M, markers; lane 1, Alfaré; lane 2, Precinutri (Pepti); lane 3, NAN PRO; lane 4, NAN HA; lane 5, Precinutri (HA); lane 6, Tummy Care. (a) Stacking gel, (b) spacer gel, and (c) separating gel.

that the allergenic residues cannot be detected by the developed sELISA. Some larger peptides/proteins (greater than 30 kDa) existed in "Tummy Care" compared with other three partially hydrolyzed infant formulas (Figure 4), which might explain why BLG content in "Tummy Care" was highest (Table 3).

Even though Tricine-SDS-PAGE and HPLC can analysis the molecular weight distribution of hydrolyzed infant formulas, the residual allergenicity is not dependent on degree of hydrolysis but the presence and stability of IgE and T cell epitopes in these formulas recognized by patients, resulting in these methods cannot assess the safety or residual allergenicity

of these hydrolysates.⁴² The basophil activation test is always used for evaluation the residual allergenicity of the hydrolyzed infant formula, but this method required basophil, IgE from cows' milk allergy infants/children, expertise, expensive equipment, and the results might be different when using different patients' sera.^{42,43} In theory, a peptide contains 35 amino acids (approximately 3.8 kDa, at least two IgE epitopes) could be able to cross-link IgE and induce the basophil response.⁴² The pAb-tBLG used here could recognize seven human major linear IgE epitopes of BLG,²³ when the peptide contains not less than two IgE epitopes and be recognized by our antibodies, the developed sELISA could detect it. In comparison with the *in vitro* cellular degranulation assays, IgE epitopes specific antibodies based ELISA is economical, time-saving, easy to use, and the results are stable. All of these merits making our proposed sELISA could be as a reliable and user-friendly method to detect BLG allergenic residues in food, and aiding in the assessment of the residual allergenicity of milk hydrolysates.

In conclusion, we have developed an antibody-PtNPs probe-based sELISA for highly sensitive detection of BLG and its allergenic residues with wide linear range. The results indicated that our sELISA exhibited good analytical performance with ultrawide linear range between 0.49 ng/mL and 1.6×10^4 ng/mL for BLG testing with excellent correlation ($r^2 = 0.9989$), and its LOD (0.12 ng/mL) was 16-times lower than that of conventional sELISA (1.96 ng/mL). The precision was calculated as intraday repeatability (RSDr in the 5.52%–12.85% range) and interday reproducibility (every 3 days for 15 sequential days; RSDr in the 7.54%–15.33% range). The recovery values ranged from 93.53% to 111.95%, indicated high recovery. In addition, BLG and its allergenic residues were detected in six hydrolyzed infant formulas. The results of PtNPs probe-based sELISA were in good correlation with those of conventional sELISA and commercial sELISA kit, implying the reliability of our developed method. Moreover, the PtNPs probe had a long useful lifetime of 9 weeks. These features prove our proposed PtNPs probe-based sELISA to be a sensitive, reliable, and user-friendly method for detection of BLG and its IgE epitopes in food samples.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b04086.

Checkerboard titration to determine concentrations of pAb-tBLG and pAb-BLG-PtNPs probe; accuracy and precision of sELISA for detecting BLG (PDF)

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Notes

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■ ABBREVIATIONS USED

ALA, α -lactalbumin; BLG, β -lactoglobulin; BSA, bovine serum albumin; α -CN, α -casein; β -CN, β -casein; κ -CN, κ -casein; HRP, horseradish peroxidase; OD, optical density; PtNPs, platinum nanoparticles; pAb, polyclonal antibody; RSDr, relative standard deviation of repeatability for intra-assay precision; RSDR, relative standard deviation of reproducibility for interassay precision; sELISA, sandwich enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, scanning electron microscope; tBLG, epitope tandem derived from β -lactoglobulin; TMB, 3,3',5,5'-tetramethylbenzidine

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Table S1. The checkerboard titration to determine the concentrations of pAb-tBLG and pAb-BLG-PtNPs probe.

Concentration of		Dilution of pAb-BLG-PtNPs probe			
pAb-tBLG (μg/mL)		1:50	1:100	1:200	1:400
Positive	3.50	2.416	1.662	<u>0.982</u>	0.547
	1.75	1.809	<u>1.079</u>	0.627	0.350
	0.88	<u>1.175</u>	0.654	0.378	0.211
	0.44	0.767	0.421	0.222	0.140
Negative	3.50	0.270	0.155	<u>0.102</u>	0.080
	1.75	0.239	<u>0.142</u>	0.100	0.073
	0.88	<u>0.180</u>	0.100	0.076	0.057
	0.44	0.124	0.071	0.054	0.056
Positive/	3.50	8.948	10.723	<u>9.627</u>	6.838
Negative	1.75	7.569	<u>7.599</u>	6.270	4.795
	0.88	<u>6.528</u>	6.540	4.974	3.702
	0.44	6.185	5.930	4.111	2.500

Note: The positive values around 1.0 and the corresponding negative and positive/negative values were underlined. Among them, the positive and negative values that produced maximum positive/negative value are presented in bold.

Table S2. The accuracy and precision of the sandwich ELISA for detecting BLG.

BLG (ng/mL)	Intra-assay ^a				Inter-assay ^b		
	Mean±SD	Bias	RSDr ^c	n	Mean±SD	RSDR ^d	n
	(ng/mL)	(%)	(%)	replica	(ng/mL)	(%)	replica
31.25	31.67±2.29	1.34	7.23	5	34.47±2.60	7.54	5
62.5	61.42±4.14	-1.73	6.74	5	65.85±7.09	10.77	5
125	136.19±16.30	8.95	11.97	5	128.98±15.89	12.32	5
250	245.73±31.57	-1.71	12.85	5	273.87±29.43	10.75	5
500	478.10±38.75	-4.38	8.10	5	499.60±76.57	15.33	5
1000	1149.07±124.08	14.91	10.80	5	1052.34±146.81	13.95	5
2000	1936.14±106.88	-3.19	5.52	5	1934.22±287.18	14.85	5
4000	4226.47±262.81	5.66	6.22	5	4185.79±367.91	8.79	5
	Mean	2.48	8.68		Mean	11.79	

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

^b Assay was performed every 3 days for 15 sequential days.

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

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