



Sensitive detection and quantification of gliadin contamination in gluten-free food with immunomagnetic beads based liposomal fluorescence immunoassay

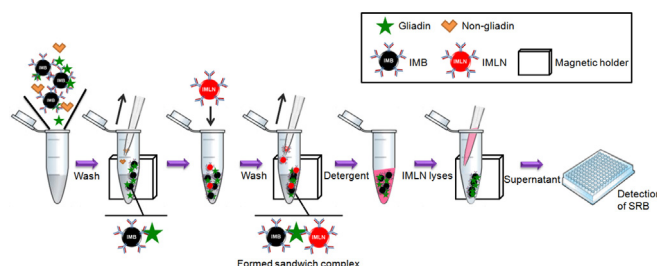
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HIGHLIGHTS

- Immunomagnetic beads are used for specifically isolating gliadin from food.
- Immunoliposomal nanovesicles are used for instantly amplifying the assay signal.
- Detection limit of this assay was lower than those of commercial ELISA kits.
- This magnetic bead detection platform is suitable for a high-throughput screening.
- Results of this assay are comparable to those of an AOAC ELISA kit in test-food.

GRAPHICAL ABSTRACT



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ABSTRACT

Gliadin from wheat is a common food allergen that can induce baker's asthma, wheat-dependent exercise-induced anaphylaxis, atopic dermatitis, and celiac disease. This gliadin assay focuses on rapidly screen and check for gluten contamination in raw materials and in the gluten-free food production process, not only for wheat-sensitive patients but also for the industries producing gluten-free foodstuffs. The developed assay incorporates the use of anti-gliadin antibody-conjugated immunomagnetic beads (IMBs) to capture the gliadin in samples and fluorescent dyes-loaded immunoliposomal nanovesicles (IMLNs) to produce and enhance the detection signal. Hence, a sandwich complex is formed as "IMBs–gliadin–IMLNs". Experimental results indicate that this detection platform exhibits good sensitivity for gliadin with a detection limit as low as $0.6 \mu\text{g mL}^{-1}$ of gliadin; as the polyclonal antibody showed slight cross-reactions with barley and rye. Excellent recovery rates were found ranging from 83.5 to 102.6% as testing the spiked samples. Moreover, the CV (%) of intra- and inter-assay of this developed assay are 4.8–10.6% and 3.5–9.9%, respectively. Based on a parallel analysis of twenty food samples, the results of this developed assay provide a good consistency with those of an AOAC-approved ELISA kit without any false-negative results. The proposed assay method is thus a highly promising alternative method for detecting the contamination of gliadin in the food industry.

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1. Introduction

Wheat protein is responsible for not only a common food allergy but also a contact allergy, owing to occupational exposure [1]. In clinical diagnosis, wheat allergy (WA) is an IgE-mediated hypersensitivity and the correlation diseases of WA include: atopic

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dermatitis, baker's asthma, wheat-dependent exercise-induced anaphylaxis, and contact urticaria [2,3]. On the other hand, the celiac disease (CD) is the T cell-mediated autoimmune disease and the result of permanent intolerance to gluten [4]. In previous studies, sufferers of WA and CD could be induced by ingestion of prolamins which were the fraction extracted by 40–70% aqueous ethanol, and is known as gliadin in wheat, as hordeins in barley, in oats as avenins, and as secalins in rye [5].

In general, gluten includes two groups of proteins known as glutenins and prolamins, and each group is account for approximately 50% of gluten [6]. In wheat, prolamins are also called as gliadins and can divide into α -, β -, γ - and ω -gliadin. In the midst of gliadin, α -gliadin and ω -5-gliadin have been considered to be the major wheat allergens [7]. In addition to above, the major wheat allergens also include the α -amylase/trypsin inhibitor (12–18 kDa); while the minor wheat allergens contain a 9-kDa lipid transfer protein in the albumin/globulin fraction and the low-molecular-weight glutenin subunits [8]. Patients must comply with a gluten-free diet, should avoid contact with barley and rye or their crossbred varieties and derivatives. The standard of gluten-free diet is described as the gluten level does not exceed 20 parts per million (ppm) in total [1].

Gliadin must be quantified accurately for proper food labeling and consumer protection. Several methods have been developed in recent years to detect gliadin, including quartz crystal microbalance (QCM) [9], mass spectrometry (MS) [10], electronic tongue (E-tongue) [11], real-time PCR [12], electrochemical immunosensor [13], and enzyme-linked immunosorbent assay (ELISA) [14]. Despite their accurate analysis, these methods must still be performed in an analytical laboratory, as well as operated by skilled technicians. Therefore, a simple detection method must be developed, characterized by rapid and sensitive analysis of gliadin in food samples. Among various assays commercially available, enzyme-linked immunosorbent assay (ELISA) is a conventional means of quantifying a target protein in food samples and possesses several advantages, such as high throughput, simplicity, and low cost [15].

Immunomagnetic separation (IMS) is a highly effective diagnostics approach, owing to its high specificity and sensitivity, as well as reduction of assay time. Immunomagnetic bead (IMB), a high-performance material, has been used to extract target analyte from liquid samples [16]. Bead-based immunoassays are generally superior to the conventional one, owing to their sample purification and concentration enabled by applying external magnetic fields. For instance, compared with the quantification of carcinoembryonic antigen (CEA) in human serum using the ELISA and the IMB-based ELISA, the limit of detection of the ELISA assay is 1.6 ng mL^{-1} which is higher than that of the one with IMB (0.5 ng mL^{-1}) [17]. Additionally, magnetic beads have a three-dimensional structure that increases the surface-to-volume ratio, subsequently providing more binding sites for target molecules than the wells of microtiter plate with a two-dimensional structure. Recently, more magnetic bead assays have been integrated into microfluidic systems because they are further easily manipulated, sorted, and concentrated in the microfluidic systems [18,19]. Besides, a novel magnetic bead detection method, a competitive immunomagnetic-proximity ligation assay (CIPLA), has been developed for small-molecules detections, which is essential for study related to drug discovery, physiological functions, and chemical residues in foods [20,21]. By using IMB, a previous study removed allergens from peanut extract by conjugating anti-peanut antibodies to the surface of magnetic beads [22]. To amplify the detection signals, the antibody-tagged liposomal nanovesicle (immunoliposomal nanovesicle; IMLN) has been used as the detection reagent for several assays. Liposomes can be defined as closed spherical vesicles consisting of one or more phospholipid bilayers surrounding an aqueous cavity [23]. The hollow sphere of liposomes can encapsulate hundreds of thousands

of dye molecules. Additionally, the release of this large amount of markers can significantly increase the assay signal immediately. To transform a liposome into a functionally labeled reagent, various biological ligands (e.g., peptides, hormones, antibodies, sugars, and nucleic acids) can be conjugated to the liposomal surface to make the targeted liposome. By altering the lipid composition, size, encapsulated materials, and tagged surface biomolecules, liposomes have been used in various applications, including drug delivery, gene transfection, and immunoassays [24–26].

This work develops a rapid and sensitive method by using IMBs as the capture reagent to isolate gliadin from food, followed by using anti-gliadin antibody tagged IMLNs as the detection reagent to quantitatively detect the contamination of gliadin in food. Moreover, these detection results are compared with an AOAC approved gliadin-ELISA kit to validate the precision and feasibility of this developed assay.

2. Materials and methods

2.1. Materials

Two different sized magnetic beads, 2.8 and 2–10 μm coupled with carboxyl acid, were purchased from Dynal Invitrogen (CA, USA) and Abnova (Taipei, Taiwan), separately. Dipalmitoyl phosphatidylethanolamine (DPPE), dipalmitoyl phosphatidylcholine (DPPC), and dipalmitoyl phosphatidylglycerol (DPPG) were purchased from NOF Corp. (Tokyo, Japan). The Mini Extruder was purchased from Avanti Polar Lipid, Inc. (AL, USA), and the 0.2 and 0.4 μm pore size polycarbonate membranes were purchased from Whatman (NJ, USA). Chloroform and methanol were purchased from Merck (Darmstadt, Germany). All chemicals used in this study were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich Co. (MO, USA).

2.2. Sample preparation

Nine crops (wheat, barley, oat, rice, foxtail millet, corn, buckwheat, soybean, and rye) were obtained from local supermarkets (Taichung, Taiwan). In order to isolate gliadin from these crops, the extraction method used in this study resembled that used elsewhere [27,28]. Briefly, samples were crushed in a mill to produce flour and then sifted through a 100-mesh screen to remove the impurities. First, water-soluble proteins were extracted to remove albumins by shaking with 50 rpm in deionized water at room temperature for 1 h. After the water-soluble proteins were extracted twice, globulins were extracted from the flour residue with 0.5 M NaCl solution. Finally, the pellet was extracted with 60% (v/v) ethanol, the mixture was centrifuged at 8000 rpm for 10 min, and supernatant was filtrated with 0.45 μm pore size filter for use as the gliadin sample. Samples were maintained at 4 °C, and pre-warmed at room temperature before analysis with the developed IMB assay.

Additionally, gliadin in food samples were also analyzed by an AOAC-approved gliadin ELISA kits (R7001, R-Biopharm, Germany) by following the manufacturer's instruction. Briefly, 2.5 mL of cocktail solution was added to the food samples, followed by shaking of the mixture for 1 h. The resulting liquid was centrifuged at 6000 rpm for 15 min and the supernatant was collected to determine the gliadin concentration by using the ELISA kit.

2.3. Production and isolation of anti-gliadin IgY from yolk

The method to purify anti-gliadin IgY from gliadin-immunized chicken eggs was mentioned in our previous study [9]. Briefly, 40-week-old white Leghorn hens were immunized in the thorax muscle at two sites using 1.5 mL of Sigma gliadin (G-3375)-complete Freund's adjuvant mixture. After 14 days, the first boost

was to inject 1.5 mL of Sigma gliadin-incomplete Freund's adjuvant. In the third boost, eggs were collected daily and stored at 4 °C until use. When the anti-gliadin IgY purification started, the egg white must be removed from the egg yolk as much as possible. Next, 3.5% polyethylene glycol 10000 (PEG10) in 0.1 M phosphate buffer saline (PBS, pH 7.6) of four times volume of the egg yolk was mixed well with the yolk and then centrifuged at 8000 rpm for 10 min. Additional PEG10 was added to the clear supernatant to obtain a final concentration of 12% (w/v) and then centrifuged as described above. The supernatant was removed and the pellet was dissolved in a PBS with 12% (w/v) PEG10 and then centrifuged as described above. The pellet was dissolved in 50% ethanol with a total volume of half of the original volume of the yolk and the pellet was obtainable after centrifugation and dissolved in 1 mL of PBS. Finally, the IgY concentration, titer, and purity were estimated with protein assay, ELISA, and Western blot analysis, respectively.

2.4. Preparation of the anti-gliadin antibody tagged immunomagnetic beads

In this work, coupling between the amino group of the anti-gliadin IgY and the carboxylic acid groups on the surface of magnetic beads is the formation of amide bonds mediated by carbodiimide activation [29,30]. One hundred milliliters of Dynal or Abnova magnetic beads were washed twice with 0.1 M N-morpholinoethanesulfonate (MES, pH 5), followed by mixing with 100 μ L of 25 mg mL⁻¹ anti-gliadin IgY in 1.5 mL eppendorf for 1 h to produce immunomagnetic beads (IMBs). After incubation, the reaction solution was further reacted with 30 μ L of 1 mM 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) solution for 30 min. After washing twice with 0.5 mL of 0.1 M phosphate buffered saline (PBS), IMBs were resuspended in PBS and stored at 4 °C until the use.

2.5. Preparation of anti-gliadin antibody-tagged liposomal nanovesicles

ATA-liposomes were prepared via a hydration/freezing-thawing/extrusion method [31]. The mixture of DPPC, DPPG, cholesterol, and DPPE-ATA was dissolved in a solution of 6 mL chloroform, 1 mL methanol, followed by drying. The dried lipid film was hydrated by 3 mL sulforhodamine B (SRB). After 5 freeze and thaw cycles, the lipid sample was extruded by the Mini Extruder through 0.4 and 0.2 μ m pore-size polycarbonate membranes. Unencapsulated SRB was removed using a Sephadex G-50 column. Next, anti-gliadin-IgY tagged liposomal nanovesicles, immunoliposomal nanovesicles (IMLN), were made by conjugating maleimide activated antibodies to sulfhydryl liposomal nanovesicles, with the starting mol% of antibodies to surface lipids of 0.5, 1.0, 2.0 and 3.0. Additionally, IMLNs were separated from unbound antibodies by a Sepharose CL-4B column. The phospholipid concentration of IMLNs was determined by Bartlett's phosphorus assay [32], and the size of IMLNs was determined by a laser diffraction particle size analyser (Coulter ScientiWc Instruments, FL, USA). Finally, the concentration of antibodies was determined using the Bio-Rad protein assay with IgG as the standard.

2.6. Assay performance

Initially, 1 mg wheat gliadin powder purchased from Sigma-Aldrich Co. was dissolved in 1 mL 60% ethanol and then diluted into varied concentrations. Subsequently, 100 μ L gliadin solution was reacted with IMBs in an eppendorf with thoroughly mixing for 1 h at 25 °C. Following removal of the supernatant, 100 μ L of IMLN solution was added and incubated for another 1 h at 25 °C. The IMB-gliadin-IMLN complexes were then washed

twice with Tris-buffered saline solution containing 0.05% Tween 20 (TBST). Next, 30 mM n-octyl- β -D-glucopyranoside (n-OG) was added with 5-s of vigorous shaking to release the entrapped SRB molecules. Additionally, the released SRB molecules were measured by a fluorescence ELISA reader (BioTek® FLx800™, VT, USA) with an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Moreover, the sensitivity of the IMLN/IMB assay was assessed using a serial dilution of gliadin solutions (1–500 μ g mL⁻¹) prepared by diluting the commercial gliadin powder in 60% ethanol for the construction of a dose-response curve. Finally, the limit of detection (LOD) for gliadin was determined from the dose-response curve as the concentration of gliadin that corresponded to the mean (plus three standard deviations) fluorescent signal of the unspiked sample [33].

2.7. Recovery rate

The recovery rate of this immunoassay was determined by using three food samples (gliadin-free pancake mix, plain crackers, and wheat toast) spiked with four various concentrations of gliadins. Food samples were extracted by following the method previously described in sample preparation. In the last step of extraction, various amounts of gliadin (10, 25, 50, and 100 μ g mL⁻¹) were spiked into the 60% ethanol using the gliadin powder purchased from Sigma-Aldrich Co. This spiked 60% ethanol was then used to extract gliadin from food samples. After extraction, the total content of gliadin in the 60% ethanol was determined by the developed IMB fluorescence assay. The recovery rate was expressed as follows: $[(C - C_0)/C_1] \times 100\%$, where C denotes the detection value of the gliadin in the spiked sample; C_0 represents the measured value of the original sample; and C_1 refers to the known spiked concentration of the gliadin in the same sample. Finally, the gliadin content was determined using this developed method.

2.8. Method comparison

Reliability of this developed IMB fluorescent assay was determined by comparing the results of gliadin-spiked gluten-free pancake mix product which was obtained from local supermarkets (Taichung, Taiwan) with the quality label "Controlado por FACE" guaranteed by the Spanish Federation of Celiac Associations, and then analyzed by the IMB assay and an AOAC-approved ELISA kit (RIDASCREEN® Gliadin kit). Firstly, different amounts of gliadin powder was spiked into 60% ethanol and then pancake mix was extracted using this spiked 60% ethanol, followed by the developed IMB assay. For preparing the spiked sample for the ELISA test, various amounts of gliadin was spiked into 80% ethanol and then pancake mix was extracted by following the manufacturer's instruction with the use of the spiked 80% ethanol, followed by the ELISA test.

2.9. Statistical analysis

All data obtained using the developed IMB assay were presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using the SAS statistical package (SAS Institute, NC, USA). A p-value of under 0.05 was considered significant.

3. Results and discussion

3.1. Antibody specificity

The performance of an immunoassay is heavily dependent on the affinity and specificity of the antigen-antibody interaction,

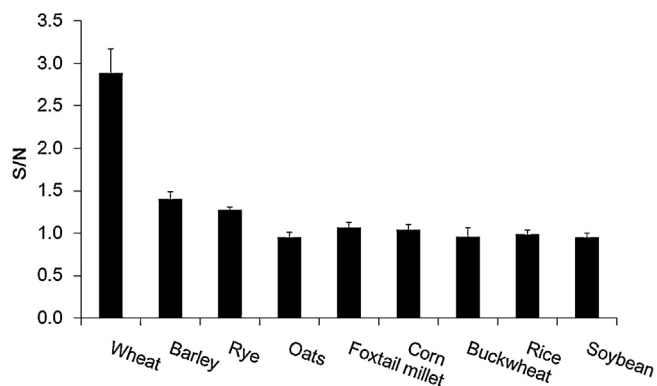


Fig. 1. Specificity of the anti-gliadin antibody in the IMB fluorescence assay and the detection fluorescence signals showed in signal-to-noise (S/N). Each crop sample (0.1 mg) was extracted and then determined their gliadin content by the use of this developed assay.

however, the cross-reactivity of antibody may reduce the specificity of immunoassays. Since the anti-gliadin antibodies from yolk used in this developed assay are polyclonal, the specificity of antibodies was evaluated. Western blot and quartz crystal microbalance (QCM) in our previous study has confirmed the specificity of the anti-gliadin IgY used in this work [9]. Experiments on real food samples demonstrated the good selectivity of anti-gliadin IgY which could analyze the gliadin from other components in mixtures without interference, thus supporting the appropriateness of this polyclonal IgY antibody for coating on magnetic beads in this work. However, different detection systems using the same antibodies might produce different results in terms of specificity. Therefore, this work was to evaluate the specificity of anti-gliadin IgY which was coated on magnetic beads. Extracts from nine frequently used crops were detected by anti-gliadin antibodies and Fig. 1 summarizes those results. The definition of a signal-to-noise (S/N) analysis is the signal of the sample (S) compared with that of the background (N). According to this figure, the anti-gliadin antibody from the yolk had the highest reactivity to the extract of wheat ($S/N = 2.88 \pm 0.29$), and no cross-reactivity was found in oats, foxtail millet, corn, buckwheat, rice, and soybean flour (the S/N ratio was approximately equal to one). However, this polyclonal antibody had slight cross-reactions with the extract of barley ($S/N = 1.40 \pm 0.09$) and rye ($S/N = 1.27 \pm 0.03$). Such occurrence may be owing to that wheat, barley and rye are from the same family (Poaceae), which have a highly similar amino acid sequence, possibly causing the non-specific binding of anti-gliadin antibody to barley and rye [34,35]. Additionally, a previous study also showed that the mouse monoclonal anti-gliadin antibodies had similar cross-reaction in detection of wheat, barley, and rye as this monoclonal antibody was manufactured by the immunization of BALB/c mice with gliadin purchased from Sigma–Aldrich Co. [36].

3.2. Optimization of IMB reaction conditions

In the traditional ELISA assay, the surface area of the wells supplies a solid phase for antibodies to bind. However, in this study, magnetic beads support the binding of anti-gliadin antibody in order to form IMBs as capture reagents for isolating gliadin from food. The magnetic beads are provided simple, effective separation when extracting a slight amount of food allergens from various food matrixes. Conventional approaches such as those using filter separation or centrifugation fail to obtain a highly pure allergen in samples [37]. To conjugate the antibody on a solid phase, most immunoassays may use protein A or G, which can specifically bind the Fc region of antibody, to coat on the solid phase first [38]. However, protein A or G cannot recognize IgY. Therefore, the carboxyl

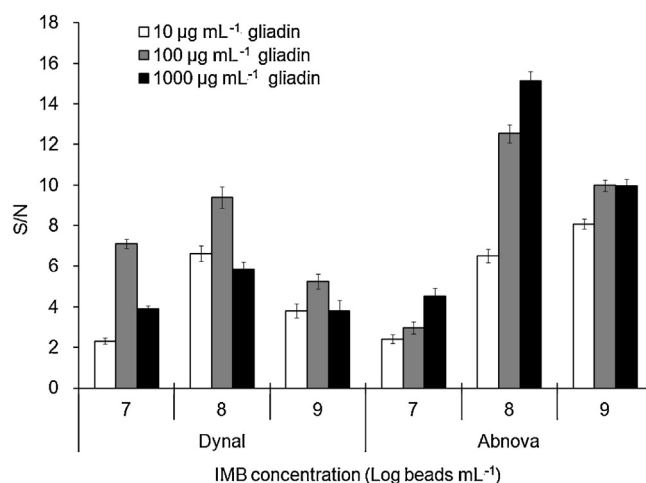


Fig. 2. Effect of bead concentration on the S/N ratios from samples spiked with various amounts of gliadin (10, 100, and 1000 µg mL⁻¹) in the IMB assay with two brands of magnetic beads (Dynal and Abnova).

group on the surface of magnetic beads is activated by a water soluble carbodiimide crosslinker for spontaneous reaction with primary amines of the antibody. As a chemical reaction, the binding of anti-gliadin antibody to carboxyl acid group of magnetic beads can obtain a high-purity IMB without adding additional biomolecules.

In this work, the concentration and volume of IMBs were optimized to reduce the cost of materials and perform a sensitive assay. According to Fig. 2, the optimized concentration of these two brands of IMB was 10^8 beads mL⁻¹, providing the highest S/N values among three different IMB concentrations (10^7 – 10^9 beads mL⁻¹). The 10^7 beads mL⁻¹ IMB concentration failed to capture all gliadin molecules in the sample, while the 10^9 beads mL⁻¹ IMB concentration might result in the steric hindrance. Thus, lower amounts of gliadin were captured as using these two concentrations of IMB and subsequently their assay signals were lower than that of the assay using 10^8 beads mL⁻¹. This figure also indicated that at the same concentration of IMB, the Abnova beads with a larger size and broad size distribution could give a higher detection signal than that of Dynal beads, which had a highly uniform size distribution [39]. Comparing these two magnetic beads revealed that Dynal beads with mono-distribution for its particle size could offer more stable results with relatively small SD values. However, the larger sized Abnova beads provided a large surface area for gliadin to be captured, as well as more magnetism to divide magnetic beads from a mixture. Hence, the optimal condition of IMB to detect gliadin used 10^8 beads mL⁻¹ Abnova beads.

Furthermore, the IMB volume was optimized with various volumes of IMB (10, 20, 30, and 40 µL) when attempting to detect gliadin. Fig. 3 plots the intensity of fluorescence signals for various volumes of Abnova IMB, based on the analysis of 0 and 10 µg mL⁻¹ gliadin. Briefly, the fluorescence signal intensity increased with an increasing IMB volume. However, the background signals also rose as the IMB volume was increased. Among four different IMB volumes, the test with 30 µL IMB gave the highest S/N ratio (6.8). Therefore, 30 µL was the optimized IMB volume and used for further assay development in this work.

3.3. Optimization of IMLNs

For immunoassay, liposomes have been successfully used as amplification systems since their large surface area can carry numbers of capture molecules, such as an antibody or a receptor. Additionally, the large internal volume can accommodate a tremendous number of reporter molecules to increase the strength of assay

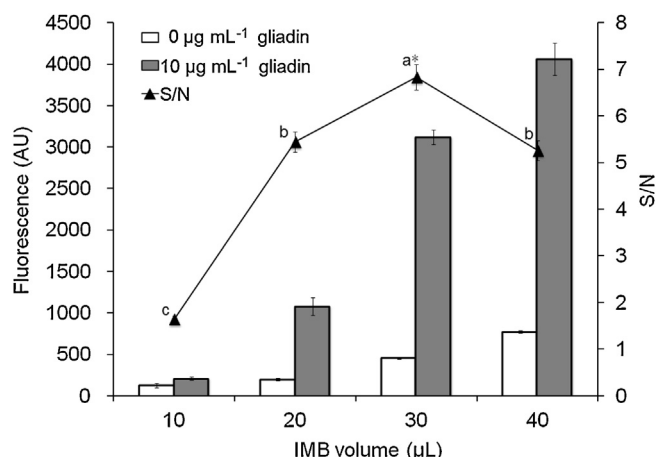


Fig. 3. Optimization of IMB volume for unspiked sample and sample spiked with $10 \mu\text{g mL}^{-1}$ gliadin in the IMB fluorescence assay. Different amounts of IMBs were incubated with a test sample at 25°C for 1 h and the bound gliadin molecules were detected with the use of IMLNs. *The S/N ratio with different letters indicates a significant difference ($p \leq 0.05$).

signals [21,40,41]. Furthermore, liposomes are rather stable, and numerous biological molecules can be conjugated on the liposome surface to make it have functional ability for targeting to a specific molecule, such as a pathogen, herbicide, or allergen [42]. In this work, IMLN was used as the detection reagent in which the water-soluble fluorescent SRB dye molecules were encapsulated and anti-gliadin antibodies were tagged on its surface. The detection efficiency of a batch of IMLN generally depends on its particle size, concentration, and tagged antibody mol% on its surface. This work also attempted to optimize the particle size and lipid concentration of IMLN by using IMLN with three particle sizes (200, 300, and 400 nm) versus various lipid concentrations (100, 200, 400, and 800 nmol mL⁻¹) in order to detect gliadin (1, 10, and 100 µg mL⁻¹ in 60% ethanol) (Fig. 4). When $1 \mu\text{g mL}^{-1}$ gliadin with these 12 batches of IMLNs was detected, the use of 400 or 800 nmol mL⁻¹ of 200 nm IMLNs or 400 nmol mL⁻¹ of 300 nm IMLNs could prove the highest S/N ratio. Moreover, IMLNs with a lipid concentration of 400 nmol mL⁻¹ displayed the highest signal at diameters of 300 nm than those of 200 nm and 400 nm when 10 and 100 µg mL⁻¹ of gliadin are detected. When the smallest size of IMLN (200 nm) was used, decline of the assay signal strength might be owing to the reduction in the number of encapsulated SRB molecules in the IMLNs with a smaller diameter. Moreover, the assay signal strength decreased when a larger sized IMLN (400 nm) was used. This decrease in the signal intensity might be owing to the steric

Table 1

Effect of tagged antibody mol% of IMLNs on the S/N ratios and the slope of each corresponding dose–response curve as detecting samples spiked with different amounts of gliadin using the developed IMB assay. The size and total lipid concentration of IMLNs used herein for the detection of gliadin were 300 nm and 400 nmol mL⁻¹, respectively.

IMLNAb (mol%)	Gliadin concentration ($\mu\text{g mL}^{-1}$)			Slope (m)
	1	10	100	
0.5	$4.1 \pm 0.4^{\text{c}}$	$7.0 \pm 0.2^{\text{d}}$	$8.8 \pm 0.2^{\text{d}}$	0.0364
1.0	$5.3 \pm 0.3^{\text{b}}$	$7.6 \pm 0.2^{\text{c}}$	$10.3 \pm 0.3^{\text{c}}$	0.0417
2.0	$6.6 \pm 0.4^{\text{a}}$	$10.5 \pm 0.4^{\text{a}}$	$12.3 \pm 0.6^{\text{a}}$	0.0423
3.0	$6.7 \pm 0.3^{\text{a}}$	$9.3 \pm 0.4^{\text{b}}$	$11.0 \pm 0.5^{\text{b}}$	0.0336

* The value of S/N ratios (mean $\pm$ SD) in the same column with different letters indicate a significant difference ($p \leq 0.05$).

hindrance with larger IMLNs. Based on these results, the IMLN with a diameter of 300 nm and lipid concentration of 400 nmol mL⁻¹ was selected for this work.

The next step to optimize IMLN focused on the conjugated antibody mol% on the liposomal surface. Four antibody mol% were tested: 0.5, 1.0, 2.0 and 3.0 mol% (Table 1). The detection sensitivity of tests with these four batches of IMLNs was illustrated by the slope (m) of the regression line from their corresponding dose–response curves. As the antibody mol% increased from 0.5 to 2 mol%, the slope increased from 0.0364 to 0.0423. Additionally, the S/N ratios also increased significantly when detecting various concentrations of gliadin (1–100 µg mL⁻¹). This finding suggests that a higher mol% of tagged anti-gliadin antibody could increase the total affinity between gliadin and IMLNs, resulting in a higher sensitivity in detection. However, when the antibody mol% reached 3.0 mol%, the slope decreased to 0.0336. This reduction might be owing to that too many antibodies were presented over a limited surface area of liposomes; they might be too crowded to provide adequate space and proper orientation to react with the bound antigen (gliadin) on the surface of IMBs. Therefore, fewer IMLNs were conjugated with the gliadin-IMB complexes, subsequently allowing for a lower assay signal. Since 2.0 mol% antibody-tagged IMLNs yielded the highest S/N ratios among the other batches of IMLNs in analysis of the same concentration of gliadin, this batch of IMLN was selected as the optimal detection reagent for this work.

3.4. Assay performance

The assay was developed as a sandwich format by using IMBs as the capture reagent to isolate gliadin from food. As the IMBs–gliadin complex was isolated from samples, IMLNs were used as detection reagents to reveal the captured gliadin. More gliadin in samples resulted in more “IMB–gliadin–IMLN complexes”. Therefore, the release SRB molecules which encapsulated in IMLNs could yield a higher intensity of the fluorescence signal. To evaluate the feasibility of this developed assay method, gliadin was detected with various concentrations (0–500 µg mL⁻¹). The LOD of this developed assay was determined according to the dose–response curve (Fig. 5). The International Union of Pure and Applied Chemistry (IUPAC) defines LOD as the concentration that corresponds to the mean of the intensity of fluorescence signal in the background plus three standard deviation (SD). The dose–response curve was $y = 88.535x + 530.83$, where y is the fluorescent intensity, x is the concentration of gliadin ($\mu\text{g mL}^{-1}$) and the correlation coefficient (R^2) is 0.998. The dynamic range of this curve was approximately 0–500 µg mL⁻¹ gliadin, and the calculated LOD value was nearly 0.6 µg mL⁻¹ gliadin, which is lower than the official limit of EU for gluten-free food, containing less than 10 µg mL⁻¹ of gliadin. There are many methods for gliadin detection, and the feature of our study is to combine the technique of IMBs and IMLNs for gliadin detection. According to the previous publication, an electrochemical magneto

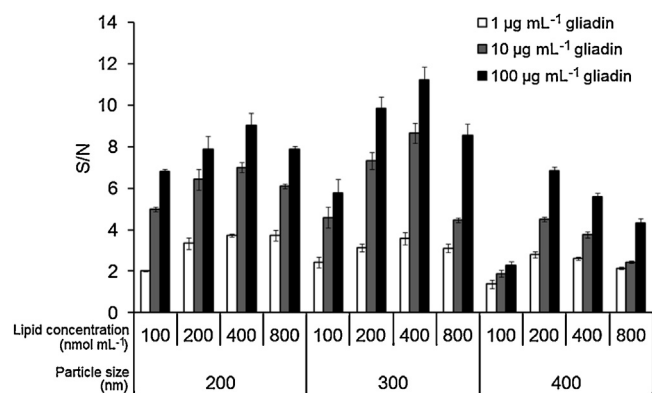


Fig. 4. Effect of IML size and lipid concentration of IMLNs on fluorescence signals from samples spiked with various amounts of gliadin (1, 10, and 100 µg mL⁻¹) in IMB fluorescence assay.

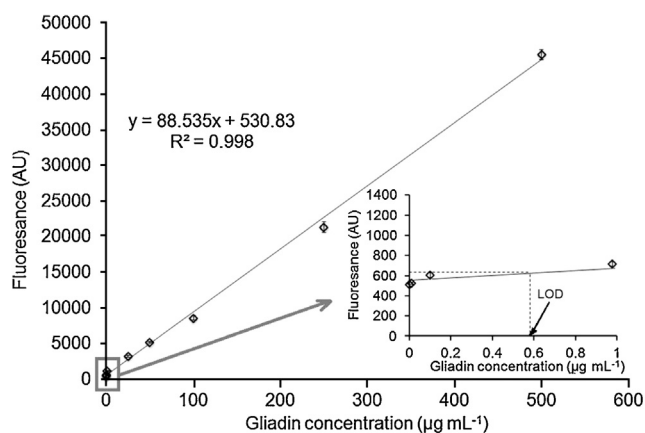


Fig. 5. Dose–response curves for the sample spiked with a serial dilution of gliadin in IMB fluorescence assay. Solid line is a one order equation curve with an R^2 value of 0.998. The limit of detection (LOD) is defined as mean of the intensity of fluorescence signal for background plus three SDs, calculated as $0.6 \mu\text{g mL}^{-1}$ of gliadin.

immunosensor for the detection of gliadin was reported and its performance was compared with a magneto-ELISA [43]. The results of LOD for gliadin of magneto immunosensor and magneto-ELISA showed similar values in the PBST buffer ($\sim 5 \text{ ng mL}^{-1}$), however, the values of magneto immunosensor in gluten-free food samples (24.2 ng mL^{-1} in skimmed milk; 21.2 ng mL^{-1} in beer) were slightly higher than magneto-ELISA (8.3 ng mL^{-1} in skimmed milk; 1.2 ng mL^{-1} in beer). In addition, a label-free gliadin QCM biosensor was given the LOD in 60% ethanol as 8 ng mL^{-1} gliadin and had an excellent correlation with the ELISA kit as analyzing gliadin in food samples [9]; an optical biosensor based on the specific binding of the glutamine-binding protein to gliadin peptides yielded the LOD as $2 \mu\text{g L}^{-1}$ [44]. Another work developed an electronic tongue for the detection of gliadin with a sensitivity of $1\text{--}2 \mu\text{g g}^{-1}$ of gliadin in baby milked flour [11]. A sandwich ELISA system based on an antibody cocktail for gluten analysis could give LOD in gluten-free food samples as $1.5 \mu\text{g mL}^{-1}$ gliadin and had higher sensitivity in gluten-free foods than those of two commercial ELISA kits (Transia and r-Biopharm Ridascreen) [45]. The LOD of a microfluorimeter with a disposable polymer chip for the detection of coeliac disease toxic gliadin in extraction buffers was $4.1 \mu\text{g mL}^{-1}$ and the microfluorimeter could obtain similar results with the colorimetric ELISA as analyzing gliadin in food samples [46]. Finally, a fluorescence correlation spectroscopy assay for gliadin in food could detect levels as low as 6 ng mL^{-1} gliadin and had higher sensitivity than R5-ELISA [47]. Based on the results of the above works, biosensors and a fluorescence correlation spectroscopy could provide a lower LOD

around 10 ng mL^{-1} gliadin; meanwhile, ELISA kits and other assays achieved a higher LOD of around $5 \mu\text{g mL}^{-1}$ gliadin. The LOD of this developed assay was $0.6 \mu\text{g mL}^{-1}$ gliadin, which was less sensitive than LOD of biosensors, yet was about 10 times more sensitive than those of conventional ELISA kits.

3.5. Recovery rate of the immunoassay applied to real food sample

The recovery rate of the target molecule must be assessed to verify the accuracy of applying the proposed immunoassay method to food [48]. Table 2 summarizes the analysis results of the gliadin recovery rate from three different food samples spiked with various amounts of gliadin by using the developed IMB assay in this study. Owing to the equal amounts of gliadin and glutenin in wheat gluten, the gluten concentration of the sample is calculated by multiplication with the factor 2 [6]. The first food sample, pancake mix, was detected with $0.4 \mu\text{g mL}^{-1}$ gliadin (equal to $0.8 \mu\text{g mL}^{-1}$ gluten); this product could thus be referred to as “gluten-free food” ($<20 \mu\text{g mL}^{-1}$ gluten). The second one, plain crackers, was detected with $40.1 \mu\text{g mL}^{-1}$ gliadin (equal to $80.2 \mu\text{g mL}^{-1}$ gluten); this product could thus be named as “low gluten food” ($20\text{--}100 \mu\text{g mL}^{-1}$ gluten). The last one was wheat toast belonging to wheat products ($>100 \mu\text{g mL}^{-1}$ gluten). The mean recovery rate of the spiked gliadin ($10\text{--}100 \mu\text{g mL}^{-1}$) in pancake mix powder and plain cracker was 99.4% and 99.8%, respectively; however, that of the spiked gliadin in wheat toast was down to 90.9%. Although this recovery rate of the spiked gliadin in wheat toast is lower, it is sufficient to prove the ingredients of gliadin in food. Hence, above results demonstrate that this developed assay can be as the first defense for food safety and process control to avoid any ingredients that might contain gliadin from diet. However, the people who are intolerant to gluten should avoid contact not only wheat but rye, barley, and even oats [49].

3.6. Assay precision

Intra-assay and inter-assay were used to describe the precision and accuracy of the proposed assay, which were obtained by the coefficients of variation (CV) of the measurement results. Samples of a gliadin-free pancake mix spiked with four gliadin concentrations ($0, 10, 100$, and $125 \mu\text{g mL}^{-1}$) were analyzed to estimate the precision of intra-assay by assessing 20 repeats of each sample panel in one analytical run. The inter-assay precision was evaluated by analyzing each sample panel in replicates of two at two separate times per day for 10 consecutive days, with the use of different lots of IMB daily [50]. According to Table 3, the CV (%) of intra-assay and inter-assay was 4.8–10.6% and 3.5–9.9%, respectively. The CV

Table 2

Recovery rates of spiked gliadin in three food samples originally containing different amounts of gliadin. Various concentration of gliadin ($10, 25, 50$, and $100 \mu\text{g mL}^{-1}$) were spiked into different food samples, extracted and then measured by the developed IMB fluorescence assay.

Food samples	Original gliadin content ($\mu\text{g mL}^{-1}$)	Gliadin concentration ($\mu\text{g mL}^{-1}$)		Recovery rate (%)
		Spiked	Measured	
Gliadin-free pancake mix	0.4	10	10.2 ± 0.4	98.5
		25	26.0 ± 0.3	102.6
		50	50.5 ± 0.4	100.2
		100	96.5 ± 0.9	96.1
Plain crackers	40.1	10	50.2 ± 0.3	100.9
		25	65.0 ± 0.4	99.8
		50	88.6 ± 2.2	97.1
		100	141.4 ± 0.9	101.3
Wheat toast	115.9	10	125.6 ± 0.9	96.8
		25	139.6 ± 3.2	94.6
		50	160.2 ± 1.4	88.6
		100	199.4 ± 7.5	83.5

Table 3

The precision of the immunomagnetic bead assay. The intra-assay was performed by running 20 replicates of each sample panel in one analytical run. The inter-assay was evaluated by measuring four food panels in replicates of two at two separate times per day for consecutive days.

Spiked gliadin concentration ($\mu\text{g mL}^{-1}$)	<i>n</i>	Measured gliadin concentration ($\mu\text{g mL}^{-1}$)	CV (%)
Intra-assay	0	0.1 $\pm$ 0.0	7.0
	10	10.3 $\pm$ 1.1	10.6
	100	98.4 $\pm$ 4.9	5.0
	250	250.8 $\pm$ 12.0	4.8
Inter-assay	0	0.2 $\pm$ 0.0	6.1
	10	10.1 $\pm$ 1.0	9.9
	100	105.3 $\pm$ 8.6	8.1
	250	254.5 $\pm$ 9.0	3.5

values of both assays were lower than 15%; the precision of this developed assay was thus deemed satisfactory [51].

3.7. Method comparison

Reliability of this developed IMB–IMLN fluorescent immunoassay was determined by comparing the results of gliadin spiked food samples (pancake mixture) analyzed by the IMB test and an AOAC-approved ELISA kit (RIDASCREEN® Gliadin kit). In this analysis, 64 food samples were spiked with gliadin concentrations ranging from 0 to 400 $\mu\text{g mL}^{-1}$ gliadin and then analyzed by the two methods. An excellent correlation between the ELISA kit and the newly developed IMB assay was found ($y = 1.0009x + 0.1258$, $R^2 = 0.999$). Moreover, the agreement between this developed assay and the ELISA kit was evaluated by using a Bland–Altman plot (Fig. 6), in which the difference between the two measurements per subject is plotted against the mean of the two measurements. In Fig. 6, the X-axis indicates the average of the measured values of the two methods and the Y-axis indicated the differences between the measured values of the two methods. The limits of agreement interval are defined as mean $\pm$ 1.96 SD, and mean and SD are the average and standard deviation of the differences between the two measurements, respectively [52]. According to Fig. 6, the mean of the differences of these two assays is $-0.2 \mu\text{g mL}^{-1}$ and the limits of agreement interval indicated as two dotted lines (-2.3 – $1.8 \mu\text{g mL}^{-1}$) include zero; therefore, no evidence suggested systematic

bias. Only 6.25% (4/64) of the values exceeding the limits of agreement interval were acceptably small. Above results demonstrate a satisfactory agreement between the developed IMB assay and an AOAC-approved ELISA kit.

3.8. Screening of food samples

Effectiveness of the proposed assay in detecting gliadin in commercial food products was assessed. Gliadin-free and wheat containing food products with different brand names were detected. Table 4 summarizes those results. Also, the gliadin concentration was analyzed using the AOAC-approved ELISA kit (RIDASCREEN® Gliadin kit). Comparing the gliadin concentrations determined revealed no statistically significant difference between these two methods for these twenty samples. Therefore, the proposed method is acceptable for determining the amount of gliadin in food samples. Of the twenty food samples, kewpie Japanese millet snack food product, pumpkin snack and sweet potato snack, which were labeled as “allergen-free” yet not certified by any official organization, were verified as gluten-free products by both IMB and ELISA assay, since their gliadin content did not exceed the legal limit for gluten-free foods ($<20 \mu\text{g mL}^{-1}$ gluten, equivalent to $10 \mu\text{g mL}^{-1}$ gliadin), as defined by the U.S. Food and Drug Administration (FDA).

Moreover, the gliadin concentration measured by this developed IMB assay was slightly higher than that by the ELISA kit. This difference might be owing to that the proposed IMB assay uses the polyclonal anti-gliadin IgY as the ELISA uses the monoclonal antibody. Additionally, polyclonal antibodies are often the preferred choice when the nature of the antigen is unknown, because polyclonal antibodies will recognize multiple epitopes of a target and have more tolerance of minor changes in the antigen than monoclonal antibodies, e.g., slight denaturation or hydrolysis. The quantification of gliadin by this sandwich ELISA kit which used monoclonal antibodies as capture and detection antibodies might fail to detect the hydrolyzed gliadin in processed foods and lower than the actual gluten content [53,54]. Therefore, this developed method with polyclonal antibodies could provide a

Table 4

Concentration of gliadin in 20 commercial food samples determined using either the developed IMB fluorescence assay or the commercial ELISA kit approved by AOAC.

Food samples	Gliadin concentration ($\mu\text{g mL}^{-1}$)	
	IMB assay	ELISA kit
Gluten-free products ^a		
All purpose plain flour	3.9 $\pm$ 0.4	3.8 $\pm$ 0.1
Chocolate mousse dessert mix	3.3 $\pm$ 0.3	3.1 $\pm$ 0.9
Chocolate muffin mix	3.4 $\pm$ 1.6	3.4 $\pm$ 0.3
Falafel mix traditional recipe	3.9 $\pm$ 0.5	3.8 $\pm$ 0.4
Kewpie Japanese millet snack	9.6 $\pm$ 0.3	9.1 $\pm$ 0.3
Pancake mix	0.4 $\pm$ 0.1	0.6 $\pm$ 0.0
Pumpkin snack	4.1 $\pm$ 0.2	3.9 $\pm$ 0.2
Skin milk	4.8 $\pm$ 0.3	4.8 $\pm$ 0.0
Spaghetti in tomato sauce	7.8 $\pm$ 0.4	7.8 $\pm$ 0.2
Sweet potato snack	3.6 $\pm$ 0.3	3.3 $\pm$ 0.2
Tomato cream soup	5.1 $\pm$ 0.3	4.8 $\pm$ 0.1
Wild mushroom cream soup	5.0 $\pm$ 0.4	4.8 $\pm$ 0.2
Wheat-containing products		
Corn cream soup	12.1 $\pm$ 0.5	11.6 $\pm$ 0.2
Fortune cookies	34.6 $\pm$ 2.1	33.3 $\pm$ 2.2
Mushroom cream soup	19.4 $\pm$ 0.4	18.7 $\pm$ 0.2
Plain crackers	40.1 $\pm$ 0.2	41.6 $\pm$ 0.9
Saltlets brezel	46.7 $\pm$ 0.9	43.8 $\pm$ 0.9
Seaweed miso soup	39.1 $\pm$ 0.4	38.2 $\pm$ 0.2
Venezia vanilla biscuit	25.9 $\pm$ 0.1	24.8 $\pm$ 0.9
Wheat toast	115.9 $\pm$ 2.0	113.8 $\pm$ 1.0

^a The gluten-free products had the quality label “Controlado por FACE” which guaranteed by the Spanish Federation of Celiac Associations.

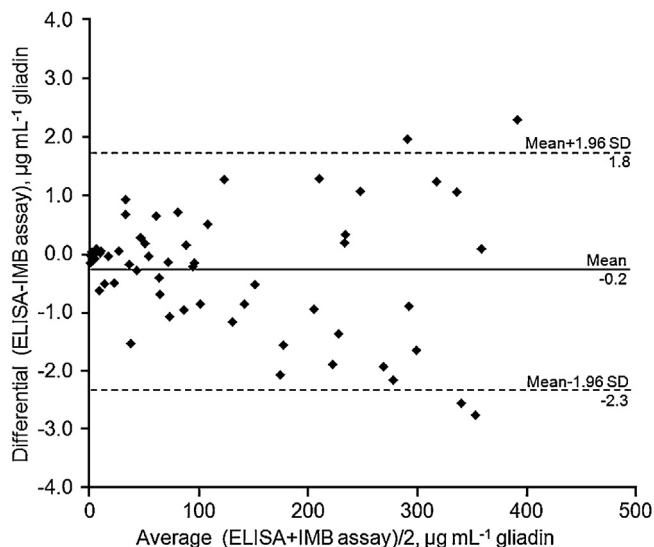


Fig. 6. Bland–Altman plots of a method comparison for food between the developed IMB fluorescence assay and the AOAC-approved ELISA kit. Mean difference (solid line) and limits of agreement (mean $\pm$ 1.96 SD, dashed line) were shown ($n = 64$).

sensitive detection level for gliadin than the sandwich ELISA kit with monoclonal antibodies. Moreover, in order to solve the problem of inaccurate determination in the sandwich method with monoclonal antibodies, a competitive ELISA using R5 monoclonal anti-gliadin antibodies was developed and it was able to accurately assess the gliadin content of hydrolyzed ingredients [55]. This kit is commercially available but has not yet been validated.

4. Conclusions

This work presents an immunoliposomal nanovesicle (IMLN) based on immunomagnetic bead (IMB) assay for detecting gliadin in foods which could produce a specific and selective immune response to the target allergen. Importantly, the developed assay could detect levels of gliadin as low as $0.6 \mu\text{g mL}^{-1}$. Moreover, the sample preparation method in this work also provides excellent recovery rates of spiked gliadin in gliadin-free and gliadin-content food samples, ranging from 83.5 to 102.6%. Additionally, the good repeatability and reproducibility of this assay are ensured by the CV (%) of intra- and inter-assay as 4.8–10.6% and 3.5–9.9%, respectively. Moreover, the performance of the proposed assay is compared with that of an AOAC-approved ELISA kit when analyzing gliadin spiked samples and ten commercial food samples. Analytical results demonstrate that this IMB-IMLN method achieves a satisfactory consistency between these two methods and no false-negative or false-positive results. Conclusively, this developed assay is highly promising for use in investigating gliadin contamination in foods efficiently and reliably.

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