



Rapid detection of fish major allergen parvalbumin using superparamagnetic nanoparticle-based lateral flow immunoassay

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ABSTRACT

A quantitative lateral flow immunoassay (LFIA) was developed based on superparamagnetic nanoparticle (SPMNP) probe for fish major allergen parvalbumin (Pa). The SPMNP probe was prepared by coupling monoclonal antibodies against allergen Pa onto the surface of SPMNP. Dispersibility of the obtained SPMNP probe was analyzed. Nitrocellulose membrane and test line coating concentration were optimized to construct the lateral flow system. Results showed that Sartorius CN 140 membrane, 0.8 mg/mL Pa was suitable for strip construction. Furthermore, a calibration curve with good linearity ($R^2 = 0.9949$) was obtained by plotting magnetic signals against series concentrations of Pa. Signals of the T-line were linear in the range from 0.01 to 100 $\mu\text{g/mL}$ Pa. LODs for qualitative and quantitative detection were 5 $\mu\text{g/mL}$ and 0.046 $\mu\text{g/mL}$, respectively. The average recoveries in clam and peanut matrices ranged from 84.6% to 97.0%, within an acceptable level (80%–120%). 29 food extract samples were separately tested by LFIA and Western Blot assay. Comparative results indicated that the relative consistency between the two methods was 93.1% (27/29). On the other hand, the magnetic signal analysis results indicated that the detection time of the LFIA method was less than 20 min while Western Blot assay typically takes about 5 h. In conclusion, the LFIA method based on SPMNP probe for allergen Pa detection is rapid, specific and simple. It would significantly improve efficiency for large-scale screening and point-of-care detections of allergen Pa.

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1. Introduction

Food allergy is a universal problem which gains more and more attention with the improvement of the living standards. Milk, egg, fish, crustacean shellfish, wheat, soybeans, peanuts and tree nuts are the 8 major allergens listed as the most common causes for food allergy worldwide (FAO, 1995). The Chinese Centre for Disease Control and Prevention (China CDC) conducted a prevalence study on food allergy situation of college students, and the statistic data indicated that 6% of the healthy Chinese people aged between 15 and 24 are allergic to aquatic products, dairy products and eggs, which is higher than the international average level (Lü & Yang, 2005).

Once exposed to the allergen (fish and fish products), the allergic individual can suffer a mild to life-threatening reaction, such as shortness of breath, difficulty swallowing, sudden onset of vomiting, diarrhoea cramps, urticaria, loss of consciousness, or even death in severe cases. Extensive researches show that the

major allergen in a variety of fishes (cod, tuna, mackerel, carp, etc.) is parvalbumin (Pa) (Bugajska-Schretter et al., 2000; Van Do, Hordvik, Endresen, & Elsayed, 2005), which is a water-soluble calcium-binding albumin protein with low molecular weight (typically 9–14 kD). Pa has already been separated by researchers from the muscle tissue of fish (Hamada et al., 2003; Van Do, Elsayed, Florvaag, Hordvik, & Endresen, 2005), frog (Hilger et al., 2004), chicken (Heizmann & Strehler, 1979) and rabbit (Capony, Pina, & Pechere, 1976). New methods for Pa detection have been reported in recent years, including PCR (Hildebrandt & Garber, 2010), cytochemical detection (Härtig, Brückner, Brauer, Seeger, & Bigl, 1996) and various immunoassays such as immunoblotting (Beale, Jeebhay, & Lopata, 2009), enzyme-linked immunosorbent assay (ELISA) (Fæste & Plassen, 2008), and surface plasmon resonance (SPR) biosensor (Lu, Ohshima, & Ushio, 2004, pp. C652–C658). PCR-based detection method is highly specific and sensitive because its detection principle is based on the amplification of a known certain DNA of allergen. However, this technology could increase the probability of false positive results because it can detect the target DNA even if the related proteins are not existed in samples. ELISA and SPR biosensor assay are time-consuming and labour-intensive as they require advanced

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equipment and professional operating techniques. Therefore, a rapid testing methodology for the presence of Pa in food samples appears particularly essential. A lateral flow immunoassay (LFIA), also called the “sol particle immunoassay (SPIA)” or the immuno-chromatographic assay, has thrived in the past decade because of its simple operation and convenient result display (Cui et al., 2008; Peng et al., 2007). Furthermore, a new labelling material – super-paramagnetic nanoparticle (SPMNPs) has been developed in recent few years. It provides an alternative to the traditional labelling materials such as colloidal gold, latex, selenium, carbon and liposomes (Wang et al., 2009). Compared with traditional lateral flow strips which utilize colour intensity to determine the signal strength, SPMNPs-based lateral flow assay can realize quantitative measurement through reading the magnetic signals by a Magnetic Assay Reader (MAR) system (Workman et al., 2009). The magnetic signals provided by the SPMNPs were quite stable, making it possible to recheck the testing results (Xu et al., 2009).

In order to develop a rapid, simple and highly specific detection method for fish major allergen Pa, monoclonal antibody (McAb) against fish major allergen Pa was firstly immobilized on the surface of the SPMNP to obtain a Pa specific SPMNP probe in this study. Then a competitive format LFIA labelled by SPMNP probe was developed. Precisions of the SPMNP probe-labelled LFIA for allergen Pa detection were also discussed.

2. Material and methods

2.1. Reagents and apparatus

Disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium tetraborate, boric acid, and calcium chloride were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Sodium azide, D-(+)-Trehalose dehydrate, sucrose, Triton X-100, Tween-20, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), 2-[N-morpholino] ethane sulfonic acid (MES), and bovine serum albumin (BSA, Albumin Fraction V), phenylmethyl sulfonyl fluoride (PMSF) and Tris base were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). All the solutions were prepared with distilled water. A MAR Assay Development System was purchased from Magna Bioscience (LLC, USA) for magnetic signal reading in this experiment. All the materials for strips construction, including SPMNPs (carboxyl group modified, 200 nm), absorbent pad, conjugate pad, support sheet, top cover film and test cassettes were provided by the MAR Assay Development System. Four kinds of nitrocellulose membranes were used in this research: CN 140 (Sartorius Stedim Biotech, Germany), HF 135 (Millipore corporation, USA), Prima 40 (Whatman, England), and FP (Whatman, England). Trichloroacetic acid (TCA) was purchased from Hongyan Chemical Regent Co., Ltd. (Tianjin, China). Goat anti-mouse secondary antibody (GAM) was purchased from Longene Biotech Co., Ltd. (Hangzhou, China).

Live aquatic specimens were used for food sample detection, including crucian carp (*Carassius carassius*), common carp (*Cyprinus carpio*), tilapia (*Tilapia nilotica*), cod (*Gadus macrocephalus*), cutlassfish (*Lepidopus caudatus*), big eye tuna (*Thunnus obesus*), Large yellow croaker (*Larimichthys crocea*), mangrove crab (*Scylla serrata*), Chinese white shrimp (*Penaeus chinensis*), Pacific white shrimp (*Penaeus vannamei*), American lobster (*Homarus americanus*), abalone (*Haliotis discus*), white clam (*Macrura veneriformis*), Pacific razor clam (*Siliqua patula*), common scallop (*Argopecten irradians*), Peru squid (*Dosidicus gigas*), and common octopus (*Octopus vulgaris*). They were purchased from TESCO supermarket in Shanghai. Duck, goose, chicken, pork, milk, egg, peanut, oat, and soybean were also purchased from TESCO supermarket in Shanghai. White

croaker (*Argyrosomus argentatus*) surimi and sea robin (*Lepidotrigla micropterus*) surimi were purchased from Zhoushan Xingye industrial group Co., Ltd. (Zhejiang, China). Fugu (*Takifugu obscurus*) was purchased from Zhongyang group Co., Ltd (Jiangsu, China).

2.2. Allergen and antibodies

EG8 McAb against fish major allergen Pa employed in this study was prepared and characterized in our previous work (Kawase, Ushio, Ohshima, Yamanaka, & Fukuda, 2001). Firstly, EG8 hybridomas were intraperitoneally injected to BALB/c mice to prepare ascites. The ascites were then purified by a protein G affinity chromatography column (GE Healthcare, USA) according to the product's operation manual. Purified EG8 McAbs were freeze-dried, re-dissolved in distilled water and quantified by Bradford protein assay (Bradford, 1976). GAM secondary antibody was diluted with deionised water to 1 mg/mL.

Pa from crucian carp muscular tissue was purified by TCA method. First, carp muscle was homogenized with five volumes of extraction buffer (w/v, 10 mM CaCl₂, 100 mM PMSF, 10 mM Tris–HCl, pH 7.5). The mixture was then centrifuged twice at 10,000 rpm for 20 min. Next, 100% TCA was added to supernatant slowly until final TCA concentration reached 3%. After the mixture being stirred at 4 °C for 10 min, 6 N NaOH was used to adjust pH value to 5.2. Then the solution was stirred gently at 4 °C for 1 h. The supernatant was obtained by centrifugation at 8000 rpm for 20 min. 3% TCA precipitate procedure was repeated. Finally, the obtained precipitate was dissolved in distilled water and quantified by Bradford method (Bradford, 1976). All the extraction procedures were carried out at 0–4 °C.

2.3. Preparation of Pa specific SPMNP probe

2 mg carboxyl-modified SPMNPs were firstly washed by MEST buffer (0.01 mol/L, pH5.0, containing 0.05% (v/v) Tween-20) for three times. Then 2 µL EDC (0.5 g/mL) and 4 µL NHS (0.25 g/mL) were mixed with the SPMNPs suspension (diluted to 500 µL), and reacted with SPMNPs for 30 min. After the SPMNPs was washed with BST buffer (0.005 mol/L, pH 9.0, containing 0.05% (v/v) Tween-20) for three times, 150 µg EG8 McAbs were added into SPMNPs suspension and reacted for 3 h at room temperature. When the coupling reaction was finished, the supernatant was collected for protein quantification, and the precipitate was washed with BST buffer for three times. Next, 1% BSA was added into the SPMNPs suspension to block the active sites of the nanoparticles through physical adsorption. After reacting for 30 min, the obtained specific SPMNPs probes were finally dispersed in the conservation solution (pH 9.0, 0.005 mol/L BST, 0.05% Tween-20, 1% NaN₃, 0.1% BSA), and stored at 4 °C.

Protein quantification was performed using the Bradford method. The content of EG8 antibody contained in the supernatant was detected before and after conjugation to measure the coupling rate. In order to demonstrate the change of particle size and dispersive property, 2 µL SPMNPs suspension collected from each conjugating procedure were diluted to 1 mL with PBS for particle size and PDI index detection.

2.4. Construction of SPMNPs probe-based lateral flow system for Pa detection

2.4.1. Lateral flow strips assembling

The main body of the test strip consists of five parts, including support sheet, sample pad, conjugate pad, wick pad and NC membrane, on which the test line and control line were sprayed (Fig. 1). Competitive format was adopted to establish the Pa test

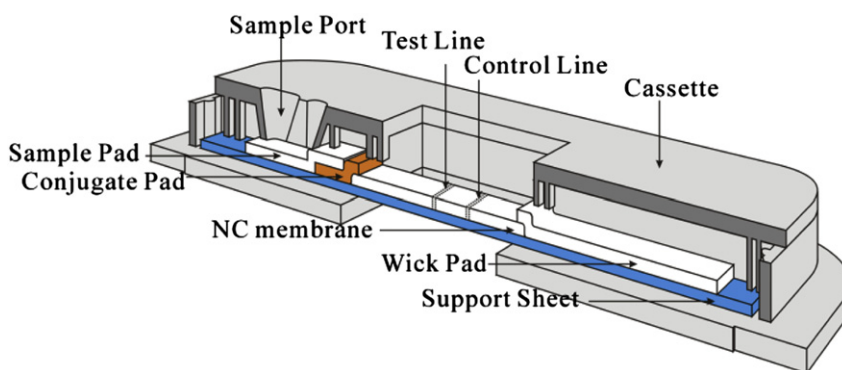


Fig. 1. Schematic view of a SPMNPs probe-based lateral flow test strip.

strips. Pa and GAM at proper concentration were firstly striped onto nitrocellulose (NC) membrane at the rate of $0.8 \mu\text{L}/\text{cm}$, respectively as T-line and C-line. Next, the NC membrane was dried at 35°C overnight. Striped NC membrane ($300 \text{ mm} \times 25 \text{ mm}$), conjugate pad ($300 \text{ mm} \times 10 \text{ mm}$), treated sample pad ($300 \text{ mm} \times 20 \text{ mm}$), and wick pad ($300 \text{ mm} \times 25 \text{ mm}$) were then pasted onto the base card ($300 \text{ mm} \times 100 \text{ mm}$). Finally, a transparent top cover was pasted on the membrane as a protection sheet. The assembled system was cut into 5 mm-wide strips for detection.

2.4.2. Lateral flow test procedure

$4 \mu\text{L}$ SPMNPs probe suspension was applied on the conjugate pad. $100 \mu\text{L}$ sample solution was added on the sample pad for detection. After chromatography for 20 min at room temperature, magnetic signals at T-line and C-line were analyzed by a MAR system.

2.4.3. Optimization of the LFIA system

NC membrane: Four kinds of NC membranes (Sartorius CN 140, Millipore HF 135, Whatman Prima 40 and Whatman FP) from different manufacturers were respectively tested according to the procedure described in 2.4.2. In this investigation, C-line was coated with $1 \text{ mg}/\text{mL}$ GAM, and T-line was coated with $0.8 \text{ mg}/\text{mL}$ Pa. 10 mM PBS buffer was used as negative sample. Followed by chromatography for 20 min, magnetic signals at T-line were measured.

T-line: A series of purified Pa ($0.05, 0.1, 0.2, 0.4, 0.8, 1.6 \text{ mg}/\text{mL}$) were immobilized in T-line. PBS was applied as negative sample and $100 \mu\text{g}/\text{mL}$ Pa solution was used as positive sample. Test procedure was operated according to 2.4.2. After chromatography for 20 min, magnetic signals at T-line of each strip were measured.

2.5. Test time of the LFIA system

Negative and positive samples used were PBS and $1 \mu\text{g}/\text{mL}$ purified Pa solution, respectively. The LFIA system was prepared according to 2.4.1, where T-line was coated with $0.8 \text{ mg}/\text{mL}$ Pa, and C-line was coated with $1 \text{ mg}/\text{mL}$ GAM. Magnetic signals at T-line were measured every minute for 30 min during the chromatography.

2.6. Calibration curve of the LFIA system

The purified Pa was serially diluted to $0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 \mu\text{g}/\text{mL}$ with 10 mM PBS. The LFIA system was prepared according to 2.4.1, where T-line was coated with $0.8 \text{ mg}/\text{mL}$ Pa, and C-line was coated with $1 \text{ mg}/\text{mL}$ GAM. Quantitative analysis was performed by MAR system. Five parallel specimens were tested for each standard Pa solution. Signal values of different samples were marked and fitted by a logarithmic function.

2.7. Stability of the magnetic signal of the LFIA system

In this investigation, series of purified Pa ($0.01, 0.1, 1.0, 10, 100 \mu\text{g}/\text{mL}$) were applied as positive samples, and 10 mM PBS was used as negative sample. The LFIA system was prepared according to 2.4.1, where T-line was coated with $0.8 \text{ mg}/\text{mL}$ Pa, and C-line was coated with $1 \text{ mg}/\text{mL}$ GAM. Test procedure was operated according to 2.4.2. Five specimens were tested in parallel. After the sample flowed for 20 min, magnetic signals at T-lines were measured by MAR system. Next, all the strips were stored at 60°C for 72 h and the magnetic signals at T-lines were measured again. Then, the strips were incubated at 60°C for another 72 h and the magnetic signals at T-lines were measured for the third time.

2.8. LFIA detection of Pa spiked food extracts

5 g food samples (pork, clam and peanut) were homogenized with 40 mL 10 mM PBS, then the solutions were boiled for 15 min. Followed by filtration, the obtained precipitate samples were centrifuged at $10,000 \text{ rpm}$ for 15 min. The supernatants collected were used as food extract samples. The purified Pa was diluted to serial concentrations ($0.1, 1, 10 \mu\text{g}/\text{mL}$) with different food extractions. Food extractions without Pa were used as negative controls. The LFIA system was prepared according to 2.4.1. Five parallel specimens were tested for each sample. Magnetic signals at T-lines were analyzed by MAR system.

2.9. Allergen detection by LFIA

Food extracts including crucian carp, common carp, tilapia, cod, cutlassfish, big eye tuna, large yellow croaker, fugu, white croaker, sea robin, mangrove crab, Chinese white shrimp, Pacific white shrimp, American lobster, abalone, white clam, Pacific razor clam, common scallop, Peru squid, common octopus, duck, goose, chicken, pork, milk, egg, peanut, oat, and soybean were prepared as procedures described in 2.8. Sample test was performed in accordance to 2.4.2. Five parallels were tested for each sample. Magnetic signals of the T-lines were analyzed by MAR system.

2.10. SDS-PAGE and Western blot

All the food samples tested by the LFIA were simultaneously detected by the following western blot assay. 14% acrylamide were used to prepare the separation gel of SDS-PAGE. $5 \mu\text{L}$ protein of each food sample was loaded to the wells of gel. After electrophoresis with a Bio-Rad Mini Protein II system, the samples in gel were transferred onto PVDF membrane using the method described by Towbin, Staehelin, and Gordon (1979). $2 \mu\text{g}/\text{mL}$ purified anti-Pa MAb was used as the primary antibody. Goat anti-mouse IgG-HRP

(1:5,000, v/v) and 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate were used for specific detection.

2.11. Judgement of the LFIA result

In order to judge the positive result detected by the LFIA method, a percent positive value is set by the following formula:

$$\%P_0 = (T_0 - 3S.D.) / T_0 \times 100\% \quad (1)$$

$$\%P = T / T_0 \times 100\% \quad (2)$$

where T_0 is the average magnetic signal at T-line of negative control sample, T is the average magnetic signal at T-line of the tested sample. The LFIA results from tested samples are classified into three levels:

“Negative”: $P > P_0$;

“Positive”: $P < 50\%$;

“Weakly positive”: $50\% \leq P \leq P_0$.

3. Results and discussion

3.1. Characterization of SPMNPs dispersibility

The changes in hydrodynamic size and polydispersity index (PDI) of SPMNPs before, after coupling and blocking were analyzed. The hydrodynamic size increased slightly from 225.7 nm to 231.4 nm after conjugation. However, PDI varied little before and after conjugation, suggesting good dispersion of the SPMNPs. Under the coupling condition described in 2.3, the couple amount of EG8 antibody was 41.5 $\mu\text{g}/\text{mg}$ SPMNPs. The increasing of nanoparticle size was probably due to the coupling of McAb and adsorption of BSA proteins.

Good dispersibility guarantees that SPMNPs would not gather together to form larger particles during the probe preparation. Large particles have adverse effect on for chromatography procedures because they might slow down the lateral flow speed or even block the pores in NC membrane.

3.2. Optimization of NC membrane

Magnetic signals at T-lines on 4 NC membranes were compared in Fig. 2. Magnetic signals of strips constructed by FP membrane were the highest, while magnetic signals of strips constructed by Prima 40 membrane were the lowest. However, detection time based on FP membrane was more than 45 min. Signals from strips based on CN 140 were weaker than those of FP, but detection time was less than 20 min. With consideration both of the test time and magnetic signal, CN 140 was selected as the most suitable NC membrane for strip preparation in the following experiments.

3.3. Optimization of Pa coating concentration at T-line

The LFIA results of negative (Fig. 3a) and positive samples at 100 $\mu\text{g}/\text{mL}$ (Fig. 3b) from strips with different T-line coating concentration displayed remarkable differences. With the increasing of Pa coating concentration (0.05–1.6 mg/mL), T-lines from negative samples changed from invisible to clearly visible (Fig. 3a). Magnetic signal values were listed in Table 1, where the signal from strips with T-line at 1.6 mg/mL showed the highest value. Quantification data of negative samples were consistent with the results of Fig. 3a. When 100 $\mu\text{g}/\text{mL}$ Pa samples were tested, 1.6 mg/mL Pa coating strips displayed a visible band at T-line, while 0.8 mg/mL Pa coating strips did not illustrate visible band at T-line (Fig. 3b).

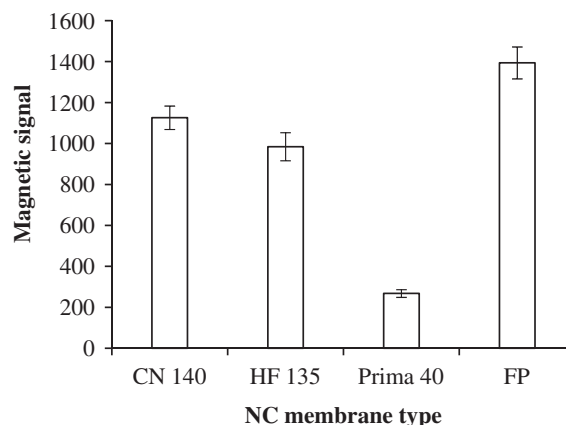


Fig. 2. Magnetic signals at T-line on different NC membranes. Magnetic signals of four kinds of nitrocellulose membranes (CN 140, HF 135, Prima 40 and FP) were detected by testing PBS buffer as negative samples.

Normally, the qualitative sensitivity of strips was judged based on whether T-line gave visible bands. According to this criterion, visual qualitative sensitivity of strips with T-line at 1.6 mg/mL was significantly inferior to those strips with T-line coating where the Pa concentration was lower than 1.6 mg/mL. Therefore, comprehensively considering the assay sensitivity and magnetic signal values for negative samples, 0.8 mg/mL was selected as the optimal Pa coating concentration at T-line.

3.4. Detection time of LFIA system

Based on the time-magnetic signal curves of negative and positive samples (data not shown), the magnetic signal of negative sample increased rapidly during the first 8 min. Between 8 and 15 min, the signal decreased down and finally entered into a plateau after 16 min. For the positive sample (Pa: 1 $\mu\text{g}/\text{mL}$), the signal rose up dramatically at first, and reached a peak point. Then, it declined to an equilibrium position at 14 min. During the increase stage of magnetic signal, SPMNP probes on the conjugate pad moved towards the T-line, and a number of nanoparticles were captured by the Pa sprayed at T-line, which caused an increase of magnetic signal. Due to the capillary action, some beads might leave the T-line, which caused a downward signal phenomenon. When the amount of SPMNPs captured by the Pa at T-line reached equilibrium, the magnetic signal became stable at last. After the sample flowed for 20 min, signals of both lines became stable. Therefore, the detection time of the LFIA system for Pa detection was considered as 20 min.

3.5. Calibration curve for Pa detection

A series of Pa samples were tested by the LFIA system to obtain a standard curve. As shown in Fig. 4a, it was obvious that the colour of T-line lightened with the increasing of Pa sample concentration. The colour of the T-line from 5 $\mu\text{g}/\text{mL}$ Pa samples (Fig. 4a, strip 8) changed obviously when compared with the control sample. Therefore, the qualitative limit of the LFIA system for Pa was set at 5 $\mu\text{g}/\text{mL}$.

MAR quantitative detection data was illustrated in Fig. 4b. There was an obvious decline in magnetic signals at T-line with the increasing of Pa concentration. A standard curve for Pa was plotted by the signals at different Pa concentrations. It showed good linearity in the range between 0.01 and 100 $\mu\text{g}/\text{mL}$ Pa with a correlation coefficient of 0.9949. LOD of quantitative detection was calculated by substituting x_L into standard curve formula. The value of x_L was given by the following equation

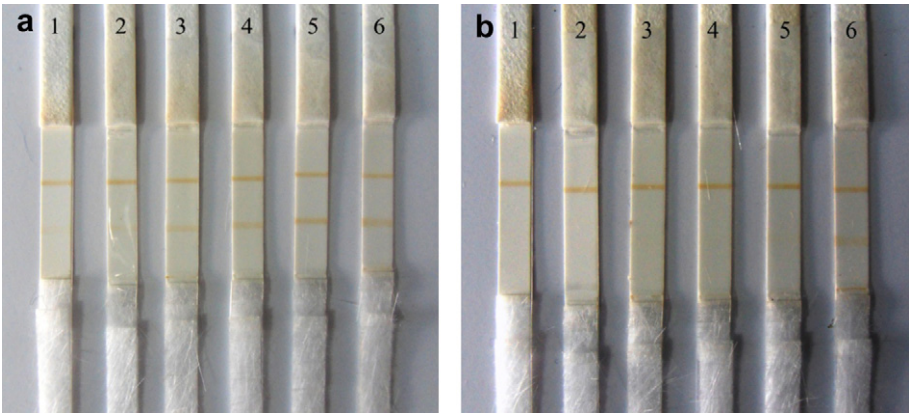


Fig. 3. Chromatographic photo of strips with different Pa coating concentration at T-lines. (a) Negative sample (PBS) was detected using strips with different Pa coating concentration at T-lines. 1: 0.05 mg/mL Pa coated T-line; 2: 0.1 mg/mL Pa coated T-line; 3: 0.2 mg/mL Pa coated T-line; 4: 0.4 mg/mL Pa coated T-line; 5: 0.8 mg/mL Pa coated T-line; 6: 1.6 mg/mL Pa coated T-line. (b) Positive sample (100 µg/mL Pa) was detected using strips with different Pa coating concentration at T-lines. 1: 0.05 mg/mL Pa coated T-line; 2: 0.1 mg/mL Pa coated T-line; 3: 0.2 mg/mL Pa coated T-line; 4: 0.4 mg/mL Pa coated T-line; 5: 0.8 mg/mL Pa coated T-line; 6: 1.6 mg/mL Pa coated T-line.

$$x_L = x_{b1} - ks_{b1} \tag{3}$$

where x_{b1} is the mean of the blank measures ($x_{b1} = 1086.0$, 10 replicates), s_{b1} is the standard deviation of the blank measures ($s_{b1} = 71.00$), and k is a numerical factor chosen according to the confidence level desired ($k = 3$). According to the calculation result, LOD of the calibration curve was 0.046 µg/mL.

3.6. Evaluation of magnetic signal stability

In order to evaluate the stability of magnetic signals of the LFIA system, the same tested strips under different store conditions were measured for 3 times. Signals of Pa negative sample and each dilution of Pa positive samples (0.01–100 µg/mL) were almost the same, showing that the signals of the LFIA system were very stable even when the strips were incubated twice at 60 °C for 72 h. It was reported that the stability of magnetic signals at 25 °C for one year is approximately equivalent to that of 60 °C for 3 days according to the empirical Arrhenius equation (Wang et al., 2009). Therefore, our LFIA system for Pa could be stored at 25 °C for about 2 years.

3.7. Accuracy evaluation of the LFIA system

As ingredients in food are more complicated than the PBS buffer, Pa recovery rates in three food matrices (pork, clam, peanut) were

calculated to evaluate the influence of food matrix on detection of target analyte. As shown in Table 2, the recoveries with the standard error in the clam and peanut matrices were 76.57%–105.99%. Therefore, the recovery rate with the standard error limits of the LFIA method was set to be 100% ± 24%, namely, the total error of the LFIA method was 24%. The average recoveries in clam and peanut matrices ranged from 84.6% to 97.0%, within an acceptable level (80%–120%). Recovery rates in pork matrix appeared higher than those in other samples. In addition, recovery rates at three levels of spiked Pa (low, mid, high) were all above 100%, showing an increasing trend with the increased amount of spiked Pa. Based on

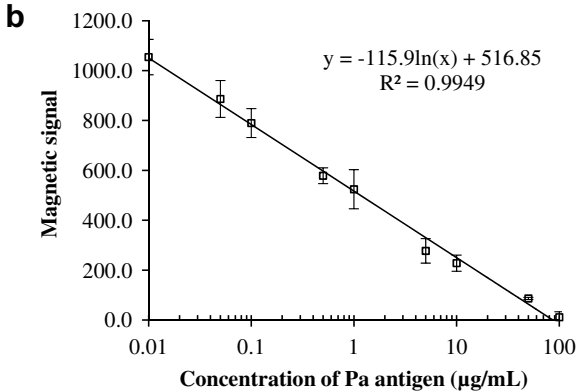
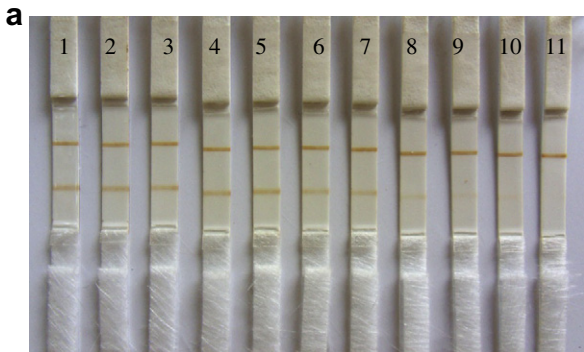


Table 1
Selecting of Pa coating concentration at T-line. The LFIA strips coated with 6 levels of Pa (0.05, 0.1, 0.2, 0.4, 0.8, 1.6 mg/mL) at T-line were tested by negative sample (PBS) and positive sample (100 µg/mL). Magnetic signals at T-lines were read by MAR system.

No.	Coated Pa concentration (mg/mL)	Magnetic signal (MS)	
		Pa concentration (µg/mL)	
		0	100
1	0.05	Average	68.4
		CV (%)	7.2
2	0.1	Average	187.6
		CV	5.3
3	0.2	Average	298.8
		CV	8.0
4	0.4	Average	769.6
		CV	4.5
5	0.8	Average	1089.6
		CV	2.5
6	1.6	Average	1155.1
		CV	3.2

Fig. 4. Calibration curve for Pa detection by the LFIA. (a) Qualitative results of Pa standard solutions detected by LFIA. 1: negative (PBS); 2: 0.005 µg/mL Pa; 3: 0.01 µg/mL Pa; 4: 0.05 µg/mL Pa; 5: 0.1 µg/mL Pa; 6: 0.5 µg/mL Pa; 7: 1 µg/mL Pa; 8: 5 µg/mL Pa; 9: 10 µg/mL Pa; 10: 50 µg/mL Pa; 11: 100 µg/mL Pa. (b) Calibration curve of Pa standard solutions detected by LFIA. The x-axis is expressed on logarithm.

Table 2

Recovery rates obtained from different food matrices. Low (0.1 µg/mL), medium (10 µg/mL) and high (100 µg/mL) level of Pa in pork, clam and peanut extracts were tested by the LFIA strips. Quantification of Pa was obtained by the calibration curve obtained in Fig. 4(b).

Sample	Measured value (MAR)	Pa calculated value (µg/mL)	CV (%)	Recovery rate (%)
Food extracts	Pa (µg/mL)			
Pork	0	956.3	0.02	5.12
	0.1	763.5	0.12	8.24
	1	488.3	1.28	5.99
	10	210.2	14.09	9.20
Clam	0	1105.9	0.01	8.91
	0.1	787.6	0.10	8.61
	1	520.3	0.97	8.99
	10	256.0	9.50	6.45
Peanut	0	1158.8	0.00	5.18
	0.1	792.2	0.09	8.78
	1	523.0	0.95	6.39
	10	269.4	8.46	8.03

our previous study, the EG8 McAb was cross-reacted with some vertebrate Pa such as frog (Lu et al., 2004). Herein, higher recovery rates in pork samples implied that the LFIA system might have false positive reactions to pork matrix. Further research is necessary to demonstrate it in the future.

3.8. Specificity of the LFIA system

In order to evaluate the specificity of the LFIA system, 29 food extracts were tested using the LFIA and Western blot method. Results were listed in Table 3.

Table 3

Comparison of 29 food samples tested by the LFIA and Western blot methods.

Food sample types	Sample names	Western blot ^a	LFIA ^b	% P value for LFIA
Fish (10)	Crucian carp	+	+	1.5
	Common carp	+	+	3.2
	Tilapia	+	+	2.4
	Cod	+	+	6.2
	Cutlassfish	+	+	4.4
	Big eye tuna	+	+	2.7
	Large yellow croaker	+	+	9.7
	White croaker	+	+	28.4
	Sea robin	+	+	44.2
	Fugu	±	±	72.5
Crustacean and molluscs (10)	Mangrove crab	–	–	87.2
	Chinese white shrimp	–	–	82.3
	Pacific white shrimp ^c	–	±	73.0
	American lobster	–	–	86.8
	Abalone	–	–	91.7
	White clam	–	–	98.8
	Pacific razor clam	–	–	101.4
	Common scallop	–	–	100.9
	Peru squid	–	–	88.6
	Common octopus	–	–	84.8
Myofibrillar protein samples of vertebrates (4)	Duck	–	–	88.7
	Goose	–	–	84.5
	Chicken	–	–	95.6
	Pork ^c	+	±	66.6
Food allergens (5)	Milk	–	–	99.6
	Egg	–	–	92.4
	Peanut	–	–	103.4
	Oat	–	–	93.2
	Soybean	–	–	87.7

^a For the Western blot result of food sample test: “–” represents “negative”; “+” represents “positive”; “±” represents “weakly positive”.

^b P_0 value was 80.40; for LFIA result of food sample test, “–” represents “ $P > P_0$, negative”; “+” represents “ $P < 50\%$, positive”; “±” represents “ $50\% \leq P \leq P_0$, weakly positive”.

^c The result of western blot was not in accordance with LFIA.

As shown in Table 3, 10 fish samples, containing fresh water fish and marine fish, showed good conformity in both LFIA and Western blot assays. Among them, %P values of robin and white croaker were higher than those of other fish samples. These two samples were also positively detected by the Western blot assay. As these two samples were prepared from surimi, we suspected that the ingredients of surimi might contain traces of Pa or its hydrolysate. For the fugu sample, both assays showed weak positive results. For the 10 crustaceans and mollusk species samples, only white prawn showed different results between LFIA and Western blot assay. For the tested 20 aquatic food samples, the consistency between LFIA method and Western Blot assay was 95.0% (19/20).

Overall, false positive results were obtained when the LFIA strips were used to test white prawn and pork muscle protein extraction, the relative consistency between the LFIA and Western blot assays was 93.1% (27/29). Our results indicated that the LFIA could be applied to detect fish major allergen Pa. However, pork and white prawn materials or their ingredients should be avoided.

4. Conclusions

In this paper a competitive LFIA based on SPMNPs probe for fish major allergen Pa has been developed. Optimization of NC membrane type and T-line was also described. The use of SPMNPs and MAR system realized a rapid quantitative detection for Pa, which require little sample manipulation and simple operation. The qualitative and quantitative results of the LFIA can be obtained within 20 min. Furthermore, this method was applicable to different matrices (clam and peanut) and allows for the detection of Pa of different food materials. Our research indicates that the LFIA method has good potential applications in areas such as large-scale screening or point-of-care detections for fish major allergen Pa.

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