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An accurate and amplifying competitive lateral flow immunoassay for the sensitive detection of haptens

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ABSTRACT

We report a new competitive lateral flow immunoassay (LFIA) for hapten detection, which uses antibodies to bind antigens from a sample and form a coloured immune complex due to artificial antigens conjugated with gold nanoparticles; these were intercepted with SPA. Here, 2,4-dichlorophenoxyacetic acid (2,4-D) and gentamicin (GM) were selected as model analytes. The visual limit of detection of the 2,4-D strip was 25 ng/mL in tap water; the GM strip was 3.13 ng/mL in milk. This approach exhibits more than a 40-fold lower detection limit for 2,4-D and 3.2-fold lower for GM, which is compared to traditional LFIA using the same reagents. This approach can accurately control the amount of antibody, protect the activity of antibody and enhance the detection signal, thus improving the sensitivity of the LFIA and providing more and more sensitive methods for the development of LFIAs.

Highlights

- This study developed a new method of LFIA that could improve the sensitivity of LFIA without adding any reagent.
- The antibodies are unlabelled; thus, the immunological properties of the antibodies are not destroyed by antibody folding during the labelling process.
- Sufficient gold-labelled antigens ensure that each antibody bound to the test line can combine two gold nanoparticles, thereby amplifying the overall signal.

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Sensitive enhancement; competitive lateral flow immunoassay; test strip; GM; 2,4-d

1. Introduction

2,4-D is one of the most successful and commonly used herbicides in modern agriculture to control the growth of broad-leaved weeds (Smith et al., 2017). The presence of residues

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of 2,4-D in agricultural products, air, water, soil can be extremely harmful to both humans and animals (Wu et al., 2005; Zhang et al., 2016). Exposure to 2,4-D elicits a variety of toxicities in rodents such as genotoxicity (Madrigal-Bujaidar et al., 2001), hepatotoxicity (Tayeb et al., 2010), neurotoxicity (Bortolozzi et al., 2001) and renal toxicity (Uyanikgil et al., 2009). As such, 2,4-D residues have gained significant attention (Wang et al., 2017). As a “probable carcinogenic compound to humans” (Group 2B), 2,4-D has been listed as a drinking water contaminant with a critical level of 0.07 mg/L by the Environmental Protection Agency (EPA). The European Union has established that concentrations of pesticides in drinking water may not exceed 0.1 µg/L or 0.1 ppb for a single pesticide and 0.5 µg/L or 0.5 ppb for total pesticides (Fusco et al., 2016). The World Health Organization (WHO) established maximum residue limits of 2,4-D to be no more than 30 µg/L in the Guidelines for Drinking-Water Quality: Fourth Edition (Organization, 2003). Hence, there is an urgent need for highly sensitive, simple and accurate detection methods.

Beyond herbicides, antibiotics are another common toxin. Aminoglycosides are a class of antibiotics that consist of two or more amino-sugars linked by glycosidic bonds to an aminocyclitol scaffold. Gentamicin is an aminoglycoside that has been extensively used in both human and veterinary medicine to treat various Gram-positive and Gram-negative bacterial infections (NV et al., 2016). GM interferes with bacterial protein synthesis. Moreover, the residual amount of GM in the foodstuff may lead to antibiotic resistance from the pathogenic bacterial strains and cause ototoxicity, nephrotoxicity, and neuromuscular blockage (McKinney et al., 2015; Mohamadi Yarijani et al., 2019). Thus, monitoring GM drug residues in food is mandatory in many countries; the United States has set an MRL of 30 µg/L in milk (Gukowsky et al., 2012).

Lateral flow immunoassay (LFIA) is a common technique for both GM and 2,4-D. LFIA uses a cellulose membrane as the carrier and a labelled antigen or antibody as a tracer. This technique has several advantages over traditional immunoassays: simplicity of the procedure, rapid operation, detection via the naked eye, low cost, and no need for a skilled technician or expensive equipment (Fu et al., 2011; Zhang et al., 2009). In the past decade, LFIA has been applied in many fields such as medicine (Liang et al., 2020), agriculture (Yang et al., 2019), and food quality control (Sun et al., 2014). In the field of food quality control, competitive LFIA is most widely applied for the detection of low molecular weight compounds. A standard competitive LFIA detects the distribution of nanoparticle-labelled antibodies between a free antigen of the tested sample and the antigen immobilized on the surface of the working membrane. Unfortunately, the sensitivity of LFIA still requires improvement in many applications.

The poor detection limits can be explained by three factors. First, in the colloidal gold labelling antibody process, the optimal ratio between the reagents is determined from the flocculation curve (Hermanson, 2007), and the antibodies are present in significant excess (from tens to hundreds of molecules per colloidal particle). They stabilize the surface of the colloid particle, but their immunological properties can change because antibody folding upon binding to the surface. In an ideal scenario, there would be an equimolar ratio of antibodies with respect to the colloidal label. However, such conjugation is methodologically challenging (Urusov et al., 2014). The second limitation is that the ability of the artificial antigen to bind antibody influences signal of test line. Thus, the concentration of the labelled antibodies should be high to increase the

signal. However, increasing the concentration of antibodies also leads to the need for more antigens in the sample, which in turn will adversely affect the detection limit. Third, self-amplification of detection signal cannot be realized.

Urusov et al. (Urusov et al., 2016; Urusov et al., 2014) proposed a new method of competitive LFIA that uses gold nanoparticle-labelled secondary antibodies to improve the sensitivity of an immunochromatographic assay. This method enables precise adjustment of the required quantities of antibodies and the colloidal gold marker. However, the test strip also has other two factors.

The objective of the current study was to develop a new method of LFIA that could solve the three factors described above and amplify the signal without adding any reagent. In the new method, antibodies were dried free at the bottom of the sample cups, the competing substances are a test sample containing an antigen molecule, and a test strip with the gold nanoparticle-labelled antigen in the binding pad of the strip. Antigen molecules that are in the sample will bind antibodies first. This results in fewer unreacted antibodies and gold nanoparticle-labelled antigen available to bind to the SPA/immobilized anti-antibodies in the test line. The combination of antibodies and artificial antigen conjugated to gold nanoparticles is used rather than antibodies conjugated to gold nanoparticles. This approach ensures that the immunological properties of the antibodies will not be destroyed without labelling and gold-labelled antigen is polymerized under the action of antibody, which increases the magnitude of the detected signal and does not affect the detection limit.

Herein, a new competitive lateral flow immunoassay was established by employing 2,4-dichlorophenoxyacetic acid (2,4-D) and gentamicin (GM) as the model analytes. 2,4-D was selected because it has good results in the competitive enzyme-linked immunosorbent assay (ELISA) (Gong et al., 2016) but it cannot provide the required detection limit in traditional competitive LFIA (Figure 3(C)). We found that the ability of the artificial antigen to bind antibody is the main factor underlying this observation. Traditional competitive LFIA for GM was successfully developed, and with a high affinity between the artificial antigen and antibody (Sun et al., 2018). Thus, GM was selected as the contrast test antigen to further expound the validity of the new method.

2. Material and methods

2.1. Chemicals and instrumentation

IgG-free/protease-free bovine serum albumin (BSA) was purchased from Jackson (West Grove, PA). 2,4-D and GM were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Casein, trehalose, and gold chloride trihydrate were purchased from Sigma Aldrich (St Louis, MO, USA). Staphylococcal protein A (SPA) and Tween-20 were purchased from Merck & Co. Inc.

Anti-2,4-D mouse monoclonal antibodies were prepared in Henan Provincial Key Laboratory of Animal Immunology. The IC_{50} was 0.801 ng/mL to 2,4-D (Gong et al., 2016). Anti-GM mouse monoclonal antibodies were prepared in the Henan Provincial Key Laboratory of Animal Immunology. The IC_{50} is 0.30 ng/mL to GM (Zhi et al., 2010).

Nitrocellulose membranes (HF13502S25, $30 \times 2 \text{ cm}^2$), absorbent pads, and fibreglass were purchased from Millipore (Bedford, MA). The purity of all other reagents was of analytical grade or higher.

A CM 4000 Cutter, XYZ Biostrip Dispenser, and TSR3000 membrane strip reader were all purchased from Bio-Dot (Richmond, CA). An ultrapure water purification system was purchased from Millipore (Bedford, MA). A zetasizer Nano ZS90 was purchased from Malvern (Worcestershire, UK).

2.2. Preparation of colloidal gold probes

To prepare the colloidal gold probes, anti-2,4-D monoclonal antibody (mAb), 2,4-D-BSA conjugate, anti-GM monoclonal antibody (mAb), and GM conjugate were labelled with gold nanoparticles (GNPs). First, GNPs with a diameter of 30 nm were obtained according to Frens (Frens, 2009). The pH of the GNP solution was adjusted to 8.5 with 0.2 mol/L potassium carbonate. The GNP-labelled proteins were then prepared according to the literature (Urusov et al., 2016). Anti-2,4-D mAb, 2,4-D-BSA conjugate, anti-GM monoclonal antibody (mAb), and GM conjugate were diluted into 1 mL colloidal gold at 5, 9, 5, and 20 µg loading levels. The resulting mixtures were incubated for 30 min at room temperature followed by the addition of a 10% aqueous solution of casein (M/V = 1:20). Ten minutes later, the mixture was centrifuged at 12,000 r/min at 4°C for 30 min and the supernatants were discarded. The final pellets were re-suspended in 400 µL gold suspension buffer (20 mmol/L borate buffer containing 1% BSA, 3% trehalose, and 0.03% sodium azide) and stored at 4°C until use.

2.3. Preparation of the LFIA

For the traditional scheme, anti-2,4-D mAb with the GNPs solution or anti-GM mAb with the GNPs solution was sprayed on the fibreglass conjugate pad (8 µL per 1 cm or 7 µL per 1 cm). The 2,4-D-BSA conjugate or GM-BSA conjugate were dissolved in PBS to a concentration of 0.9 mg/mL or 0.02 mg/mL applied on nitrocellulose membranes (NC membranes) as the test line. The SPA was dissolved in PBS to a concentration of 0.4 mg/mL applied on the NC membranes as the control line.

For the new scheme, the 2,4-D-BSA was conjugated with the GNPs solution. Alternatively, the GM-BSA conjugate with the GNPs was sprayed on the fibreglass conjugate pad (8 µL per 1 cm). SPA (broad-spectrum secondary antibodies) were dispensed on the NC membrane as the test line (Figure 1). Next, anti-2,4-D mAb or anti-GM mAb were diluted to 0.005 mg/mL via an antibody stabilization solution (10 mmol/L PBS (pH 7.4) containing 1% BSA, 10% trehalose, 0.5% Tween20, and 0.03% sodium azide). The diluted monoclonal antibodies were added into sample cup and then lyophilized until the antibodies were fixed at the bottom of the cup (Figure 1). The sample pad, probe pad, absorbent pad, and working NC membrane were pasted on the plastic packing sequentially with 1–2 mm overlap and then cut into 3-mm-wide strips. The strips were then sealed and stored at room temperature in a sealed package containing desiccant.

2.4. Immunochromatographic analysis of 2,4-D and GM

For the traditional scheme, 100 µL of the test sample was added into the sample cup and examined by a traditional immunochromatographic test strip. In the improved scheme,

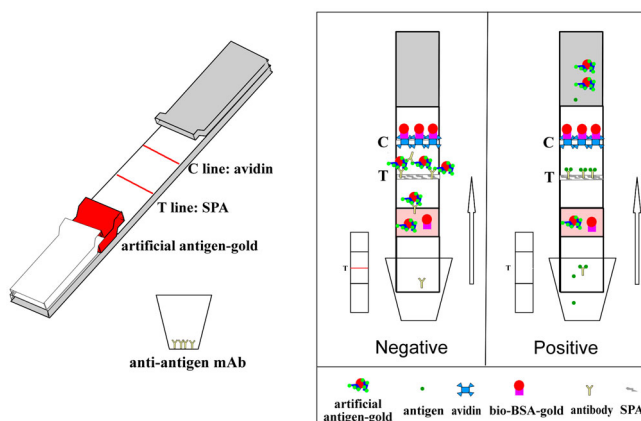


Figure 1. Schematic illustration of the new competitive strip.

100 μL of the test sample was added into the sample cup and incubated for 3 min at room temperature and then examined with the new LFIA.

The test strips (for both the traditional and new schemes) were examined at 2,4-D dilutions ranging from 1.56 to 1000 ng/mL in tap water or GM dilutions ranging from 1.31 to 10 ng/mL in milk. After 10 min, the strips were observed with visual detection.

3. Results

3.1. Justification of the new LFIA format

Figure 1 is a schematic illustration of the new LFIA showing the separate stages of the competitive interaction and labelling of the immune complex. The test-strip NC membrane was exposed to a mixture of samples and specific antibodies against the antigen followed by an artificial antigen conjugated with gold nanoparticles. If the antigen was present in the sample at a concentration above the threshold, it will bind to the specific antibodies, thus hampering their reactivity to the gold-labelled artificial antigen. No coloured band will appear in the NC membrane where SPA is coated. If the antigen in the sample is missing, or its concentration is below the detection limit, then specific antibodies will bind to the secondary antibodies in the test line and form a coloured band via the labelled artificial antigen. This approach needs the addition of some reagents to establish the control line (C-line), which does not react with any component of the strip^[7] like biotin–avidin. We did not set the C-line to avoid introducing additional influencing factors and to better illustrate the principles and advantages of the new method using the simplest model.

The main difference between the new LFIA with the traditional LFIA is that the former requires drying antibodies at the bottom of the sample cups; artificial antigen labelled with colloidal gold; and SPA coated on the test line. There are three models of LFIA shown in Figure 2: A is the new model, B is the traditional model, and C is an ideal traditional model with an equimolar ratio of antibodies with respect to the colloidal gold label. To better compare the above models, we quantified the antigen–antibody response. Figure 2(A1, B1, and C1) show that the strip needs to achieve a consistent colour signal (T lines intercept

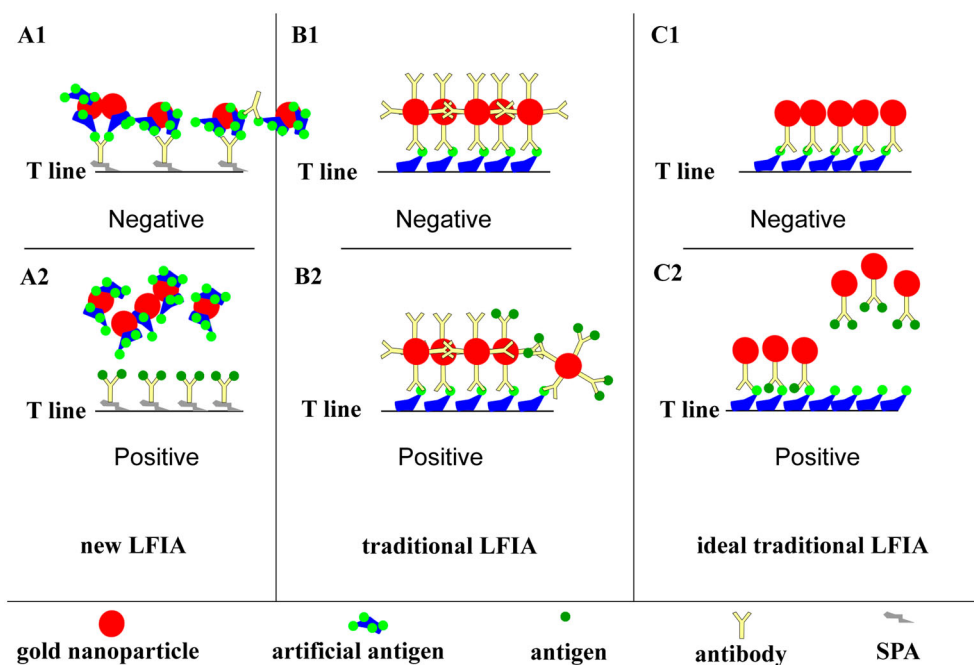


Figure 2. Comparison of the new and traditional LFIA. A is the new model, B is the traditional model, and C is an ideal traditional model with an equimolar ratio of antibodies with respect to the colloidal gold label.

five gold nanoparticles), the traditional model requires more than five antibodies, and the new model requires only three to five antibodies. The new LFIA are based primarily on colloidal gold-labelled artificial antigens to enhance the detection signal. When the sample contains eight antigens (Figure 2(A2, B2, and C2)), the colour of the T lines in the new model is completely cut off. However, it was only partially removed or not changed at all in the traditional model. Therefore, the new LFIA can accurately control the amount of antibody, aggregate gold-labelled antigen and enhance the detection signal, thus improving the sensitivity of the LFIA.

3.2. Development and characterization of the traditional LFIA

Traditional 2,4-D strips were developed and optimized. The sensitivity of the strips is shown in Figure 3(C). The detection limit for 2,4-D was 1000 ng/mL according to visual detection. The colour of the test lines is light because the ability of the artificial antigen to bind the antibody is very weak.

Similarly, the GM LFIA prepared according to a traditional scheme was developed and optimized. The sensitivity of the strip is shown in Figure 4(C). The detection limit for GM was 10 ng/mL based on visual detection.

3.3. Development and characterization of the new LFIA

The 2,4-D LFIA based on the new scheme was developed and optimized. Conditions including the concentration of SPA in the NC membrane, concentration of 2,4-D

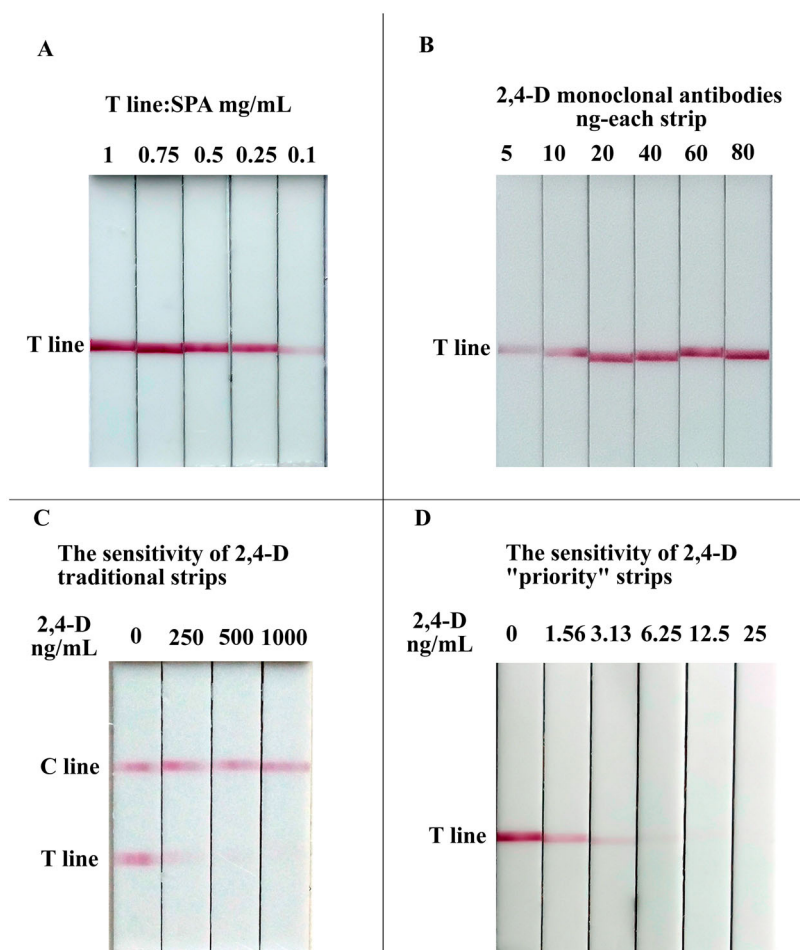


Figure 3. Optimized 2,4-D new strips in contrast to the sensitivity with 2,4-D traditional strips. The concentration of SPA in the NC membrane (A), and the concentration of 2,4-D monoclonal antibodies in the sample cups (B) were optimized. The sensitivity of the 2,4-D traditional scheme strip (C), and the sensitivity of the 2,4-D new strip (D).

monoclonal antibodies in the sample cups, and reaction time were all evaluated. The concentration of SPA in the NC membrane and concentration of the 2,4-D monoclonal antibodies in the sample cups were optimized concurrently. Reaching the minimal limit of detection was a criterion for choosing the optimal concentrations in both of these cases. In this experiment, the concentrations of 2,4-D monoclonal antibodies in the sample cups were the main factors influencing the strip sensitivities. In a certain range, the 2,4-D monoclonal antibodies decreased with increased SPA concentration, thus implying improved strip sensitivity. The results of the SPA concentration in the NC membrane are in Figure 3(A). When SPA concentration was 0.75 mg/mL, the signal intensity in the test line was uniform and saturated; the detection limit was minimal. The 20 ng per sample cup was chosen as the appropriate system for 2,4-D monoclonal antibodies because it offers a bright and uniform T line. A similar

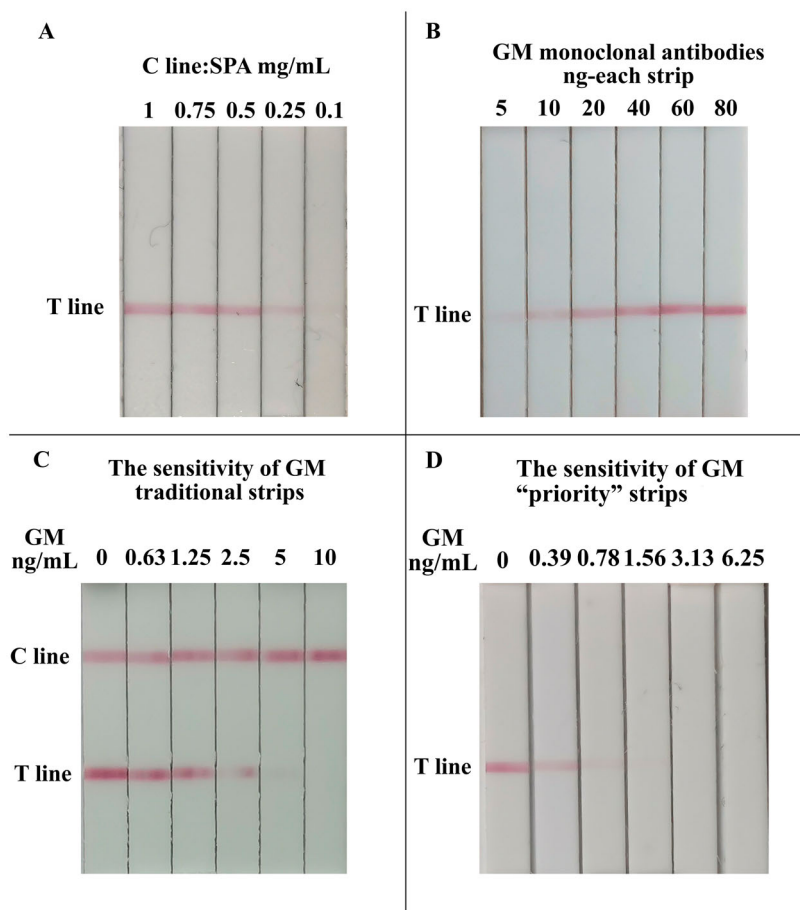


Figure 4. Optimized GM new strips versus traditional strips. The concentration of SPA in the NC membrane (A) and the concentration of GM monoclonal antibodies in the sample cups were optimized (B). The sensitivity of the GM traditional strip (C) and the sensitivity of the GM new strip (D).

optimization was carried out for the incubation time in the sample cup between 2,4-D and antibody. The sample in the sample cup was mixed and became uniform with a time ranging from 0 to 30 min with 3-minute intervals; this led to the detection of new strips. The optimal incubation time is 3 min. Further incubation did not improve the sensitivity. After optimization, the system produced the experimental results presented in Figure 3(D). The detection limit for 2,4-D was 25 ng/mL by naked eye. This is a 40-fold enhancement versus traditional test strips.

The GM new LFIA was similarly developed and optimized (Figure 4). The concentration of SPA in the NC membrane is shown in Figure 4(A); 1 mg/mL was chosen as the appropriate SPA concentration for the T line. Next, 40 ng of GM monoclonal antibodies per sample cup were used with a 3 min reaction time; these were chosen as the appropriate conditions. The results were obtained after the optimization [Figure 4(D)]. Visual detection showed a detection limit for GM of 3.13 ng/mL. Compared to the traditional test strips, the visual detection limit decreased by 3.2-fold.

4. Discussion

LFIA has been applied in many fields because it is fast, low cost, and simple. As the number of controlled compounds and stricter requirements on the levels of toxic substances increase, highly sensitive LFIAs are urgently needed. The existing approaches to lower the detection limit for LFIA are incorporating different kinds of nanoparticles (NPs) to enhance the detection signal as reporters (Huang et al., 2016). Currently, the analytical sensitivity and/or limit of detection (LOD) of LFIA has mainly enhanced three types of NPs as alternative labels: coloured [e.g. GNPs, carbon NPs (CNPs), and colloidal selenium NPs (SNPs)]; luminescent [e.g. quantum dots (QDs), up-converting phosphor NPs (UCPs), and dye-doped NPs]; and magnetic NPs (MNPs) (Chun, 2009; Faulstich et al., 2009; Huang et al., 2016). These NPs can also be applied to this method to improve sensitivity further.

The new model improved the LFIA detection limit due to several mechanisms.

- Monoclonal antibodies are fixed in the sample cup, there are three distinct advantages. First, the amount of antibody can be precisely controlled. Second, the antigen in the sample reacts with the antibodies earlier than the gold-labelled antigens. Third, the antibodies are unlabelled; thus, the immunological properties of the antibodies are not destroyed by antibody folding during the labelling process.
- Artificial antigens were conjugated to gold nanoparticles and used rather than being coated on the test line. This weakened the effect of the artificial antigen. Sufficient gold-labelled antigens are polymerized by antibody, thereby amplifying the overall signal and improving the sensitivity of LFIA.
- SPA was coated on the test line instead of the artificial antigen to significantly increase interception efficiency and reduce the needed antibody concentration. Secondary antibodies can be used in the test lines as well. This reduces interference when detecting animal serum samples.

Herein, a new competitive LFIA was established by employing 2,4-D and GM as the model analytes. The results showed that the sensitivity of the new strips for the two analytes was higher than the traditional strips. This new approach therefore exhibits a 40-fold lower detection limit for 2,4-D, and a 3.2-fold lower limit for GM versus traditional LFIA using the same reagents. In the 2,4-D traditional strips, 2,4-D-BSA as a test line exhibits a poor ability to intercept antibodies, which affects the sensitivity of the strip. However, GM traditional strips do not have this problem. Thus, the sensitivity of the 2,4-D strips improved more relative to GM using the new strip system.

The drying of antibodies in the sample cup is an important step that directly affects the uniformity and stability of the strip. BSA, trehalose, Tween-20, and freeze drying were used to protect the antibody activity in the drying process and antibody solubility after drying.

5. Conclusions

We report a new immunochromatographic assay based on competitive interactions. This approach uses antibodies to bind antigens from a sample and form a coloured immune

complex due to artificial antigens conjugated with gold nanoparticles; these were intercepted with SPA. This approach needs incubation for 3 min and can be examined in 10 min; the limit was 25 ng/mL in tap water for 2,4-D; and the limit was 3.13 ng/mL in milk for GM. This approach exhibits a 40-fold lower detection limit for 2,4-D and 3.2-fold lower limit for GM versus traditional LFIA using the same reagents. This approach can improve the sensitivity of the LFIA and providing more and more sensitive methods for the development of LFIAs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Yaning Sun: Conceptualization, Methodology, Software, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Funding acquisition. Suzhen Yang: Methodology, Formal analysis. Jifei Yang: Formal analysis, Software. Xiaofei Hu: Formal analysis. Yao Wang: Formal analysis. Qiang Wei: Formal analysis. Yunrui Xing: Methodology. Linlin Chen: Methodology. Xinxin Chen: Formal analysis. Ruiguang Deng: Supervision. Gaiping Zhang: Conceptualization, Supervision, Project administration, Investigation, Resources.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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