



Short communication

Rapid detection of β -conglutin with a novel lateral flow aptasensor assisted by immunomagnetic enrichment and enzyme signal amplification



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ABSTRACT

A simple, rapid and economic lateral flow immunochromatographic assay (LFICA) was designed for ultra-sensitive detection of β -conglutin. Instead of antibodies and gold nanoparticles (AuNPs) used in conventional LFICA, a cognate aptamer duo, binding to β -conglutin and Fe_3O_4 @Au core-shell nanoparticles, was applied in this study. An enzyme signal amplification strategy was used to enhance sensitivity. In addition, a new magnetic enrichment strategy was employed to further enhance sensitivity of the assay, slowing down movement of the capture probe (i.e., Fe_3O_4 @Au nanostructures) using an external magnetic field. The novel LFICA assay can be completed within 20 min and achieved a detection limit of 8 fM, a thousand-times lower than similar assays without magnetic focusing. Overall, our results demonstrated the potential for the proposed LFICA sensor in rapid detection of β -conglutin without any special analytical expertise or instrumentations.

1. Introduction

Lupin, a leguminous plant, is being used increasingly as an attractive alternative for gluten-free foodstuffs. Due to its nutritional benefits and unique biological activities, lupin flour has been used widely in bread, pastry, sauces and other food products (Jauset-Rubio et al., 2017; Mairal, Nadal, Svobodová, & O'Sullivan, 2014). However, ingestion of lupin has been associated with allergic reactions ranging from mild to severe, some of which have been life-threatening (Brennecke, Becker, Lepp, & Jappe, 2007; Jauset-Rubio, Svobodová, Mairal, McNeil, et al., 2016; Matheu et al., 1999; Moneret-Vautrin et al., 1999). The European Union (EU) stipulates that all lupin products sold in EU must be labeled correctly. The two major lupin storage proteins are α -conglutin and β -conglutin, accounting for 33% and 45% of total protein content. α -Conglutins, also known as legumine-like protein 11S, are oligomeric proteins that consist of hexamers of disulphide-linked subunits, which include acidic subunits with high molecular weights (MWs), and basic subunits with lower MWs. Prior to pro-polypeptide proteolytic conversion to mature subunits, the prevalent form is trimeric. The acidic subunits of α -conglutin, unlike most

other 11S globulins, are *N*-glycosylated. The β -conglutin, or vicilin-like globulin 7S, is a trimeric protein in which monomers consist of a number of polypeptides ranging from 16 to more than 70 kDa, with no disulphide bridges linking them. The precursor of β -conglutin (64 kDa) is *N*-glycosylated (Duranti, Sessa, & Carpen, 1992). There are two additional minor globulins, γ - and δ -conglutin, which account for 5% and 12% respectively. γ -Conglutin, a peculiar basic 7S protein, consist of two heterogeneous disulphide linked subunits of 17 and 30 kDa, which are monomeric at acid pHs but tend to form tetramers or hexamers at a neutral pH. Finally, the monomeric δ -conglutin is similar to other 2S albumins, consisting of two disulphide linked chains of about 4 and 9 kDa (Restani, Duranti, Cerletti, & Simonetti, 1981).

β -Conglutin, the predominant globulin, also known as Lup an 1, was identified as an allergen by the International Union of Immunological Societies (IUIS) in 2008 (Jauset Rubio, Svobodová, Mairal, Schubert, et al., 2016). Currently, since there is no cure for food allergies, only strict avoidance of food allergens can prevent serious health consequences (Jauset-Rubio et al., 2017). Rigorous surveillance for trace amounts of β -conglutin in food products is, therefore, essential.

For a long time, no method is capable of selectively detecting β -

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conglutin. In recent years, Sullivan et al. (Jauset Rubio, Svobodová, Mairal, Schubert, et al., 2016; Jauset-Rubio, Svobodová, Mairal, McNeil, et al., 2016; Jauset-Rubio et al., 2017; Mairal et al., 2014; Nadal, Svobodová, Mairal, & O'Sullivan, 2013) did a lot of work to detect β -conglutin accurately using aptamers specifically for β -conglutin. However, the methods developed need either recombinase polymerase amplification or fluorescence spectrometer, making detection complicated and unsuitable for high-throughput methods. It is imperative that an alternatively rapid and efficient detection method for trace β -conglutin detection in cereal products is developed.

Lateral flow immunochromatographic assays are inexpensive, rapid, simple to operate and can be performed on-site, and have attracted a great deal of attention (Cho, Bhunia, & Irudayaraj, 2015; Masiri et al., 2016). However, their significant lower analytical sensitivity compared with other detection methods hinders further application, which is why there are so few lateral flow immunochromatographic assay (LFICA) detection kits are available commercially available. Some approaches have been used to improve the sensitivity of LFICA including elaborately designed capture probes and highly sensitive instruments. However, the labels are difficult to synthesize and detection requires sophisticated instruments, tedious chemical processes, and prolonged analytical times, which contradict directly with the advantages of LFICA, i.e. low cost, simplicity and in-situ detection. LFICA methods that rely on quantifying color change can avoid the problem. However, detection limits are not usually satisfactory although many attempts have been made to enhance sensitivity through pre-enrichment or amplification, making them less attractive and harder to commercialize.

According to Narahari, Dendukuri, and Murthy (2017), concentration and intensity of the LFICA end product is predictable, based on the rate at which the analyte flows past immobilized receptors. Flow control is, therefore, paramount in the sensitivity of LFICA devices. Considerable efforts have been devoted to developing flow control techniques. However, these are either expensive or suffer from poor reproducibility, and require custom-built manufacturing equipment. Thus, new flow control strategies are needed urgently.

Most recent LFICA are based on antibodies. However, antibodies are expensive, temperature sensitive, and target affinity can be reduced by method modifications (Ahmad Raston, Nguyen, & Gu, 2017). In comparison, aptamers, as chemical antibody, possess many inherently appealing features such as small size, stability, lack of immunogenicity, easy synthesis, low cost, ready adaptation, and cell-free production (Chen & Yang, 2015), making them more suitable for use in LFICAs.

In this work, a pair of β -conglutin aptamers were used as alternatives in a LFICA. By combining a magnetic enrichment strategy and enzyme signal amplification, an ultrasensitive LFICA aptasensor was developed. The novel approach proposed in this study could enable rapid on-site detection of β -conglutin in foodstuffs for food industry and government laboratories.

2. Materials and methods

2.1. Extraction of globulin proteins

α , β , γ and δ conglutins were extracted and purified from *Lupinus albus* according to the protocol reported by Pedro Nadal, Canela, Katakis, and O'Sullivan (2011).

2.2. Preparation of Fe_3O_4 @Au core-shell nanoparticles

Magnetic nanoparticles were synthesized using a chemical co-precipitation method reported previously (Liang, Yao, Fan, & Qiu, 2012) with slight modifications.

2.3. Preparation of magnetic signal probe

The second aptamer was stabilized by mixing 10 mL of secondary aptamer (50 mM) with 2 mL of TCEP (10 mM) at room temperature for 1 h. The detailed preparation procedure of signal probe (Fe_3O_4 @Au-secondary aptamer conjugate) was in accordance with Ahmad Raston and Gu (2015) with slight modifications.

2.4. LFICA for β -conglutin detection

The LFICA detection of β -conglutin was carried out according to a method described previously (Cho et al., 2015).

2.5. Cross-reactivity with other lupin conglutins

To evaluate selectivity of the proposed LFICA platform for β -conglutin detection, the lateral flow results were compared with those obtained for three other lupin conglutins, i.e., α , γ and δ conglutins. The concentration of β -conglutin and the other three conglutin subunits, were 50 pM and 50 nM, respectively. They were all tested using the same analytical procedure described above. Each measurement was performed at least three times.

3. Results and discussions

3.1. The working principles of LFICA platforms

Three LFICA platforms were used in this study for the detection of β -conglutin, as illustrated in Fig. 1.

Fig. 1a shows the working principle for the conventional LFICA method. Typically, the sample containing the analyte was dropped on to the sample pad. Under capillary action, a flow in the longitudinal direction was initiated. When the analyte conjugated with signal probes (i.e., AuNPs-secondary aptamer conjugate) and passed into the detection zone, the immobilized capture aptamer (i.e., primary aptamer) formed an aptamer-antigen-aptamer sandwich immunocomplex. Qualitative or quantitative detection was based on the red color generated by the accumulated AuNPs in test zone. However, poor LOD is a key limitation in this approach.

Making the captured AuNPs more 'detectable' is a good way to solve this problem. Herein, nanoparticles modified with a reasonable amount of horseradish peroxidase (HRP), via streptavidin-biotin link, were used as signal probes for more sensitive detection of β -conglutin (Fig. 1b). With the addition of 3,3',5,5'-tetramethylbenzidine (TMB), a relatively rapid catalytic reaction between HRP and TMB occurred, resulting in an enhanced detection signal (blue color) and sensitivity.

Increasing the amount of analyte captured by the immobilized aptamer also increases analytical sensitivity. One of essential reasons for poor sensitivity is the low target interaction ratio between the analyte and capture aptamer in the detection zone. As shown in Fig. 1c, instead of the conventionally used gold nanoparticles (AuNPs), magnetic Fe_3O_4 @Au core-shell nanoparticles (Fig. 2) were used as signal probes in this study. An external magnet under the strip provided a magnetic field that interacts with the Fe_3O_4 @Au nanoparticles, reducing flow speed and increasing interaction time, between the probe-labelled targets and the receptors immobilized at the detection zone increased. As a result, amounts of the target captured in the test zone increased and sensitivity enhanced. By combining enzyme amplification and magnetic concentration, much lower concentration of β -conglutin can be detected without sophisticated instrumentation or specialized analytical skills.

3.2. Comparison of the results from three different LFICA platforms

Fig. 3 shows results for the conventional enzyme-based signal amplification method and the method developed employing enzyme

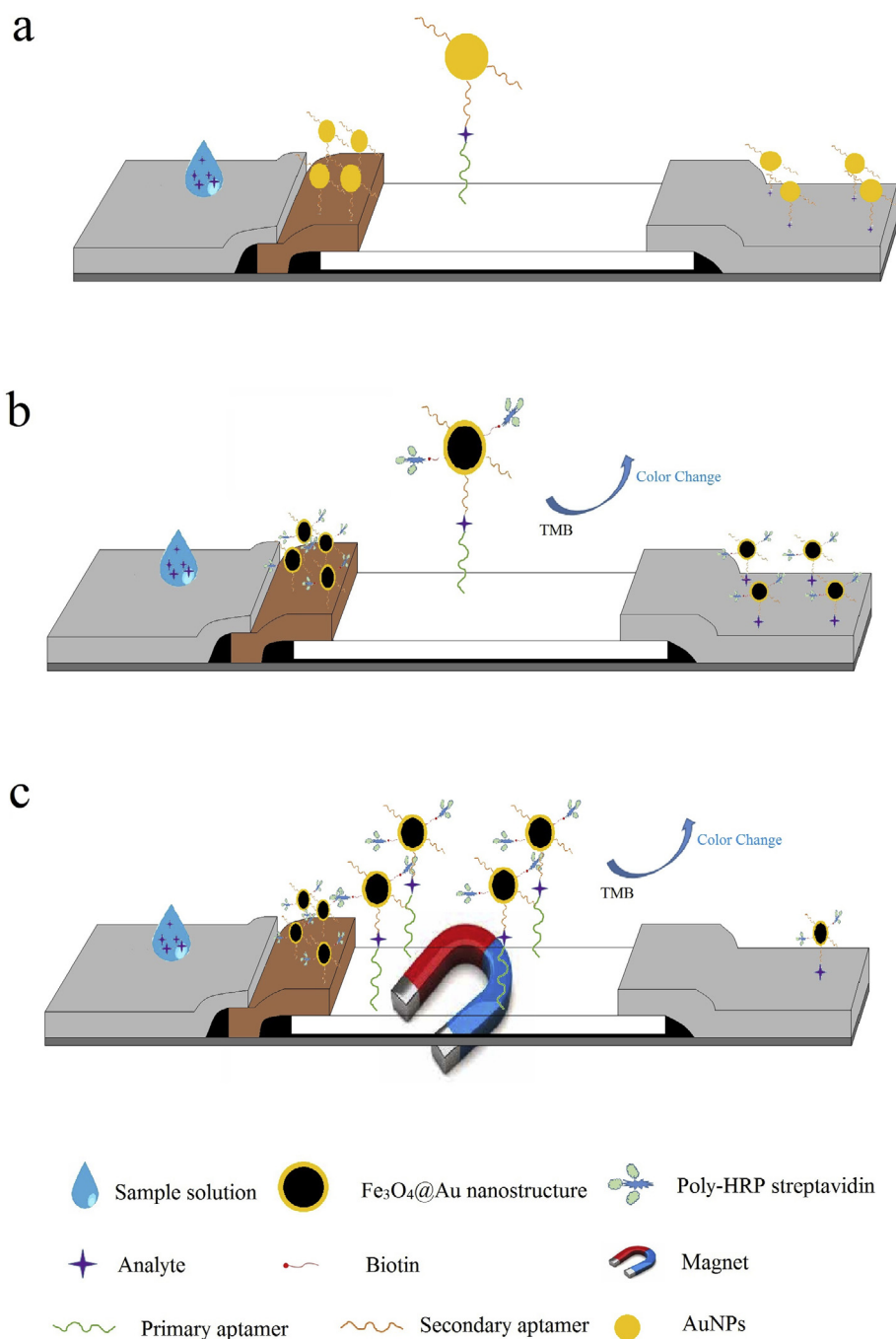


Fig. 1. Schematic illustration of the principles of the traditional AuNPs-based LFICA method (a), the enzyme signal amplification-based LFICA method (b) and the Fe₃O₄@Au nanoparticles based LFICA method assisted with enzyme amplification and external magnetic field (c).

amplification and magnetic enrichment strategies. When the concentration of β -conglutinin was 5 nM, no visual identification was achieved with the traditional AuNPs-based LFICA method while blue color signals were observed on strips for the alternative LFICA platform. It is worth noting that the concentrations of β -conglutinin used with the conventional AuNPs-based and enzyme amplification-assisted methods were 1000 times higher (5 nM) than that used with the proposed alternative (5 pM). Compared with the enzyme amplification-based LFICA, the color of the proposed LFICA was far more intense, revealing that the magnetic enrichment strategy increased detection sensitivity by more than 1000 times. This can be attributed to significant interaction between the aptamer-signal probes conjugates and the aptamer immobilized on the strip facilitated by the external magnetic field. The

results demonstrate the surprising capacity of an external magnetic field to enhance LFICA sensitivity.

3.3. Analytical performances

Visual readout in the presence of different concentrations of β -conglutinin are shown in Fig. 4a. As can be seen in the figure, color intensities of the blue dots generated by the HRP-catalyzed TMB reaction increased with the increasing in concentrations of the target. A smartphone camera was used to record images of the strips, which were analyzed using ImageJ software to calculate intensities of the blue dots. As shown in Fig. 4b, a regression curve in the range 10 fM–100 pM was used to determine these intensities were proportional to the

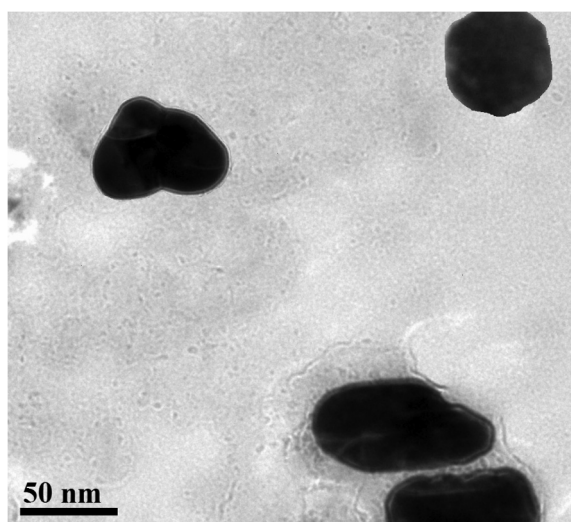


Fig. 2. TEM image of $\text{Fe}_3\text{O}_4\text{@Au}$ nanoparticles.

concentrations of β -conglutin. A strong linear correlation was obtained with R^2 of 0.9940 and LOD of 8 fM.

3.4. Evaluation of selectivity

The selectivity of the proposed method for β -conglutin was evaluated by comparing the color changes caused by β -conglutin and its structural analogues. As illustrated in Fig. 5a, an obvious blue color dot was observed with the addition of β -conglutin. However, changes in color intensities with α -, γ - and δ -conglutins were negligible. This is because the β -conglutin aptamer bound specifically to β -conglutin, demonstrating the LFICA assay was highly specific and sensitive for β -conglutin alone.

The stability of this method was also assessed by monitoring color changes in strips stored for 1 day, 20 days and 60 days, respectively. As shown in Fig. 5b, after adding the same amount of β -conglutin, no significant differences were observed among these strips during storage, meaning the method is stable as well as sensitive and specific.

This proposed strategy overcomes the limitation of low surface reaction between target and capture probe (antibody/aptamer) in LFICA, enhancing sensitivity. The resulting assay is rapid, simple and low cost. More importantly, specialized equipment is not needed with this method and detection took less than 20 min using low sample volumes ($\sim 100 \mu\text{L}$).

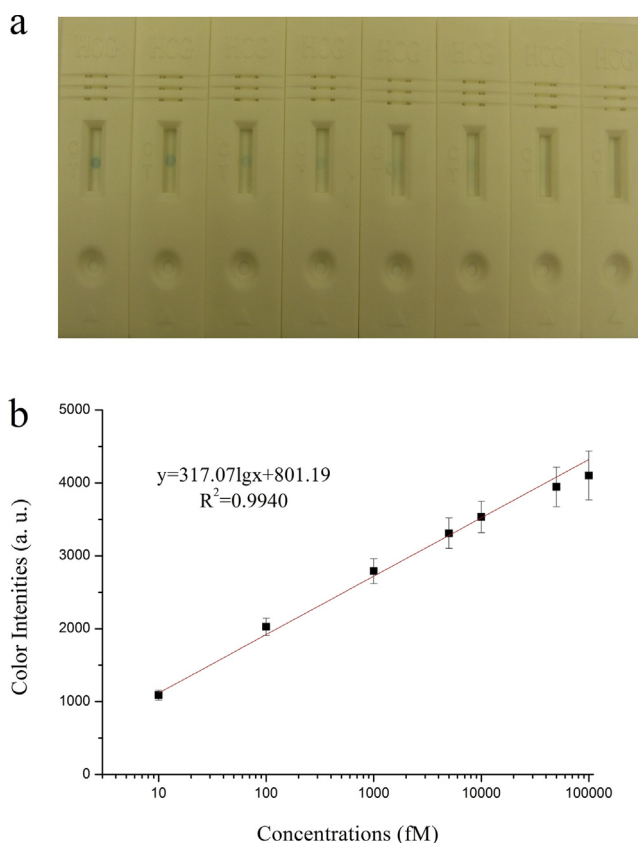


Fig. 4. Typical recording outputs for detecting various concentrations of β -conglutin using the proposed immunoassay (a) and calibrated color intensities as a function of the concentrations of β -conglutin (b).

4. Conclusions

A novel LFICA assay combined enzyme amplification and magnetic enrichment for determination of β -conglutin in food samples was established, which was highly selective, had wide linear range and a very low LOD. Compared with the enzyme amplification-based LFICA assay, the novel LFICA assay was 1000-fold more sensitive, demonstrating great potential in the detection of β -conglutin in food samples.

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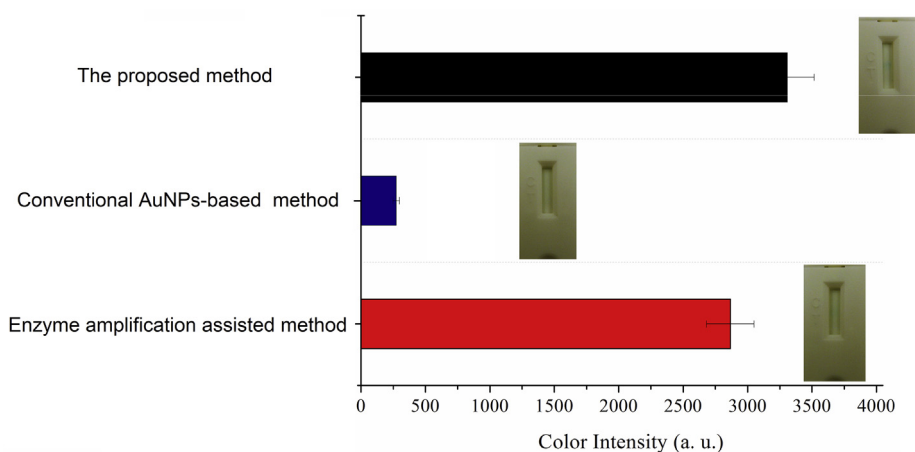


Fig. 3. Comparison of the detection results (color intensity) of three LFICA platforms based on different working principles.

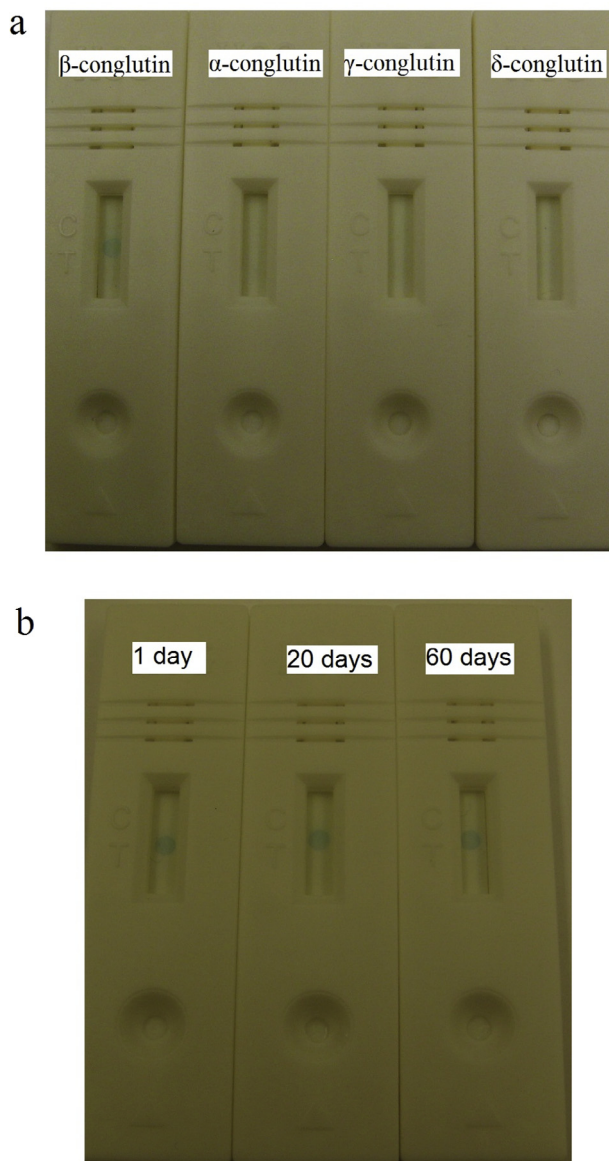


Fig. 5. Selectivity (a) and stability (b) analysis of the proposed assay.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.foodchem.2018.07.011>.

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Supporting Information

Rapid detection of β -conglutin with a novel lateral flow sensor assisted by immunomagnetic enrichment and enzyme signal amplification

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Running title: Ultrasensitive detection of β -conglutin by LFICA

Chemical reagents

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 6-Mercapto-1-hexanol (MCH), hydrochloroauric acid, phosphate-buffered saline (PBS, pH = 7.4) and TMB were purchased from Sigma (Saint Louis, MO, USA). Bovine serum albumin (BSA) was from SeraCare Life Sciences, Inc. (Milford, MA, USA). Sulfo-NHS-LC-Biotin was purchased from ApexBio Technology (Houston, TX, USA). Iron (III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), Poly-HRP streptavidin and sodium chloride (NaCl) were obtained from Fisher Scientific (Waltham, MA, USA). Iron (II) chloride hydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$) was purchased from Alfa Aesar Co., Inc. (Tewksbury, MA, USA).

The detailed sequences of the oligonucleotides are as follows:

Primary aptamer:

5'-biotin-agctccagaagataaattacaggggccgggggtggctcaggcaaggggttgacctgtcgtaggattgtttaacaactaggatactatgacccc-3'.

Secondary aptamer: 5'-thiolated- ggt ggg ggt gg-3'

Preparation of Fe_3O_4 @Au core-shell nanoparticles

Generally, in a three-neck flask, 11.2 mM FeCl_3 and 5.6 mM FeCl_2 were dissolved in 180 mL aqueous solution and heated to 50 °C under the gentle flow of nitrogen. The mixture was then mixed with 12.5 mL of $\text{NH}_3 \cdot \text{H}_2\text{O}$ and vigorously stirred for 30 min, followed by the collection of the resultant on the vessel wall under the external magnetic force. After that, the precipitate was washed with ethanol and water three times, respectively to neutral pH. The collected Fe_3O_4 nanoparticles were re-dispersed in water.

The Fe_3O_4 @Au core-shell nanoparticles were fabricated based on the approach reported by

Wu, He, Chen, Tang, and Nie (2007). Briefly, under ultrasound condition, 20 mL of pre-synthesized Fe_3O_4 nanoparticles and 15 mL of 1% HAuCl_4 solution were mixed for 20 min. Then, 15mL of 20 mM sodium citrate was dropped into the mixture and sonicated until dark red solution was obtained. Finally the precipitated product was washed with ethanol and re-dispersed in ethanol and water three times, respectively and re-dispersed in water for further use.

Preparation of magnetic signal probe

Typically, 1 mL of prepared $\text{Fe}_3\text{O}_4@\text{Au}$ nanostructures was first mixed with 10 mL of secondary aptamer which had been stabilized with TCEP in a tube. Then, the mixture was incubated for 20 h at room temperature before the addition of buffer solution containing 500 mM Tris acetate (pH = 8.2) and 1 M NaCl and incubated for another 24 h at room temperature. To efficiently block the residual surface (i.e., unoccupied region) of $\text{Fe}_3\text{O}_4@\text{Au}$, 10 mM MCH was added to the solution containing second aptamer-modified $\text{Fe}_3\text{O}_4@\text{Au}$ nanostructures and incubated for 30 min at room temperature. After that, the solution was centrifuged at $5000 \times g$ for 30 min to remove unbounded molecules. The produced precipitates were washed and re-dispersed in 5mM Tris-HCl (pH = 7.4) solution. 10 μg of Sulfo-NHS-LC-biotin was added in the obtained solution and shaken gently in a shaken unit for 1 h to biotinylate the secondary aptamer modified $\text{Fe}_3\text{O}_4@\text{Au}$. Finally, after centrifugation, the resultant conjugates were washed with 5 mM Tris-HCl for at least three times and stored in 4 °C until further use.

Synthesis of AuNPs

AuNPs were prepared by the reduction of hydrogen tetrachloroaurate (HAuCl_4) using trisodium citrate according to a previously reported method (Feng, et al., 2016) with slight modification. Initially, an aqueous solution of chloroauric acid tetrahydrate (100 mL, 0.25 mM) was brought to the boil with vigorous stirring and refluxed. After that, 1.8 mL of freshly prepared aqueous trisodium citrate solution (1%) was quickly added. Ten minutes later, the color of the

reaction solution changed from blue to wine red. Finally, the resultant solution was cooled to room temperature and stored at 4 °C for further use.

LFICA for β -conglutinin detection

To perform the detection, 100 μ L of samples with different concentrations (10 fM, 100 fM, 1 pM, 5 pM, 10 pM, 50 pM and 100 pM) were first mixed with 2 μ L of the magnetic signal probe and 0.5 μ L of streptavidin Poly-HRP for 5 min. Then the mixture was dropped on the sample pad of the as-prepared LFICA strip and allowed the sample flowed through the strip with an external small magnet placed right under the detection zone of the strip. Thereafter, 30 μ L of water was introduced to the strip followed by the addition of 30 μ L TMB to generate the colorimetric signal with three minutes of incubation at room temperature. After that, 30 μ L water was used to stop the reaction. The produced blue color signals were recorded by direct photography with a smartphone and were saved in JPEG format. The images were further processed by the ImageJ program (a public-domain, Java-based image processing program developed at the National Institute of Health). Images were split into red, green and blue channels using the RGB stack command. In this research, the images were performed through red channel following the discard of the blue and green channels since this channel showed less background level.

Characterization of nanoparticles

All UV-Vis spectra were acquired using a commercial spectrometer TU-1900 (Purkinje General Corporation, Beijing, China) at room temperature. The size and morphology of nanoparticles were investigated by high-resolution transmission electron microscope (TEM, JEM-2100, JEOL, Japan) equipped with a charge-coupled device (CCD) camera operating at an acceleration voltage of 200 kV with a point resolution better than 0.23 nm. The TEM samples were prepared by dropping NPs solution onto copper grids coated with a thin carbon film and then it was allowed to dry under ambient condition.

Data analysis

All data were expressed as mean $\pm$ standard deviation (SD) of at least three determinations throughout the study, and spectra drawings were performed using Origin 8.5 (OriginLab Inc., USA). Data was analyzed using one-way analysis of variance and means were compared by Duncan's multiple range tests using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The probability value of $p < 0.05$ was considered significant.

Preparation of Lateral-flow strips

Before the detection of β -conglutin, the lateral flow strips were pretreated as follows. 20 μ L of biotin-primary aptamer (0.5 μ M) were incubated with 30 μ L of streptavidin (100 μ g/mL) for 1 h at 4 $^{\circ}$ C. Then 1 μ L of aptamer-conjugated streptavidins was dropped on the nitrocellulose (NC) membrane to be used as capture probe and dried at 60 $^{\circ}$ C for 30 min for immobilization. After that, PBS buffer containing 50 μ g/mL of BSA was flow through the membrane for blocking before the membrane was dried again and the strips (0.4 cm wide) were assembled and stored in 4 $^{\circ}$ C until further use.

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