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SHORT COMMUNICATION

A new kind of highly sensitive competitive lateral flow immunoassay displaying direct analyte-signal dependence. Application to the determination of the mycotoxin deoxynivalenol

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Abstract
A new kind of competitive immunochromatographic assay is presented. It is based on the use of a test strip loaded with (a) labeled specific antibodies, (b) a hapten-protein conjugate at the control zone, and (c) antibodies interacting with the specific antibodies in the analytical zone. In the case where a sample does not contain the target antigen (hapten), all labeled antibodies remain in the control zone because of the selected ratio of reactants. The analytical zone remains colorless because the labeled antibodies do not reach it. If an antigen is present in the sample, it interferes with the binding of the specific antibodies in the control zone and knocks them out. Some of these antibodies pass the control zone to form a colored line in the analytical zone. The intensity of the color is directly proportional to the amount of the target antigen in the sample. The assay has an attractive feature in that an appearance in coloration is more easily detected visually than a decoloration. Moreover, the onset of coloration is detectable at a lower concentration than a decoloration. The new detection scheme was applied to the determination of the mycotoxin deoxynivalenol. The visual limit of detection is 2 ng·mL^{-1} in corn extracts (35 ng per gram of sample). With the same reagents, this is lower by a factor of 60 than the established test strip. The assay takes only 15 min. This new kind of assay has wide potential applications for numerous low molecular weight analytes.

Keywords Immunochromatography · Test strip · Competitive immunodetection · Deoxynivalenol control of toxic contaminants · Increased sensitivity · Competitive enhancement · Gold nanoparticles · On-site testing

Introduction

Immunochromatographic assay (ICA) has been in use for a long time. The first commercial tests and publications appeared in the 1980s [1]. The technique's ease of use, low cost, and visual detection capability has made it popular worldwide for solving problems in medicine, agriculture, and food quality control [2–7].

When analyzing low molecular weight compounds, ICA uses a competitive scheme, immobilizing the antigen–protein conjugate in the analytical zone of the test strip, which competes with the sample antigen for the binding sites of labeled specific antibodies. When no compound is detectable in the sample, specific antibodies bind at the analytical zone, forming a colored band. As the concentration of the antigen in the sample increases, the antigen blocks some of the antibodies, which do not bind in the analytical zone. As a result, the intensity of staining decreases. The visual limit of detection is the antigen concentration at which the coloration of the analytical zone disappears completely [8, 9]. Lower antigen concentrations only partially reduce label binding; they cannot be reliably detected visually. The instrumental registration of coloration allows reduction of the detection limit. However, the reliable registration of a slight decrease in the staining intensity is a complex task, even for instrumental detection. Thus, ICA is inferior to ELISA in sensitivity in the majority of cases (when using the same reagents) [2, 3, 10].

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This work proposes a new scheme of competitive ICA. This study suggests registering labeled antibodies that bind to the sample antigen. Such antibodies are knocked out of the first zone (with antigen-protein conjugate) and pass it to be accounted for by the next zone of the test strip (with anti-species antibodies, protein A/G, etc.) Thus, the intensity of staining increases with the growth of the antigen concentration. Visual detection of the color's appearance is easier than its palliation. The scheme enables visual detection of lower concentrations.

This article presents experimental confirmation of the new approach's effectiveness. The authors chose as an example mycotoxin deoxynivalenol (DON), a contaminant often found in feed and food that poses a significant threat to the health of farm animals and humans [11, 12]. Maximum residue levels (MRLs) of DON in different types of food products are 200–1750 ng·g^{-1} (Commission Regulation [EC] No. 1881/2016). For this reason, simple, inexpensive, and highly sensitive methods for DON detection are necessary.

Materials and methods

Chloroauric acid, sodium azide, and Tween-20 were from Sigma-Aldrich (St. Louis, MO, USA; www.sial.com). DON, ochratoxin A, aflatoxin B1, zearalenone, T-2 toxin, and fumonisin B1 were from Chromresurs (Moscow, Russia; www.chromresurs.ru). Bovine serum albumin (BSA) was from MP Biomedicals (Santa Ana, CA, USA; www.mpbio.com). Mouse monoclonal antibodies against DON and DON–BSA conjugate were from Biotez (Berlin, Germany; www.biotez.de). Goat anti-mouse polyclonal antibodies (GAM-IgG) were obtained from Arista Biologicals (Allentown, PA, USA; www.aristabiologicals.com). All other reagents were of analytical grade purity or greater.

Deionized water (18 MΩ·cm at 25 °C, Simplicity Millipore, Billerica, Massachusetts, USA; www.millipore.com) was used to prepare all solutions.

The immunochromatographic test strips were fabricated from Hi-Flow Plus KIT (Millipore, Billerica, MA, USA; www.merckmillipore.com) membranes. The application of reagents on the membranes was carried out by IsoFlow dispenser (Imagene Technology, Hanover, NH, USA; www.imagenetechnology.com), and Index Cutter-1 (A-Point Technologies, Gibbstown, NJ, USA, www.manta.com) was used for cutting.

Immobilization of antibodies on gold nanoparticles

Gold nanoparticles (GNPs) with a concentration of 50 μg·mL^{-1} and diameter of 30 nm (A520 = 1) were obtained according to the Frens method with modifications [13, 14]. The pH of the GNP solution was adjusted to between 8.5 and 9.0 with potassium carbonate, followed by the addition of antibodies (10 μg·mL^{-1} of GNP solution) diluted in 10 mM Tris buffer, pH 8.5. The resulting mixture was incubated for 45 min at room temperature, after which a 10% aqueous solution of BSA (VGNP:VBSA = 40:1) was added and the mixture was stirred vigorously for 15 min. The GNPs were pelleted by centrifugation at 15,000×g for 15 min at 4 °C. The precipitate was collected and re-suspended in 10 mM Tris buffer, pH 8.5, which contained 1% BSA, 1% sucrose, and 0.05% sodium azide (TBSA); the solution was stored at 4 °C.

Preparation of the immunochromatographic test strips

The DON–BSA conjugate was dissolved in PBS to a concentration of 0.25–1.5 mg·mL^{-1}, and GAM-IgG were dissolved in PBS to a concentration of 0.25 mg·mL^{-1}. These compounds were applied to nitrocellulose working membranes (Millipore HF120) fixed on a plastic support at a rate of 0.1 μL·mm^{-1} using an IsoFlow dispenser.

A specific antibody—GNP conjugate in TBSA +0.05% Tween-20 with A_{520} = 0.008–1.000—was applied to the glass fiber conjugate pad (3.2 μL·mm^{-1}).

The working membrane, pad(s), and absorbent membranes (both the initial and final membranes for the sample) were assembled on a plastic surface and cut into strips with a width of 3.5 mm. Ready tests were stored at 20–22 °C in sealed packages containing silica gel.

Preparation and validation of samples to be tested

The corn was bought at a store. Milled grains were mixed with an extraction solution (methanol/water 70:30, *v*/v) at a ratio of 1:5 and incubated with gentle stirring at room temperature for 1 h in accordance with [15]. After centrifugation, the supernatants were collected and stored at 4 °C. DON solutions were introduced into the extracts before the immunoassays.

To characterize the corn samples, common HPLC protocol was used, as described in [16]. No DON was detected in the initial corn samples, which provided possibility to characterize artificially spiked samples.

Immunochromatographic analysis of mycotoxins and data processing

The test strips were vertically immersed in a series of DON-spiked samples (2.0–31.3 ng·mL^{-1}) of diluted corn extracts (3.5-fold with PBS + 0.05% Tween-20). After 15 min of incubation, the strips were scanned in a flatbed scanner (Canon Lide 90) at a resolution of 600 dpi without applying modes for contrast and color correction.

To process the images of the test strips, the Total Lab software package (TotalLab, Newcastle upon Tyne, UK; www.totallab.com) was used. A rectangular area 90% of the width and length of the working membrane was selected on the digital images of the test strips. This region was analyzed to determine the presence and intensity of the stained areas in comparison with the background. For statistical processing, all measurements were performed in triplicate.

Results and discussion

Justification of the new immunochromatographic format

The competitive scheme of ICA is usually used to determine low molecular weight compounds. It is effective but has significant disadvantages:

- Sensitivity is inferior to other immunochemical methods with the same antibodies. Moreover, 1 to 2 orders of sensitivity are lost in the case of visual (non-instrumental) registration of the analysis results because reduction of coloring intensity cannot be registered visually (maximal and lesser staining cannot be distinguished without a calibrator). However, the absence of staining and the appearance of weak staining is easily apparent.
- The control zone does not provide complete control. The binding of antibodies that occurs within it does not depend on the antibodies' ability to interact with the antigen, leading to false positive results.

This study proposes a new scheme of competitive immunochromatographic analysis. The localization of reactants at the test strip is the same (see Fig. 1a), namely: (a) labeled specific antibodies at a special pad that is in contact with the strip's sample pad, (b) hapten-protein conjugate as first (lower) binding zone at the working membrane, and (c) antibodies interacting with the specific antibodies as second (higher) binding zone at the working membrane.

However, the concentration of hapten-protein conjugate increases significantly. For DON, the model antigen, an 8-fold increase is proposed, (i.e., from 0.25 mg·mL^{-1} in traditional test strips to 2 mg·mL^{-1} in the new strips), which thus changes the functions of the binding zones (see Fig. 1b). When the target analyte is absent in the tested sample, all labeled antibodies are bound at the strip's first binding zone, whereas the second binding zone remains colorless. When the target analyte is present, it blocks binding sites of some specific antibodies. Thus, some labeled antibodies move along the first binding zone and interact at the second zone. The assay results in the coloration of two lines. Actually, in the new test strip, the first binding zone becomes the control, whereas the second zone is

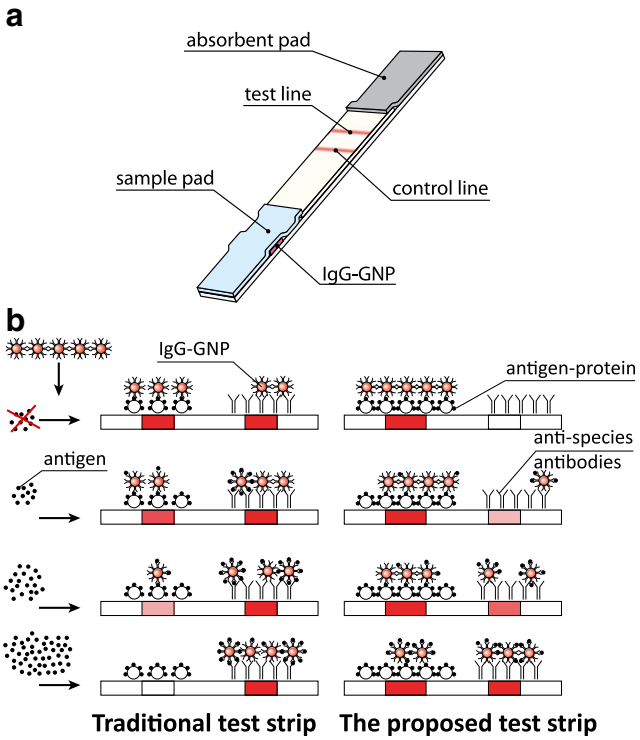

Fig. 1 Scheme of the new kind of lateral flow immunoassay. **a** Composition of the strip. **b** Comparison of the assay results for various concentrations of analyte for traditional and the new assay schemes

analytical one. Table 1 summarizes the binding zone properties for the traditional and the new ICA formats.

An essential property of the new scheme is the coloration on the second (analytical) zone from the analyte content. Consequently, the samples that cause only partial blocking of the antigen-binding sites qualify as positive (containing

Table 1 The comparison of binding zones for the traditional and the new ICA

Location of zones in the accordance of flow movement	First zone	Second zone
Immobilized reactant	Antigen-protein conjugate	Antibodies interacting with the specific antibodies (GAM-IgG)
Traditional ICA		
Cause of zone's coloration	Absence of low content (<LOD of the given ICA) of antigen in the sample	Stored functional properties of reactants
Function of the zone	Analytical zone	Control zone
Proposed ICA		
Cause of zone's coloration	Stored functional properties of reactants	Presence (>LOD of the given ICA) of antigen in the sample
Function of the zone	Control zone	Analytical zone

analyte) in contrast with the traditional assay, where complete blocking (by higher concentration of the analyte) is necessary to visually assess the assay results as positive. In the new assay, the coloration value in the absence of the analyte is near zero and has minimal absolute variations. The appearance of coloration may be simply distinguished from its absence both visually and instrumentally. This enables the achievement of more sensitive analyte detection than in traditional techniques.

The first (control) zone provides confirmation of reactant stability (i.e., storage of their immunochemical properties) in accordance with common immunochromatography demands [8, 17]. Moreover, the new test binding in the control zone involves strictly checking the functionality of the antigen-binding sites of specific antibodies. In contrast, the traditional assay binding in the control zone is based on an alternate site of antibody molecules that is responsible for their recognition by anti-species antibodies. Thus, the proposed modifications make the functionality assessment more accurate. Wetting the membrane during the analysis and subsequent distribution of coloration serve to simply visually control the test strip's mechanical defects and stop the reactants from flowing.

Optimization of the test system

With the traditional test strip as a basis. The length of the working membrane and the location of the first and the second binding zone were not changed. Choice of these parameters was based on the authors' previous experience in test strip development for mycotoxin detection [18–20].

To use the second analytical zone for the detection of unbound antibodies, its staining in the absence of the antigen should be reduced to zero. For this purpose, the concentration of the DON–BSA conjugate on the first control zone was increased. Moreover, reaching high assay sensitivity required reducing the concentration of labeled antibodies, which necessitated optimizing these parameters simultaneously. The Electronic Supplementary Material provides corresponding data and figures.

The authors found that only high concentrations of the DON–BSA conjugate in the control zone (≥ 1 mg·mL^{-1}) lead to the disappearance of the analytical zone coloration. At the same time, the analytical zone does not completely disappear, even with a high concentration of DON–BSA (1.5 mg·mL^{-1}). The optimal concentration of the adsorbed conjugate was 1 mg·mL^{-1} (see Fig. S1). Higher concentrations lead to background staining and extra consumption of reagents. The preliminary choice of the specific antibody–GNP conjugate concentration was about $A_{520} = 0.25$.

An additional finer optimization of the conjugate concentration was carried out in experiments with the free DON (competing analyte) presence. The assay is sensitive to the concentration of the specific antibody–GNP conjugate. Increasing it to $A_{520} = 0.45$ (bottom row of Fig. S1) leads to a background staining in the analytical zone at zero antigen content. Low concentrations ($A_{520} \leq 0.25$) lead to a decrease in coloration intensity. The selected optimal content of the conjugate accord with $A_{520} = 0.35$ (see Fig. S2).

Finally, the following experimental conditions were found to give best results: (a) a concentration of the DON–BSA conjugate on the membrane of 1 mg·mL^{-1} and (b) a concentration of the specific antibody–GNP conjugate that accords with $A_{520} = 0.35$.

Determination of the analytical characteristics of ICA

The prepared corn extracts (methanol/water—70:30 *v*/v) were free from DON as shown by HPLC. Based on previous studies [10], the ICA was implemented with spiked diluted extracts containing 20% (v/v) methanol. Figure 2 shows the DON detection results.

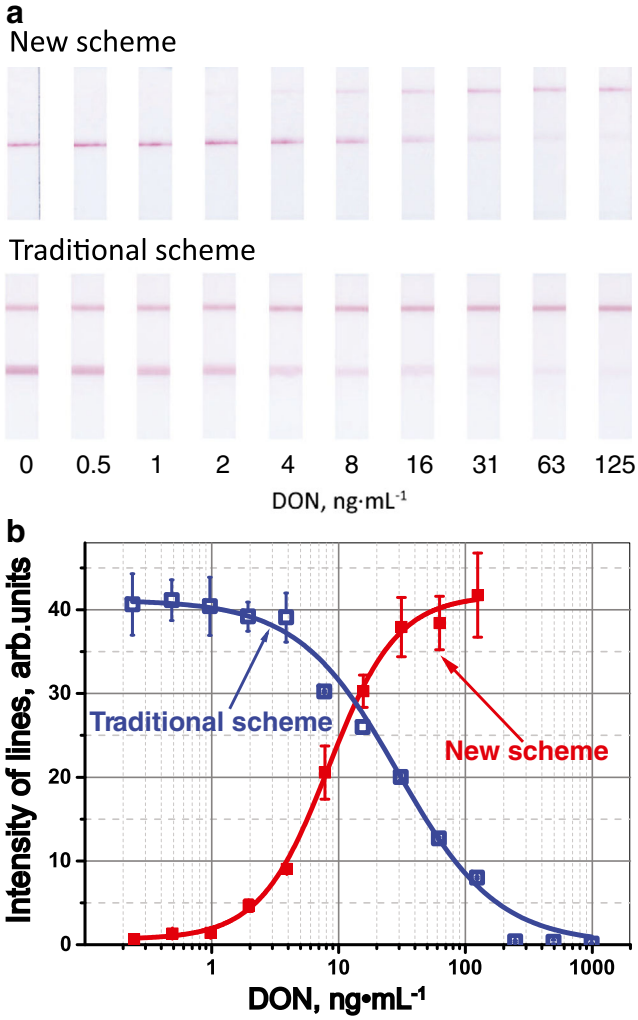

Fig. 2 Immunochromatographic detection of DON in corn extracts. **a** The test zones in the new and traditional schemes after analyzing samples with different DON concentrations. **b** Calibration curves for immunochromatographic detection of DON by traditional scheme (blue line, hollow squares) and new scheme (red line, filled squares)

Table 2 Revealing of DON in the spiked corn samples ($n = 3$) by the new ICA

Added DON, ng·mL^{-1}	Found DON, ng·mL^{-1}	Degree of revealing, %
31.3	34.3	110
15.6	15.0	96
7.8	8.0	103
3.9	3.6	92
2.0	2.1	105

The visual limit of the assay was 2 ng·mL^{-1} (or 35 ng of DON per gram of sample) at 15 min assay duration. The detection limit of the traditional test system with the same reagents was more than 125 ng·mL^{-1} (or 2.2 μg per gram of the sample; see Fig. 2a).

When the spiked samples were tested in triplicate, the analyte recovery ranged from 92% to 110% (see Table 2). The average variation of the measured DON content was no more than 10%.

The MRL of DON in baby foodstuffs is 200 ng·g^{-1} (Commission Regulation [EC] No 1881/2016). This test can thus be used to determine DON in baby food products. The test created here exceeds the existing solutions among scientific publications (see Table 3). Its sensitivity is significantly higher than that of other immunochromatographic systems with assay result visual/photometric registration. The detection limits for fluorescent and electrochemical systems are often lower, but these types of analysis are more time consuming and lengthy and require special equipment.

Specificity of the assay is based on the properties of used immunoreactants (anti-DON antibodies and DON-BSA conjugate) and was confirmed experimentally. No interactions with other widely presented mycotoxins such as ochratoxin A, aflatoxin B1, zearalenone, T-2 toxin, or fumonisin B1 were found for their 500-fold excess by weight in comparison with DON both for ELISA and the ICA.

The development of assay does not require the synthesis of new reagents. Manufacturing highly sensitive tests requires changing only the component ratio. Direct calibration is easy to use. The appearance of coloration corresponds to the minimum concentration of the working range (the instrumental detection limit does not differ from the visual one). The analytical zone with the hapten–protein conjugate became the control zone in the new test. Moreover, the control zone of the test is more accurate, and its intensity depends on the antigen-binding ability of the conjugated antibodies. A limitation of the new approach is the need for pure antibody preparations. Thus, the approach cannot be realized with the use of polyclonal antibodies, where the proportion of specific immunoglobulins is small. In addition, the concentrations of labeled antibodies should be carefully selected to achieve the necessary sensitivity without background coloration.

Conclusions

A new scheme for determining low-molecular antigens with direct dependence of staining-antigen concentration was proposed. Using the same reagents, the new scheme allows visual registration of lower antigen concentrations than traditional assays. The test strip for DON detection showed a 60-fold reduction in the visual detection limit (up to 2 ng·mL^{-1}). Changes in the concentrations of specific components are sufficient to transform any traditional test into a test for the new

Table 3 Overview of recently reported nanomaterial-based methods for detection of deoxynivalenol

Materials used	Method applied	LOD	Reference
gold nanoparticles	lateral flow immunoassay	2 ng· mL^{-1}	our work
gold nanoparticles with silver staining	lateral flow immunoassay	40 ng·mL^{-1}	[15]
gold nanoparticles	lateral flow immunoassay	50 ng·mL^{-1}	[21]
gold nanoparticles	lateral flow immunoassay	200 ng·mL^{-1}	[22]
gold nanoparticles	lateral flow immunoassay	50 ng·mL^{-1}	[23]
gold nanoparticles	lateral flow immunoassay	200 ng·mL^{-1}	[24]
gold nanoparticles and polypyrrole-electrochemically reduced grapheme oxide nanocomposite film	electrochemical immunosensing	8.6 ng·mL^{-1}	[25]
a composite from fullerene, ferrocene and the ionic liquid	electrochemical immunosensing	0.3 pg·mL^{-1}	[26]
gold nanoparticles-dotted 4-nitrophenylazo graphene	electrochemical immunosensing	0.3 μg·mL^{-1}	[27]
single-walled carbon nanotubes/chitosan	electrochemical immunosensing	5 pg·mL^{-1}	[28]
upconversion nanoparticles	fluorescence bioassay	0.001 ng· mL^{-1}	[29]
quantum dots	fluorescent immunosorbent assay	0.16 ng·mL^{-1}	[30]
magnetic nanoparticles	magnetic chemiluminescent enzyme immunoassay	0.032 ng·mL^{-1}	[31]

analysis scheme. Thus, any traditional immunochromatographic test strip for a low molecular weight antigen can be converted into a highly sensitive test with visual detection and direct dependence of coloration from the analyte content.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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