Chapter 1
Chromogenic Platform-Based Lateral Flow Immunoassay

Na Liu and Aibo Wu

Abstract In general, several instrumental analytical methods (i.e., TLC, HPLC, CE-MS, LC-MS, MS-MS) of chromatography and rapid detection approaches of ELISA and FLA formats have been widely developed and proved to be sensitive and reliable for diverse occasions and purposes. In the recent decade, there is a still increasing interest in developing new strategic platforms which are simple, portable, disposable, and inexpensive, enabling to rapidly and simultaneously detect the target analytes in the fields of clinical diagnosis, environmental monitoring, and food safety control. Especially, those expected detection formats are extremely useful and highly welcome in remote settings, with high specificity and affinity to the target molecules showing more prospective to fulfill the above objective for these typed rapid analysis, which had been proved to be applicable for detecting clinical diseases and for monitoring environmental and food-based mycotoxins. We here propose some schematic immunoassay via a chromogenic platform-based FLA, attaining to the abovementioned expectations regarding developing ideal rapid tests of mycotoxins, especially for large-scale monitoring for industrial uses.

Keywords Mycotoxin · Rapid detection · Immunoassay · Lateral flow assay · Chromogenic quantitation

1.1 Introduction

Up to now, the most classic and long-term applicable analytical methods are commonly referring to thin layer chromatography (TLC) (Swanson et al. 1984), high-performance liquid chromatography (HPLC) (Tanaka et al. 1985), gas

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chromatography (GC) (Kinani et al. 2008), liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Cavaliere et al. 2005), enzyme-linked immunosorbent assay (ELISA) (Friguet et al. 1985), and lateral flow assay (Smits et al. 2001). As we are all known, there are some unavoidable drawbacks for the individual analysis. To be specific, TLC has poor separation efficiency, as well as low sensitivity and accuracy, which actually limits its application. With the availability of chromatographic instruments of HPLC and GC and linked with different detectors or MS/MS, more satisfactory performances in terms of accuracy, precision, sensitivity, and reproducibility are certainly achieved. However, due to the indispensable highly expensive equipment and strong expertise, as well as laborious procedure for sample preparation before loading, they are not appropriate for large-scale and on-site fast screening in short time. ELISA is one of the main rapid methods for screening analysis, but still in need of reader and relatively complicated sample pretreatment.

Since its initial development in the early 1980s, the paper-based lateral flow immunoassay (LFA) has gained wide applications in rapid clinical diagnosis, food safety, environmental monitoring, and agricultural field for its outstanding advantages of clearly visible result observation, user-friendly format, easy storage, and convenient transportation. The technology of lateral flow immunoassay based on the specific and sensitive antigen-antibody reaction generally provides a simpler and more economical alternative to instrumental approaches for the determination of chemical compounds, including mycotoxins, virus, and large analyses. However, the lateral flow immunoassay has achieved a broad penetration in various areas for its excellent advantages of more reproducible, more sensitive, easier to manufacture, and easier to operate in the last three decades.

To be simplified, LFA strips at least physically consisted of an absorbing pad, a gold conjugate pad, a sample pad, and a nitrocellulose membrane (Fig.1.1). The immunoassay formats can be distinguished into competitive and direct immunoassay according to the character of analytes. Competitive formats are typically applied when detecting for the small molecules, such as antibiotics and mycotoxins which would be exemplified in this paper. In this case, a positive result could be derived from the absence of a test line. In stark contrast, direct formats are always used for

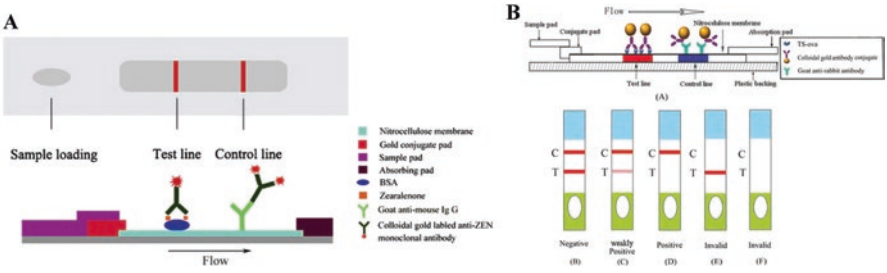

Fig. 1.1 The simplified schematic design, inner structure, and lines for reading results of the classic format of lateral flow immunoassay (LFA). (**a**) Different sectors on the horizontal plat of LFA; (**b**) the detection results read by visible lines for small molecules

testing large compounds, such as hCG. In this format for a single assay, a positive result is indicated by the presence of a test line.

In the latest decade, LFA has been widely used for rapid detection of multiple mycotoxins as small molecules, including aflatoxin B1 and B2 (Tang et al. 2009); ochratoxin A (Lai et al. 2009; Laura et al. 2011); deoxynivalenol (Kolosova et al. 2008; Xu et al. 2010); fumonisin B1, B2, and B3 (Li et al. 2012); zearalenone (Kolosova et al. 2007; Liu et al. 2012); and T-2 and HT-2 (Molinelli et al. 2008). Nevertheless, in the previously reported cases for LFA against the target mycotoxins, they are commonly for qualitative analyses and visible reading via naked eyes. It is not well known whether the feasible and applicable candidate LFA-related approach simultaneously fulfill the needs for both rapid analysis and sensitive quantitation in this aspect. Also, when we select the suitable antibody as sensing materials for the given toxin, we have to comprise the high sensitivity, assay specificity (cross-reactivity), and wide detection range, plus the complex matrices.

To overcome the above posed difficulties, the ultimate goal of chromogenic platform-based lateral flow immunoassay is to open a novel channel based on the most ubiquitously applicable LFA; to develop one combinatorial means for rapid and sensitive quantitation of ZEN contamination, which could be commonly acceptable for a wide variety of agricultural matrices, with simple sample preparation, capability for quantitation, satisfactory detection limit, sufficient accuracy, and stability; and to meet the higher requirements when applying in practical uses on some specific occasions.

1.2 The Selected Antigen and Antibody

The technical basis of LFA is the interaction between antibodies and antigens. Thus the availability of antigens and antibodies with good characteristics in terms of sensitivity, affinity, specificity, and purity will consequently ease the rest of the establishment of LFA strips. Immunogenicity and purification are key factors of antigens. Indeed, impurities will give rise to relatively low-immune response and poor quality of the antibody. Antigens of the larger molecular size usually give a good immune response, while smaller ones could not. For this reason, antigens of smaller size are generally coupled to proper carrier proteins, often bovine serum albumin (BSA), ovalbumin (OVA), or keyhole limpet hemocyanin (KLH).

The availability of a new highly sensitive monoclonal antibody against the target molecule plays a key role in LFA systems, highly determining the detection sensitivity and specificity for cross-reactivities. During the whole procedure of preparing the antibody, at the first-run screening, more than 50 positive clones against the selected coating antigen (-BSA or -OVA) were firstly screened. As followed, the clones with desirable performance to recognize the antigen are selected and characterized by SDS-PAGE. As shown in Fig. 1.2a, the anti-ZEN had good purity revealed by the apparently clear heavy and light chains at 50 and 20 KD, respectively. For further analyses, the competitive indirect ELISA system was applied to test the

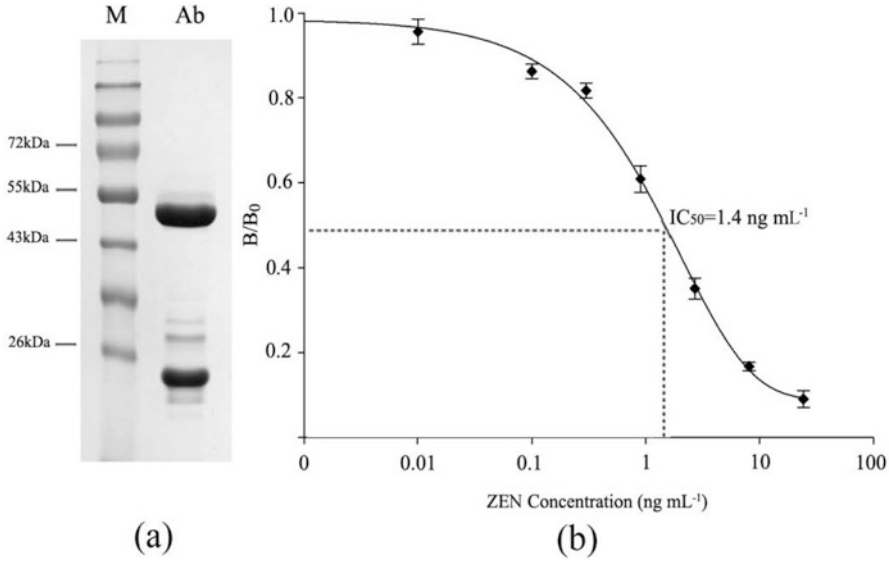

Fig. 1.2 Characterization of the selected monoclonal antibody on SDS-PAGE (**a**) and the performance of the anti-ZEN using the competitive ELISA. Each point represents the mean of three replicates (**b**). (Liu et al. 2012)

sensitivity of more anti-ZEN, in order to get the best clone which could produce the anti-ZEN with the highest sensitivity to ZEN. Finally, as indicated in Fig.1.2b, the IC50 value was 1.4 ng mL^{-1}, and the antibody titer was 1:256,000 (Liu et al. 2012).

Recently, for replacement of routine mouse monoclonal antibody, rabbit has been used to produce rabbit monoclonal antibody (RabMAbs) due to the bigger spleen and better responsiveness for broad classes of antigens (Yu et al. 2010; Spieker-Polet et al. 1995). Moreover, RabMAbs were considered to be superior to MAbs from mouse origin owing to various advantages such as a wider repertoire, simpler structure, higher binding affinity, more robust reproduction, and easier to be humanized (Feng et al. 2011; Rocha et al. 2008).

Combinatorial strategies for a single mixture immunization, screening, and rabbit hybridoma cell technology were described (Fig.1.3). Six fluoroquinolones (FQs) were conjugated with bovine serum albumin and used as immunogens for subsequent immunization, while a mixture of all was injected for co-immunization. The hybridomas against the individual and multiple FQs were obtained via elaborated screening and used for the production of diverse varieties of rabbit monoclonal antibodies (RabMAbs) against the target analytes. The approach opens a new way for simultaneously obtaining functional monoclonal antibodies which are capable of recognizing both individual and multiple analytes in a single preparation circle.

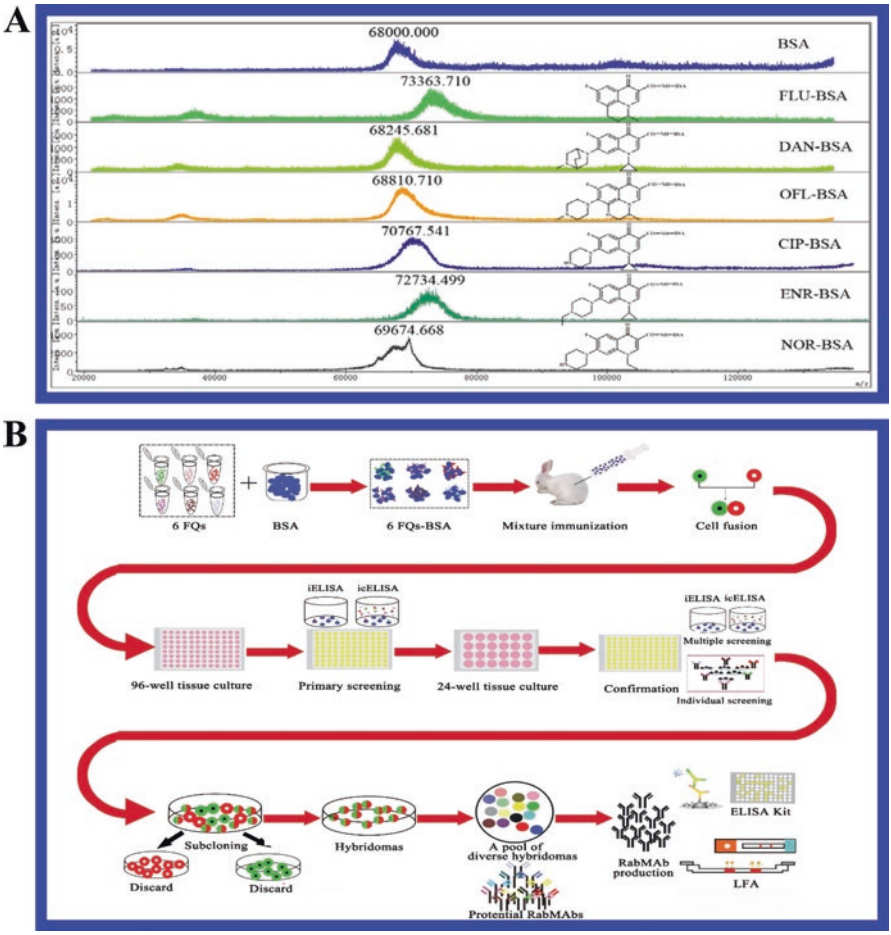

Fig.1.3 (**a**) MALDI-TOF-MS identifications of BSA, FLU-BSA, DAN-BSA, OFL-BSA, CIP-BSA, ENR-BSA, and NOR-BSA from the top down. (**b**) The scheme of simultaneous acquisition of various RabMAbs with diverse recognition functionalities against the target small molecule of fluoroquinolones (FQs). (Liu et al. 2016)

1.3 Chromogenic Reader Designed for LFA Applications

In order to overcome the limitation of misjudgments of positive samples caused by visual difference of individuals, convenient devices were invented by recording and comparing the color intensity values between the test line and control line.

1.3.1 LFA Applied for the Rapid Determination

In this case, ZEN, known as an essential mycotoxin, was screened by a colloidal gold-based competitive LFA integrated with a CHR-100 chromogenic reader (Kaiwood, Taiwan, China).

Practically, to test the accuracy of the established chromogenic platform -based FLA, we select series standard solutions of ZEN of quantitation with different CI values (Fig. 1.4). The lowest level was set at 1.0 ng/mL, which was obviously lower than the limits of ZEN in Chinese standards or even worldwide regulation concentrations. Of course, the final check using real samples contained ZEN was also consistent with the results of in-parallel LC-MS/MS analyses.

With the availability of three class-specific monoclonal antibodies, a multiplex LFA platform for simultaneous multi-class qualification or semi-quantification of AFB1, ZEN, DON, and their analogs (AFs, ZENs, DONs) was established (Fig.1.5). Moreover, the technical platform offers multiple advantages for simplicity, rapidity, sensitivity, cost-effectiveness, and time-efficiency.

Matrix effect is one of the major problems, which significantly affects the real application of the developed rapid detection platforms. For the evaluation of matrix effects, four varieties of blank samples to the blank matrices of different matrices were prepared and pretreated.

The achieved data (not shown) indicated that the samples of wheat and baby food displayed negligible matrix effects, and the sensitivity of detection also remained at

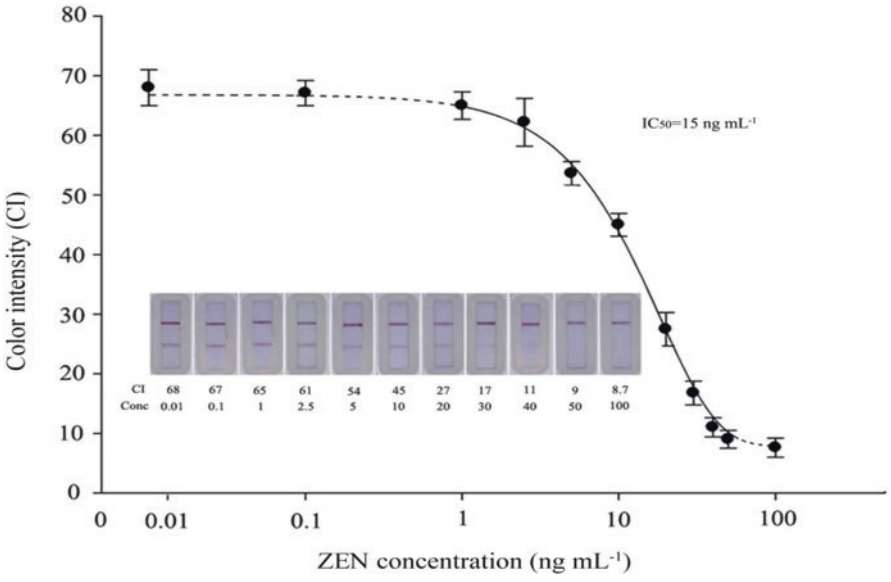

Fig. 1.4 LFA of a series of ZEN concentrations (0.01–100 ng mL^{-1}) in PBS. All points are means±s.d. of four independent measurements for each concentration. (Liu et al. 2012)

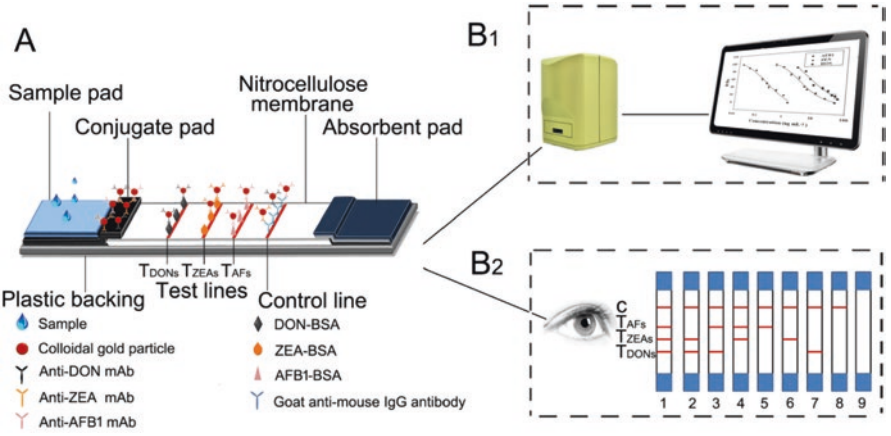

Fig.1.5 A multiplex LFA platform for qualitative and semi-quantitative determination of aflatoxin B1 (AFB1), zearalenone (ZEN), deoxynivalenol (DON), and their analogs (AFs, ZENs, DONs). (**a**) Scheme of the multiplex lateral flow immunoassay (LFA) for multiplex mycotoxins. (B_1) Semi-quantitative analysis platform for LFA. (B_2) Qualitative analysis platform for LFA. Strips 1–9 are the schematic illustrations of detection results. (1) AFs (−), ZENs (−), DONs (−); (2) AFs (+), ZENs (−), DONs (−); (3) AFs (−), ZENs (+), DONs (−); (4) AFs (−), ZENs (−), DONs (+); (5) AFs (−), ZENs (+), DONs (+); (6) AFs (+), ZENs (−), DONs (+); (7) AFs (+), ZENs (+), DONs (−); (8) AFs (+), ZENs (+), DONs (+); (9) invalid result. (Song et al. 2014)

a similar level. On this contrast, the detection of ZEN could be seriously affected by the matrices of feed and maize extracts since the curves constructed in these two matrices were different from the curve constructed in PBS. Despite the existence of the serious matrix effects, the developed test strips based on the chromogenic platform, other than the naked eyes, were still sensitive enough (1 ng mL^{-1}) to recognize the detectable ZEN in most cases, and the concentrations of ZEN could be accurately calculated in those complicated matrices.

1.3.2 Comparative Analyses of LC-MS/MS and Chromogenic LFA in Real Samples

To finally assess the accuracy of the developed chromogenic membrane-based LFA, the proper tests for its detection performance were thoroughly verified by in-parallel LC-MS/MS analyses in real samples which could contaminate the target mycotoxins.

In total, 32 food and feed samples were selected for via LC-MS/MS evaluation, where 7 positive ones were found, with the contamination levels ranging from 14.3 to 131.9 μg kg^{-1}, while the values of chromogenic LFA were 20.4–94.0 μg kg^{-1}. According to case-by-case comparison, the data and detection results are almost identical and consistent. This is the most compelling evidence to demonstrate that the developed chromogenic platform-based LFA is fast, reliable, and sensitive, supporting its application for both qualitative measurement and quantitative analyses.

1.3.3 Storage Stability

Normally, the developed LFA strips have above 12–18-month shelf-life and even could be used as far as it is calibrated before use. Moreover, the measured CI values indicate that the LFA strips remain almost 85% efficiency, supporting its effectiveness.

1.4 Conclusion

LFA strips have been proved to be the most ubiquitously applicable formats of rapid detection in practical uses, in China and other developing countries or even in developed countries for large-scale screening analyses in industrial plants. Previously available LFA strips have been used to qualitative assays against single molecule on result observation as positive or negative via naked eyes, with the commonly acceptable cutoff values. The existed insufficiencies on sensitivity and incapability for quantitation seriously limited the wide applicability of LFA, which could not fulfill the currently strictest regulation requirements in mycotoxin monitoring. This combinatorial means of LFA and chromogenic device present enables to perform quantitative analysis against three target molecules in a wide range of biomatrices, simultaneously carrying the desirable accuracy, which was thoroughly confirmed via in-parallel LC-MS/MS analyses. More importantly, this methodological platform that will benefit not only to rapid analysis of small molecules or proteins but also provide new insights to utilize the developed LFA for remote quantitative determination counting on the output images, which would be proved generally applicable to a wide range of biological systems, related to food safety management, environment monitoring, and diagnostically clinical services.

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