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Advances in Visual Immunoassays for Sensitive Detection of Mycotoxins in Food—A Review †

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Abstract: Mycotoxins are the toxic secondary metabolites naturally produced by fungi, their contamination in agricultural products and food severely threaten food safety and public health worldwide. The reliable, efficient, and sensitive quantification of mycotoxins in food have become increasing challenging to tackle due to the complexity of food matrices and their low level. Visual detection has emerged as a popular trend toward miniaturization and simplification of mycotoxins assays yet is constrained with their limited sensitivity. In this review, we mainly focus on the various kinds of the visual immunoassays by utilizing nanomaterials for loading enzyme and nanozyme. These enzymes have been as signal amplification for the improved sensitivity of mycotoxins detection through the various enzymatic catalytic reaction. Besides, the underlying principle and the advantages of the visual immunoassays for mycotoxins have been proposed. And the challenges and perspectives have been proposed to develop improved efficient catalytic detection strategies for mycotoxins in food.

Keywords: mycotoxins; nanomaterials; catalysis; immunoassay; visualization

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1. Introduction

Mycotoxins are toxic secondary metabolites secreted by fungi under suitable temperature and humidity in pre- and/or post-harvest [1-3]. Mycotoxins can affect the quality and safety of agriculture products, the associated processed foodstuffs, feedstuff, and animals. Over 400 mycotoxins have recently been identified, the worldwide occurrence of mycotoxins involving aflatoxin (AF), ochratoxin (OT), zearalenone (ZEN), deoxynivalenol (DON), fumonisin (FB), and T-2 toxin [4,5]. It is well known that aflatoxin is the representative mycotoxins, including AFB_1, AFB_2, AFG_1, and AFG_2, which has been confirmed to be immunosuppressive, teratogenic, and mutagenic [6,7]. Meanwhile, AFB_1 could be metabolized into the toxic hydroxyl metabolite of AFM_1, which are widespread presence of milk and dairy products. Additionally, ZEN with strong estrogenic effect, and OTA with the neurotoxicity and hepatotoxicity could impose adverse effects on animal and humans. To protect the humans from exposure mycotoxins, strict standards of limiting mycotoxin level in food and the associated products have been regulated in many countries worldwide[8]. The monitoring of mycotoxins has been recognized as the significant way to safeguard food safety. Yet the mycotoxins detection in food matrices is challenging due to their low levels, and complex food matrices. Accordingly, it is highly

desirable to conduct the effective, reliable and sensitive analytical strategy for screening mycotoxins in food matrices.

Nowadays, many efforts have been made to detect mycotoxins in food, involving instrumental analysis[9-13] and immunoassays[14-16]. The instrumental analysis requires expensive sophisticated instruments, time-consuming sample preparation process and well-trained staff, which is not suitable for rapid screening numerous samples, and preclude their wide application in resource-constrained regions [17]. Immunoassays have been extensively identified as promising specific recognition for quantifying mycotoxins thanks to their sensitivity, on-site, as well as high-throughput screening capability. The specific recognition interaction between antibody and antigen have generally favored for highly selective and reliable monitoring mycotoxins. Various signal transduction techniques have currently been utilized to conduct mycotoxins immunoassays, such as fluorescence [18-20], electrochemistry [21-24], chemiluminescence [25] and colorimetry [26-28]. Attractively, visual detection, a popular trend toward miniaturization and simplification analysis, is capable of direct observing the results by the naked eye without other sophisticated instruments [29-31]. Currently, various immunoassays involving enzyme-linked immunosorbent assay (ELISA)[32,33], lateral flow immunoassay (LFI) [34, 35], flow injection immunoassay[36], and flow immunoassay[37], have been demonstrated as an excellent platform for discrimination of mycotoxins[7]. Among them, ELISA and LFI served as the representative visual immunoassay, have attracted continuous interest due to their advantages of simple, and on-sites for rapid screening mycotoxin. Yet, the sensitivity of these conventional visual detection requires to be improved for monitoring trace amounts of mycotoxins in complex food matrices. Thus, numerous studies have currently been devoted to the construction of the visualized immunoassays for enhancing sensitive sensing mycotoxins *via* signal amplification.

Recently, the robust enzyme catalytic amplification has been confirmed to enhance the sensitivity of immunoassays. Particularly, elaborate enzymatic strategies for improving the limited enzyme amount and the catalytic activity, have been engineered as efficient and sensitive immunoassays for high-performance sensing targeted analytes. The emerging nanomaterials with unique optical, electrical, magnetic, and catalytic properties provides new opportunities for improving enzymatic immunoassays[38-42]. More evidences have revealed that the integration of novel nanomaterials promoted the sensitivity improvements on mycotoxins detection[43-45]. For instance, Au nanoparticles (AuNPs) functionalized with antibodies, which can effectively discriminate the immune complex and enzyme to catalytic reaction substrate, significantly elevated their analytical performance[46-48]. Accordingly, a combination of nanomaterials and enzymatic immunoassays provides a potent signal amplified platform for highly sensitive and specific rapidly screening of mycotoxins. Herein, we summarize the improvements on signal amplified immunoassays of mycotoxins by the integration of nanomaterials and enzymatic signal amplification. The improvements on sensitivity of mycotoxin in food were emphasized with the assistance of nanomaterials for encapsulation enzyme, enzyme-mediated nanomaterials as the amplified signal readout, and nanomaterials for enzyme-mimics. Challenges and outlook of mycotoxin detection have been proposed to develop the improved efficient visual immunoassays in food.

2. The Signal Amplified Strategies

ELISA as a classical enzyme-based visual immunoassay, mainly involves the sorbent substrate, immuno-recognition and enzyme labels. Typically, the antigen or antibody serves as sorbent substrate to immobilize onto the supporting material, enzyme-labeled molecule then immobilized to sorbent *via* the formation of a bioconjugation, the resultant detectable signal is recorded with the assistance of chromogenic reagent [49]. The sensitivity of ELISA could be effectively enhanced through improving the absorbent substate, the recognition element, enzyme-label, or chromogenic reagent. Among them, enzyme represents the robust signal amplification, which have been extensively utilized to

develop the highly sensitive immunoassays for trace level mycotoxins because of the catalytically amplified signal.

In the conventional ELISA, peroxidase activity of horseradish peroxidase (HRP) has been extensively served as signal amplification for catalysis H_2O_2 into hydroxyl radical (•OH), which can react with the colorless chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB), 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) or o-phenylenediamine (OPD) into blue TMB_{ox}, green $ABTS^{+\bullet}$, or yellow OPD_{ox} under acidic condition. The colorimetric signal intensity is associated with the anchored HRP-labeled antigen or antibody for catalysis chromogenic substrates [50]. Accordingly, the analytes can be quantified through a direct method or an enzyme-labeled secondary antibody. In the previous studies, HRP-labeled antibodies were the most commonly used in the traditional ELISA to realize the various mycotoxin detection in foods [51-55]. The aforementioned ELISA adopted enzyme-labeled secondary antibodies through chemical conjugation to generate signal. Yet the chemical conjugation of enzyme might result in the loss of enzyme activity, low stability for reagents labeling, and decreased sensitivity and specificity of the ELISA [56]. More evidences have revealed that the fusion protein has been recognized as an immunological agent for mycotoxins detection since its good antigen binding and enzyme activity. Clearly, a nanobody-alkaline phosphatase (ALP) fusion protein has been revealed as improved sensitivity for detection of FB_1 and OTA in actual argo-products [57-59].

Note that the enzyme-labeled antigen or antibody revealed the limited enzyme molecules. For instance, HRP-labeled conjugate always presented the limited HRP molecules with approximately 2-3 HRP per antibody [60], which remarkably weaken the enzymatic signal amplification and the sensitivity of immunoassays. Besides the limited enzyme molecules, the low economy of the conjugated enzyme might lead to increase the production cost of the immunoassays [61-64]. Meanwhile, enzyme-label is susceptible to decrease or even loss its catalytic activity upon practical detection [65]. Thus, the efficient strategies of augment enzyme amounts contribute to amplify the sensitivity of visual immunoassay. Various enzymatic signal amplification immunoassays by using nanomaterials as robust scaffold for enzyme immobilization, enzyme-mediated nanomaterials for amplified signal readout, and nanozyme as an alternative for natural enzyme have recently used to improve the enzyme loading and catalytic activity.

2.1. Immobilized Natural Enzymes on Nanomaterials for Amplification

Increasing the enzyme amounts in the final antigen-antibody-enzyme complex facilitates the catalysis of the substrate and signal amplification in a single recognition reaction (Figure 1A). Attractively, nanomaterials can execute as excellent carriers for loading and immobilizing enzymes by virtue of their large surface area-to volume ratio, high loading capacity, facile fabrication, ease of functionalization, and high chemical stability. The multienzymes and antibodies-immobilized on the surface of single nanomaterial to effectively amplify the detectable signal and thus enhance the sensitivity [66]. The emerging nanomaterials of metal/metal oxides nanoparticles, silica nanoparticles [67], carbon nanomaterials, and metal organic frameworks have been demonstrated as the excellent carriers for immobilizing natural enzyme for sensitive analysis. For instance, Zhu et al utilized botryoid-shaped Au/Ag nanoparticles loading HRP–IgG to construct indirect competitive ELISA for amplified ochratoxin A (OTA) sensing in wheat four samples. The high loading amount of HRP–IgG onto the Au/Ag nanoparticles contributed to an improved sensitivity of OTA with the IC_{50} of 0.05 ng/mL, which revealed a 30-fold improvement compared to the conventional ELISA [68]. Besides, Li et al [69] developed indirect competitive ELISA for the total amount of FB_1, FB_2, and FB_3 detection in maize samples based on AuNPs immobilized HRP-goat anti-mouse IgA. The enhanced sensitivity was approximately 10 times compared to the conventional ELISA (Figure 1B). Liu et al [70] developed MOF-loaded HRP and goat anti-mouse IgG for ZEN detection in argo-products. The LOD of this immunoassay achieved 0.5 ng/L for ZEN detection, which showed an approximately

126-fold enhancement relative to conventional HRP-based immunoassay (Figure 1C). Besides to single nanomaterials, polymer-coated nanomaterials as enzyme container have demonstrated to be the amplified strategies of conventional nanomaterials for further elevating the enzyme loading capacity of nanomaterials. SiO_2 NPs carrying poly (acrylic acid) brushes as a "CAT container" were used to amplify the sensitivity of OTA. Xiong's group [71] presented a competitive ELISA for OTA in various argo-products by using CAT-catalyzed the changed plasmonic signal readout of AuNPs. The LOD by naked eye and microplate reader was 10^{-18} and 5×10^{-20} g/mL, which was 7 and 8 orders of magnitude lower than that of CAT-based ELISA (10^{-11} g/mL by the naked eye) and HRP-based conventional ELISA (10^{-11} g/mL by the microplate reader) (Figure 1D).

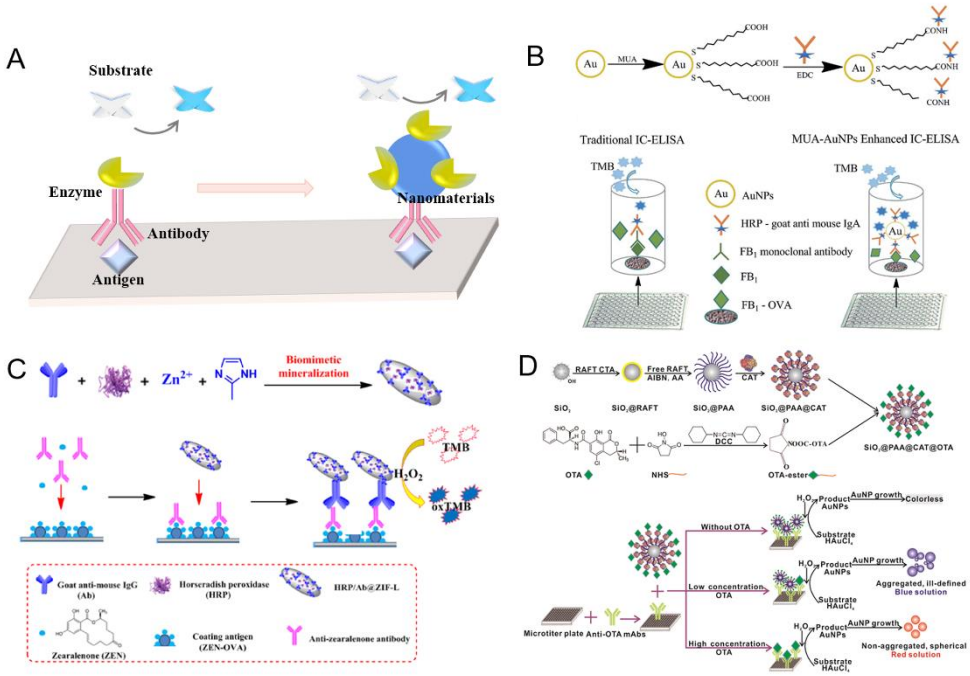

Figure 1. (A) The improved immunoassays using nanomaterials for immobilization natural enzymes; (B) AuNPs-HRP-goat anti-mouse IgA enhanced ELISA for FB_1. Reprinted from ref [69]. Copyright 2018 Royal Society of Chemistry. (C) Zeolitic imidazolate framework-encapsulated HRP-based ELISA for ZEN. Reprinted from ref [70]. Copyright 2021 Elsevier. (D) SiO_2 NPs carrying poly (acrylic acid)@CAT-based ELISA for OTA. Reprinted from ref [71]. Copyright 2016 American Chemical Society.

2.2. Natural Enzyme-Mediated Nanomaterials for Amplified Signal Readout

In addition to the typical chromogenic substrate, natural enzyme-catalyzed products enable regulate the color change of nanomaterials, especially for plasmonic property of AuNPs, achieving the visual detection of mycotoxins (Figure 2A). For instance, Xiong's group [72] developed a direct competitive ELISA through CAT-mediated AuNPs aggregation using HRP + H_2O_2 + tyramine system. In this case, phenol polymerization of tyramine by •OH from HRP-catalyzed H_2O_2 triggered AuNPs aggregation. The competitive antigen of OTA-labeled CAT was employed to catalyze H_2O_2 into H_2O and O_2. AuNPs appeared monodisperse (red) without OTA, while the AuNPs aggregation (blue) were observed with OTA. The combined advantages of ultrahigh CAT catalytic activity and color change of AuNPs contributed to sensitively detect OTA in corn sample. The IC_{50} and LOD (IC_{10}) of OTA was 84.75 and 17.8 pg/mL, which revealed a 2.9- and 2.7-fold enhancement compared with the conventional ELISA (Figure 2B). Meanwhile, this group also utilized the GO_x-catalyzed product of H_2O_2, which reduce Au^{3+} into Au^0 on the surface of Au seeds with an obvious color change for a direct competitive ELISA for FB_1 detection in

maize samples. The IC_{50} was 1.86 ng/mL, which was approximately 13-fold lower than that of HRP-based conventional ELISA [73]. Apart from AuNPs, enzyme-assisted etching of AuNRs triggered visual detection of mycotoxins. HRP-assisted AuNRs-etching direct competitive ELISA was developed to sensitively detect AFB_1 in corn samples. The competitive antigen of AFB_1-labeled GO_x could catalyze glucose molecule into H_2O_2, and HRP simultaneously catalyze H_2O_2 to form •OH. The rod-like morphology AuNRs was chemically etched to spherical morphology by •OH, leading to visual signal output. The etching process of AuNRs efficiently occurred without AFB_1, yet the blocking of AuNRs etching was clear presented in the presence of AFB_1. The method allowed sensitive determination of AFB_1 with IC_{50} of 22.3 pg/mL, which enhanced 32 times compared to the traditional ELISA [74].

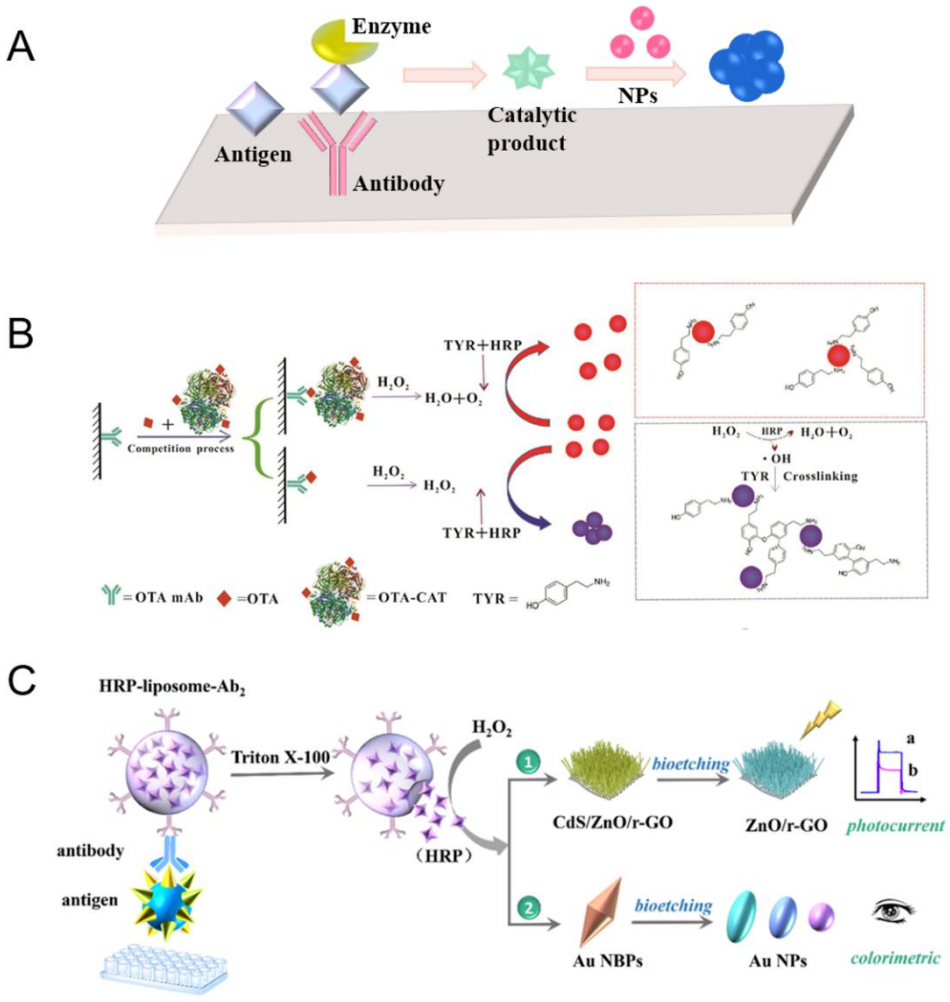

Figure 2. (A) The enzymes-catalyzed products-mediated nanomaterials for signal readout; (B) CAT-mediated AuNPs aggregation-based ELISA for OTA. Reprinted from ref [72]. Copyright 2018 Elsevier. (C) HRP-mediated Au nanobipyramids etching process-based immunoassay for ochratoxins. Reprinted from ref [78]. Copyright 2019 American Chemical Society.

Although these approaches achieved the superior sensitivity, most of them relies on traditional single-signal readout mode. And these strategies might encounter the limitation of inaccuracy for mycotoxins evaluation, which was partly ascribed to external interferences, such as nonstandard test process, different operators or diverse surrounding environment [75-77]. Recent development in mycotoxins immunoassays enable the integration of visual and various signal transduction techniques into dual-signal strategies, and thus offering multi models for mycotoxins detection because of their self-calibration.

Typically, by using the changed multiple color and LSPR shifts of Au nanobipyramids etched by •OH generated from HRP-catalyzed H_2O_2, Wei et al [78] developed an improved colorimetric and photoelectrometric immunoassay for ochratoxins. The nanoliposomes as the vehicle for carrying more secondary antibody and encapsulating HRP significantly amplified the detection signal, allowing the sensitive simultaneous detection of three ochratoxins (OTA, OTB, and OTC). The dual-modality immunoassay presented a high sensitivity with LOD of 0.7 and 1.7 ng/L, respectively. Attractively, the dual-modality response immunoassays showed a more accurate and reliable outcome compared with single modality (Figure 2C).

2.3. Nanozyme for Signal Amplification

Natural enzymes are extensively used in countless laboratories, medical and food safety, whereas their activities were susceptible to the extreme environment (e.g., Heat, pH, organic solvents, mechanical stress, heavy metal) and the limited practical applications (e.g., the preparation, reaction, and storage requirements), leading to their poor operational stability, low recyclability and high expense [79-81]. Nanomaterials-based artificial enzymes (nanozyme) are particularly attractive since the discovery of Fe_3O_4NPs with peroxidase-like activity by Yan's group in 2007 [82]. Nanozyme are excellent candidates for alternative natural enzyme due to their high stability, economy, durability and functionalization. Various nanozyme have been served as catalytic label for multi-category signal amplification in newly developed immunoassays. Nowadays, numerous studies revealed that metal NPs (Au, Ag, Pt, Pd) [83,84], metal oxide NPs (Fe_3O_4, CeO_2, MnO_2, CuO) [85-90], carbon-based (graphene oxide, carbon nitride, carbon dots)[91-94], and MOF-based nanomaterials [95-97] with peroxidase-, catalase-, oxidase-, superoxide dismutase-mimicking properties. And these nanozyme have been designed to amplified sensing of mycotoxins (Figure 3A). For example, Xu et al [98] developed an indirect competitive MOF-linked immunosorbent assay for the high throughput and sensitive detection of AFB_1 grain drinks. Peroxidase-like activity of MOF (MIL-88) was conjugated to secondary antibody to substitute natural HRP-labeled secondary antibody. The MOF-based immunoassay allowed to sensitively detect AFB_1 with the LOD of 0.009 ng/L with 20 times improvement compared to the conventional ELISA. The enhanced sensitivity might arise from their well dispersity, more active sites and pores of MOFs-labeled antibody promoted the catalytic reaction between MOFs-labeled antibody nanozyme and substrate. Significantly, the immunoassay could successfully decrease the occurrence of false positives and false negatives during the detection of AFB_1 (Figure 3B). Besides, Zhu et al [99] developed a competitive ELISA was constructed to sensitively monitor OTA in millet samples (LOD: 0.47 ng/L) through octahedral Cu_2O nanoparticles etching of Au nanobipyramids. Peroxidase-mimicking activity of Cu_2O could oxidize TMB in the presence of H_2O_2, and the yellow product TMB^{2+} could etch the Au nanobipyramids, triggering a significant longitudinal peak blue shift of local surface plasmon resonance. In this case, dopamine-coated microplate was used to capture OTA antigens, and followed by the immunoreaction between OTA antibodies and the Cu_2O-labled secondary antibody. The growing concentration of OTA resulted in a decrease of Cu_2O-labled secondary antibody amount, further imposing adverse effects on the generation of catalytic product TMB^{2+} and the etching process of AuNRs (Figure 3C).

Apart from the single nanozyme for signal amplification, multienzyme-based cascade catalysis is another important strategy for signal transduction and amplification. In the cascade catalytic system, the decreased diffusion path of intermediates between the enzymes enables the improvement of unstable intermediates, which facilitated their efficiency and specificity [100,101]. Meanwhile, the single substrate can be converted into more signal molecule through the multienzyme-associated continuous catalysis reaction, and contributes to the signal amplification [66,81,102]. Lai et al [103] proposed a competitive cascade amplified immunoassay for AFB_1 detection in peanut samples by combination of AO_x/anti-AFB_1 antibody-labeled AuNPs and oxidase-mimics MnO_2. With

assistance of ascorbic acid (AA), blue MnO_2-TMB system was converted into colorless because of the dissolution of MnO_2 into Mn^{2+}. Once introduced AO_x, the color change could be suppressed since AO_x catalysis AA to dehydroascorbic acid. The cascade signal amplification remarkably improved the sensitivity of AFB_1 with LOD of 6.5 pg/mL, which approximately enhanced 15-, 7-, and 38-fold comparative to the existing commercialized AFB_1 kits (e.g., QuickingBiotech:100 ppt; Max Signals: 50 pg/mL; MyBioSource:250 pg/mL) (Figure 3D). Similarly, Lai further developed a competitive immunoassay for sensitive screening AFB_1 (LOD: 0.1 ng/mL) based on the just-in-time generation of an oxidase-mimics MnO_2 through the reaction $KMnO_4$ and Mn^{2+} with the assistance of AO_x [104].

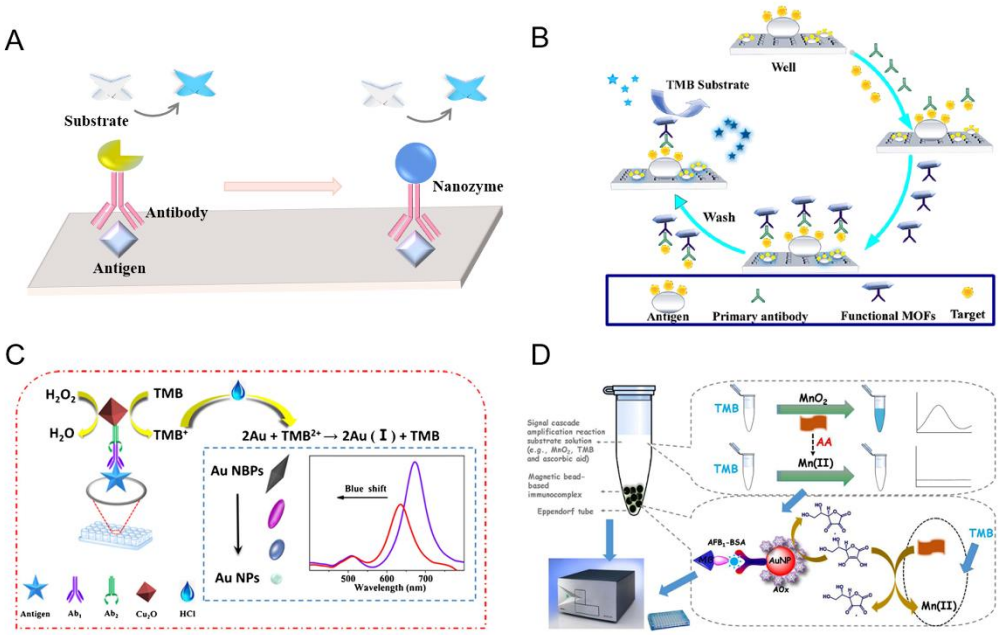

Figure 3. (A) Nanozyme-based immunoassays; (B) MOF-linked immunosorbent assay for AFB_1 detection. Reprinted from ref [98]. Copyright 2021 Elsevier. (C) Peroxidase-like activity of Cu_2O-based immunoassay for OTA detection. Reprinted from ref [99]. Copyright 2021 Springer Nature. (D) MnO_2-AO_x cascade amplified immunoassay for AFB_1 detection[103] Reprinted from ref [103]. Copyright 2017 Elsevier.

Similar to ELISA, LFI is another important visual immunoassay for nanomaterials-labeled one-step immunochromatographic paper-based point of care tests. LFI is widely adapted to detect mycotoxins in food safety owing to its low cost, rapidly, and ease of use [105-107]. The components of LFI mainly include sample pad, nitrocellulose (NC) membrane containing the test and control zones, conjugate and absorbent pads from cellulose, and a polyvinyl chloride backing card for assembling the components [108]. Once dropped sample solution to the sample pad, it can migrate along the strips driven by capillary forces [109]. And then, the sample dissolves the detection reagent in the conjugation pad, followed by flows along the strip within the porous membrane, where the analyte and the signal reporter were captured on the test line, thereby leading to the generation of a detectable signal. AuNPs is the common signal labeled material for visual output through non-covalent electrostatic adsorption of antibodies or antigens[110]. Au nanomaterials-based LFI have been extensively developed for analysis multiplex mycotoxins including FB_1[111], AFB_1[112], OTA[113], ZEN[114] etc. Besides, natural enzymes also provide signals through conjugating to mycotoxin-protein, and executed as the signal transducer to achieve visual detection [115], such as HRP-labeled antibodies or /antigen for immunological recognition for construction LFI[116,117]. Nowadays, numerous nanozyme have been used to label antibody or antigen for visual rapid detection in LFI. The evidence of Fe_3O_4 nanozyme for enhanced detection Ebola virus with 100 times

enhancement compared to the conventional AuNPs-based LFI, revealing the signal amplification ability of nanozyme[118]. Various fascinating nanozyme, such as AuPt nanoflowers[119], Pt nanocatalyst[120], Pt-Ni(OH)$_2$ nanosheets[121], Prussian blue NPs (PBNPs)[122], been used to construct LFI, and realized the their widely application in food safety. For example, Tian et al. developed PBNPs as a marker signal LFI platform for OTA in soybeans samples. The new signal of PBNPs can be amplified *via* the TMB cascaded signal. The colorimetric signal of PBNPs accumulated on the test line through specific immune interactions, triggering the formation of a visible blue line. Meanwhile, the colorimetric signal could be further amplified *via* the peroxidase mimic property of PBNPs. This proposed LFI significantly improved the sensitivity of OTA with 2-3 orders of magnitude relative to commercial AuNPs-based LFI[123].

3. Conclusions and Outlook

Mycotoxin contamination is a continuous global concern for food safety. Visual immunoassays remain simple, rapid, on-site detection of mycotoxins contamination as alternative to traditional sophisticated techniques. The combination between conventional visual immunoassays and nanomaterials, novel visual immunoassays tend to be popular for mycotoxins by using the signal amplified strategies for tackling their inherent limited sensitivity. The representative immunoassays based on various nanomaterials could achieve the enhanced sensitive detection of mycotoxins using the enzyme-nanomaterials catalytic strategies. Enzyme-immobilized onto nanomaterials, enzyme-mediated nanomaterials for amplified signal readout, nanomaterials-based artificial enzyme for amplifying the sensitivity of mycotoxins detection.

Although the aforementioned sensitive strategies for visual mycotoxins immunoassays have revealed the outstanding analytical performance and a fascinating prospect, while there are still many challenges needing to be tackled. (1) The visual signal is obtained by the naked eye, yet the reliance on manual observation rather than instrumental measurement might cause large subjective uncertainty, as well as difficulty in reporting quantitative data. The integration of digital technology [124] (e.g., machine vision) to simulate human visual ability and objective perception, the accurate and reliable results could be easily quantified, and thus might reduce subjective errors in manual observations; (2) compared to the traditional immunoassays, the limited reproducibility and stability of nanomaterials-based immunoassays is the important obstacle for further application in food analysis due to their experimental and systemic factors. The standardization of nanomaterials preparation could effectively guarantee the reproducibility and stability of nanomaterials-based immunoassays; (3) most of visual immunoassays are developed for single mycotoxin detection, while mycotoxins always co-occurred with the others in actual food samples. The simultaneous monitoring multi-mycotoxins by combing the multi-recognition elements in immunoassays facilitate to shorten time, save cost and alleviate labor force; (4) integration the multi analysis technologies (e.g., magnetic, optical, and thermal properties, etc) by coupling to visual analysis technology, multi-signal immunoassays of mycotoxins contribute to minimum background signal and false positive errors.

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