Affinity Biosensors for Detection of Mycotoxins in Food

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Abstract

This chapter reviews recent achievements in methods of detection of mycotoxins in food. Special focus is on the biosensor technology that utilizes antibodies and nucleic acid aptamers as receptors. Development of biosensors is based on the immobilization of antibodies or aptamers onto various conventional supports like gold layer, but also on nanomaterials such as graphene oxide, carbon nanotubes, and quantum dots that provide an effective platform for achieving high sensitivity of detection using various physical methods, including electrochemical, mass sensitive, and optical. The biosensors developed so far demonstrate high sensitivity typically in subnanomolar limit of

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detection. Several biosensors have been validated in real samples. The sensitivity of biosensors is similar and, in some cases, even better than traditional analytical methods such as ELISA or chromatography. We believe that future trends will be focused on improving biosensor properties toward practical application in food industry.

1. INTRODUCTION

Biosensors are portable analytical devices that utilize biochemical recognition elements for sensitive and selective capture of analyte molecules on the transducer interface (Thevenot, Tóth, Durst, & Wilson, 1999). Since the first article was published in the biosensor area in 1962 (Clark & Lyons, 1962), great efforts have been made to their commercialization and use in medicine, pharmacy (Kirsch et al., 2013), agriculture (Antonacci, Arduini, Moscone, Palleschi, & Scognamiglio, 2018), food industry (Rotariu, Lagarde, Jafferesiz-Renault, & Bala, 2016), and environmental monitoring (Arduini, Cinti, Scognamiglio, Moscone, & Palleschi, 2017). The attention paid to biosensors is related to remarkable advantages they promise, e.g., autonomous operation, simple assembling and detection protocols, and user-friendly measurement mode.

Biosensors in food industry and environmental monitoring are mainly considered as a kind of warning devices designed for detection of dangerous levels of contaminants or for their semiquantitative determination on the limited threshold levels (Scognamiglio, Arduini, Palleschi, & Rea, 2014). The use of biosensors does not assume competition with conventional laboratory equipment like chromatographs or mass spectrometers that provides higher sensitivity and a broader list of analytes determined. Meanwhile, biosensors are quite appropriate as point-on-demand devices used outside chemical laboratory. They do not require well-trained personnel or specific auxiliary equipment. Instead, biosensors diminish risks related to unexpected contamination of foodstuffs, falsification of drugs, and potential hazards in industrial emergencies and accidents.

Besides chemical contamination caused by industry, mycotoxins are of great concern as common toxic species present in feedstocks and foodstuffs. Mycotoxins are secondary metabolites produced by molds of *Aspergillus* family, which exert toxic effects on vertebrates including humans (Kumar, Mahato, Kamle, Mohanta, & Kang, 2017). They are frequently present in many agricultural products, especially cereals and cereal-based foods, oilseeds, tree nuts, and spices (Pinotti, Ottoboni, Giromini, Dell'Orto, & Cheli, 2016;

Shephard et al., 2012). The European Food Safety Authority (EFSA) estimated risks related to mycotoxins poisoning within 2002–12 as 15% against 20% of persistent organic pollutants (Alexander et al., 2002). Mycotoxins lead to severe complications in the human health due to high hepatotoxicity, teratogenicity, and immunotoxicity. A large scale of the agriculture production and stability of mycotoxins in the food processing conditions including fermentation and pasteurization complicate control of the mycotoxins contamination.

Immunoanalysis, a conventional tool for specific bioassay, is frequently applied in medical diagnostics (Wu, 2006). In them, target species (antigens) are bonded to specific proteins (antibodies) excreted by T-lymphocytes as a part of immune system of the organism. The formation of antigen–antibody complex is very specific and makes it possible to distinguish individual compounds or structurally relative species depending on particular goals of the analysis and affinity of antibodies used. The antibody binding is commonly performed on a solid support and the target interactions are detected by specific labels introduced in the structure of the reactants. ELISA (enzyme-linked immunosorbent assay) is a "golden standard" of immunochemical detection that shows both high specificity and reliability of the response (Morgan, 1989). The enzyme labels are sensitively detected by changes in optic properties or electrochemical reactions following the addition of specially designed substrates. The immunoassay makes it possible to detect ultra-low amounts of target species. Nevertheless, there are some problems referred to the long duration and multiple stages of a single measurement, relatively low stability of antibodies during their storage and application and difficulties in antibody selection and mass production, especially in case of recognition of low molecular compounds (haptens). *Immunosensors* are intended to simplify conventional immunoassay modes by reduction of the reagent quantities and stages of their stepwise addition/washing and by making easier detection of the signal produced by specific labels (Duffy & Moore, 2017; Ricci, Volpe, Micheli, & Palleschi, 2007). Regarding mycotoxin detection, further progress in immunosensor design involves the selection of more specific antibodies and elaboration of novel protocols for their immobilization and signal detection. The latter stages should take into account a small size of aflatoxins and their modest influence on the label characteristics and immunosensor interface. The screening of new approaches to the detection of such analytes belonging to hapten family remains in the scope of immunosensor development efforts (Piro & Reisberg, 2017).

Aptamers are artificial oligonucleotides synthesized from a random library of RNA/DNA nucleotides followed by selection of suitable sequences against analyte molecules by the affinity chromatography (SELEX protocol) (Famulok & Mayer, 1999). Aptamers are more stable toward hydrolysis and easily modified than antibodies. Their use can prolong the storage period of appropriate biosensors called *aptasensors*. The efficiency of aptamer-based analyte binding depends on the architecture of the aptamer molecule and the way it is implemented in the biosensor assembly (Groff et al., 2015). In some cases, appropriate binding constants are comparable with those of antibodies. For this reason, aptamers are called as "artificial antibodies" and considered as their replacement in immunoassay (Toh, Citartan, Gopinath, & Tang, 2015). As in the case of immunosensors, aptasensors call for further improvement of the aptamer structure and immobilization protocol and new approaches to the enhancing sensitivity of analyte detection.

In this review, the application of affinity biosensors based on antibodies and aptamers has been described for the determination of mycotoxins as food contaminants.

2. MYCOTOXINS AND CONVENTIONAL ANALYTICAL METHODS FOR THEIR DETECTION IN FOOD

Conventional analytical methods for determining mycotoxins mainly include chromatographic techniques, namely thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC) as well as antibody-based fast screening methods such as ELISA (Anfossi, Giovannoli, & Baggiani, 2016; Rahmani, Jinap, & Soleimany, 2009; Shephard et al., 2008; Zheng, Richard, & Binder, 2006). Independently from the detection technique employed, the following common sequence of analytical steps will be followed in mycotoxin detection: sampling, homogenization, pretreatment (extraction, cleanup), immunological or chromatographic analysis, and finally result interpretation (Fig. 1) (Anfossi et al., 2016; Meneely, Ricci, Egmond, & Elliott, 2011; Turner et al., 2015). Most of the chromatographic methods require a preanalysis step, whereas for immunological methods it may not be needed. This step can add extra cost and time to an analytical method, but the improvements in sensitivity, as well as benefits to robustness of a technique (e.g., reducing column blockage and contamination) are important (Turner et al., 2015). Below, we will briefly discuss the methods applied for mycotoxin detection and analysis.

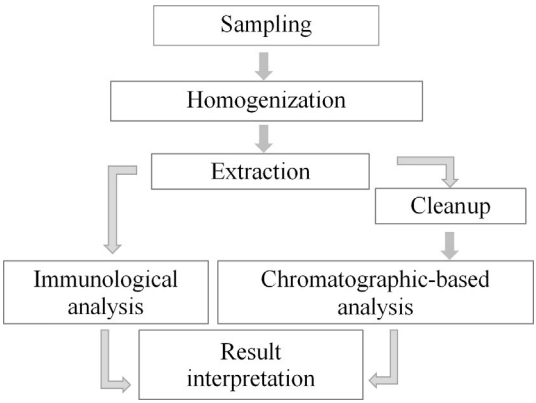


Fig. 1 Sequence of analytical steps for chromatographic-based and immunological methods in mycotoxin detection.

2.1 Methods Based on Chromatographic Techniques

Chromatographic techniques have been at the core of mycotoxin analysis for over 50 years (Turner et al., 2015). These methods are generally used to confirm samples deemed as positive during the screening process and to quantify accurately the concentrations of mycotoxins within food or feed samples (Meneely et al., 2011).

HPLC is perhaps the most frequently and widely used method for the determination of mycotoxins, while TLC and GC analyses are often considered to be less practical, or to have insufficient sensitivity compared with LC methods (Turner et al., 2015). Both normal and reverse-phase HPLC are used for separation and purification (Roseanu, Jecu, Badea, & Evans, 2010). Application of HPLC coupled to specific detectors (ultraviolet, fluorescence, mass spectrometry) allows for a large range of mycotoxins to be determined with the highest sensitivity and selectivity even at very small amount of this substance. This is crucial, due to high toxicity of mycotoxins (Anfossi et al., 2016; Meneely et al., 2011; Turner et al., 2015). Up to date, several well-established, reliable, robust, and sensitive HPLC methods with fluorescence detection have been applied for the determination of mycotoxins with natural fluorescence, e.g., aflatoxins, ochratoxin A (OTA), and citrinin (Kralj Cigić & Prosen, 2009). Mycotoxins without natural fluorescence, such as fumonisin, require derivatization that can be performed by employing *o*-phthalaldehyde or 9-(fluorenylmethyl) chloroformate (Roseanu et al., 2010).

HPLC hyphenated to mass spectrometry (HPLC-MS) has opened new methodologies for routine analysis of mycotoxins and advanced to the status

of the reference and definitive method in the field of mycotoxin analysis in the last 10 years (Kralj Cigić & Prosen, 2009). The introduction of HPLC-MS instrumentation has made possible the development of multitoxin methods suitable for a range of structurally diverse toxins in a single chromatographic run. Moreover, HPLC with MS detection has eliminated the need for sample derivatization for fluorescence activity enhancement and therefore provided a great opportunity for the analysis of mycotoxins (Rahmani et al., 2009).

Overall, as an analytical tool, HPLC offers the advantage of a high resolution, limit of detection, with the possibility to be coupled to multiple detection automated systems. However, it should be noted that most of the chromatographic assays are rather expensive and require sophisticated equipment and time-consuming sample extraction and cleanup procedures.

2.2 Immunological Methods

Immunological methods are based on monoclonal or polyclonal antibodies binding assays raised against toxins (antigens), which can be performed as immunoaffinity column-based analysis or ELISA (Roseanu et al., 2010). Because of simplicity and cheapness coupled to sensitivity and selectivity, immunoassays are preferably employed for the first-level screening and survey studies on mycotoxin contamination (Anfossi et al., 2016). Immunological methods are often called rapid methods as they are simple and easy to use; thus they can be used by nonspecialists and are relatively fast when compared to chromatographic techniques (Zheng et al., 2006).

ELISA methods for mycotoxin assay have been commercially available for more than a decade for important mycotoxins like aflatoxins, fumonisins, and trichothecenes. This technique is based on the antigen–antibody reaction, where mycotoxins play an antigen role. The microtiter plate immunoassay (ELISA format) is most frequently used rapid test for mycotoxins. The assay is mostly performed in a 96-well plate, allowing simultaneous analysis of up to 45 samples in duplicate. A conventional microtiter plate ELISA requires equilibrium of the antibody–antigen reaction that would require an incubation time of approximately 1–2 h. Currently, most of the commercially available ELISA test kits for mycotoxins are working in the kinetics phase of antibody–antigen binding, which reduces the incubation time to minutes.

ELISAs are available in a number of formats, including direct assay, competitive direct assay, and competitive indirect assay (Shephard, 2008).

The direct competitive ELISA is usually the first choice in mycotoxin analysis. After mycotoxin extraction from ground sample a portion of the sample extract and a conjugate of an enzyme coupled mycotoxin are mixed and then added to the respective antibody-coated microtiter wells. Any mycotoxin in the sample extract or control standards competes with the enzyme-conjugated mycotoxin for the antibody-binding sites. Any unbound enzyme conjugate is then removed in a washing step. After washing, an enzyme substrate is added and bound enzyme conjugate converts the substrate into a colored, fluorescent, or chemiluminescent product. The intensity of the resulting color is inversely proportional to the concentration of mycotoxin in the sample or standard. Further, a solution is added to stop the enzyme reaction. In case of horseradish peroxidase (HRP) labeling, the developed yellow color is usually measured spectrophotometrically using an ELISA reader with an absorbance filter of 450 nm (Kralj Cigić & Prosen, 2009; Zheng et al., 2006).

Although conventional analytical methods are known for their accurate and precise detection of mycotoxin in food or feed samples and are widely used in all around the world, they require skilled operators, extensive sample pretreatment, and considerable amount of laboratory equipment and may lack accuracy at low analyte concentration. Therefore, a rapid, sensitive, and specific assay technique is required for the routine analysis of foods and beverages (Chauhan, Singh, Sachdev, Basu, & Malhotra, 2016). Typical limit of detection of mycotoxins for above-mentioned conventional methods is typically in subnanomolar concentration range.

3. ANTIBODIES AND DNA APTAMERS AS RECEPTORS FOR MYCOTOXIN RECOGNITION

As it was mentioned earlier, antibodies are frequently used for immunoseparation and detection of mycotoxins due to rather cheap mass production, high specificity of target interactions, and universal format of signal detections. Due to their small size, mycotoxins should be linked to polymeric carrier like bovine serum albumin (BSA) to exert immune response. Most of the immunosensors developed for mycotoxin determination are based on the direct or indirect competitive assay. Based on the production protocol, antibodies are classified as polyclonal, monoclonal, and recombinant. Polyclonal antibodies are obtained by immunization of animals followed by extraction from blood. Being inexpensive and easy produced in mass scale, they can show insufficient selectivity toward structurally

relative mycotoxins and proteins used as conjugates on the immunization step. Monoclonal antibodies are produced from hybridomas by fusing myeloma or spleen cells from immunized mice. They are more uniform in affinity toward analytes against polyclonal antibodies. The use of recombinant microorganisms excludes necessity of animals for antibody production and makes cheaper the mass production of both receptors and appropriate immunosensors. The functional gene of antibody is cloned and transmitted into prokaryotic or eukaryotic organisms from positive hybridoma or spleen cells (Zeng, Shen, & Mernaugh, 2012). Then appropriate antibody is expressed and collected.

In mycotoxin assay, G-type immunoglobulins (IgG) are most frequently used (Fig. 2).

They consist of two pairs of identical proteins (light L and heavy V chains) that are folded into globular domains assembled with each other. The domains are denoted in Fig. 2 as V_L, C_H1, C_H2, and C_H3. The specificity of antigen binding is achieved due to the variation in the N-terminal part of the protein sequences (variable domains). The opposite C-terminal end of the chain is constant.

Despite high sensitivity and specificity of analyte recognition, antibodies described show some limitations, i.e., need of animals for their production, at least at first stages, susceptibility to denaturation and hydrolytic degradation, and incompatibility with many organic solvents required for mycotoxin extraction. The use of modern methods for modification of antibodies by changing their native structure can avoid at least some of the problems related to the standard immunoassay technique.

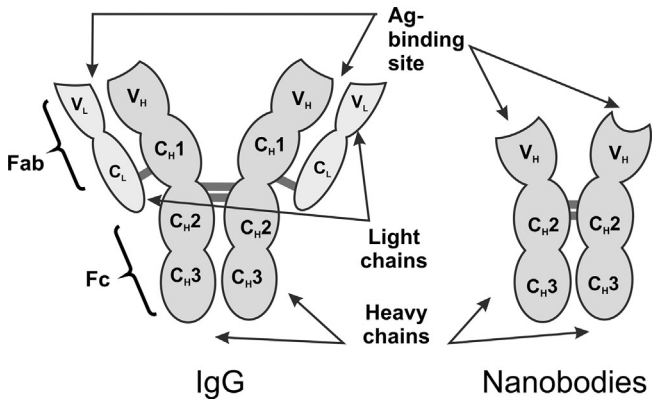


Fig. 2 Schematic outline of IgG structure and derived fragments used in immunoassay of mycotoxins. *C*, constant domains of heavy (H) and light (L) chains; *V*, variable.

Besides whole IgG molecules, their fragments obtained by specific cleavage with proteolytic enzymes or produced by recombinant bacterial cells are used in the assembly of immunosensors (Maragos, 2009). Reduced parts of immunoglobulins can contain the tips of Y-shape molecule (Fab) or single-chain variable fragments (scFv) of both heavy and light chains. Reduced IgG parts are much smaller than intact antibodies and can be easily implemented in the immunosensor assembly. For the same reason, their application minimizes steric hindrance in heterogeneous systems including immunosensors (Romanazzo et al., 2010).

In addition to fragmented natural antibodies, immunoglobulins consisted of heavy chains only (nanobodies) have found increasing attention in the past decade (Harmsen & De Haard, 2007). Found first in camelid species, they can be produced by genetically modified microorganisms, e.g., yeasts. They are more stable than conventional immunoglobulins and easily adapted to various applications, e.g., fusion with markers for detection purpose (Winter, Griffiths, Hawkins, & Hoogenboom, 1994).

The scFv antibodies have been recently reported for aflatoxin determination. The examples of nanobody-based immunoassay are presented later in the description of appropriate immunosensors.

3.1 Zearalenone

The scFv against zearalenone expressed in *Escherichia coli* showed IC_{50} of 14 ng/mL (Yuan et al., 1997). Recombinant antizearalenone scFv fragment was obtained by cloning in plants using *Arabidopsis* seeds (Yan, Hu, Pestka, He, & Hart, 2000). The IC_{50} value achieved was 11.2 ng/mL, which was comparable to that of bacterially produced scFv fragments and parent monoclonal antibody. The antizearalenone scFv produced in recombinant *E. coli* was utilized in direct competitive ELISA with HRP conjugate (Wang, Du, Lin, Huang, & Wang, 2008). The detection limit of 1 mg/L was achieved. A synthetic gene coding for scFv antibody against zearalenone was designed and expressed in *Pichia pastoris* (Chang, Choi, & Chun, 2008). Binding activities of two selected clones to zearalenone were assessed using surface plasmon resonance (SPR) analysis and found comparable or better results against commercial monoclonal antibody (Sigma).

3.2 Deoxynivalenol

The scFv against deoxynivalenol was described for competitive ELISA assay based on HRP conjugate (Wang et al., 2007). The IC_{50} (8.2 ng/mL) and

cross-activity toward nivalenol and T-2 mycotoxins were similar to that of monoclonal antibodies. Multiform scFv derivatives with different linkers and bispecific scFv were obtained by constructing specific expression vector expressed in *E. coli*. The affinity constant increased with the linker length from $9.6 \times 10^{10} M^{-1}$ (no linker) (Wang et al., 2007). In a similar manner, variable segments of the heavy and light chains of antideoxynivalenol were amplified from the pDisplay-4-Fab template and assembled into the scFv gene (Maragos, Li, & Chen, 2012). After polymerase chain reaction (PCR) amplification and purification, it was subsequently cloned into the phagemid pDisplay-2. The plasmid was transformed into *E. coli* host cells. Clones capable of binding the conjugate of deoxynivalenol with BSA were identified by noncompetitive ELISA. The IC_{50} values were found to be 36.1 and 13.8 ng/mL for assays based on the scFv and monoclonal antibodies, respectively. The deoxynivalenol-specific scFv antibody was produced in recombinant *E. coli* (Choi et al., 2004). The variable regions of the heavy and light chains cloned from the hybridoma 3G7 were connected with a flexible linker using an overlap extension PCR. Expression was made by gene fusion and chaperone coexpression providing soluble expression of the scFv. The scFv antibody fused with hexahistidine residues was purified by affinity chromatography. The SPR analysis of specific interactions showed significant but weaker analyte binding against whole antideoxynivalenol antibodies.

3.3 Fumonisin B_1

The scFv was cloned from the hybridoma first used for the monoclonal antibodies production and then expressed in recombinant *E. coli* (Min et al., 2010). The IC_{50} of fumonisin was equal to 220 ppb, 12 times lower than that of parental antibodies. A similar hybridoma-based approach was utilized for construction of scFv fragments from monoclonal antibodies (Zou, Li, He, Chen, & Wang, 2013). Appropriate DNA-coding sequences were amplified using splice overlap extension PCR. Resulting scFv DNA was cloned into the phagemid and functional scFv fragment was identified by phage display. Prokaryotic expression vector pET22b-scFv was constructed to prepare scFv fragments tested in ELISA on standard solutions and spiked corn samples (limit of detection: 8.3 μg/kg).

3.4 Aflatoxin B_1

An antiaflatoxin B_1 monoclonal antibody from the hybridoma 2C12 was produced together with the scFv fragment formed in recombinant *E. coli*

after cloning the scFv-coding gene from hybridoma 2C12. The scFv formed inclusion bodies in the cytoplasm of *E. coli* required in vitro refolding. The IC_{50} obtained by ELISA was equal to 8 ng/mL and cross-reactivity against other mycotoxins less than 4%. The dissociation constant determined by SPR analysis showed the values 17 times higher than that of parent monoclonal antibody (1.16×10^{-7} M vs 6.95×10^{-9} M).

3.5 Aptamers

In mycotoxin analysis, the selection of specific aptamers is complicated due to the low molecular mass of analytes (<1 kDa). First aptamer against OTA was synthesized in 2008 (Cruz-Aguado & Penner, 2008). The aptasensor showed nanomolar detectable concentrations of the mycotoxin. In 2010, a series of 18 aptamers against fumonisin B_1 (FB_1) were selected with the highest affinity of 100 nM (McReague et al., 2010). So far, aptamers against other mycotoxins, e.g., aflatoxin B_1 (AFB_1), aflatoxin B_2, aflatoxin M1, and zearalenone, have been selected and characterized. The examples of appropriate structures are presented in Table 1.

The number of aptamers selected is rather limited taking into account the variety of potential targets and binding sites utilized. The optimization of the aptamer structure is mainly based on the comparison of appropriate binding constants determined using electrophoresis, SPR technique, or other conventional methods developed for such a purpose in immunoassay. The complexity of primary library compiling and SELEX product selection demanded the use of additional screening methods based on computation of aptamer 3D structure and relative affinity capabilities toward certain analytes (Schuster, 2006). Many of the aptamers designed for mycotoxin detection change their configuration after the analyte binding. Guanine reaches sequences that are transformed from the linear structure to G_4-quadruplex, a flat fragment consisting of four guanine residues coordinated with a central K^+ ion (Fig. 3A) (Rhodes & Lipps, 2015).

Such a transformation often defined as aptamer folding results in the charge and density changes within the surface layer that can be monitored using electrochemical impedance spectroscopy (EIS) (Mejri-Omrani et al., 2016), SPR (Yoshida, Saito, Yokoyama, Ferri, & Ikebukuro, 2013), or circular dichroism techniques (Zhao, Dong, Jiang, Zhang, & Liu, 2014) depending on the support nature. Besides, folding affects detection of labels attached to terminal groups of an aptamer.

Among G_4-quadruplexes, aptamer sequences can contain self-complementary fragments to form one or few loops that are unfolded to

Table 1 Aptamers Selected Against Various Mycotoxins for Their Determination

Mycotoxin	Aptamer Structure	Sensitivity	References
Aflatoxin B_1	5′-GTT GGG CAC GTG TT GTC TCT CTG TGT CTC GTG C CCT TCG CTA GGC CCA C-3′	LOD 0.1 ng/mL	Seok, Byun, Shim, and Kim (2015)
	5′-CTC GTC TCG TTC TGT CAG TGT TCT TCT GGC TTG GTG GTT GGT GTG GTG GCT TGA TTT GGT AGA CAC GAA GAA GAA GGA AGG A-3′	K_D = 50.4 nM, LOD 20 ng/mL	Setlem, Mondal, Ramlal, and Kingston (2016)
	5′-TGG GGT TTT GGT GGC GGG TGG TGT ACG GGC GAG GG-3′ 5′-GTT GGG CAC GTG TTG TCT CTC TGT GTC TCG TGC CCT TCG CTA GGC CCA CA-3′	LOD 0.2 ng/mL	Goud, Sharma, et al. (2016)
Aflatoxin B_2	5′-AGC AGC ACA GAG GTC AGA TGC TGA CAC CCT GGA CCT TGG GAT TCC GGA AGT TTT CCG GTA CCT ATG CGT GCT ACC GTG AA-3′	K_D = 9.8 nM, LOD	Ma et al. (2015)
	5′-AAA AAA AAA AGT TGG GCA CGT GTT GTC TCT CTG TGT CTC GTG CCC TTC GCT AGG CCC ACA-3′	LOD 4.5 ppb	Joo et al. (2017)
Aflatoxin M_1	5′-ACT GCT AGA GAT TTT CCA CAT-3′	LOD 5 ng/kg	Sharma et al. (2016)
	5′-GTT GGG CAC GTG TTG TCT CTC TGT GTC TCG TGC CCT TCG CTA GGC CCA CA-3′	K_D = 10 nM	Hamula, Guthrie, Zhang, Li, and Le (2006)

Table 1 Aptamers Selected Against Various Mycotoxins for Their Determination—cont'd

Mycotoxin	Aptamer Structure	Sensitivity	References
Ochratoxin A	5′-GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-3′	0.8 ng/mL	Cruz-Aguado and Penner (2008)
	5′-GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-3′	LOD 0.01 ng/L	Chrouda et al. (2015)
	5′-GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-3′	LOD 24.1 nM	Guo, Ren, Wang, and Wang (2011)
Fumonisin B_1	5′-AAT CGC ATT ACC TTA TAC CAG CTT ATT CAA TTA CGT CTG CAC ATA CCA GCT TAT TCA ATT-3′	$K_D = 100$ nM	McKeague et al. (2010)
Zearalenone	5′-AGC AGC ACA GAG GTC AGA TGT CAT CTA TCT ATG GTA CAT TAC TAT CTG TAA TGT GAT ATG CCT ATG CGT GCT ACC GTG AA-3′	LOD 0.5 ng/mL	Goud, Hayat, Satyanarayana, et al. (2017)

K_D, dissociation constant; *LOD*, limit of detection.

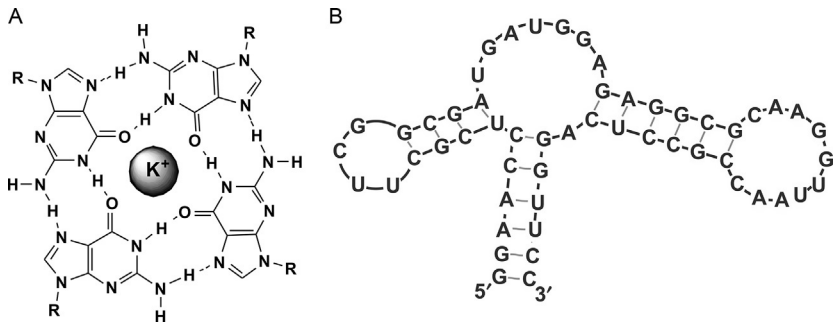


Fig. 3 (A) Structure of G_4-quadruplex stabilized with central K^+ ion; (B) pinhole RNA aptamer against sulforhodamine B. *Partially adapted from Werner, A., Konarev, P. V., Svergun, D. I., & Hahn U. (2009). Characterization of a fluorophore binding RNA aptamer by fluorescence correlation spectroscopy and small angle X-ray scattering.* Analytical Biochemistry, 389, *52–62, with permission of Elsevier.)*

linear conformation in target interactions (see example in Fig. 3B; Werner et al., 2009). The appropriate formats of the signal transduction based on such pinhole aptamers are discussed in the following sections.

4. BIOSENSORS FOR MYCOTOXIN DETECTION

4.1 Immobilization of Antibodies and Aptamers at Surfaces

Implementation of biochemical receptors in the assembly of immuno- and aptasensors is one of the most important stages of the biosensor design. The immobilization protocol should provide a well-reproducible structure of the surface layer with antibodies and aptamers attached to the solid surface (Kokkinos, Economou, & Prodromidis, 2016). The necessity of immobilization stage is attributed to the solution of the following problems:

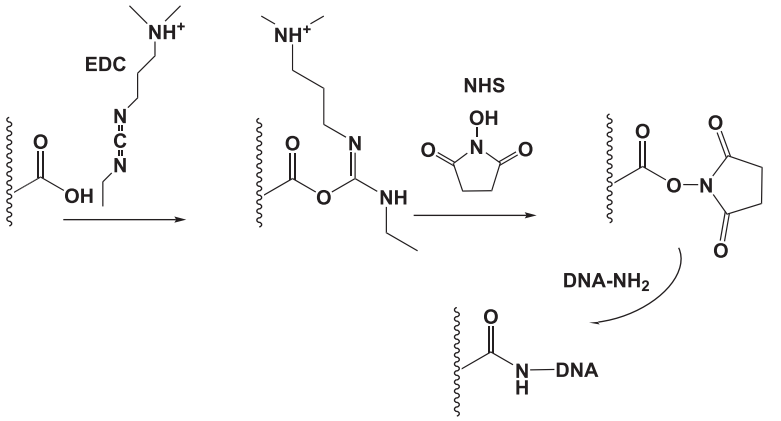
- stabilization of biochemical reactants for prolonged lifetime of the biosensor and decreased drift of the signal during the storage period. The immobilization makes it easier to retain native structure of the antibodies and aptamers in comparison with their solutions especially outside chemical laboratory;
- separation of the recognition products (antigen–antibody or aptamer–analyte complexes) and their purification from excessive reagents;
- sensitivity enhancement due to preconcentration of the analyte on the biosensor interface and separation of the analytes from interferences due to their electrostatic or steric distinguishing.

Contrary to enzyme sensors that mostly require immobilization to reach multiple use of the same enzyme and hence decrease the measurement cost, affinity biosensors are mostly single-use devices and do not assume recovery of the receptors after their contact with the sample tested. Nevertheless, immobilization protocol improves biosensor performance because the target interactions are moved from the solution on the sensor interface where the signal is generated. Besides, many immunosensors follow conventional techniques of heterogeneous immunoassay, mostly ELISA, that also utilize solid supports for reactant separation (Bučko et al., 2012).

Various immobilization protocols have been elaborated for affinity biosensor design. Among many others, site-directed methods become privilege due to higher reproducibility of the sensing layer and lower steric hindrance of the reactions within the layer. The immobilization techniques

are classified in accordance with the reactions and processes providing implementation of the reagents in the surface layer (Balamurugan, Obubuafo, Soper, & Spivak, 2008).

Covalent immobilization provides the most stable product obtained by covalent bonds between functional groups of biochemical reactants and carrier. Carbodiimide binding is the most popular in such biosensors. In this protocol, carboxyl group reacts with amino group to form amide bond that is rather stable toward hydrolysis and oxidation in conventional storage/measurement conditions. An example of the reactions with participation of EDC (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride) and NHS (*N*-hydroxysuccinimide) is presented in scheme (1). Carboxylic groups involved in such an immobilization are provided by carbonaceous materials like carbon black, carbon nanotubes, and graphene oxide. They can be used in suspension with the following deposition on the sensor interface. Instead of EDC, bis(cyclohexyl)- and cyclohexylmorpholinoethyl derivatives are also used for the same purpose (East, Mulvihill, Todd, & Bruse, 2011):

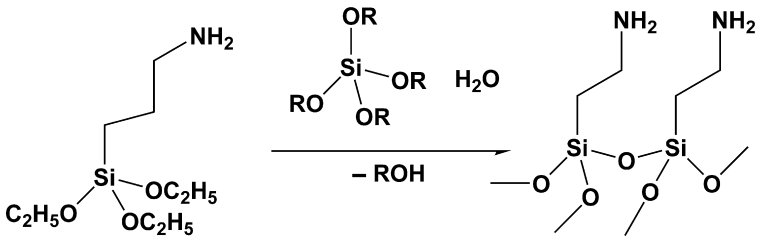


(1)

Glassy carbon and pyrographite electrodes can be preoxidized to form oxygenated surface groups or covered with carboxylated particles that are intended to further carbodiimide binding of biochemical receptors. Antibodies are directly attached to the carboxylated materials "activated" by EDC/NHS treatment, whereas aptamers should be preliminarily modified with primary amino group at their ends.

Aminated carriers suitable for carbodiimide binding are obtained from 3-aminopropyltriethoxysilane (APTES) that is involved in polycondensation

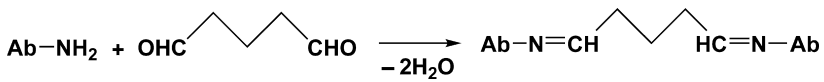
reaction (2) to form silicate layer bearing aminopropyl group (Vashist, Dixit, McCraith, & O'Kennedy, 2011):



(2)

The reaction catalyzed with residues of strong acid can be performed on various hydrophilic supports or in sol–gel immobilization protocols together with chlorosilanes and organosilicates (Yuan et al., 2008). The use of APTES and related amino-functionalized materials in biosensor format is limited by low permeability and insulation properties of the oligocondensation products. This is critical for electrochemical sensors due to disruption of the electron transduction. The limitation is partially avoided by the synthesis of small silicate particles instead of dense film, which can be implemented in electroconductive films (Wang, Ahmed, et al., 2015).

Carbodiimide binding is the most popular but not the only protocol of covalent immobilization. The immobilization of antibodies can be also performed by cross-linking with glutaraldehyde that forms Schiff bases in reaction (3) with two amino groups of proteins or carrier, e.g., aminated cellulose (Isoda, Ichihara, Ryujin, Urushibara, & Shimizu, 2017):



(3)

Besides aldehyde groups, glutaraldehyde reacts with less efficiency with thiols and primary hydroxide. During the storage, glutaraldehyde is reversibly converted into a mixture of reactive oligomers partially soluble in water and able to bind aldehyde groups of the biopolymers (Kuznetsova, Mishaeva, & Gudkin, 1999). All this makes glutaraldehyde cross-linking less reliable and reproducible than the site-specific carbodiimide binding.

Meanwhile, glutaraldehyde is often used for development of protein matrices for antibody immobilization with BSA or gelatin that are first added to the antibody solution and then treated with glutaraldehyde either in water

or in vapors. Then, the products of glutaraldehyde binding can be stabilized by chemical reduction of >C═N bonds with $NaBH_4$ and by treatment with glycine able to bind with excessive aldehyde groups of the products.

The formation of self-assembled monolayers of thiolated molecules on Au films or particles is another example of site-specific immobilization often used in affinity biosensors (Mandler & Kraus-Ophir, 2011). The reaction of Au—S formation is spontaneous and results in the formation of a rather rigid regular film that can be destroyed by UV irradiation or oxidation with strong oxidants. Besides thiolated aptamers, —SH groups of cysteine residues of the proteins can be involved in the reaction. The use of thiols that differ in length of aliphatic linker and charge of terminal groups allows varying hydrophobicity and functional groups of the SAMs and is easily combined with some other immobilization techniques. The examples of the compounds applied in the SAMs for biosensor assembling are presented in Fig. 4.

Thus, the use of mercaptoundecanoic acid provides an easy and reliable method for carbodiimide binding of aminated receptors. This approach is widely used in SPR sensors with a thin golden film as recognition element (Oh, Kim, Park, Lee, & Cgoi, 2004) and in SERS sensors utilizing Au nanoparticles (Ma & Harris, 2012). Mercaptohexanol is mostly applied for the suppression of nonspecific adsorption of proteins and analytes on the Au surface.

Affine immobilization assumes the formation of molecular complexes between native receptors and ligands. For this purpose, avidin (streptavidin)–biotin pair is mostly used. The high affinity of this interaction (dissociation constant $K_D \sim 10^{-14}$–10^{-15} M) makes binding very efficient (Dupont-Filliard, Billon, Livache, & Guillerez, 2004). Each protein molecule can bind

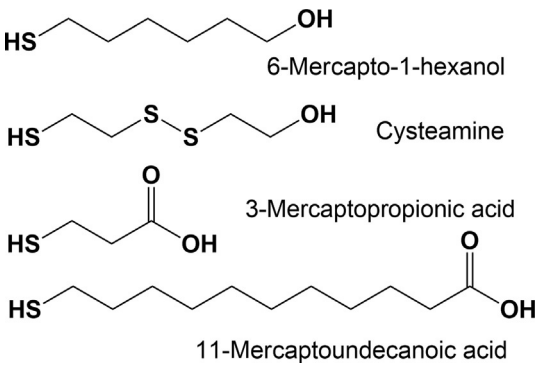
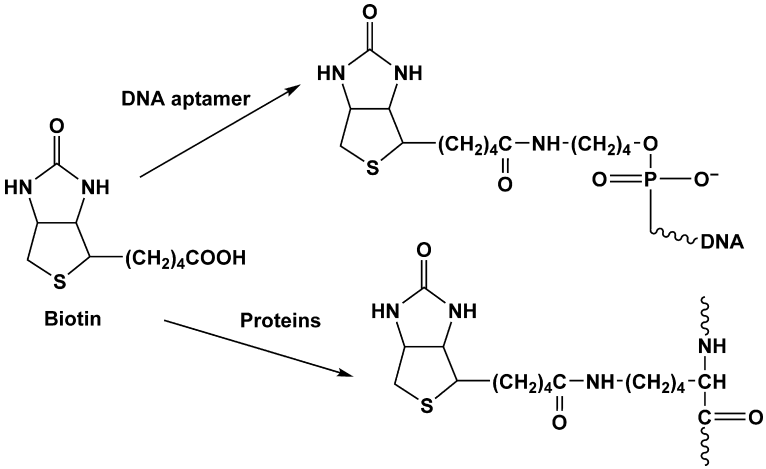


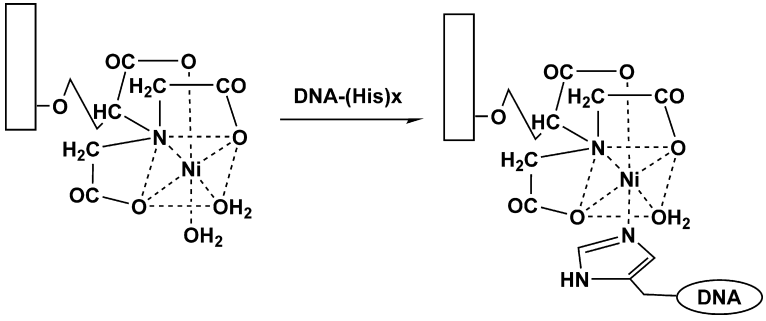
Fig. 4 Thiolated compounds applied for the synthesis of SAMs in biosensor assemblies.

up to four biotin molecules. This allows assembling multilayer complexes with appropriate components including biotinylated enzymes used as labels in ELISA protocols and DNA aptamers. The reactions of biotin introduction for the following affinity binding are presented in scheme (4). Deglycosylated avidin molecules (NeutrAvidin) retain high affinity to biotin and meanwhile can be easily immobilized on golden supports via surface thiol groups:



(4)

Complexes of nitrilotriacetic acid with Ni or Cu cations offer the affinity toward biomolecules bearing histidine tags similar to that of avidin–biotin pair (Baur et al., 2010). The complex coordinates histidine residues in the equatorial position of a complex plane (5):



(5)

Commonly, 5–10 histidine fragments are attached to the DNA aptamer or protein molecule to reach the efficiency of the binding necessary for biosensor development. The silicate sorbent bearing nitrilotriacetic acid is commercially available.

Protein G is another specific receptor used in immunoassay for IgG binding. This is a cell surface protein isolated from *Streptococcus* sp. It consists of three domains one of which can specifically bind to the Fc domain of IgG subtypes. This leads to efficient immobilization of the Fab fragments or whole immunoglobulins on appropriate support (Perotta et al., 2012).

Affine immobilization is site specific and hence exactly determines the structure of the final product. Single-point binding offers a maximal access of the biological targets to the active sites and a minimal influence on the conformation of the protein or DNA molecule.

4.2 Electrochemical Biosensors

4.2.1 Electrochemical Immunosensors

Fig. 5 represents principal protocols for detection of the antibody–mycotoxin interactions. Indirect competitive immunoassay (Fig. 5A) assumes two stages, i.e., homogeneous reaction between a mycotoxin and an access of specific (capturing) antibodies. After that, the mixture is added to the immunosensor where a transducer is covered with the conjugate synthesized from the analyte and inert protein as high molecular support. BSA is frequently used for conjugate synthesis. The reaction results in the binding of the capturing antibodies left free in the mixture. After that, signaling antibodies bearing labels are added to quantify the unbonded analyte molecules in the conjugate layer. The signal of a label can be measured by various electrochemical techniques, preferably by voltammetry. In the second protocol (label free or direct measurement), diffusionally free indicators (redox probes) are used that can be oxidized on the electrode. Implementation of

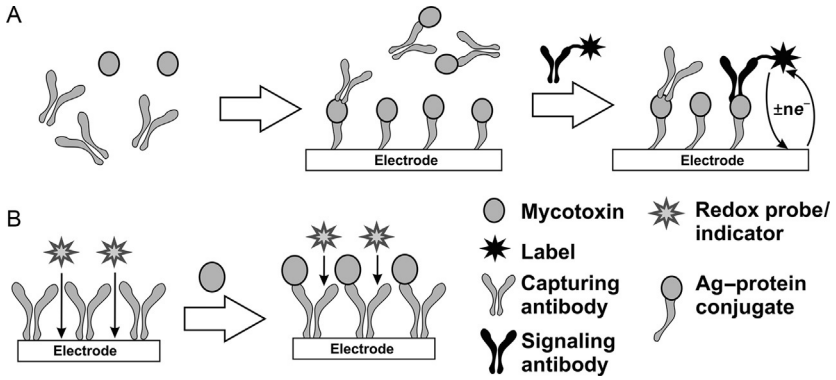


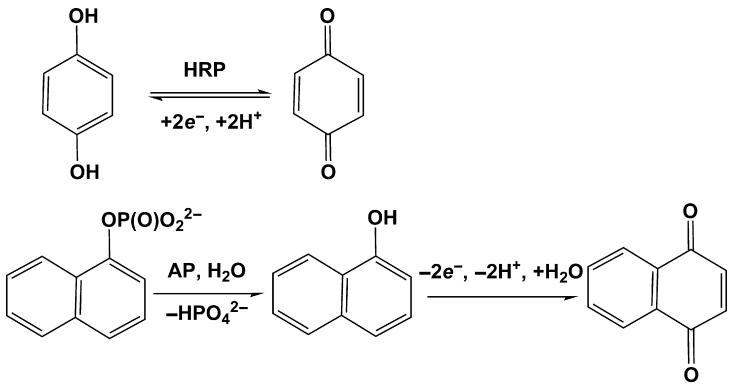
Fig. 5 Outline of detection modes applied in immunosensors for mycotoxin determination. (A) Competitive assay; (B) direct measurement based on partial surface blocking.

mycotoxin molecules in the surface layer changes the rate of diffusional transfer of the indicators to the electrode and hence the rate of electron exchanges (Fig. 5B). The signal can be measured by EIS or voltammetry with ferricyanide ions as indicators. Although mycotoxins are sufficiently smaller in size than high-molecular antigens commonly detected as antigens, this protocol provides required sensitivity probably due to additional changes in the hydrophilicity of the surface layer.

The characteristics of immunosensors developed for the detection of mycotoxins are summarized for the period from 2012 to 2017 in Table 2.

As shown in Table 2, monoclonal antibodies are mainly utilized in electrochemical immunosensors due to higher affinity and selectivity toward target molecules against polyclonal antibodies. The antibodies are immobilized via carbodiimide binding and thiol groups to appropriate carriers, e.g., carbon nanotubes and Au nanoparticles. Other modifiers are mainly implemented in the sensing layer to enhance the signal recorded by voltammetry and EIS.

The selectivity of the signal is mostly established on the stage of antibody selection. The signal transduction mode does not affect the selectivity but seriously influences sensitivity of the assay. The use of electrocatalytic systems or enzyme labeling improves the LODs against other detection systems. Alkaline phosphatase (AP) and HRP are traditionally exploited in the immunosensors due to high sensitivity of their detection and reliable protocols for conjugate synthesis. Hydroquinone and α-naphthylphosphate are commonly used as their substrates. Their reactions on the electrode (6) include their reversible conversion to quinones:



(6)

Cross-selectivity toward other mycotoxins is mentioned only in few articles. Thus, the possible influence of OTA on the signal toward AFB_1 has

Table 2 Analytical Characteristics of Immunosensors for Mycotoxin Determination

Surface Layer Assembly	Signal Measurement Protocol, Samples Tested	LOD, Linearity Range	References
Aflatoxin B1			
Graphene/Au nanoparticles; monoclonal antibodies immobilized in chitosan matric containing ionic liquid	Direct assay, DC measurement of $[Fe(CN)_6]^{3-}$ redox probe; rice, milk, flower, soybean testing	LOD 1 fM, conc. range 3.2 fM to 0.32 pM	Linting et al. (2012)
Carbodiimide binding of monoclonal antibodies onto carbon nanotubes	DC, $[Fe(CN)_6]^{3-/4-}$ redox probe	LOD 0.08 ng/mL, conc. range 0.25–1.375 ng/mL	Singh et al. (2013)
Preoxidized CNTs were mixed together with ionic liquid and antibodies and placed on glassy carbon electrode	Direct assay. DC measurements of $[Fe(CN)_6]^{3-/4-}$ redox probe, olive oil	LOD 0.03 ng/mL, conc. range 0.1–10 ng/mL	Yu et al. (2015)
Reduced graphene oxide, pyrrole–pyrrolopropionic acid copolymer; antibodies attached via carbodiimide binding	Direct assay. DC measurements of $[Fe(CN)_6]^{3-/4-}$ redox probe, corn testing	LOD 10 fg/mL, conc. range 10 fg/mL to 10 pg/mL	Wang, Hu, et al. (2015)
Glassy carbon electrode covered with Au nanoparticles with attached antibody in chitosan matrix; Fe_3O_4 nanoparticles covered with Au and catechol derivative	Competitive assay with DC measurement of catechol redox activity	LOD 0.2 ng/mL, conc. range 0.6–110 ng/mL	Masoomi et al. (2013)

Continued

Table 2 Analytical Characteristics of Immunosensors for Mycotoxin Determination—cont'd

Surface Layer Assembly	Signal Measurement Protocol, Samples Tested	LOD, Linearity Range	References
Reduced graphene oxide on transparent glass with antibodies immobilized onto polylysine	Capacitance measurement with a field effect capacitor	LOD 0.1 fg/mL, conc. range 0.1 fg/mL to 1 pg/mL	Basu, Datta, and Chaudhuri (2015)
Au/reduced graphene layer with monoclonal antibodies attached by carbodiimide binding	Direct assay. DC measurement of electrocatalytic current	LOD 0.1 ng/mL, conc. range 0.1–12 ng/mL	Srivastava et al. (2015)
Porous carbon electrode decorated with Pt nanoparticles	Microfluidic detection by electron transport assessment in the pores	Conc. range from 10^{-12} to 10^{-7} g/mL	Mondal, Ali, Srivastava, Malhotra, and Sharma (2016)
Au electrode covered with poly(diallyldimethylammonium chloride) and Pd–Au nanoparticles and antibodies	Direct assay. DC measurement of $[Fe(CN)_6]^{3-/4-}$ redox probe	LOD 0.03 ng/mL, conc. range 0.05–25 ng/mL	Zhang, Shen, et al. (2016)
Electrode covered with Au nanoparticles with antibodies. Aflatoxin–BSA conjugate labeled with Cu apatite	Competitive assay with stripping voltammetric detection of Cu(II) ions	LOD 0.2 pg/mL, conc. range 0.001–100 ng/mL	Wang, Zhang, et al. (2016)
Poly(ethylene dioxythiophene) modified with Au nanoparticles and antibodies	EIS measurements of charge transfer resistance, maize testing	LOD 0.0156 ng/mL, conc. range 1–25 ng/mL	Sharma, Kumar, and Khan (2017)
Au electrode modified with cysteine, carbon nanotubes, and antibodies by carbodiimide binding	EIS measurement of charge transfer resistance, corn flour testing	LOD 0.79 pg/g, conc. range 0.1–20 pg/g	Costa, Frias, Andrade, and Oliveira (2017)

Aflatoxin M1			
Fe_3O_4-graphene—capturing antibody for magnetic separation, signaling antibodies on carbon nanotubes with CdTe nanodots	Electrochemiluminescence of quantum dots, milk testing	LOD 0.3 pg/mL, conc. range 1.0 pg/mL to 100 ng/mL	Gan et al. (2013)
Ochratoxin A (OTA)			
Electrode covered with magnetically accumulated magnetic beads modified with protein G and monoclonal antibodies	Direct assay with Ochratoxin A–horseradish peroxidase conjugates added to the sample. Amperometric measurement of pyrocatechol formed in HRP reaction, red wine	LOD 0.008 μg/L, conc. range 0.01–20 μg/L	Perrotta, Arévalo, Vettorazzi, Zón, and Fernández (2012)
Electrode covered with magnetically accumulated magnetic beads modified with streptavidin and monoclonal antibodies	Direct assay with ochratoxin A–horseradish peroxidase conjugates added to the sample. Amperometric measurement with hydroquinone as enzyme substrate, red wine testing	LOD 0.11 ng/L (0.12 ng/L in wine), conc. range 0.26–8.87 ng/L (0.24–8.33 in wine)	Vidal, Bonel, Ezquerra, Duato, and Castillo (2012)
Deoxynivalenol			
Electrode modified with Au nanoparticles/graphene oxide bearing 4-nitrophenylazo implemented in Nafion film, monoclonal antibody covalently attached to reduced nitro group of the dye	EIS measurement with Ru bipyridine complex as redox probe	LOD 0.032 ng/mL, conc. range 6–30 ng/mL	Sunday et al. (2015)

Continued

Table 2 Analytical Characteristics of Immunosensors for Mycotoxin Determination—cont'd

Surface Layer Assembly	Signal Measurement Protocol, Samples Tested	LOD, Linearity Range	References
Electrode modified with carbon nanotubes in chitosan matrix and deoxynivalenol–BSA conjugate, signaling antibodies labeled with alkaline phosphatase	DPV measurements with α-naphthylphosphate as enzyme substrate, corn testing	LOD 5 pg/mL, conc. range 0.01–1000 ng/mL	Qing et al. (2016)
Electrode modified with Au nanoparticles/polypyrrole/ antibodies	DPV measurement of intrinsic polypyrrole activity	LOD 8.6 ppb, conc. range 0.05–1 ppm	Lu, Seenivasan, Wang, Yu, and Gunasekaran (2016)
Fumonisin B_1			
Electrode covered with carbon nanotubes in chitosan matrix and fumonisin–BSA conjugate. Signaling antibody labeled with alkaline phosphatase	Competitive assay with DPV measurement with α-naphthylphosphate as enzyme substrate, corn testing	LOD 2 pg/mL, conc. range 0.01–1000 ng/mL	Yang et al. (2015)
Electrode modified with magnetic beads covered with protein G, fumonisin–horse radish peroxidase conjugate	Competitive assay, amperometric measurements with hydroquinone as enzyme substrate, maize testing	LOD 0.33 μg/L, conc. range up to 1000 μg/L	Jodra, López, and Escarpa (2015)
Electrode modified with Au nanoparticles/polypyrrole/ antibodies	DPV measurement of intrinsic polypyrrole activity, corn testing	LOD 4.2 ppb, conc. range 0.2–4.5 ppm	Lu et al. (2016)

Zearalenone			
Capture antibody adsorbed on nitrogen-doped graphene nanosheets, signaling antibodies labeled with Pt—Co nanoparticles	H_2O_2 signal measurement in DC mode, pig feed testing	LOD 2.1 pg/mL, conc. range 0.005–25 ng/mL	Feng et al. (2013)
Electrode covered with dispersion of mesoporous carbon and Au@AgPt nanorattles and antizearalenone antibodies physically adsorbed	Direct assay. Square-wave voltammetry of $[Fe(CN)_6]^{3-}$ ions, milk testing	LOD 1.7 pg/mL, conc. range 0.005–15 ng/mL	Liu et al. (2014)
Electrode covered with polyethylene imine–carbon nanotubes and then Au and Pt nanoparticles followed by immobilization of monoclonal antibodies	Direct assay, DC measurement of $[Fe(CN)_6]^{3-/4-}$ redox probe	LOD 1.5 pg/mL, conc. range 0.005–50 ng/mL	Liu et al. (2017)
Electrode covered with carbon nanotubes in chitosan matrix and zearalenon–BSA conjugate. Signaling antibody labeled with alkaline phosphatase	DPV measurements with α-naphthylphosphate as enzyme substrate, corn, wheat, and fodder testing	LOD 4.7 pg/mL, conc. range 10 pg/mL to 1000 ng/mL	Xu et al. (2017)

been described in the mixtures (Singh et al., 2013) and individual standard solutions (Liu et al., 2009). Regarding the assay of spiked and real samples, corn is frequently tested with mycotoxins added to the extracts. The extraction and extract purification are mostly derived from the appropriate HPLC analysis techniques.

Most immunosensors described involve conventional steps of immunoseparation and rather universal approaches to the signal generation. This is especially true for label-free techniques that are easily adapted to various analytes by simply replacing the antibodies. This might be useful for the development of multiplex immunosensors providing detection of a number of analytes from a single sample. This can reduce the overall duration of the analysis, critical for field applications of the immunosensors. Surprisingly, the number of such multiplexed immunosensors is rather modest.

4.2.2 Electrochemical Aptasensors

The detection schemes of many aptasensors utilize universal approaches previously elaborated for immunoassay. Thus, direct label-free measurements suggest the limitation in the redox probe access due to implementation of the mycotoxin in the surface layer. Contrary to antigen–antibody interactions with domination of steric factors, electrostatic interactions play a significant role in case of aptamer–mycotoxin binding. Negative charge of phosphate residues limits transfer of ferricyanide ions and hence the charge transfer resistance measured with EIS. Electrostatic attraction is used for self-assembling of the surface layer by consecutive deposition of oppositely charged molecules (Evtugyn, Porfireva, & Stoikov, 2017). An interesting case has been described in Sun, Zhang, et al. (2017). In this work, thionine has been covalently attached to an aptamer and mixed with carboxylated graphene oxide. The complex thionine–aptamer–graphene was suspended in the solution. Thionine involved in the complex does not produce the redox signal due to electrostatic repulsion of negatively charged complex from the electrode. In the presence of OTA, the complex is dissociated and OTA is bonded to aptamer–thionine. As a result, the signal appears. Treatment with DNAzyme releases OTA and results in amplification of the signal.

Other signal detection protocols utilized in aptamers for mycotoxin detection are presented in Fig. 6.

In displacement-based sensors, aptamer is involved in hybridization with a short auxiliary DNA probe. In signal-off sensors, second (signaling) DNA probe labeled with redox groups is attached to form sandwich complex

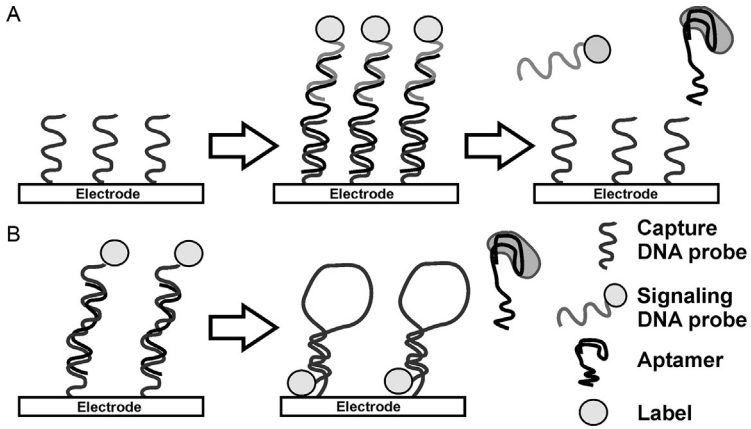


Fig. 6 Schematic outline of aptasensors for mycotoxin determination. (A) Sandwich mode (signal-off sensors); (B) aptasensors on the base of pinhole aptamers (signal-on sensors).

closely to the electrode. As a result, the redox signal of the label is recorded. The reaction with an analyte results in dissociation of triple complex and binding aptamer with an analyte molecule. Labeled DNA probe is removed from the electrode and label signal disappears. In signal on sensors, the aptamer is involved in complementary interaction with double-labeled auxiliary sequence (pinhole DNA probe) that is attached to the electrode by one terminal group (commonly via Au—S binding). The reaction with an analyte removed aptamer from the double-stranded DNA structure. Left free, DNA probe converts to a pinhole structure due to partial self-complementarity of its structure. As a result, second label is moved to the electrode surface so that its redox signal appears. In such signal-on sensors, Methylene blue is frequently used as a signal generating label. Instead of two DNA probes, the same signal-on mode can be realized with an aptamer able to reversibly convert from linear to folded conformation. The displacement-based aptasensors are rather sensitive to the steric limitations of conformational switches. To avoid limitations related to incomplete folding, optimal surface concentration of aptamers should be specified. Besides, aliphatic linkers can be introduced between the electrode surface and an aptamer to move the conformational transfers from the electrode surface.

The examples of application of various measurement protocols to electrochemical aptasensors for mycotoxins determination are summarized in Table 3 for the period from 2012 to 2017.

Table 3 Analytical Characteristics of Aptasensors for Mycotoxin Determination

Surface Layer Assembly	Signal Measurement Protocol, Samples Tested	LOD, Linearity Range	References
Aflatoxin B_1			
Polyelectrolyte layers of poly(neutral red) and carboxylated macrocycle bonded to redox label and aminated aptamer	Changes in DC voltammetry and EIS with $[Fe(CN)_6]^{3-/4-}$ redox probe, peanuts, cashew nuts, white wine, and soy sauce testing	LOD 0.1 nM (DC), 0.05 nM (EIS), conc. range 0.1–100 nM	Evtugyn et al. (2014)
Cystamine–aptamer binding on Au electrode with aptamer cross-linking with glutaraldehyde	Changes in DC voltammetry and EIS in the presence of $[Fe(CN)_6]^{3-/4-}$ redox probe, peanut–corn flakes testing	LOD 0.04 nM, conc. range 0.1–10 nM	Castillo et al. (2015)
Signal-off sensor with Au nanoparticles bearing aptamer with Methylene blue as redox indicator	Telomerase/EXO III two round amplification cycle, square-wave voltammetry measurement of Methylene blue signal	LOD 0.6×10^{-4} ppt	Zheng et al. (2016)
Aptamer monolayer on electrode obtained via diazonium coupling	EIS measurement with $[Fe(CN)_6]^{3-/4-}$ redox probe, beer, and wine testing	LOD 0.12 ng/mL, conc. range 0.125–16 ng/mL	Goud, Catanate, et al. (2016)
Aptamer immobilized on electrode covered with Au nanoparticles modified with luminol, auxiliary DNA probe labeled with HRP, and Au nanoparticles	Simultaneous changes in the HRP activity measured with Methylene blue and electrochemiluminescence in the presence of H_2O_2	LOD 0.43 (square-wave voltammetry) and 0.12 pM (electrochemiluminescence), conc. range 5.0 pM to 10 nM	Wu et al. (2017)
Signal-on sensor with Methylene blue-tagged aptamer in SAM layer on graphene oxide modified electrode	DPV signal of Methylene blue, beer, and wine testing	LOD 0.05 ng/mL, conc. range 0.05–6.0 ng/mL	Goud, Hayat, Catanate, et al. (2017)

Aflatoxin M_1			
Aptamer immobilized onto Fe_3O_4-polyaniline composite by cross-linking with glutaraldehyde	EIS measurements with $[Fe(CN)_6]^{3-/4-}$ redox probe, milk testing	LOD 1.98 mg/L, conc. range 6–60 ng/L	Nguyen et al. (2013)
Aptamer immobilized onto electrode by diazonium binding	Direct EIS and DC measurement in the presence of $[Fe(CN)_6]^{3-/4-}$ redox probe, milk testing	LOD 1.15 ng/mL conc. Range 2–150 ng/mL	Istamboulié et al. (2016)
Ochratoxin A			
Thiolated aptamers attached to Au nanoparticles in dendrimer matrix on electropolymerized neutral red	EIS measurements with $[Fe(CN)_6]^{3-/4-}$ redox probe, beer	LOD 0.02 nM, conc. range 0.1–100 nM	Evtugyn, Porfireva, Stepanova, et al. (2013)
Carboxylated magnetic beads modified with aminated aptamers by Invitrogen protocol	Competitive assay in flow regime with alkaline phosphatase modified ochratoxin A, electrochemical detection of α-naphthol, beer samples	LOD 0.05 μg/L	Rhouati, Yang, Hayat, and Marty (2013)
Glassy carbon electrode modified with poly(neutral red) and Ag nanoparticles modified with macrocyclic ligands bearing catechol fragments and thiolated aptamer	EIS measurements with $[Fe(CN)_6]^{3-/4-}$ redox probe, beer samples	LOD 0.05 nM, conc. range 0.3–30 nM	Evtugyn, Porfireva, Sitdikov, et al. (2013)
Capture DNA probe covalently attached to Au electrode, reduced graphene oxide and Au nanoparticles as labels of signaling DNA probe	Displacement assay, EIS measurements of charge transfer resistance affected by dissociation of triple complex (capture DNA probe–aptamer–signaling DNA probe) in ochratoxin A presence, real red wine samples	LOD 0.3 pg/mL, conc. range 1 pg/mL to 50 ng/mL	Jiang et al. (2014)

Continued

Table 3 Analytical Characteristics of Aptasensors for Mycotoxin Determination—cont'd

Surface Layer Assembly	Signal Measurement Protocol, Samples Tested	LOD, Linearity Range	References
Au electrode modified with cysteamine and reduced graphene oxide with Au nanoparticles covalently attached via 2-aminothiophenol	EIS measurements with $[Fe(CN)_6]^{3-/4-}$ redox probe	LOD 0.03 ng/mL, conc. range 1.0–200 ng/mL	Qian et al. (2014)
Self-assembling of aptamer on Au electrode, followed by consecutive hybridization with two auxiliary pinhole DNA sequences	DPV measurement of alkaline phosphatase label attached via avidin–biotin binding with α-naphthylphosphate as enzyme substrate	LOD 2 pg/mL, conc. range 0.005–100 ng/mL, cereal testing	Qing et al. (2017)
Magnetic separation of the beads with aptamer specific to aflatoxin B_1 and ochratoxin A labeled with silica coated with quantum dots (CdTe and PbS)	DPV signal of Cd^{2+} and Pb^{2+} ions after quantum dots dissolution	Conc. range 10 pg/mL to 10 ng/mL (ochratoxin A) and 50 pg/mL to 50 ng/mL (aflatoxin B_1)	Wang et al. (2017)
Thionine-labeled aptamers attached to graphene oxide nanosheets, thionine as redox indicator	DPV signal of thionine increased with ochratoxin A binding, additional triggering by DNase I recycling, wheat testing	LOD 5.6 pg/mL	Sun, Zhang, et al. (2017)

Thiolated aptamer immobilized on Au electrode; carbon nanotubes bearing Methylene blue are used as signal amplifiers	Displacement assay with hybridization of aptamer and DNA probe attached to carbon nanotube with auxiliary DNA sequence; ochratoxin destroys the hybridization product due to competitive aptamer binding, DPM measurement, grape and blood serum testing	LOD 134 (serum) and 58 (grape) pM	Abnous et al. (2017)
Au electrode treated with cysteamine, porous carbon, and Au nanoparticles followed by aptamer binding	Displacement protocol with auxiliary complementary DNA probe removed from the aptamer by mycotoxin binding, Methylene blue DPV signal as a measure of double-stranded product	LOD 5 fg/mL, conc. range 5 fg/mL to 0.05 ng/mL	Wei and Feng (2017)

Most of the aptasensors described are devoted to the determination of OTA. The appropriate aptamer changes its conformation from linear to G_4-quadruplex, while the analyte is bonded (Hayat, Yang, Rhouati, & Marty, 2013). This suppresses the permeability of the surface layer toward ferricyanide ions applied as redox probes in EIS measurements. The application of magnetic beads as aptamer carriers is stimulated by their commercial availability (Dynabeads®, SureBeads™, etc.) and similarity to parent protocols of appropriate immunoassay. As in the case of immunosensors considered earlier, selectivity of the assay depends on the suppression of unspecific sorption. The use of self-assembled monolayers for aptamer immobilization simultaneously solves this problem.

Increased density of binding sites on the transducer surface can be achieved by the use of dendrimers and polycarboxylated carriers like graphene oxide and macrocycles (pillar[5]arene and thiacalix[4]arene). However, the use of such carriers needs to be supported by some measures to enhance the electron transduction in the surface films, e.g., coimmobilization of redox-active dyes or Au nanoparticle deposition.

4.3 Mass-Sensitive Biosensors

Mass-sensitive or acoustics immunosensors or aptasensors are based on the piezoelectric effect. These sensors are also known as quartz crystal microbalance (QCM) or thickness shear mode aptasensors. The AT-cut type transducers are used for this purpose. These transducer poses shearing oscillation parallel to the quartz surface following application of high-frequency voltage to the electrodes sputtered at both sides of the crystal. The oscillation frequency depends on the transducer thickness and is typically in the range from 5 to 20 MHz. These transducers provide highly stable frequency which does not depend on temperature at typical experimental conditions used (the temperatures around 20–30°C). The antibodies or aptamers are immobilized at one side of the crystal using various methods mentioned earlier (see Section 4.1). In contrast with electrochemical method, there are no special requirements for the support in relation to conductivity, although the modified QCM method–electrochemical quartz crystal microbalance-based biosensor uses mass-sensitive detection combined simultaneously with amperometry. The most convenient experimental setup is composed of the flow cell connected to the syringe or a peristaltic pump. The signal is applied and analyzed by the network analyzer (Poturnayova et al., 2017). However, more simple configuration is also possible and rather often used. It is based on the oscillation circuit which induces crystal oscillation and frequency counter

that measures the oscillation frequency. The detection principle is based on the Sauerbrey equation (7) (Sauerbrey, 1959):

$$\Delta f = -2f_0^2\rho_S\left(\mu_q\rho_q\right)^{-1/2} = -2.26\times10^{-6}f_0^2\left(\Delta m/A\right) \tag{7}$$

where f_0 is the fundamental frequency of the quartz, ρ_S is the mass density, $\mu_q = 2.95\times10^{10}$ Pa is the shear stiffness of quartz, $\rho_q = 2650\,\mathrm{kg\,m^{-3}}$ is the quartz density, and A is the sensor area. In Eq. (7), the fundamental frequency is in Hz and the surface mass loading ($\Delta m/A$) is in $\mathrm{g\,cm^{-2}}$. As it can be seen from this equation, the changes in the frequency of crystal oscillation are indirectly proportional to the mass changes. Addition of the analyte at the surface of the crystal modified by antibodies or aptamers results in increase of its mass that causes the decrease of resonant frequency. However, the Sauerbrey equation is strongly valid only for crystal in vacuum. In a liquid where the analyte–receptor interactions are studied, however it is necessary to take into account also viscous forces that took place due to friction between sensing layer and surrounding solvent. These forces affect the oscillation frequency. Therefore, most correct measuring setup is based on analyzing both resonant frequency and motional resistance which reflect the contribution of viscous forces. Mass-sensitive biosensors are rather popular due to high sensitivity. They do not need any labels. However, detection of mycotoxins is rather complicated due to low mass of these compounds. Probably this is one of the main reasons why only two papers were reported on mycotoxin detection using mass-sensitive biosensors. Spinella et al. (2013) reported immunosensor for detection of AFB_1, OTA, and FB_1. Mycotoxin-specific antibodies were covalently bound to a gold layer of QCM transducer using DSP (3,3′-dithiodipropionic-acid-di-*N*-hydroxysuccinimide ester). The changes of the frequency were rather small; therefore only higher concentrations of mycotoxins (around μM concentrations) can be detected. Most recently, Karczmarczyk, Haupt, and Feller (2017) reported detection of OTA using QCM-D technique, allowing simultaneous measurement of frequency (Δf) and dissipation (ΔD) changes. ΔD value is responsible for viscosity effect. In this work, secondary antibodies conjugated with gold nanoparticles were used for signal amplification. This allowed to reach rather high sensitivity of detection with LOD of 0.16 ng/mL (0.39 nM), which is one order of magnitude lower than LOD specified by European Union legislation. The sensor has been validated in wine. The matrix effect caused by the presence of polyphenols in wine has been eliminated by pretreatment of the wine with the addition of 3% poly(vinylpyrrolidone).

4.4 Optical Biosensors

Optical biosensors provide a powerful and attractive alternative to conventional analytical methods such as ELISA and chromatographic techniques which are widely used for detection of mycotoxins. They allow very sensitive, nondestructive, and real-time operating analysis of food toxins without needing extensive sample preparation (Bhand, Kanungo, & Pal, 2017). Optical biosensors can employ numerous optical methods to detect analyte of interest. Those methods are usually based on light absorbance, fluorescence, light polarization, and rotation or vibration spectroscopy measurements. Among optical techniques, SPR and fluorescence resonance energy transfer (FRET) are frequently used.

Fig. 7 shows the main principle of SPR for biosensing. SPR offers label-free and real-time detection molecules resulting changes in refractive index (Damborský, Švitel, & Katrlík, 2016). SPR is based on the excitation of surface plasmons at the metal surface sputtered at the glass prism by visible or infrared light. In the most common Kretschmann configuration, the metal (gold or silver) is sputtered at the surface of glass prism. The prism is illuminated by visible light (usually laser beam) at certain angle. The beam induced evanescent wave is penetrated through the metal film. The plasmons are excited at the outer side of the film at the metal/air or metal/water interface. At certain angle of incidence the resonance takes place. The resonance is accompanied by minimum reflectivity at the plot of light reflectivity vs angle of incidence. The surface plasmons are sensitive to adsorption phenomena at the metal surface. Therefore they can be used for detection affinity interactions

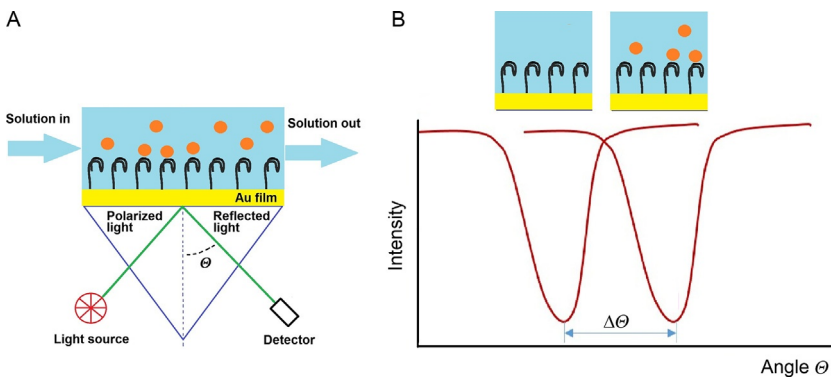


Fig. 7 The scheme of SPR aptasensor. Aptamers are immobilized at the gold surface sputtered on glass prism (*left*). Addition of the analyte resulted in shift of resonance angle (*right*) (Hianik, 2018).

between receptors (aptamers or antibodies) immobilized at the metal surface and the analyte. The binding of analyte will result in shifting of resonance angle. Therefore the plot of the changes of the angle as a function of analyte concentration can serve as a calibration curve for determination of various compounds. In the dependence on the biorecognition element such as antibody or aptamer, the biosensors can be divided into the groups of immunosensors and aptasensors, respectively. Very recent researches (2012–nowadays) focused on developing of optical biosensors for detection mycotoxins are presented.

4.4.1 Optical Immunosensors

Label-free immunosensor based on quenching of intrinsic fluorescence of antibodies by specific antigen for detection of AFB_1 was developed (Li, Byun, Kin, Shin, & Kim, 2013). FRET method used overlap of AFB_1 absorption spectrum with emission spectrum of tryptophan in antibodies upon binding. It has been found that Fab fragment, the antibody-specific binding site, increased sensitivity of this method. The LOD for assay employing complete antibody was 0.85 ng/mL, while for the Fab fragment LOD was 10 times lower.

QDs due to their unique properties such as high quantum field, tunable spectrum, and stability are more often used in FRET-based immunoassay as donors (Mahdu et al., 2016; Xu, Xiong, Lai, Xu, & Li, 2014; Zekavati et al., 2013).

Immunosensor for detection AFB_1 was based on FRET between fluorophore-labeled analyte and antibody attached to cadmium telluride (CdTe) QDs. Rhodamine-labeled AFB_1-albumin was bound to CdTe QDs conjugated antibody resulting higher fluorescence intensity due to FRET. Addition of AFB_1 led to a reduction in fluorescence intensity. LOD 0.02 nM was achieved (Zekavati et al., 2013). Both antibody and antigen AFB_1 were labeled by different-sized QDs. Variable parameters such as ratio donor/acceptor pair, pH, and ionic strength of buffer as well as reaction time affected the sensitivity of sensor. Immunosensor was validated in rice extract with LOD 0.04 ng/mL (Xu et al., 2014).

Magnetic silica nanoparticles amplified FRET between CdTe QDs coated by antibodies against OTA and fluorescent dye Rhodamine 123. This competitive immunoassay for detection of OTA allowed detection limit 0.8 pg/mL (Mahdu et al., 2016).

Beloglazova et al. (2014) presented multiplex QDs-based immunosorbent assay for mycotoxins, such as deoxynivalenol, zearalenone, AFB_1, T2 toxin, and FB_1. Single-analyte approach used multiwell plate with

one sample pretreatment. LODs were 3.2, 0.6, 0.2, 10, and 0.4 ng/mL for deoxynivalenol, zearalenone, AFB_1, T2-toxin, and FB_1, respectively. Two types of antibodies were immobilized into one single well for simultaneous screening of zearalenone and AFB_1 labeled with green- and orange-emitted QDs, respectively, but this double-analyte approach for determination of zearalenone and AFB_1 did not obtain LOD as low as single-analyte multiplex.

SPR-based immunosensor enables label-free detection of AFB_1 (Park, Kim, et al., 2014). The protein cross-linker has shown strong binding to Fc segment in antibodies and can be used for immobilization of antibody on gold surface instead of usual alkanethiols.

Indirect detection of OTA in red wine was provided using gold nanoparticles (AuNPs)-enhanced SPR (Karczmarczyk, Reiner-Rozman, et al., 2016). Secondary antibodies were conjugated with AuNPs and interacted with antibody–OTA complex on gold surface of sensor chip immobilized via self-assembled thiol monolayer with. LODs were estimated in PBS buffer and red wine samples as 0.068 and 0.19 ng/mL, respectively. A similar AuNP-enhanced SPR immunosensor was constructed for determination of aflatoxin M1 (AFM_1) in milk and dairy products (LOD 18 pg/mL) (Karczmarczyk, Dubiak-Szepietowska, et al., 2016). Polymer poly(2-hydroxyethyl methacrylate) marked as p(HEMA) brush was utilized for immobilization of AFM_1 and primary antibody on gold surface to avoid nonspecific adsorption.

An immunosensor for detection deoxynivalenol based on SPR combined with ionization mass spectrometry was fabricated (Joshi, Zuilhof, Beek, & Nielen, 2017). Gold sensor chip was coated with monoclonal antibodies against deoxynivalenol and high voltage was applied to generate spray for mass spectroscopy analysis of analyte.

Although immunosensors are very sensitive and detection limits are sufficient for practical applications, the production of antibodies requires animals and it is very expensive. Instead of antibodies, aptamers can be easily synthesized and modified by functional groups.

4.4.2 Optical Aptasensors

Upon specific binding of aptamer and target molecule the three-dimensional structure and simple modification of aptamers are used for the development of aptasensors. Label-free aptasensors have been developed employing gold nanorods (AuNRs) for detection of OTA using localized SPR (Park, Byun, et al., 2014). The aptasensor (0.4 ng/mL) has shown good reusability by heating in methanol at 70°C. Other label-free aptasensors using SPR have

been reported for direct detection of OTA (Zhu et al., 2015) and AFB_1 (Sun, Wu, & Zhao, 2017). Biotin-labeled aptamers were bound to streptavidin via strong binding affinity on gold surface of sensor chip.

Evanescent wave all-fiber method was employed for development of portable aptasensors against OTA (Liu, Zhou, & Shi, 2015; Wang, Xiang, et al., 2015).

Most of fluorescent sensors are based on signal-on detection of analyte. Usually, fluorophore-labeled aptamer and quencher molecule are approached very close. After the formation of aptamer and target complex, fluorescence intensity is increasing. Zhao, Geng, and Wang (2013) studied fluorescein (FAM)-labeled aptamer against OTA. Aptamers were designed with different positions of FAM resulting in decreasing or increasing fluorescence intensity after specific OTA binding. Dissociation constant of the OTA-aptamer binding was determined by measuring the fluorescence anisotropy.

The signal-on fluorescent aptasensor for detection of OTA (LOD 20 pg/mL) based on Tb^{3+} and switching structure of aptamer was reported (Zhang, Zhang, Yang, Chen, & Wang, 2013). Aptamer was immobilized on magnetic beads (MBs) through biotin–streptavidin interaction and complementary sequence of two single-stranded DNA was hybridized to form a duplex structure. In the presence of OTA, aptamer formed G-quadruplex leaving two single-stranded DNA in supernatant, which interacted with Tb^{3+} and increased fluorescence intensity of Tb^{3+}.

Application of Ag^{+} instead of Tb^{3+} led to the formation of DNA-scaffold silver nanoclusters (AgNCs) and 10 times lower LOD was obtained (Chen, Zhang, Cai, Wu, & Chen, 2014). Further signal-on fluorescent aptasensor based on AgNCs coupling with zinc(II) ion has been constructed for simultaneous detection of OTA and AFB_1. LOD was achieved for OTA 0.2 pg/mL and AFB_1 0.3 pg/mL (Zhang, Xia, et al., 2016). Thiolated aptamer-conjugated MBs with Au shell and CdTe QDs as labels were exploited for construction of the sensitive aptasensor for OTA detection (5.4 pg/mL) (Wang, Qian, et al., 2015). The binding aptamer and OTA released CdTe QDs from MBs into bulk solution, where they were separated by magnet. Fluorescence intensity of CdTe QDs in supernatant increased regarding to OTA concentration. Biosensor employing luminescence resonance energy transfer method (LRET) used upconversion nanoparticles (UCNPs) as donors and AuNPs as acceptors for detecting OTA. LOD 27 pg/mL was achieved and tested in beer samples (Dai, Wu, Duan, & Wang, 2016). Despite that, single-walled carbon nanohorns (SWCNHs) are very promising as

quencher material (Lv et al., 2016). SWCNHs-based aptasensor was able to detect OTA only at concentration as low as 17.2 nM (7 ng/mL).

Most recently, aptasensor against OTA (LOD 2.5 pg/mL) based on FRET between colloidal cerium oxide nanoparticles (nanoceria) and graphene QDs has been reported (Tian et al., 2018). FRET occurred between QDs and nanoceria due to electrostatic interaction. Two DNA probes were designed to be complementary with aptamer against OTA and attached to nanoparticles and QDs allowing FRET. In the presence of OTA, aptamer changed conformation and energy transfer was interrupted.

The ultrasensitive aptasensor has been developed using fluorescent real-time PCR for detection of OTA and achieved detection limit of 1 fg/mL (Ma et al., 2013). Complementary single-stranded DNA to aptamer specific for OTA was utilized for PCR amplification and fluorescence signal was measured.

Colorimetric assays enable simple detection based on color changes that can be observed by eye and change in the absorption spectrum. DNAzyme hairpin-based aptasensors for detection of OTA were reported (Yang, Lates, Prieto-Simón, Marty, & Yang, 2012; Yang, Lates, Prieto-Simón, & Marty, 2013; Wang, Dong, et al., 2015). Guanine-rich DNA sequence interacted with hemin that acting as HRP-mimicking DNAzyme, which enhanced catalytic activity. Then DNAzyme activity is followed through H_2O_2-mediated oxidation with tetramethylbenzidine. Potassium and magnesium ions affected on DNAzyme activity (Yang et al., 2012).

AuNRs have been used for construction of optical aptasensor (LOD 0.22 ng/mL) for detection of OTA (Xu, Xu, & Ying, 2016). Linker aptamer with sequence selective for OTA was dispersed in solution and two thiolated DNA molecules with complementary sequence to side sites were assembled on the AuNRs. Due to specific optical properties of AuNRs and conformational changes of aptamer in the presence of OTA, the absorption changes were observed.

AuNPs-doped Fe_3O_4 (Au@Fe_3O_4) NPs as signal indicator and magnetic separator were utilized in colorimetric assay (LOD 0.03 ng/mL) for OTA (Wang, Qian, et al., 2016). Aptasensor was based on hybridization reaction of aptamer and complementary DNA attached to Au@Fe_3O_4 NPs and peroxidase-like activity of Fe_3O_4 NPs.

5. CONCLUSION AND FUTURE PERSPECTIVES

Affinity biosensors utilizing antibodies and specific aptamers offer excellent opportunities for reliable detection of mycotoxins on their maximal

permissible levels. Although many of the biosensors described exploit general principles of assembling and signal recording previously elaborated for high-molecular analytes, e.g., proteins or pathogenic microorganisms, small volume and structural similarity of many mycotoxins offer additional requirements to the performance of appropriate biosensors. Commonly, subnanomolar LODs are expected in accordance with legislation requirements. Then, the analysis should be performed with minimal sample treatment and in reasonable time. The latter requirements also take into account the tendencies to reduce the number of the stages of reagent addition and intermediate washing. Meanwhile the highest sensitivity of the biosensors described often is achieved by rather long and complicated procedures including application of DNAzymes and nucleases for signal amplification in case of aptasensors. The alternative "fast and inexpensive" vs "long and super-sensitive" leaves space for the choice depending on particular analytical problem and consumer expectations.

The selection of aptamers and antibody fragments for mycotoxin detection increases the number of biochemical receptors implemented in the biosensors. This is particularly true for the aptamers for deoxynivalenol not available at the moment. The use of polyclonal antibodies tends to decrease due to the higher selectivity and increasing availability of monoclonal and recombinant antibodies. Meanwhile, nanobodies consisting of heavy chains are not used yet in mycotoxin analysis. The applicability of bioreceptors in the contact with organic solvent needed for extraction of mycotoxins from foodstuffs in another problem to be solved in the future.

Further progress development of signal transduction principles is directed to a higher application of nanomaterials and microfluidic devices that ideally result in fully automated analysis protocol including sampling, extraction, extract purification/dilution, and analysis. Graphene oxide, carbon nanotubes, QDs have found place in new biosensors such as aptamer/antibody carriers, signal amplification, and signal generation systems. It should be also mentioned that combined sensors exploiting electrochemical and optic detection systems, e.g., electrochemiluminescence and SPR assay, seem very promising to achieve a reliable and sensitive signal with self-checking and self-calibration abilities.

The development of universal biosensing systems and multiplex assay is another trend in the development of mycotoxin biosensors. Although it can be achieved in many cases by replacement of bioreceptor, the number of appropriate multianalyte biosensors is very limited. Moreover, even for single-analyte biosensors, the selectivity of the signal is rarely tested using other mycotoxins. The reason for that is not clear yet. Probably, some foodstuffs like milk and cheese in case of aflatoxin M1 or cereals in case of AFB_1

do not require the determination of all the mycotoxins known at the moment. However, food safety especially in third world countries calls for extension of the analytes determined on preliminary stages of the potential risk assessment.

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