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Current Immunoassay Methods for the Rapid Detection of Aflatoxin in Milk and Dairy Products

Emmanuel Matabaro, Nestor Ishimwe, Eric Uwimbabazi, and Byong H. Lee

Abstract: The presence of mycotoxins in foodstuff causes serious health problems to consumers and economically affects the food industry. Among the mycotoxins, aflatoxins are very toxic and highly carcinogenic contaminants which affect the safety of many foods, and therefore endanger human health. Aflatoxin M_1 (AFM_1) found in milk results from the biotransformation of aflatoxin B_1. Many efforts have been made to control the source of AFM_1 from farmers to dairy product companies. However, AFM_1 escapes ordinary methods of food treatment such as cooking, sterilization, and freezing, hence it appears in milk and dairy products. The presence of high levels of AFM_1 constitutes an alarming threat as milk and dairy products contain essential nutrients for human health, especially for infants and children. For this reason, there is a pressing need for developing a fast and reliable screening method for detecting trace aflatoxins in food. Several analytical methods based on high-performance liquid chromatography (HPLC) and mass spectroscopy have been used for aflatoxin detection; however, they are expensive, time-consuming, and require many skills. Recently, immunoassay methods, including enzyme-linked immunosorbent assay (ELISA), immunosensors, and lateral flow immunoassay (LFIA), have been preferred for food analysis because of their improved qualities such as high sensitivity, simplicity, and capability of onsite monitoring. This paper reviews the new developments and applications of immunoassays for the rapid detection of AFM_1 in milk.

Keywords: aflatoxin M_1, antibody, dairy product, immunoassay, milk

Introduction

Mycotoxins are harmful secondary metabolites produced by certain types of filamentous fungi or molds (Zain 2011). Under suitable humidity and temperature conditions, fungi grow on feeds and foodstuffs, and then produce toxins such as aflatoxins (AFs), ochratoxin A, deoxynivalenol, zearalenone, and fumonisins. Mycotoxins affect humans and animals, leading to serious diseases and death (Hymery and others 2014; Heshmati and Mozaffari-Nejad 2015). In recent years, AFs have drawn the attention of researchers due to evidence of their carcinogenicity in humans as well as in other animals; leading especially to liver cancers, immune suppression, and DNA and intestinal damage (Do and Choi 2007; Cuccioloni and others 2009; Coppock and others 2012; Anfossi and others 2013a). AFs are produced by *Aspergillus* species (Haschek and Voss 2013), and the common types are AF B_1, G_1, G_2, M_1, and M_2, which present a very similar heterocyclic structure (Figure 1). Among these AF types, AFB_1 is considered as the most carcinogenic one (Roda and others 2010; Bbosa and others 2013). AFM_1 is the main product of AFB_1 hydroxylation, and it is found in the milk and urine of mammals after they have fed on contaminated feed (Kaniou-Grigoriadou and others 2005; Coppock and others 2012; Monbaliu and others 2012; Kamkar and others 2014a). For many animal species, including humans and cattle, it has been found that 0.5% to 5% of ingested AFB_1 are converted into AFM_1.

In humans, AF consumption has been reported to cause liver cancer (Caloni and others 2006), and infants are the most affected since milk is their major nutritious food (Scaglioni and others 2014). The International Agency for Research on Cancer (IARC) has put AFM_1 among "type I carcinogens" (Anfossi and others 2013a), highlighting its potential risks on human health (Kourousekos 2011; Tsakiris and others 2013). Unfortunately, due to their high stability, AFs cannot be reduced by pasteurization, ultra-high temperature (UHT), cooking, ionizing radiation, addition of enzymes and food additives, or other ordinary methods of food treatment (Omar 2013; Mashak and others 2016). This is shown by the fact that several commercial dairy products and milk tested for AFM_1 contained higher amounts than the allowed quantities (Fallah 2010; Kabak and Ozbey 2012; Tavakoli and others 2012; Er and others 2014; Kamkar and others 2014b; Rahimirad and others 2014; Mashak and others 2016; Karczmarczyk and others 2017; Pérez and others 2017).

CRF3-2017-0064 Submitted 3/9/2017, Accepted 6/14/2017. *Author Matabaro is with Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan Univ., Wuxi, Jiangsu 214122, China. Author Ishimwe is with Hefei Natl. Laboratory for Physical Sciences at Microscale and School of Life Sciences, Univ. of Science and Technology of China, Hefei, Anhui, 230027, China. Author Uwimbabazi is with School of Food Science, Jiangnan Univ., Wuxi, Jiangsu, 214122, China. Author Lee is with Dept. of Microbiology and Immunology, McGill Univ., Montreal, QC H3A 2B4, Canada. Author Ishimwe is with the Dept. of Chemistry, College of Science and Technology, Univ. of Rwanda, Rwanda. Author Lee is with Dept. of Food Science and Biotechnology, Kangwon Natl. Univ., Chuncheon 200701, South Korea. Direct inquiries to author Lee (E-mail:* byong.lee@mail.mcgill.ca*).*

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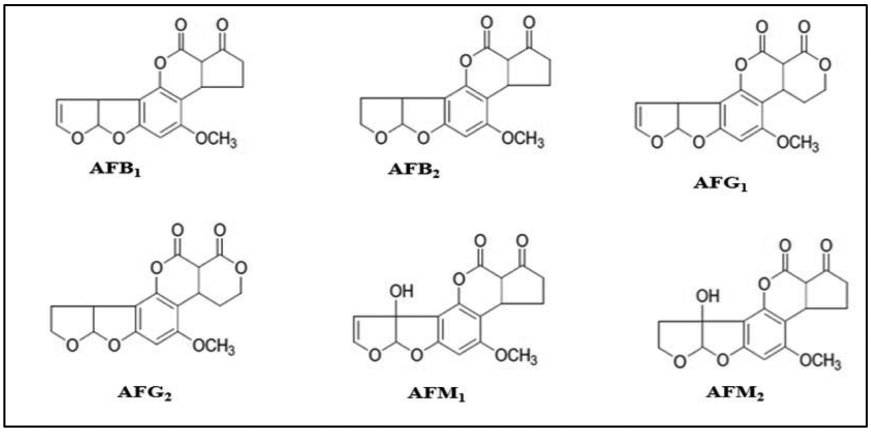

Figure 1–Chemical structures of major aflatoxins.

Table 1–Current AFM_1 legal limits in some selected countries.

Country	Raw milk (μg/kg)	Milk for dairy products (μg/kg)	Dietary foods for infants and medical cases (μg/kg)	References
Argentina, Indonesia, Taiwan	0.5	0.5	–	(FAO[a] 2004; INSA[b] 2009)
Israel	0.05	0.05	0.05	(FAO 2004; Britzi and others 2013)
Malaysia, Singapore, Vietnam	0.5	0.5	0.025	(FAO 2004; ASEAN[c] 2015)
EU[d], Bosnia Turkey, and Herzegovina	0.050	0.05	0.05	(European Commission 2010; Codex 2011)
Honduras	0.05	0.05	0.02	(FAO 2004)
GCC[e] countries	0.2	0.2	0.05	(GSO 1997; FAO 2004)
MERCOSUR[f] countries, Mexico	0.5	0.5	–	(Mazumder and Sasmal 2001; FAO 2004)
Morocco	0.05	0.05	0.03	(FAO 2004)
Switzerland	0.05	0.05	0.02	(FAO 2004)
CODEX, India, Kenya, USA, Russia, China, Iran	0.5	0.5	–	(FAO 2004; FDA 2010; China GB 2015; FSSAI 2015; Mashak and others 2016)
South Africa	0.05	0.05	0.05	(FAO 2004)

[a] Food and Agriculture Organization.
[b] Indonesian National Standardization Agency.
[c] Association of South East Asian Nations.
[d] European Union.
[e] Gulf Cooperation Council.
[f] Mercado Común del Sur.

To reduce such health risks, many control measures have been suggested in order to stop AFB_1 contamination of feeds for dairy cattle, especially lactating cows. These include prevention of fungal growth and AFB_1 formation in agricultural commodities by maintaining low moisture levels, suitable aeration and temperature during drying, and proper storage conditions as well as avoiding crop stress and damage by drought, insects, and rodents (Valencia-Quintana and others 2012). The use of chemoprotection methods to increase the animal detoxification processes, such as suppression of epoxide production, was also proposed (Lizárraga-Paulín and others 2013). Somme food additives, including cinnamaldehyde, glucomannose, and various yeast extracts may inhibit fungal growth and AFB_1 biosynthesis (Zain 2011; Davoodi and others 2013; Sun and others 2015). However, these methods alone cannot remove the threats caused by AFs. To counteract AFM_1 contamination in milk and dairy products, China, the European Union (EU), and various countries have set allowable limits, which are generally below 0.5 μg/kg (Table 1). On the other hand, a continuous risk assessment is needed to control and prevent any possible contamination by effective screening methods (Santini and Ritieni 2013; Hassan and Kassaify 2014). Such methods should be cost-effective, fast, and suitable for high-throughput screening (Do and Choi 2007; Turner and others 2009).

Several analytical methods for detecting AFs in food samples have been mostly based on high-performance liquid chromatography (HPLC)/fluorescence, tandem mass spectroscopy, and real-time mass spectrometry (DART-MS) (Rahmani and others 2009; Iqbal and Asi 2013; Busman and others 2015; Mashak and others 2016). However, these methods present some limitations, such as very expensive equipment, extensive sample pretreatment, slowness, and the need of advanced human skills. Recently, new immunochemical methods have been developed for the rapid detection and measuring of AFM_1 in milk samples (Rosi and others 2007; Tavakoli and others 2012).

Immunoassays rely on the reaction between the specific antibodies and the analyte, and therefore present such advantages as high sensitivity and selectivity. The most widely used include lateral flow immunoassays (LFIAs), microplate-based immunoassays, and different immunosensors (Goryacheva and De Saeger 2011; Anfossi and others 2013b). Immunoassays have recently attracted researchers' interests, since they are cost-effective, rapid, simple, and applicable to the onsite monitoring (Goryacheva and De Saeger 2011). The aim of this review was to investigate the currently available immunoassays for the rapid detection of AFM_1 in milk and dairy products.

Biosynthesis of AFM_1 and effects of AFs in the body

In the presence of high humidity, Aspergillus flavus and Aspergillus parasiticus grow on poorly stored food spices, cereal grains (wheat, maize, and rice), cotton, peanuts, and other

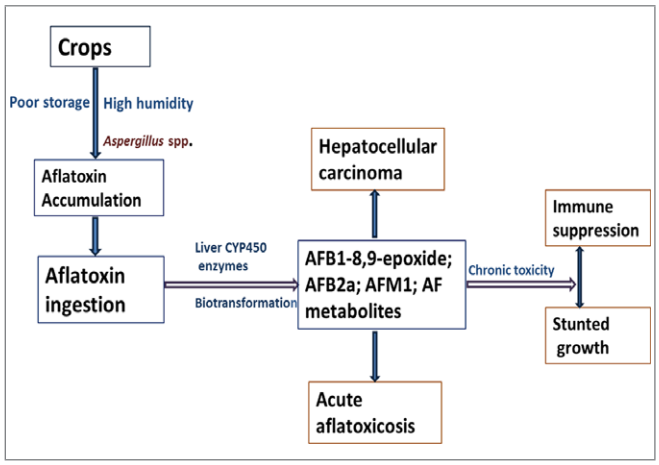

Figure 2–The effects of aflatoxin on human health.

agricultural crops, and then they produce AFs (Afsah-Hejri and others 2013; Mozaffari-Nejad and others 2014; Baranyi and others 2015; Eslami and others 2015; Vettorazzi and López de Cerain 2016;). AFs are ingested through food originating from contaminated crops (Davoodi and others 2013). After intestinal absorption, AFs are metabolized by microsomal enzymes of the cytochrome P450 (CYP450) superfamily, which are mostly concentrated in hepatic cells in humans and animals. The most predominant CYP450 required for the metabolism of different types of AFs are CYP1A2, CYP1A1, CYP3A4, CYP3A5, CYP3A7, and CYP2A6 (Bbosa and others 2013). In humans, CYP3A4 and CYP1A2 are mostly required for AFB_1 transformation into different metabolites through hydration, hydroxylation, epoxidation, and demethylation (Marin and others 2013). These 2 enzymes are required for the conversion of AFB_1 to aflatoxin-exo-8,9-epoxide.

However, CYP1A2 also catalyzes both the epoxidation and hydroxylation of AFB_1 into aflatoxin endo-8,9-epoxide and AFM_1, respectively. On the other hand, CYP3A4 is responsible for the detoxification of AFB_1 into the less toxic AFQ_1. Moreover, AFB_1 hydration gives AFB_{2a}, while o-demethylation of AFB_1 forms a detoxified product called AFP_1. AFP_1 is also reduced by cytosolic-NADPH-dependent enzymes into aflatoxicol (AFL), which can induce the DNA adducts (Yabe and others 2012).

AFB_1 8,9-epoxide is highly reactive and, therefore, it binds to the DNA and albumin, and then it forms *N*7-guanine and albumin adducts in the blood serum, respectively. The detoxification process of AFB_1 is through the conjugation of glutathione with AFB_1 8,9-epoxide, a reaction which is mediated by glutathione S-transferase (GST). Animals with higher GST resist to AF carcinogenesis, and their GST activity is about 5 times higher than that of vulnerable species (Santini and Ritieni 2013). Unfortunately, human GST activity is lower compared to that of other reported animals such as mice. Consequently, humans are more vulnerable than these animals (Gopalan and others 1992; Zain 2011).

AF consumption causes acute and chronic effects on the body. AFs cause severe damage to the body, including hepatocellular carcinoma, stunted growth, immune suppression, and even death (Figure 2). Acute aflatoxicosis leads to death, while chronic aflatoxicosis causes immune suppression and cancer (Davoodi and others 2013). Apart from the formation of DNA and albumin adducts, AFs also cause serious genetic damages including gene mutation and the creation of micronuclei (Lizárraga-Paulín and others 2011).

In the human liver, AFB_1 causes G249T transversion in the *p53* tumor-suppressor gene and, eventually, causes liver tumors such as hepatocellular carcinomas in experimented animals (Roda and others 2010; National Toxicology Program 2013; Santini and Ritieni 2013). Additionally, AFs may also cause teratogenic effects, causing congenital malformation and, therefore, affecting normal growth in children (Hussein and Brasel 2001; Brera and others 2008).

Besides several health risks caused by AFs (Figure 2), AFM_1 was also reported as a contaminant of human breast milk. While assessing the exposure of newborns to AFs in Ankara, Turkey, Gürbay and others (2010) found that the amounts of AFM_1 in breast milk were higher than 60 ng/L. Many researches have also shown a positive correlation between the amounts of AFM_1 in lactating mothers and the AFB_1 concentration in their food. Therefore, there is a need for a more coordinated and continuous risk assessment of AFs in feeds and foodstuffs (Polychronaki and others 2007; Adejumo and others 2013; Atasever and others 2014).

AFM_1 Regulations Worldwide

Due to the harmful effects of AFM_1 on human health, many countries have set specific limits for AFs in feed and food products. With the recent advances in science and technology, new methods are now able to detect and trace mycotoxins in food and feed, but resource constraints limit their availability in developing countries (Campagnollo and others 2016). Setting mycotoxin legal limits in food and feed has been a complex task due to several factors, including distribution of mycotoxins in different foodstuffs, available analytical methods and toxicological data, and the will of governmental institutions (FAO 2004).

More than 100 countries have already set limits for mycotoxins in food and feed. However, other countries, especially developing countries, do not have clear regulations (Flores-Flores and others 2015). In addition, allowable limits for AFM_1 in milk and dairy products also vary from one country to another (Table 1). In most EU countries, Bosnia, Herzegovina, and Turkey, the specific limit of AFM_1 in raw and processed milk is 0.050 μg/kg, while in the milk intended for infants and medical purposes the limit of AFM_1 has been set at 0.025 μg/kg. Other expert bodies such as MERCOSUR (Mercado Común del Sur), ASEAN (Association of South East Asian Nations), Gulf Cooperation Council (GCC), and the U.N. Codex Alimentarius have set limits at around 0.5 μg/kg AFM_1 for raw milk. In general, current legal limits for AFM_1 in milk and dairy products range between 0.02 and 5 μg/kg, as it is illustrated in Table 1.

Immunoassay Methods for the Rapid Detection of AFs

Preparation of immunogens and specific antibodies

An immunoassay is an analytical method based on an antibody-antigen (Ab-Ag) reaction. One reactant is normally marked, while the other one is immobilized on a platform. Immunoassays use specific antibodies to detect immunogens, which contain the targeted chemical structures. However, AFs are smaller molecules which are not immunogenic. Therefore, they need to be chemically modified into haptens. AF hapten includes a spacer arm (with the length of 3 to 6 carbon atoms) terminating with a reactive group (–COOH and $–NH_2$) for combining with the carrier protein (Li and others 2009a, b). Thus, it is important to design and synthesize a hapten which mimics the physicochemical properties of the target molecules (Sheedy and others 2007; Tabari and others 2011). Immunoassay methods usually use 3 types of

antibodies: monoclonal antibodies (mAbs), polyclonal antibodies (pAbs), and recombinant antibodies (rAbs).

mAbs are produced from hydridoma obtained by fusing spleen cells from immunized mouse or rabbit with myeloma cells (Pei and others 2009; Chadseesuwan and others 2016). Animals such as goat, rabbit, or horse are used to produce polyclonal antibodies. The 1st step is to immunize the animal with a protein-antigen conjugate possessing various epitopes. After several months, antibodies are produced by different plasma cells, and then collected from the blood. In contrast, rAbs are generally produced by expressing an antibody gene in a prokaryotic or eukaryotic organism (bacterial, yeast, or mammalian cells). This allows the creation of antibody libraries, which are then used to select antibodies with desired characteristics. Similarly, functional fragments of antibodies have been obtained from intact antibodies, or through recombinant DNA technology. After PCR amplification, the antibody fragment DNA is ligated into a plasmid or a phage, and then, it is expressed in a host cell. The most used antibody fragments include single-chain variable fragments (scFvs), which comprise the variable parts (light (V_L) and heavy (V_H) chains), and single-chain Fab antibody (scFab) made of light chain (L_C) and fragment difficult (F_d). In addition, their variants have been produced including single domain antibodies, diabodies, and tribodies (Nelson 2010). However, the most used antibodies for the AF detection are mAbs and pAbs due to the challenges in rAbs preparations (Puchades and Maquieira 2013).

Molecular signal markers for immunoassays

Immunoassays rely on molecular markers for the signal enhancement during AF detection. Several types of molecular markers have been developed, including sensitive optical and electronic components, gold nanoparticles, enzymes, radioisotopes, and fluorophores (Sharma and others 2014a).

Microplate-based immunoassays use different label markers to detect food contaminants. The enzyme-linked immunoassay (EIA) is based on enzymatic conversion of chromogenic substrates into colored end-products.

The intensity of the developed color or emitted light correlates with the concentration of the analyte in the sample. In that case, the choice of the enzyme depends on its ability to preserve its catalytic activity when it is conjugated to the probes such as antibodies. The horseradish peroxidase (HRP) and alkaline phosphatase (ALP) conjugates are mostly used for signal amplification in EIA. HRP is the most popular enzyme marker used in enzyme-linked immunosorbent assay (ELISA)-based methods because it is cheaper, easily available, and it reacts with a wide range of substrates, mostly 3,3“,5,5“-tetramethylbenzidine (TMB) and luminol. HRP reacts with H_2O_2, which, in turn, reacts with the substrate, resulting in a color change (Contreras-Medina and others 2013). Contrary to HRP, ALP is very sensitive and more stable during the assay process, and thus giving a linear response rather than HRP. Additionally, ALP shows higher sensitivity to commonly available substrates, including p-nitrophenyl phosphate (pNPP) and 4-methylumbelliferyl phosphate (MUP). However, ALP use is limited by the fact that it is expensive and mostly applicable to high-molecular-weight conjugates.

Radioimmunoassays (RIAs) are highly specific and sensitive methods which use radioactive atoms such as ^{125}I, ^{3}H, and ^{14}C for signal enhancement. However, they require special and expensive equipment due to the harmful effects caused by gamma rays on biological tissues. Therefore, to avoid health risks, other labels are preferred. Fluorescent labels are among the most widely used labels in immunoassays for AF detection. Good fluoro labels are water-soluble, chemically stable, less expensive, and highly fluorescent. Additionally, they should have –COOH, $–NH_2$, or –CN as functional group, which are capable of combining with antibodies and haptens (Sharma and others 2014b; Byrne and others 2015).

The most used fluorescent markers include organic fluorescent dyes (for example, fluorescein and cyanine dyes), fluorescent marker proteins (for example, phycoerythrin), metal complex markers (for example, Eu^{3+}, Tb^{3+}), noble metal ions, and nanoparticles.

Nanoparticles have recently gained a unique interest in food safety and medical research due to their unique physicochemical and biological properties (Alarcon-Angeles and others 2016). Their properties include relatively small size, large surface-to-volume ratio, biocompatibility, high stability, easy preparation, and strong ultraviolet-visible (UV-Vis) absorption spectra (Zhu and Zhou 2010; Tang and others 2013). The most used nanoparticles are semiconductor quantum dots (QDs), hybrid nanostructures, and various nanoparticles made from gold, carbon, and silica, and magnetic elements. Gold nanoparticles (5 to 200 nm) are extensively used in immunoassays because they are cost-effective and can be combined with enzymes, antibodies, or DNA for high signal amplification (Wang and others 2016). Furthermore, gold nanoparticles (GNPs) have been used as nanocatalysts, especially for silver sensors and as reducing agents of organic compounds such as 4-nitrophenol (Liu and others 2014).

There is a growing interest in developing immunoassays using magnetic particles. They especially rely on magnetic properties of magnetic elements like iron (Fe), nickel (Ni), and cobalt (Co), and also their compounds such as the iron oxides Fe_3O_4, Fe_2O_3, Co Fe_2O_4, $MnFe_2O_4$, $NiFe_2O_4$, and $MgFe_2O_4$ (Kim and Park 2005). Magnetic particles can be used both as solid carriers and signal reporters when they are combined with enzymes, antibodies, fluorescent materials, and nanoparticles. Besides their use for signal amplification, magnetic materials can also have enzymatic activity (Gao and others 2007). The previous research study by Liu and others (2015) developed a LFIA based on immunomagnetic nanobeads. The resulting immunoassay showed a lower detection limit at 0.02 ng/mL AFM_1 in milk samples.

Carbon nanomaterials are among the promising nanoparticles, and they present excellent properties when harnessed in modern immunoassays. Carbon nanoparticles exist in various forms, mainly graphene, carbon nanotubes (CNTs), and carbon dots (Cdots). Graphene consists of a one-atom single layer, which forms a hexagonal lattice as a result of sp^2 hybridization of carbon atoms, whereas CNTs are cylindrical tubes made of a sheet of graphite (Yang and others 2015). Carbon nanomaterials show incomparable qualities such as high mechanical strength and electrothermal conductivity.

In normal conditions, graphene is hydrophobic, impermeable to liquids and gases. However, graphene derivatives, including graphene oxide and reduced graphene oxide, can be dispersed in aqueous media, and capable of covalently immobilizing antibodies due to their rich oxygen-containing functional groups such as carboxyl group (Pérez-López and Merkoçi 2011; Chen and Inbaraj 2016; Wang and others 2016).

QDs constitute another group of preferred nanomaterials because of their improved qualities. They are nanocrystals (2 to 10 nm) synthesized from atoms of the group II to VI (CdTe, CdSe, CdS, ZnSe), or group III to V elements (InP and InAs) of the periodic table. QDs are inorganic semiconductor nanocrystals which emit fluorescence when they are excited by either light or

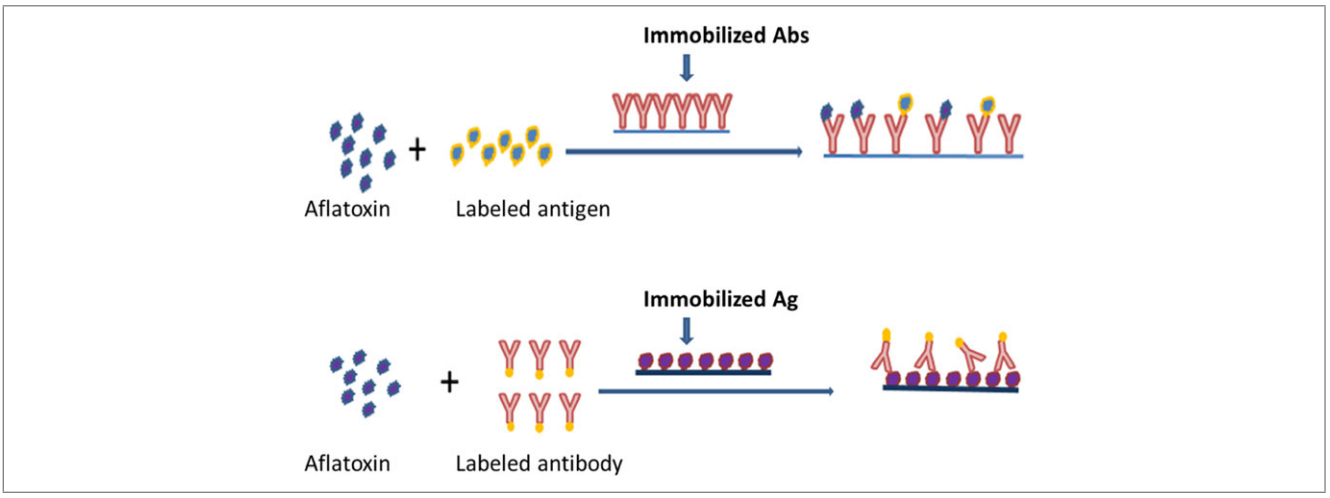

Figure 3–Competitive immunoassay formats for aflatoxin detection.

electricity. They also present many unique qualities, including photochemical stability and a broad excitation spectrum (Beloglazova and others 2014; Bahadır and Sezgintürk 2016; Wang and others 2016). Gan and others (2013) developed an electrochemiluminescent immunoassay based on magnetic Fe_3O_4-graphene oxides as the absorbent and antibody-labeled CdTe QDs. The assay showed a detection limit of 0.0003 ng/mL AFM_1, which is far better compared to ordinary ELISA-based methods (Gan and others 2013).

Lower concentrations of other mycotoxins were also detected using the QDs (Beloglazova and others 2013; Speranskaya and others 2014). For example, Zekavati and others (2013) used fluorescence resonance energy transfer (FRET) from anti-AFB_1 to rhodamine 123 to develop a FRET-based immunoassay, which showed a high detection of 2×10^{-11} M AFB_1 (Zekavati and others 2013).

The new technological advances have allowed the utilization of signal markers for designing effective immunoassays. However, immunosensors based on surface plasmon resonance (SPR) and some electrochemical methods are capable of detecting Ab-Ag interaction without the need of labels (Bacher and others 2012; Chauhan and others 2015). Apparently, label-free immunoassays seem to be low-cost and easier to design. However, other aspects, including sensitivity, specificity, and speed of operation, must also be considered. Therefore, in mycotoxin detection, labeled immunoassays are still preferred to label-free immunoassays.

Immunoassay formats

Immunoassays are grouped into competitive and noncompetitive immunoassays, depending on the size of the molecules that they can detect. That is either they are used for detecting small molecules such as toxins and drugs or big molecules such as proteins and cells, respectively (Luppa and others 2001; Dzantiev and others 2014; Sharma and others 2014a, b). In a competitive assay, unlabeled analytes compete with an antigen to bind the antibody (Figure 3). Competitive assays are generally used for AFs due to their small size. In an indirect competitive assay, specific antibodies are added into a buffer containing immobilized protein-AF conjugate (Ag) and the sample to be tested. A part of these antibodies bind the immobilized conjugate, while others remain with the analyte in the buffer (Muzyka 2014). Secondary labeled antibodies are finally released into the buffer to bind the 1st antibody, which has attached to the immobilized conjugate.

In contrast, for the direct competitive immunoassay, the secondary labeled antibody is not needed. This is because either the antigen or a specific antibody is immobilized, and then the signal material is added. Subsequently, antibody and antigen compete for labeled materials. Finally, some labeled materials stay bound to the immobilized material, while others attach to the sample analyte (Xu and others 2015). On the other hand, for noncompetitive assays, the binding of the sample analytes to the immobilized antibodies results in an optical or electrical variation, which can be detected. The sandwich formats in which the sample is first mixed with bovine serum albumen (BSA) as a carrier protein for AF molecules are, however, preferred. The resulting BSA–AF complex is then captured by immobilized antibodies, resulting in optical or electrical changes (Chu 2003;). Immunoassays may also be grouped into homogeneous and heterogeneous. In a homogeneous immunoassay, all the reagents are put into one experimental vessel, and no separation steps are needed; thus, the assay is easier and faster. However, this format is normally applicable to small analytes. For heterogeneous immunoassays, one or many separation and washing steps are needed to remove unreacted reagents, unbound components, or interfering substances after Ab–Ag binding and adsorption of one of the reagents (Dinis-Oliveira 2014).

There are now commercially available immunoassay test kits, which allow portability and rapid analysis of AFM_1 in dairy products. Their efficiency will, however, depend on many factors, including device design, reagents used (antibodies, signal-generating labels, and separation matrices), and immunoassay formats (Turner and others 2009). The current immunoassays are believed to be simple, rapid, applicable to routine analyses under field conditions, possible for automation, highly sensitive, and easy for sample preparation (Tisone and O'Farrell 2009; Goryacheva and De Saeger 2011). The advantages and disadvantages of important methods have been discussed in Table 2.

Current Immunoassay Techniques

ELISA, microtiter plate, and microplate reader

In analyzing AFs, microtiter plate and reader-based immunoassays bind proteins such as a specific antibody against AFs or secondary antibody. They allow multiple and simultaneous analyses of many samples, as they use 96-well or 384-well plates

Table 2–Comparison of different immunoassay techniques.

Immunoassay	Main properties	Advantages	Drawbacks	References
ELISA	Use of an enzyme as a label Based on color change after Ab–Ag reaction	High-throughput Long shelf-life High sensitivity Low cost	High cost per sample False positive due to low specificity and color change Time-consuming Not suitable for onsite monitoring	(Tavakoli and others 2012; Campagnollo and others 2016; Fallah and others 2016; Liu and others 2016)
Fluoro-immunoassays	Antibodies are labeled with fluorescent probes. Measurement of fluorescent intensity	High sensitivity Rapidity May be adapted	Nonlinearity of response due to change in concentration Need optimize assay conditions	(Li and others 2009b; Wheeler 2013; Byrne and others 2015; Turner and others 2015)
Chemiluminescence immunoassays	A chemical reaction results in electronic excited state Presence of an emitter and a coreactant	High sensitivity Wide range of detection High-throughput Short time	High reagent consumption Low selectivity and linearity due to interfering reagents	(Zhao and others 2009; Wang and others 2012; Vdovenko and others 2014; Turner and others 2015)
Radioimmunoassays	Use radioactive elements as labels	High sensitivity Good resistance to matrix effect Detection of trace amounts of analyte	Radioactive elements are expensive and harmful Short half-life Adulterants may cause false positive Expensive equipment Difficult to dispose of	(Chu 2003; Li and others 2009b; Sharma and others 2014a, b; Turner and others 2015)
Lateral flow immunoassays	Based on the transport of fluid by capillary Formation of Ab–Ag binding Visual	Fast and simple Suitable to onsite monitoring No separation steps Long shelf-life Use of nanoparticle labels may enhance accuracy	Low sensitivity due to matrix effect False positive Not automated No high-throughput Subjective color perception	(Anfossi and others 2013b; Liu and others 2015; Turner and others 2015; Raeisossadati and others 2016; Zhang and others 2016)
Immunosensors	Based on transducers which detect changes resulting in Ab–Ag interaction Detection of Changes in: electrical current (electrochemical), mass (Piezoelectric immunosensor), or optical signal (color or fluorescence)	Fast detection Possibility of reusability Possibility of automation High-throughput Possibility of label-free detection (for example, SPR-based) high selectivity and sensitivity Good stability	Electrode not easy to maintain Difficulty in buffer preparation Expensive labels Need advanced skills	(Micheli and others 2005; Ricci and others 2007; ·la Farré and others 2008; Vidal and others 2013; Chauhan and others 2016; Karczmarczyk and others 2016; Karczmarczyk and others 2017)
Flow injection immunoassays	Based on displacement of the labeled analyte	High Selectivity Simplicity Flexibility High sampling rate Possible reusability	Need definite conditions and optimized buffers Regeneration may lead to loss of sensitivity May need fresh sample (for example, milk)	(Badea and others 2004; Bartolomeo and Maisano 2010; Qi and others 2011)

(Puchades and Maquieira 2013; Selvaraj and others 2015). Microtiter readers use data processing software to report either optical absorbance, chemiluminescence, or fluorescence, which are then processed into the amount of analyte in the sample. The most used microplate-based immunoassays include ELISA, chemiluminescence, and fluorescence-based assays (Radoi and others 2008; Kim 2013; Sheng and others 2014). All these methods use signal markers such as HRP, QD, luminol, or fluorescein isothiocyanate.

ELISA-based methods have been extensively used to measure food contaminants. ELISA uses a chromogenic substrate and enzymes such as 3, 3′, 5, 5′-TMB/HRP. In recent years, tremendous improvements have been made in designing ELISA methods for AFM_1. ELISA kits with different detection limits are also available, and they have been used in different countries (Rama and others 2015; Bilandžić and others 2016; Fallah and others 2016; Hashemi 2016; Ismail and others 2016). In recent years, several ELISA-based immunoassays, mainly competitive formats, have been developed for improved detection of AFM_1 in milk and dairy products (Table 3). ELISA was also used for detecting AF in cheese. Tavakoli and others (2012) found high amount of AFM_1 (0.04 to 0.374 ng/mL) in 60% (30/50) of tested cheese samples. Similarly, much higher levels of AFM_1 ranging from 0.070 to 0.770 ng/mL were found in 28.3% of tested Turkey cheeses (Kav and others 2011). Despite its simplicity, in some cases, ELISA may present low sensitivity due to low color change and enzyme stability.

For an EIA, the enzyme is conjugated to analyte or antibody, and its reaction with the chromogenic substrate releases a colored product. The change in color and the signal intensity correlate with the analyte concentration in the sample (Darwish 2006). A chemiluminescence immunoassay based on ELISA may have a higher sensitivity than ELISA itself. For instance, a direct competitive chemiluminescent ELISA (CL-ELISA) has been developed for improved sensitivity, a detection limit of 0.001 ng/mL AFM_1 has been achieved (Vdovenko and others 2014).

Microtiter plate-based immunoassays present many advantages such as low detection limit, the possibility of improving the signal, and high-throughput. However, they are sometimes limited by their low selectivity and specificity due to Ab–Ag reaction and antibody-matrix components, and poor color change during detection (Chu 2003; Selvaraj and others 2015; Xu and others 2015).

LFIAs

The LFIA, also known as immunochromatographic (IC) strip test and dipstick test, involves simple devices; it does not need

Table 3–List of some ELISA-based immunoassays developed for detecting aflatoxins in milk and dairy products.

Assay methods	Signal detection	LOD ng/mL	Reference
Competitive ELISA	UV	0.005	(Fallah and others 2016)
Competitive ELISA	UV	0.021	(Hashemi 2016)
Competitive ELISA	UV	0.001	(Ismail and others 2016)
Competitive direct ELISA	UV	0.022	(Liu and others 2016)
Indirect ELISA	UV	0.035	(Peng and others 2016)
Competitive ELISA	UV	0.003	(Kamkar and others 2014b)
Competitive ELISA	Chemiluminescence	0.001	(Vdovenko and others 2014)
Competitive ELISA	UV	0.021	(Tavakoli and others 2013)
Indirect ELISA	UV	0.0016	(Jiang and others 2012)
Competitive ELISA	UV	0.003; 0.006	(Guan and others 2011)
Sandwich ELISA	UV	0.005	(Kanungo and others 2011)
Competitive direct ELISA	UV	0.014	(Wang and others 2011)
Competitive direct ELISA	UV	0.028	(Wang and others 2011)
Competitive ELISA (RIDASCREEN KIT)	UV	0.005	(Heshmati and Milani 2010)
Competitive ELISA	UV	0.043	(Rahimi and others 2010)
Indirect ELISA	UV	0.04	(Pei and others 2009)

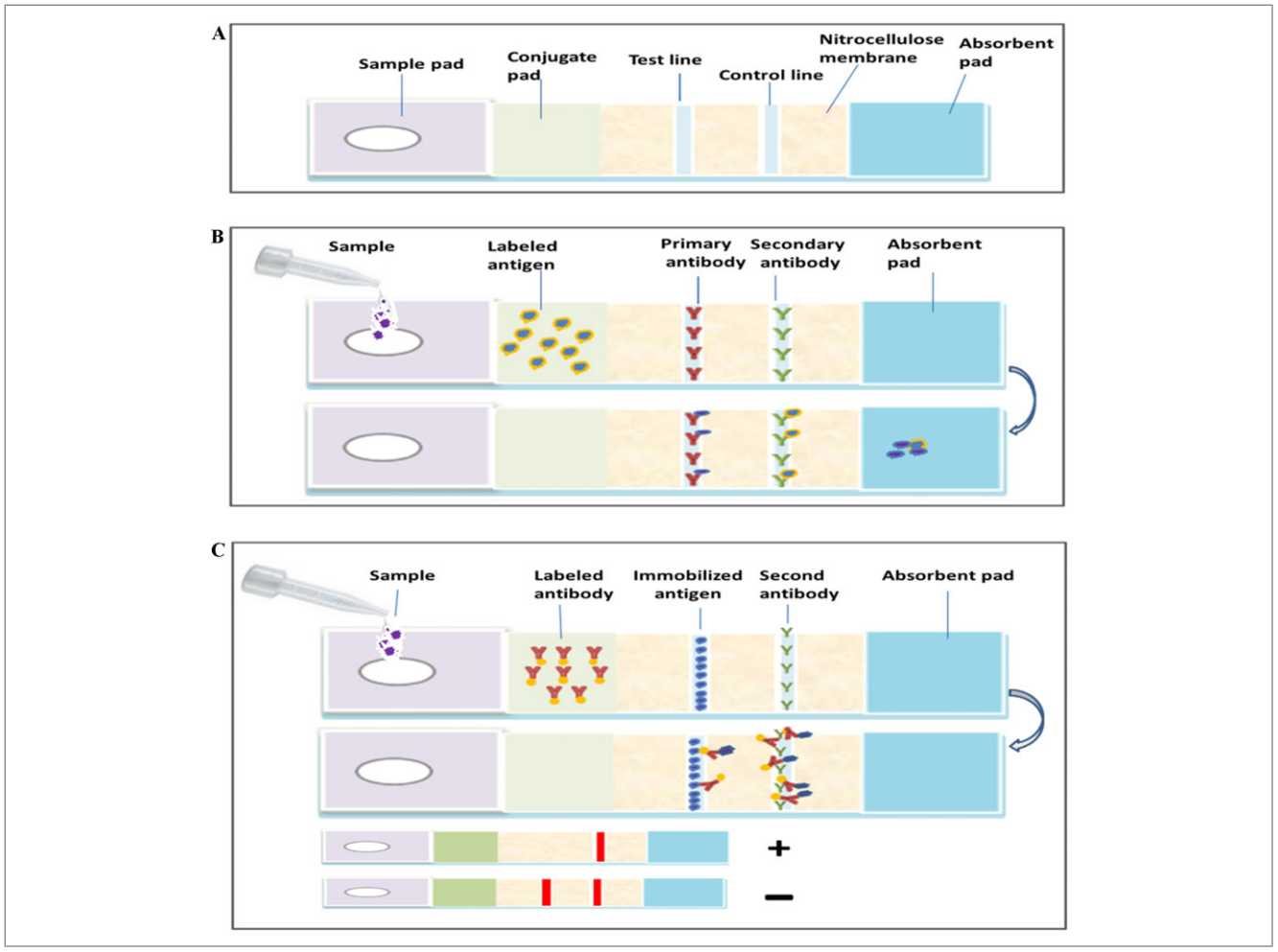

Figure 4–Principles of competitive assay in colloidal gold-based LFIA format. (A) Basic parts of a lateral flow immunoassay. (B) The labeled primary antibody is immobilized on the conjugate pad. (C) The labeled antigen is immobilized to the conjugate pad.

reagent or instrumentation because it is a one-step method (Anfossi and others 2013a; Rivas and others 2014). Most of the strips used have gold (Au) or enzyme-antibody labels. LFIA are basically made of 4 membrane pads: sample application pad, conjugate-release pad, nitrocellulose membrane, and absorbent pad (Figure 4) (Anfossi and others 2013a). For Au-based LFIA, when the sample is introduced to the absorbent pad, it migrates toward the Au conjugate-release pad, then the antibody specifically binds the analyte. The resulting complex flows to the nitrocellulose membrane, and its reaction with the immobilized antigen generates a visible signal. If the test sample has the target compound, the complex flows and binds the immobilized secondary antibody on the control line, resulting in the absence of red color on the test line (Figure 4A).

Table 4–List of some immunoassay developments for the rapid detection of AFM_1.

Type	Main characteristics/signal marker	Limit of detection (LOD) ng/mL	Detection time (min)	Reference
El Biosensor	Use of modified Au screen-printed electrodes (AuSPE)	0.037	30	(Karczmarczyk and others 2017)
LFIA	Use fluorescent microsphere	0.042	35	(Wu and others 2017)
MPWFI-immunosensor	Cyanine 5 (Cy5) as a signal report	0.045	20	(Guo and others 2016)
SPR biosensor	GNPs were used as signal markers; prevention of fouling by p(HEMA); SPFS	0.018	55	(Karczmarczyk and others 2016)
LFIA	Based on GNPs	0.1	10	(Liu and others 2016)
LFIA	One-step assay with 2 cut-off values of aflatoxin M_1; based on GNPs	0.05 and 0.5	15	(Wu and others 2016)
LFIA	Based on fluorescent microsphere	0.0044	30	(Zhang and others 2016)
LFIA	Immunomagnetic nanobeads as signal marker	0.02	10	(Liu and others 2015
LFIA	Immunomagnetic nanobeads as signal marker	0.1	10	(Huang and others 2014)
El immunosensor	Silver (Ag) wire electrodes used for impedance change	0.001	10	(Kanungo and others 2014)
LFIA	GNPs for signal enhancement	0.05	15	(Reybroeck and others 2014)
LFIA	GNPs as signal marker	0.02	17	(Anfossi and others 2013b)
Biosensor	Based on bioelectric recognition assay (BERA)	0.005	3	(Larou and others 2013)
El biosensor	Use of Ag-wire electrodes and 11-marcaptoundecanoic acid (11-MUA) as a mediator to AFM_1-mAbs	0.001	20	(Bacher and others 2012)
LFIA	Fluorescent microsphere as a signal report	0.3	15	(Zhang and others 2012)
El immunosensor	Use of HRP as a tag, and MPMS as the mediator, and screen-printed carbon electrodes	0.01	–	(Paniel and others 2010)
El immunosensor	Use of disposable multichannel microplate coupled to intermittent pulse amperometry (IPA)	0.001	–	(Neagu and others 2009)
El immunosensor	Use of (TMB)/H_2O_2 and HRP	0.039	–	(Parker and Tothill 2009)
SPR-biosensor	Excitation of long range surface plasmons (LRSPs) for fluorescence signal enhancement; SPFS	0.0006	53	(Wang and others 2009)
Impedimetric immunosensors	Gold-catalyzed deposition of silver on graphite screen-printed electrodes	0.012	2	(Vig and others 2009)
Flow injection immunosensor	Detection of HRP label for monitoring tracer Ag in the eluate	0.011	60	(Badea and others 2004)

Au, gold; LFIA, lateral flow immunoassay; El, electrochemical; TMB, 3,5′,5′-tetramethylbenzidine dihyrochloride; SPFS, surface plasmon-enhanced fluorescence spectroscopy; MPWFI, multiplexed planar waveguide fluorescence immunosensor; HRP, horseradish peroxidase; MPMS, 5-methylphenazinium methyl sulfate; SPR, surface plasmon resonance; p(HEMA), poly(2-hydroxyethyl methacrylate).

In the absence of the analyte in the sample, the labeled antibodies are captured on the test line, and the rest of the antibodies migrate toward the control line, where they bind the 2nd antibody (Rivas and others 2014). However, an enzyme-strip differs from the Au-strip; 1st, the enzyme label is in the buffer, but not on the label pad; 2nd, the color results from the oxidation reaction between the enzyme and the substrate; and, finally, biotinylated tyramine (B-T) and avidin-enzyme conjugate may be used to improve the strip sensitivity (Qian and Bau 2004; Goryacheva and others 2009).

In general, 2 alignment models are possible for LFIA, which are based on molecular signal markers. For the 1st alignment, the labeled primary Ab is immobilized on the conjugate pad, while the Ag-carrier molecule such as BSA (AFM_1-BSA) is coated on the test line. The target analyte and immobilized Ag compete to bind labeled primary Ab. The target analyte binds to the labeled Ab, and together they make a complex. The complex then moves to the control line, and it binds the 2nd Ab, resulting in a red color at the control line (Figure 4B). In the 2nd alignment, the labeled antigen is immobilized to the conjugate pad. If a sample containing AFM_1 is applied to the sample pad, it flows with labeled Ag. The unlabeled analyte competes with the labeled antigen to bind the primary Ab. The unlabeled analyte binds the 1st Ab, while the labeled antigen binds to the 2nd Ab. This generates a red color at the control line. For a negative sample, the labeled analyte binds on both control and test lines (Figure 4C).

Recently, there have been significant improvements in the design of strip tests for both medical and food research. Their accuracy and sensitivity have sometimes been compared to HPLC and other methods, but LFIAs are more rapid, and they do not require pretreatment steps. Anfossi and others (2013b) developed a highly sensitive strip test, with a LOD of 0.020 ng/mL AFM_1, and the detection time was 10 min.

A 2-step assay LFIA based on immunomagnetic nanobeads made by Liu and others (2015) showed an enhanced sensitivity for AFM_1 (0.02 ng/mL) in just 17 min (Liu and others 2015). Other various LFIAs and immunosensors with improved LODs and detection time have been designed (Table 4).

New technological development in the LFIA design makes it possible to improve sensitivity and rapidity, and to avoid the subjective perception of color. However, the reduction of the matrix effect and fouling remain as a major challenge to scientists. Therefore, various factors must be considered, including membrane properties, buffer type, and quality of the antibody (Qian and Bau 2004; Goryacheva and others 2009).

Immunosensors

Immunosensors are devices in which the Ag–Ab reaction is detected by a transducer. The basic parts of an immunosensor include a bioreceptor, a transducer, and an electronic system which enables signal amplification, processing, and display (Figure 5). A bioreceptor is a biological element such as antibody, antibody fragment, and enzymes which interact with the analyte in the sample. This bioreceptor is attached to a surface made of glass, polymer, or a metal element. The Ab–Ag interaction results in changes of electrons, photons, masses, heat, or pH. These changes are recognized by a transducer, which transforms them into measurable signals. The signals are then processed and displayed by an electronic system, which allow their results' acquisition and interpretation.

Immunosensors combine the most advanced technologies based on the binding properties of antibodies, which improve the sensitivity and the rapid detection during analysis (·la Farré and others 2008; Rasooly and Herold 2011). Generally, antibodies specifically recognize the antigens and form a stable complex, which is then detected and analyzed (Taitt and others 2005; Byrne and others 2015). The most used types are electrochemical,

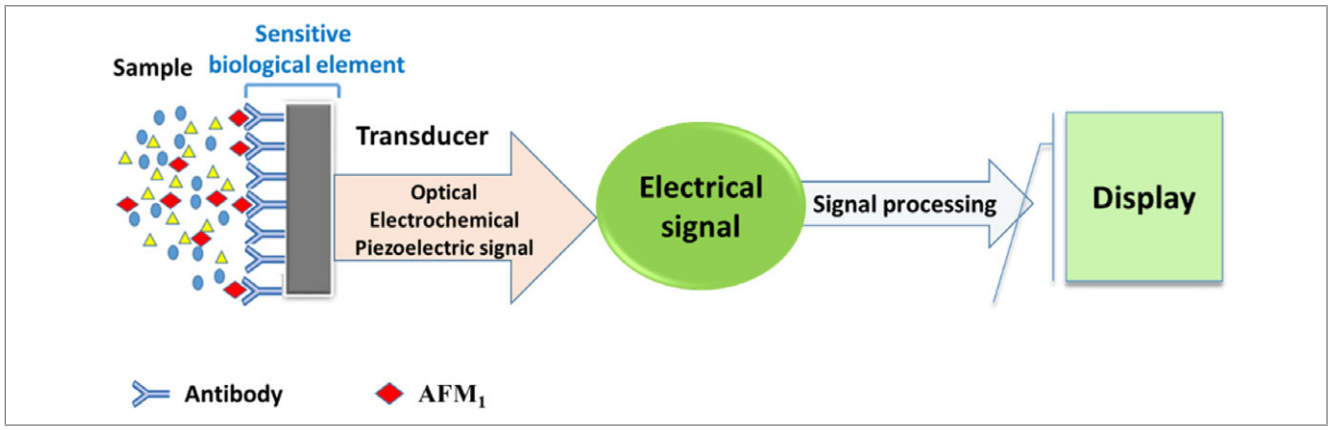

Figure 5–General basic parts of immunosensors.

optical, micromechanical, and piezoelectric (PZ) immunosensors (Aliofkhazraei and Ali 2014; Chauhan and others 2015). Contrary to other immunoassay methods, new developed transducers, such as quartz crystal microbalance (QCM) transducer do not necessarily need the use of labels for detecting the analyte. Therefore, immunosensors are classified into labeled or label-free, depending on whether or not they use labels for analyte detection.

They also exist in both noncompetitive and competitive formats. Since immunosensors are based on Ab–Ag interaction, which causes different property changes, transducers are required and needed for the measurement of electrons, photons, and masses (Wang and others 2016). Though free-label noncompetitive formats are rapid and simple, they may give a false positive response due to the nonspecific Ab–Ag or Ab–matrix binding. To overcome this difficulty, matrix properties are optimized by incorporating metals and metal oxide nanoparticles such as PtCo, CeO_2, Fe_3O_4, and TiO_2, or by introducing an appropriate conducting polymer (Chauhan and others 2016; Wang and others 2016).

Taking into account these measurable elements, there are mainly 3 types of transducers: electrochemical transducers which rely on electrical signal measurement, and they may thus be amperometric, potentiometric, and conductometric transducers; optical transducers in which optical signal such as color, fluorescence, or any optical property changes as a result of Ab–Ag complex formation; and PZ transducers which detect the changes in mass, as Ab–Ag complex formation results in a slight but detectable mass increase. Nevertheless, electrochemical and optical transducers are the most used for AF analysis (Luppa and others 2001; Ricci and others 2007; Sharma and others 2014a, 2014b).

Electrochemical immunosensors

Much progress has been made in designing electrochemical immunosensors for AF analyses. For electrochemical immunosensor based on direct ELISA, the enzyme conjugate is free, while a specific Ab or Ag is immobilized on the electrode. The competitive reaction is followed by the binding of the enzyme to the electrode, and the reaction between enzyme and substrate generates the current, which is proportional to the enzyme density. Electrochemical biosensors used for AF detection exist in various forms, most notably amperometric, impedimetric, and potentiometric (Luppa and others 2001; Micheli and others 2005).

Various nanomaterials are used for signal transduction and amplification during the detection process. They are also used as carriers for immobilizing antibody, Ag-protein conjugate, enzymes, and other biomolecules. Vig and others (2009) developed a free-label electrochemical immunosensor using GNPs and silver electrodeposition, and its detection range was between 0.015 and 1 ng/mL of AFM_1 in different samples. Conversely, GNPs and HRP were used as labels for the specific antibody and the 2nd antibody, respectively, and TMB as an electroactive dye for developing an immunosensor with a LOD of 2 ng/mL AFM_1 in milk (Ahirwal and Mitra 2010). Disposable electrochemical immunosensors for AFM_1 have also been designed. By optimizing many parameters, Micheli and others (2005) came up with an electrochemical technique for the direct analysis of AFM_1 in milk, with a LOD of 25 ng/mL.

Optical immunosensors

Optical immunosensors are similar to electrochemical sensors, but they often use SPR. In SPR, an optical-sensitive surface such as a layer of gold or a glass surface is used to immobilize Ab or Ag. As the amount of AFM_1 binding to the Au layer or glass surface increases, the angle of spectral power distribution (SPD) also increases (Li and others 2012). In order to improve sensitivity, optical immunosensors can combine competitive-direct ELISA and IC assay methods (Contreras-Medina and others 2013).

A plasmon-enhanced fluorescence spectroscopy may also be used to detect the intensity of the fluorescence signal produced by resonant excitation of surface plasmons. Based on this approach, Wang and others (2009) developed a biosensor with lower LOD of 0.006 ng/mL.

Flow injection immunoassay (FIIA)

FIIA consists of a system that possesses an immunoaffinity column in which specific antibodies are immobilized. The column is filled with a solution containing labeled antigens, which are then bound to the immobilized antibodies. Afterwards, a sample containing unlabeled analyte is also injected into the column. As the labeled antigen has lower affinity to the immobilized antibody, compared to unlabeled analyte in the sample, it is displaced. The intensity of the removed antigen correlates with the amounts of the analyte (Gübitz and Shellum 1993; Fintschenko and Wilson 1998; Bartolomeo and Maisano 2010; Qi and others 2011). Badea and others (2004) used this method to detect AF in fresh milk. The sample is incubated with a specific concentration of anti-AFM_1 Ab, and AFM_1-HRP (Ag) is used as a tracer until the equilibrium is reached. The AFM_1 in the sample and AFM_1-HRP compete for antibody, then the mixture is injected into the system column. The immobilized Protein G on the column retains the Ag–Ab complexes, and then the eluted enzyme label is detected,

which correlates with the amount of AFM_1 in the sample. This method shows a LOD of 0.011 ng/mL AFM_1, and a concentration range between 0.02 and 0.5 ng/mL (Badea and others 2004). FIIA presents a relatively high sensitivity and rapid detection; however, it requires untreated fresh milk, because AFM_1 bound in stored milk is difficult to detect (Ricci and others 2007).

Conclusion

Recent immunoassay techniques have shown promise as effective methods to detect AFM_1 traces in feeds and foodstuffs. Compared to other ordinary methods, immunoassays are simple, rapid, sensitive, portable, and cost-effective. Most of them may even be applied to onsite monitoring while preserving their specificity and high sensitivity. However, these methods may present some limitations due to many factors, including low specificity of antibodies to antigens or to matrix, and poor color change. Therefore, future research should focus on the optimization of the matrix components, nanoparticle technology, specific antibody production, automation, and miniaturization of equipment. Additionally, future immunoassays for AFM_1 should be rapid, low-cost, and accessible in both developed and developing countries. Since crops may be contaminated at any stage of processing, there is also a pressing need for a coordinated onsite monitoring and evaluation of AFs. However, this requires the exploration of new advances in food biotechnology and agricultural science, which will ensure not only the effective control of both AFM_1 in feeds and foodstuffs, but also the prevention of *Aspergillus* proliferation on crops.

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