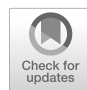

Application of Nano-ELISA in Food Analysis 15

Long Wu

Abstract

ELISA is a widely applied technique with good reliability, sensitivity, and specificity. Compared to other immunoassays, ELISA has been intensively used in many fields like biology, toxicology, immunology, and medical diagnosis due to its simple operations and high reliability. Recently, ELISA has been widely used in food safety and control. Though ELISA has so many applications and superior advantages, it encounters a lot of restrictions, especially the relatively low sensitivity and stability. Based on this, abundant work has been done to improve the detection performances of conventional ELISA (c-ELISA), including the limit of detection (LOD), accuracy, and stability. Fortunately, combined with nanomaterials, various ELISA-based methods have been developed to address the limitations of c-ELISA. The nanomaterials-based ELISA (nano-ELISA) behaves additionally mechanical, electrical, magnetic, optical, and catalytic properties. Based on this, in this chapter, we summarize ELISA methods and provide an overall description of the history, principles, designs, and applications in analysis of food contaminants, which is expected to help facilitate the food safety and control in compliance with legislation and consumers' demands.

Keywords

ELISA · Nanomaterials · Detection · Food analysis · Biomarker

L. Wu (✉)
College of Food Science and Engineering, Hainan University, Haikou, Hainan, China

Key Laboratory of Tropical Fruits and Vegetables Quality and Safety for State Market Regulation, Hainan Institute for Food Control, Haikou, China

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P. Chandra, P. S. Panesar (eds.), *Nanosensing and Bioanalytical Technologies in Food Quality Control*, https://doi.org/10.1007/978-981-16-7029-9_15

15.1 Introduction

It is known that ELISA is a popular biochemistry assay using a solid-phase enzyme immunoassay to analyze a target with a form of antibody-antigen recognition model, in a plate well or solutions (Voller et al. 1978; Butler 2000). As a golden standard in immunoassay, ELISA has been widely applied in laboratories and industries, which acts as a verification method, or a detection means in sample quality tests (Buss et al. 1997; Salomone et al. 2004; Török et al. 2015). Owing to its advantages such as good convenience, high specificity and feasibility, ELISA or ELISA-based method has been regarded as a powerful tool in analytical science (Gao et al. 2019a, b). Yet, c-ELISA suffers from inherent shortcomings like low efficiency, complicated operations, and single detection mode (usually antigen-antibody detection). Thus, it is vital to develop an effective method to solve the problem.

Since the extensive applications of ELISA, different ways have been tried to solve the existing problems. For instance, many studies have been carried out to enhance the LOD and detection accuracy of c-ELISA (He et al. 2016; Fadlalla et al. 2020). So far, not only the range of application of ELISA but also its detection performance has been developed a lot (Byer et al. 2008; Jaria et al. 2020). To be specific, from c-ELISA to avidin-biotin ELISA (ABS-ELISA), the immunoassay has been extended to in vivo detection with higher sensitivity (Peng et al. 2014). Besides that, it is an ultimate goal to achieve higher stability, better accuracy, simpler operations, and lower cost of the ELISA method.

Recently, a lot of work related to ELISA have been reported in the applications of food analysis. In particular, Zhang et al. summarized the application of ELISA in pesticide residues detection in food products, which emphasized the accuracy and universality of ELISA in pesticide residues analysis (Zhang et al. 2008). Besides, Aydin et al. gave a detailed description on the history, working principles, and different classifications of ELISA, as well as how to analyze peptide or protein using an ELISA method, including discussing what we can do with ELISA analytical errors (Aydin 2015). To be specific, Toh et al. introduced the application of aptamers in ELISA, that is aptamer-based ELISA, which uses aptamers to recognize the analytes or give signal outputs (Toh et al. 2015). After that, based on the localized surface plasmon resonance of nanomaterials such as nanogold or nanosilver particles, Satija et al. discussed the plasmonic-ELISA that used in visual detection applications (Satija et al. 2016). Later, Wang et al. reported the advances in ELISA for antibiotics detection in food matrices involving different immunosensors from electrochemical ELISA to fluorescence-ELISA (Wang et al. 2017). At present, nano-ELISA has been developed by integrating nanotechnology with ELISA, which brings much more convenience and superiority in food analysis. For example, Wu et al. introduced the development of c-ELISA in combination of nanomaterials (Wu et al. 2019a, b), especially the latest development of nano-ELISA and their applications in food safety. By modifying c-ELISA with nanomaterials, it shows superior performance with lower LOD and cost, higher stability, and accuracy. Thus, it is vital to develop nanomaterials (nanopolymer, nanoantibody, nanoprobes)-based ELISA and applied them in food analysis.

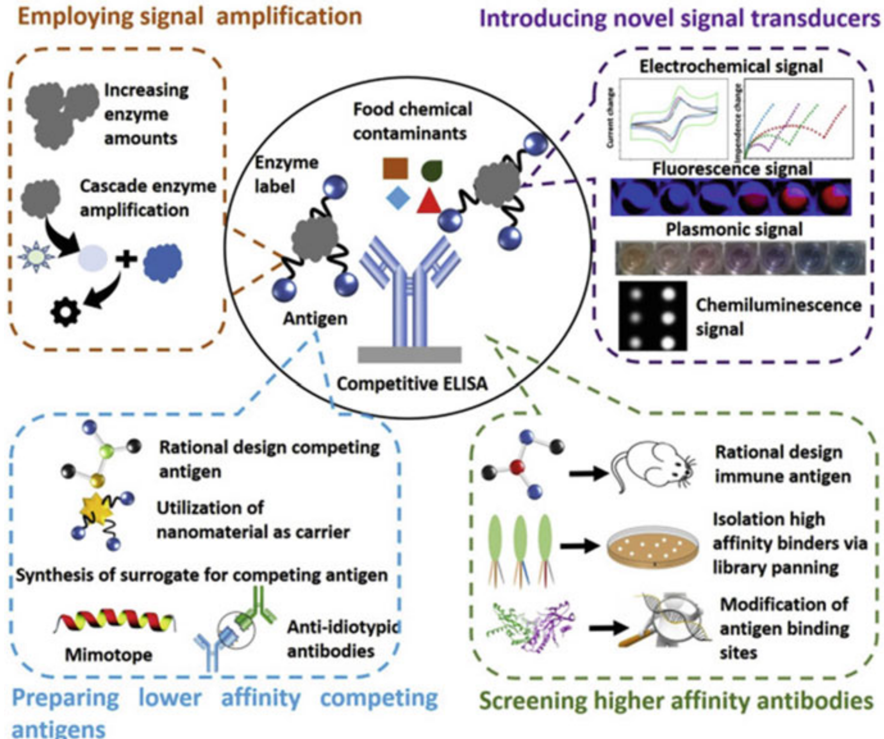

Fig. 15.1 Schematic presentation of emerging strategies for enhancing the sensitivity of c-ELISA. Reproduced from Ref. (Xiong et al. 2020) with permission from Elsevier

Based on the above background, the development of c-ELISA via nanomaterials (nano-ELISA) is given in this chapter, mainly referring to the four construction units of ELISA: substrate of sorbents, recognition models, enzyme labels, and chromogenic agents (Fig. 15.1). Also, advantages and disadvantages of c-ELISA and nano-ELISA are summarized and discussed. Different nanomaterials are described to improve or rebuild c-ELISA, which offer valuable guidance and strategies to design and construct nano-ELISA. In addition, different applications of newly developed nano-ELISA are described in food samples. Finally, challenges and perspectives on nano-ELISA are discussed, as well as their applications in food analysis and safety verification.

15.2 History and Development of ELISA

Before the development of the ELISA, radioimmunoassay was the only choice to carry out an immunoassay, which adopted radioactively labeled antigens or antibodies. At first, radioimmunoassay was proposed to achieve detection of

endogenous plasma insulin (Yalow and Berson 1960). In the method, radioactive isotope like iodine-125 as labeled signal can indicate the existence of target in the sample, usually a specific antigen or antibody. The innovative idea brings convenience to the identification of certain targets. However, as radioactivity may do potential harm to human health, a safer alternative must be developed.

As the radioactivity of such label can pose potential risks on human health, the urgent affair is to replace the radioactive signal with safer labels. Fortunately, a color change occurs as peroxidase (e.g., HRP) reacts with substrates like OPD or TMB, which can be used as signal equals to radioactive isotope. The limitation is that the color variations must be initiated by certain enzyme, so enzymes linked with antibody was developed to provide labels that react with substrates, which was defined as enzyme immunoassay (EIA) (Nakane and Pierce Jr 1967). To conveniently remove the references through simple washing procedures, antibody/antigen must be anchored on the bottom of plate wells. Based on a sorbent substrate and enzyme-labeled antibody, the prototype of enzyme-linked immunosorbent assay (ELISA) was reported for the quantitative detection of IgG for the first time (Engvall and Perlmann 1971).

Typically, the signal transducer of conventional ELISA consists of enzyme and substrates that can react to produce color changes when analyte exists (Johnson et al. 1992; Hosseini et al. 2018). Based on the fundamental structures of ELISA, optical, electrochemical, and magnetic reporters are frequently used to generate signals for other ELISA-like techniques, which can outcompete c-ELISA in sensitivity, flexibility, and stability (Bouças et al. 2008; Phillips and Abbott 2008; Al Ghounaim et al. 2016). In technical terms, some of the assays cannot be classified as ELISAs, as they are not linked with enzyme or absorbed on a solid surface of well plates. However, they are instead linked to some nanozymes or anchored on other solid surfaces. Generally, their working principles are the same, so we accepted them as ELISAs. Till the year of 2012, De La Rica reported an ELISA using AuNP as a reporter to achieve the colorimetric detection of prostate-specific antigen and HIV-1 capsid antigen, which is the very beginning of nano-ELISA (De La Rica and Stevens 2012).

15.3 The Working Ways of ELISA

Conventionally, the specificity of antigen-antibody type reaction is used because it is easy to raise an antibody specifically against an antigen in bulk as a reagent. (Gaastra 1984). Taking food sample detection as an example, the specific substance to be detected (an analyte) is anchored on a solid substrate with specific recognition captured by antibody (a "sandwich" mode) (Fig. 15.2). After the analyte is anchored on the solid plate, a liquid sample is added onto a stationary solid phase with special binding properties, followed by multiple liquid reagents that are sequentially added, incubated, and washed. Finally, color development with some optical change can be observed in the final liquid in the well, which can be used to analyze the amount of analyte qualitatively and quantitatively.

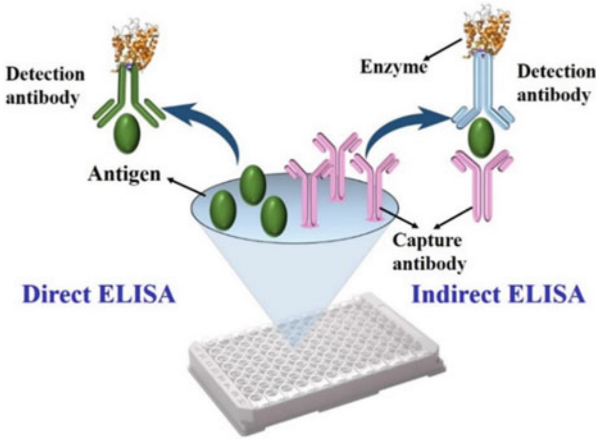

Fig. 15.2 Scheme illustration of the principle of direct ELISA and indirect ELISA

In addition, the analyte is also called the ligand because it will specifically bind or ligate to a detection reagent, thus ELISA falls under the bigger category of ligand binding assays (Ma and Shieh 2006). The ligand-specific binding reagent is usually coated and dried onto the transparent bottom and sometimes also an interface to generate a signal. In this regard, the recognition element-like aptamer can also be an alternative. On the other hand, for each washing step, the signal label and nonspecific or unbound components are washed away, but the reaction products immunosorbed on the solid phase (Heaney et al. 2020). That's to say, the ligand, immobilized antibody are parts of the plate, which is difficult to be developed into reusable ELISAs.

Generally, as a heterogenous assay, ELISA separates some components of the analytical reaction mixture by adsorbing certain components onto a solid phase which is physically immobilized (Kwong et al. 2002). In the most simple form of an ELISA, antigens from the sample to be tested are attached to a surface. Then, a matching antibody is applied over the surface so it can bind the antigen. This antibody is linked to an enzyme and then any unbound antibodies are removed. In the final step, a substance containing the enzyme's substrate is added. If there was binding, the subsequent reaction produces a detectable signal, most commonly a color change.

15.4 Structure of ELISA

As described above, from bottom to up, ELISA method consists of four main parts: solid substrates, sorbent antibody/antigen, enzyme labels, and chromogenic reagents (Fig. 15.3). To conduct the ELISA detection, the supporting substrate like microplate well provides a surface for antibody/antigen to bound to, then the enzyme-linked complementary biomolecules (a new specific antibody or a secondary antibody) will bind with the primary antibody/antigen and generate a bioconjugation (Waritani

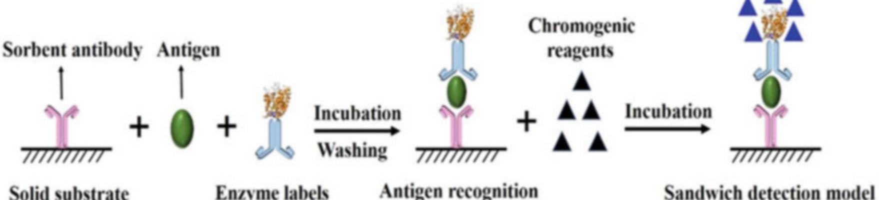

Fig. 15.3 Structure of ELISA for the detection of analytes (indirect ELISA method, **E**: enzyme)

et al. 2017). To realize visual detection, chromogenic reagents are used to generate visual detection signal (chemiluminescence, fluorescence color, etc.). In the ELISA structure, solid substrate acts as bounded foundation, biomolecules recognition as the framework, labeled enzyme as the reaction initiator, and reagent substrate as the signal indicator (Wu et al. 2019a, b).

In this part, to make it more understandable in designing the ELISA-based methods, the construction of c-ELISA will be introduced from the solid substrate to biomolecules recognition, then to the color development, as well as the washing and blocking steps. Based on the fundamental units, the improvement of c-ELISA with other new technology is introduced and discussed, especially the nanotechnology-directed ELISA method, also known as nano-ELISA.

15.4.1 Nanomaterials-Based Substrate

The most common solid substrate of ELISA is polystyrene used for most optical detection microplates (Hosseini et al. 2018). It can be colored blue by the addition of TMB/H_2O_2 for optical absorbance or luminol/H_2O_2 for luminescence detection or black by adding silver nanoparticles for biological assays (Wu et al. 2019a, b). Typically, a microplate has 6, 12, 24, 48, 96, 384, or 1536 sample wells, which is designed to allow low-volume and high-throughput assay for the samples. The plate well provides a solid surface for analytes, antibody, antigen, or other reagents to attach on, which are usually physically immobilized. The physical binding force between the bottom surface and the adsorbent provides a bridge for biomolecules conjugation, thus called "immunosorbent." In this regard, the solid substrate can be regarded as the foundation of signal recognition to be connected. So, an ideal substrate should meet the requirements of low cost, high light transmission, and weak-nonspecific adsorption.

As mentioned above, nonspecific adsorption is not welcome in the ELISA methods. However, most of the cases, the binding force of adsorbents comes from the physical adsorption of plate wells. So, the nonspecific adsorption is inevitable, which can pose great effects on the detection results, for example, false positive or negative, depending on the ELISA detection type. Thus, it is vital to develop appropriate substrate by using new materials to build the ELISA method. For

instance, poly (dimethylsiloxane) (PDMS) membrane (Wang et al. 2013), nanofibers (Pan et al. 2015), molecularly imprinted polymers (MIPs) (Li et al. 2017) and magnetic nano-beads (Al Hamshary et al. 2020) are popular and reliable adsorbent substrate in ELISA.

15.4.2 Recognition Models

When adsorbents are bound to the surface of plate wells, the recognition between antibodies and antigens should be constructed for the detection of targets. To meet the requirements of practical assay, different models are designed with different detection intentions. For example, small molecules like pesticides are firstly conjugated with BSA and coated on the plate wells, then an antibody labeled with enzyme is applied to detect analyte, which is called direct ELISA. On the other hand, for the bigger molecules such as proteins, primary antibody firstly adsorbed on the plate wells, then the proteins are recognized and captured by the primary antibody, finally a second antibody labeled with enzyme is applied to give signals, which is called indirect ELISA. Thus, direct ELISA, indirect ELISA, sandwich ELISA, and competitive ELISA are the four main models used in immunoassay.

For the direct ELISA, several steps should be followed: (1) the antigen to be tested for is added to a microtiter plate and incubate for a certain time; (2) the block agent-like bovine serum albumin (BSA) or casein, is introduced to each well to cover any uncoated surface in the well; (3) the primary antibody labeled with enzyme is added to the well in order to specifically combine with the antigen; (4) a coloring substrate-like TMB/H_2O_2 is added to generate color variations. Obviously, the higher level of primary antibody present, more significantly the color changes. The major problem of the direct ELISA is that it lacks sensitivity using antigen immobilization. Taking serum detection as an example, when serum acts as the source of test antigen, all proteins in serum may adhere to the plate well, only a small amount of analyte in serum compete with other serum proteins. As a result, the color variations will not be that significant, which is the limitation for the detection sensitivity and accuracy.

Indirect ELISA can solve the above issue by using a labeled secondary antibody. Similar to the direct ELISA, antigen is bound by the primary antibody and then detected by a second antibody. A sandwich ELISA is distinct from an indirect ELISA by the recognition model of antibody-antigen-antibody. Despite from direct ELISA, the other ELISA methods are based on two antibodies. Nevertheless, antibody itself suffers from some disadvantages, such as high cost, hard to store long term, easy denaturation, and limited application conditions. Besides that, due to the steric effect of antibody, it cannot be easily applied to ELISA easily when come to the detection of small molecules. Thus, a special class of nucleic acid molecules that named aptamers has been developed to specifically recognize small molecules, which are poised to replace the monoclonal antibodies in therapeutics, diagnostics, and drug development (Toh et al. 2015; Lee and Zeng 2017; Wu et al. 2020; Her et al. 2017).

15.4.3 Nanozyme Labels

The enzyme acts as an amplifier to accelerate chemical reactions of substrate. Due to the high catalytic activity of enzyme, even though few enzyme-linked antibodies are bound, they will produce many signal molecules that can give special color. The more enzyme-labeled antibody is bound, the faster the color will develop. To a large extent, the quality of enzyme plays a crucial role in the construction of ELISA methods. Thus, the enzyme label should have the characteristics including high purity, good specificity, excellent stability, and long-term activity.

Among the natural enzymes, horseradish peroxidase and alkaline phosphatase, are the most commonly used antibody labels. Both can produce a colored, fluorimetric, or luminescent derivative when incubated with a proper substrate, allowing it to be detected and quantified. Compared to ALP, HRP is much better in ELISA applications as it is smaller, more stable, and less expensive. The enzyme label is directly related with the signal output, so it is the most concerned part in ELISA method. However, natural enzymes suffer from the limitations such as hard to be separated and purified, hard to store long term and mass produce, high cost, easy denaturation, and limited application conditions.

To solve the problem, nanozymes, a kind of nanomaterials with peroxide activity, have been introduced to replace natural enzymes. At the same time, due to the large specific surface area, nanozymes can also act as loading substrate to achieve signal amplification, thus enhancing the detection sensitivity of ELISAs. Compared with natural enzymes, nanozymes are easier to be modified and purified. Moreover, a variety of new nanomaterials have been found to mimic the activity of enzymes, such as metal–organic frameworks (MOFs), covalent organic frameworks COFs, and Prussian blue (PB). Given the advantages like low cost, recyclable utilization, high catalytic activity and stability, nanozymes are widely used in food safety. For instance, based on MOFs, an indirect competitive ELISA method was developed by replacing natural enzyme with MOFs nanozymes for the sensitive detection of AFB1 (Xu et al. 2021). To achieve high sensitivity, Tian et al. proposed a cascade reaction-based colorimetric aptasensor for the detection of OTA (Tian et al. 2019).

15.4.4 Enzymatic Markers

When the structure of ELISA is well constructed, enzymatic markers are needed to provide the readout signal for the targets. Usually, the markers are the catalytic substrate of the labeled enzyme, which can be oxidized by certain enzyme and produce the colored, fluorescent, or luminescent product. Most of the substrates (TMB, ABTS, OPD, etc.) are used in the presence of hydrogen peroxide (H_2O_2). For instance, the commonly used HRP enzyme could oxidize TMB when H_2O_2 exists, generating a blue color variation that is detectable. Based on the principle of electron donor and receptor, ABTS and OPD with H_2O_2 also behave a color change in the presence of peroxidase. Owing to its good stability, low toxicity and high sensitivity, TMB/H_2O_2 as a coloring system is widely applied in the colorimetric assays.

Till now, most of the ELISA methods adopt the TMB/H_2O_2 coloring system for signal output. For the visual detection, the system is only a single-color measurement, which rely on the gradation of color development to indicate the analyte. However, the single-color mode requires higher demand for the naked eye and is difficult to achieve multi-sample detection. In the view of visual detection, multicolor assay is easier to realize sensitive and accurate detection. Therefore, it is of great importance to develop new chromogenic mode or multi-color reagents.

To achieve multi-color signal output, metallic nanoparticles such as gold or silver are intensively studied. Based on their localized surface plasmon resonance properties, they behave specific optical performance such as red shift in spectra. For instance, based on the principle that TMB^{2+} can etch gold nanoparticles, Guo et al. proposed a dual-color response for prostate-specific antigen, with color variations from wine red to colorless and then to yellow (Guo et al. 2016a, b). On the basis of the principle of iodine etched gold nanorods, an ALP-based plasma ELISA strategy was developed for the sensitive detection of human immunoglobulin G (Zhang et al. 2017a, b). Both methods provide examples of nanoparticles as enzymatic markers.

15.4.5 Washing and Blocking Agents

Actually, to obtain accurate, stable, and sensitive ELISA results, washing and blocking steps are indispensable. As the ELISA method is constructed based on the immunosorbent strategy, the nonspecific adsorption and instable adhesion are inevitable, which could lead to false-positive or negative results. Thus, in each step of the establishment of ELISA, washing step is essential to remove excess antigen or antibody. The washing agent we used is a kind of buffer solution with 0.05% Tween-20 in PBS solutions. When antigen or antibody are attached to the well surface, washing buffer is needed to wash away the unbound biomolecules. Similarly, in the antibody-antigen recognition step, the washing is also acquired to remove excess biomolecules and remain the reacted ones. A relatively low concentration detergent can be used because high dosage of detergent would cause damage to biomolecules.

Usually in a direct or sandwich ELISA, a solution of nonreacting protein is added to each well to block any other surface in the plate wells. This kind of protein solution is known as blocking agent, which is prepared by introducing certain proteins in the washing buffer. The proteins can be all kinds of animal serums such as bovine serum albumin (BSA), rabbit serum, horse serum, or casein. After a known quantity of capture antibody is attached on the well surface, the blocking procedures are carried out to block any nonspecific-binding sites on the surface. The blocking step is usually performed once in the ELISA construction process. Typically, the blocking step can be completed by incubating certain volume of blocking agent in the wells for 2 h at 37 °C.

It was reported that various proteins as blocking agents can produce quantitative differences. So, many researches have been conducted to explore the effects of different proteins on ELISA performance. For example, Vogt Jr. et al. explored

the influences of instantized dry milk, serum albumin, casein, gelatins on blocking nonspecific adsorption of ELISA, indicating that casein and instantized milk showed the best blocking property (Vogt Jr et al. 1987). Moreover, Xiao and Isaacs reported that different BSA preparations used as a blocking agent in an ELISA can give different amounts of nonspecific binding of ELISA reactants (Xiao and Isaacs 2012). The report reminded that critical controls are needed to ensure that ELISA reactants are appropriately bound to the blocking agent.

15.5 Applications of Nano-ELISA in Food

15.5.1 Detection of Biotoxins

Biotoxins are toxic secondary metabolites produced by living organisms, which are usually harmless to the organism itself, but after consumption, it will affect the health of humans or animals (Fletcher and Netzel 2020). Biotoxins can be divided into five categories according to their sources: mycotoxins, bacterial toxins, marine toxins, animal toxins, and phytotoxins. They can enter food through various ways, causing people to appear food poisoning and other phenomena, which are serious or even fatal. Therefore, how to efficiently and quickly detect these toxins is particularly important. On the other hand, nanomaterial-modified ELISA overcomes the relatively low stability and sensitivity of c-ELISA. Through its inherent nanostructure, stability and specificity are improved, and a more efficient and rapid detection method can be constructed (Fig. 15.4).

15.5.1.1 Detection of Mycotoxins

Mycotoxins, a kind of secondary organic metabolites, are produced by distinct fungi like *Aspergillus*, *Penicillium*, and *Fusarium* (Avery et al. 2019). In food, mycotoxins are toxic metabolites produced when fungi contaminate food, and they are also the most commonly seen toxins among different food toxins (Moretti et al. 2018). Generally, the most common mycotoxins include aflatoxin, ochratoxin, trichothecenes, zearalenone, and fumonisins. Humans and animals ingest food containing high level of mycotoxins at one time will cause acute poisoning, and long-term intake of food containing mycotoxins will also cause chronic poisoning, and even cause cancer and teratogenic effects.

Aflatoxins are common mycotoxins that contaminate food and agricultural products. The nano-ELISA based on direct competition and indirect competition can detect aflatoxins in grains and milk more sensitively, quickly, and specifically. Combining immunomagnetic beads (IMBs) with direct competition ELISA, Zhang et al. proposed a strategy using monoclonal antibody 5H3-modified immunomagnetic beads as capture probes, and AFB1-CMO-labeled horseradish peroxidase as probes. Competing with free aflatoxin, the total amount of aflatoxin in corn samples was determined (Zhang et al. 2017a, b). Subsequently, Zhou Xu et al. developed an indirect competitive ELISA based on an MOF material MIL-88, to construct AFB1 antigen, AFB1 antibody, and MIL-88-modified antibody solid-

Fig. 15.4 Schematic illustration of nano-ELISA analytical techniques for biotoxins in food. Reproduced from Refs. with permission from Elsevier (A: Xu et al. (2020); B: Liu et al. (2019); C: Chen et al. (2011); D: Orlov et al. (2013))

phase antigen-tested antibody-enzyme label secondary antibody complex (Xu et al. 2020). By adding a substrate solution of TMB and H_2O_2 to observe the degree of color development, it can be used for the high-throughput determination of aflatoxin B1 in peanut milk and soy milk. The linear range of this method is 0.01–20 ng mL^{-1}, and the detection limit is 0.009 ng mL^{-1}.

In addition, the combination of various detection methods with ELISA is also a new trend in aflatoxin detection. Based on nanoenzymes, aptamers, and Fe_3O_4 magnetic nanoparticles (MNP), Long Wu has established a simple operation and separation of nanoenzymes and aptamer immunosorbent assay (NAISA) for aflatoxin B1 (AFB1) detection. In this work, mesoporous SiO_2/Au-Pt (m-SAP) was used as a signal marker with high catalase activity, and AFB1 in peanut was specifically recognized by an aptamer, and MNP was used to achieve magnetic separation. In order to verify the performance of NAISA, traditional ELISA (c-ELISA) and enhanced ELISA (e-ELISA) based on MNP and m-SAP nanozymes were applied to the detection of AFB1. The lowest detection limit of NAISA method is 5 pg mL^{-1}, which is 600 times and 12 times lower than c-ELISA (3 ng mL^{-1}) and e-ELISA (0.06 ng mL^{-1}), respectively (Wu et al. 2020). Zherdev immobilized the antibody on the surface of magnetic particles, changed the solid phase of ELISA, and constructed a microplate-based enzyme-linked immunoassay. The immobilized antibody reacts with the natural antigen and the labeled antigen in solution, thereby shortening the interaction time to 5 min without affecting the analysis results. The adsorption of immunoglobulins on the surface of magnetic nanoparticles increases their stability in water-organic media, thereby minimizing the degree of dilution required for

samples. The detection of barley and corn extracts showed that the detection limit of aflatoxin B1 was 20 pg mL^{-1} with a total detection time of 20 min (Urusov et al. 2014). Pang et al. combines electrochemistry with enzyme-linked immunosorbent assay, introduces rolling circle amplified DNAzyme and covalent organic framework to modify the electrode to improve and expand the electrochemical response signal, and then a sandwich structure was formed via primer-antigen-aptamer and anti-AFM1 antibody to specifically recognize AFM1 in milk (Pang et al. 2020).

Similarly, fumonisins, as one of the most common mycotoxins in cereal products, have established a detection method similar to that of aflatoxin. Lu et al. developed a competitive fluorescent enzyme-linked immunosorbent assay (cFELISA) based on CdTe quantum dots (MPA-QDs) to detect fumonisin B1 (FB1) in corn (Lu et al. 2018). They labeled the analyte FB1 on catalase (CAT), outputted MPA-QDs sensitive to H_2O_2 as a signal, and adjusted the fluorescence conversion of MPA-QDs to achieve high-sensitivity detection. The linear range of this method is 0.39–12.5 ng mL^{-1}, and the detection limit is 0.33 ng mL^{-1}. On the other hand, Li et al. constructed an enhanced indirect competitive enzyme-linked immunosorbent assay (IC-ELISA) based on gold nanoparticles modified with mercaptoundecanoic acid (AuNPs-MUA) (Li et al. 2018a, b). Three hybridoma cell lines were obtained by immunization and cell cloning methods, which secreted monoclonal antibodies against fumonisin B1 (FB1); AuNPs-MUA was used as horseradish peroxidase (HRP)-goat antibody. The mouse IgA vector is used to observe the degree of color development by adding a substrate solution of TMB and H_2O_2, so as to quantitatively detect the total content of fumonisins (FB1, FB2, and FB3) in corn. The detection limit of this method is 0.078 ± 0.013 μg L^{-1}.

Ochratoxin A is a natural mycotoxin, which has been found in several food matrices. Due to its high toxicity, effective monitoring of its presence in food is particularly important. Zhu et al. prepared botryoid-shaped Au/Ag nanoparticles (BSNP) via a tailored galvanic reaction. In the presence of ascorbic acid, the silver nanoprism-BSA complex is used as a template to react with $HAuCl_4$. The formed BSNPs-HRP-IgG was used as a carrier of HRP-IgG to amplify the detection signal of indirect competitive ELISA against ochratoxin A. The linear range of this BSNPs-enhanced ELISA method is 0.016–0.05 ng mL^{-1} (Zhu et al. 2017). Karczmarczyk et al. used gold nanoparticles for QCM-D signal enhancement and successfully established an indirect competitive bioassay for the detection of ochratoxin A in red wine (Karczmarczyk et al. 2017). Based on the form of indirect competition, a specific rabbit PAb and a second goat anti-rabies PAb labeled with gold nanoparticles were used for signal amplification. The linear range of the method is 0.2–40 ng mL^{-1}, and the detection limit is 0.04 ng mL^{-1}.

Mak et al. reported an ultra-sensitive magnetic nanoparticle immunoassay that can detect more than one mycotoxin (Mak et al. 2010). The use of magnetic nanoparticles as the solid phase allows a significantly increased surface area for the immobilization of reactants and their uniform distribution in the entire volume of the reaction medium, thereby eliminating the diffusion limitation of traditional ELISA. The application of the magnetic field allows the reactants to be separated simply and quickly and simplifies the washing steps required for traditional

microplate-based ELISA. Taking advantage of these advantages, an MNP-based immunoassay protocol was developed and implemented in the wells of ELISA microplates to detect AFB1, zearalenone, and HT-2 mycotoxins.

15.5.1.2 Detection of Bacterial Toxins

It is reported that more than half of food-borne diseases are caused by pathogenic bacteria, among which botulism caused by Clostridium botulinum, gastroenteritis, and staphylococcal poisoning caused by E. coli strains are the most common (Hernández-Cortez et al. 2017). In order to reasonably control-related food pollution, it is necessary for people to strictly manage food from the primary production to the final consumption. Therefore, it is necessary to construct feasible analytical techniques to monitor bacterial toxins in food samples.

Bhairab Mondal uses staphylococcal enterotoxin B (SEB)-binding body (SEB2) as capture, and unmodified gold nanoparticles (AuNPs) as colorimetric probes to construct a simple, sensitive, and specific detection of SEB (Bhairab et al. 2018). This method is based on the color change from red to purple caused by the conformational change of the aptamer in the presence of SEB, and the aggregation of AuNPs induced by salt, which can be monitored with the naked eye or UV-Vis spectrometer. The results show that AuNP can effectively distinguish SEB-induced conformational changes of nucleic acid aptamers at a certain high salt concentration, and the stability is effectively tested in artificially added milk samples. The linear range of this method is 50 μg mL^{-1} ~ 0.5 ng mL^{-1}, the lower limit of visual detection (LOD) reaches 50 ng mL^{-1} within a few minutes, and the spectrophotometric method increases to 0.5 ng mL^{-1}.

Orlov et al. determined two staphylococcal toxins in milk by a magnetic sandwich immunoassay (Orlov et al. 2013). The capture monoclonal antibody is fixed on the 3D fiber solid phase in the kit, and the recognition monoclonal antibody is conjugated to the magnetic nanoparticles through the biotin-streptavidin binding. When the interlayer is formed, the magnetic particles are captured by the cartridge and detected with extremely high sensitivity and selectivity by combining frequencies. The signal is read from the entire volume of the non-transparent 3D fiber structure used as the solid phase, providing a large reaction surface, rapid reagent mixing, and antigen immunofiltration directly during the measurement process. This method showed that the limits of detection (LOD) of Staphylococcal Enterotoxin A (SEA) and Toxic Shock Syndrome Toxin (TSST) were as low as 4 and 10 pg mL^{-1}, respectively.

15.5.1.3 Detection of Marine Toxins

Marine toxins are a kind of toxic natural active micromolecules that exist in marine organisms and have high toxicity. According to the different carrier, it can be divided into shellfish toxin, tetrodotoxin (TTX), and cigar toxin (CTX) (Wang et al. 2020a, b). After they enter the human body through food, they will act on the nervous or digestive system, causing food poisoning symptoms such as diarrhea, paralysis, and even lead to death (Grasso et al. 2019). These marine toxins not only affect food safety and human health, but also cause serious economic losses.

Therefore, in order to prevent the occurrence of food-borne marine toxin poisoning, the early diagnosis and detection of marine toxins is of great significance.

Campbell has developed a nano-array planar waveguide biosensor for detecting tetrodotoxin (TTX). The technology consists of a nanoprinted toxin-conjugate array constructed by an indirect competitive immunoassay, and it is used to analyze pufferfish samples under high flow conditions. By studying the matrix effect and the toxin recovery rate, the applicability to natural samples was studied. The biosensor can detect TTX in 0.4–3.29 μg g^{-1} puffer fish tissue (Reverte et al. 2017).

Liu et al. combined ELISA with nanozymes and established a sensitive colorimetric immunosensor to visually detect microcystin-LR (MC-LR) (Liu et al. 2019). The microchip is modified with flaky nickel silicate-coated silica nanospheres (SiO_2@Ni Silicate) to immobilize the antigen. The copper hydroxide nanozyme acts as a marker to capture the secondary antibody used for immune response and couples with the G-quadruplex/heme DNA enzyme to form a dual integrated mimic enzyme, which reflects the peroxidase activity of ABTS. Greatly improve the visual signal. The linear range of this method is 0.007–75 μg L^{-1} with the LOD of 6 ng mL^{-1}.

15.5.1.4 Detection of Phytotoxins

Phytotoxins are natural phytochemicals or secondary metabolites formed by plants, which can protect themselves from various threats, such as bacteria, fungi, insects, and natural enemies (Bucheli 2014). According to different chemical structures, they can be divided into three main chemical structures: alkaloids, terpenes, and phenols. Among them, furanocoumarin, lectin, carbohydrate alkaloid, and pyrrolidine nuclear alkaloid are the most studied (Gunthardt et al. 2018). In food, due to non-edible plant pollution, it can be specifically divided into two types: phytotoxins inherent in food crops and phytotoxins that enter food. For example, alkaloids and cyanogenic glycosides are phytotoxins inherent in potatoes and cassava (Mol et al. 2011). In order to effectively control food safety, it is of great necessity to construct a variety of detection methods for analyzing phytotoxins in food.

The ELISA method based on colloidal gold particles significantly shortened the measurement time of ricin. Xu et al. modified the gold-coated AFM tip with polyethylene glycol derivatives to add anti-ricin antibodies to identify ricin. The sensitivity is as high as the level of fg mL^{-1}, and the LOD is as low as 1 ng mL^{-1} (Chen et al. 2011). Christopher et al. used sensitive electrochemical biochip technology combined with ELISA to detect ricin. The capture antibody immobilized on the gold electrode facilitates the specific binding of ricin. The detection of bound ricin is achieved by applying an antibody-enzyme conjugate and measuring the current of the enzymatic redox reaction on the electrode. Among them, the high conversion of the enzymatic reaction facilitates signal amplification, and the built-in redox cycle program provides a second signal amplification, which can be very sensitive to identify ricin at about 50 °C within 20 min (Phlmann et al. 2017).

15.5.2 Detection of Pesticide Residues

Pesticide is a chemical agent used in agriculture to prevent plant diseases and insect pests and regulate plant growth (Xu et al. 2017a, b). There are many varieties of pesticides, which can be divided into nine categories according to their usage, including insecticides, acaricides, nematicides, molluscicides, rodenticides, fungicides, herbicides, defoliants, and plant growth regulators (Bhandari et al. 2019). The sources of pesticide residues in food mainly include three aspects: (1) the direct pollution of crops by pesticides; (2) the absorption of pesticides by crops from the polluted environment; (3) the accumulation of pesticides in organisms due to the effect of food chain. Foods containing residual pesticides can cause habitual headaches, dizziness, fatigue, sweating, depression, memory loss, weakness, and other hidden effects. Long-term consumption can cause cancer, arteriosclerosis, cardiovascular diseases, and other diseases (Samsidar et al. 2018; Silva et al. 2019). Therefore, so as to ensure the food safety of consumers, it is crucial to analyze pesticide residues in food samples.

So far, the applications of ELISA to mainly detect pesticide residues in food are insecticides, fungicides, and herbicides (Fig. 15.5). ELISA is a detection method based on the specific and reversible binding reaction of antigen and antibody. Highly selective antibodies can be obtained by preparing antigen hapten and its carrier conjugate, so that ELISA for detecting pesticide residues can be established, including indirect competition method, direct competition method, and labeled antigen competition method (Wu et al. 2019a, b). Therefore, nano-ELISA is becoming increasingly popular in food contaminants analysis. It can detect pesticide residues

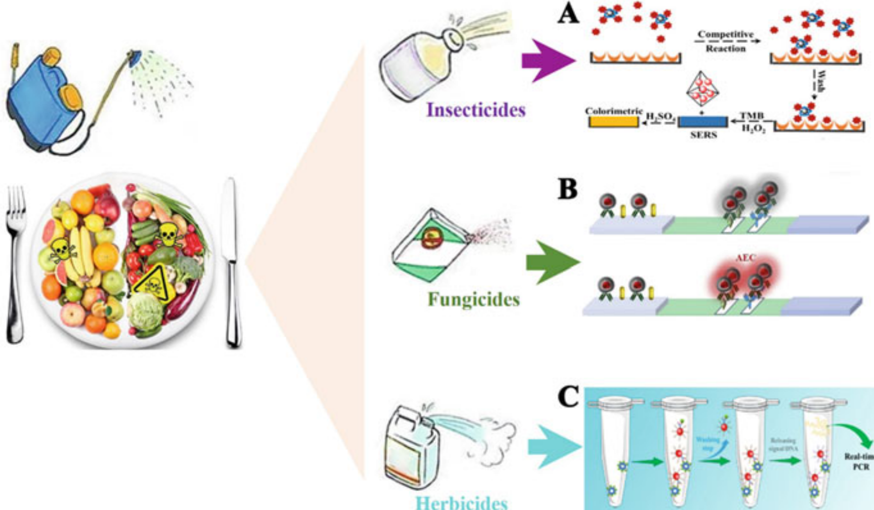

Fig. 15.5 Schematic illustration of nano-ELISA analytical techniques for pesticide residues in food samples. Reproduced from Refs. with permission from Elsevier and American Chemical Society (A: Yan et al. (2019); B: Wei et al. (2020); C: Guan et al. (2021))

in vegetables and fruits more sensitively, quickly, and specifically, and its detection level can reach ng or even pg level (Jia et al. 2009).

15.5.2.1 Insecticides

Insecticides were used to eliminate or reduce any kind of pests, such as insects and wheat aphids. Nowadays, different types of pesticides are widely used in agriculture to achieve high yields. The application of pesticides has ensured nearly one third of the world's crop production. Pesticides improve food production to meet the needs of a growing population (Nsibande and Forbes 2016). The prevention and control of plant diseases and insect pests is conducive to the prevention of harmful diseases of crops. Pesticide residues were found in many food samples, such as pyrethrins, dimethoate, imidacloprid, and triazophos. For example, In order to detect the residual content of triazophos in food, Yan et al. designed a biomimetic nano-ELISA based on molecularly imprinted polymers (MIP) and nanoenzyme markers for the detection of DDT (Yan et al. 2019). Wherein, MIP as a biomimetic antibody, Pt@BSA-hapten as a competitive probe to recognize the binding site of MIPs, and AuNP as a substrate for SERS enhancement. The detection limit of this method is 1 ng mL^{-1}. Based on competitive binding and biological barcode amplification, Zhang et al. designed an immunoassay method for the detection of triazophos (Zhang et al. 2018). The Au nanoparticles (AuNPs) are modified by monoclonal antibodies and single-stranded thiol-oligonucleotides labeled with 6-carboxyfluorescein, then bound to ovalbumin with antigenic haptens that coated on the bottom of the microplate to compete with triazophos in the sample. So, the fluorescence intensity of 6-FAM quenched by AuNPs was negative correlation to the triazophos concentration. The linear range of this method is 0.01–20 μg L^{-1}, and the limit of detection (LOD) is 6 ng L^{-1}. The recovery rate was 85.0%–110.3%, and the relative standard deviation was 9.4%–17.4%. This method performed competitive fluorescent biological barcode immunoassay in water, rice, cucumber, cabbage, and apple samples. These methods provided an idea for the rapid detection of other small molecule pesticide residues. Application the enzyme-like activity of nanomaterials and the quenching effect of fluorescent dyes, SERS, and fluorescence may bring prospects for the detection of other pollutants.

Acetylcholinesterase (AChE) activity was inactivated by the presence of acetamiprid. Based on AChEand choline oxidase (CHO), Wu et al. proposed a dual-enzyme-mediated Fe^{2+}/Fe^{3+} determination of acetamiprid residues in vegetables and fruits by an Fe^{2+}/Fe^{3+} conversion magnetic relaxation switch method (Wu et al. 2021). The linear range of this method was 0.01–1000 μg L^{-1} ($R^2 = 0.99$), and the detection limit is 2.66 ng mL^{-1} ($S/N = 3$, $n = 3$), which is better than the traditional enzyme inhibition method (0.89 μg mL^{-1}) increased by 335 times. This method provides a simple and convenient analytical tool for detecting pesticide residues in food.

15.5.2.2 Fungicides

Fungicides were a class of chemical reagents, which used to inhibit or kill pathogenic spores to protect crops, including bactericidal streptomycin, carbendazim,

bordeaux mixture, zinc methylarsenate, and so on. For example, streptomycin (STR) is an antibiotic extracted from the culture broth of Streptomyces griseus (Yin et al. 2017). STR residues in food can have serious effects on human health, such as nephrotoxicity and ototoxicity. In order to detect aminoglycoside streptomycin, Wei et al. established a new type of lateral flow immunoassay (LFA) platform with Au@Pt as a marker with enzyme-like activity, with the limit of detection (LOD) of 0.1 ng mL^{-1} (Wei et al. 2020). This method was applied to the content of streptomycin in milk. LFA based on nanoenzymes is a promising tool for detecting pesticide residues in food.

15.5.2.3 Herbicides

Herbicides can inhibit the growth of weeds in the field and increase the yield of crops. For example, atrazine, 2,4-D, trifluralin, and glyphosate (GLYP) are the main pollutants of soil and water ecosystems. For example, atrazine molecules can be fully degraded (100%) by Fe_3O_4-TiO_2/rGO nanozyme under irradiation of natural sunlight. Based on competitive ELISA, Kwon et al. developed a peroxidase-like Pd@Pt nanoparticle-conjugated primary antibody as an enzyme marker to detect atrazine (Kwon et al. 2020). The method has high sensitivity, LOD is 0.5 ng mL^{-1}, and the recovery rate is between 99% and 115%, indicating that the immunoassay can detect atrazine and other small molecule herbicides and pesticides. Wang et al. developed a direct competitive enzyme-linked immunosorbent method (Wang et al. 2016). Using ovalbumin-2,4-D (OVA-2,4-D)-modified nano-silica (OVA-2,4-D-SiO_2 NPs) as a capture probe, horseradish peroxidase labeled with anti-2,4-D antibody as a probe, and competed 2,4-D in the samples. Thus, 2,4-D was detected to prevent the abuse of 2,4-D in the commercial production of bean sprouts. The linear range of this method was 1–350 ng mL^{-1}, and LOD was 0.079 ng mL^{-1}.

Further, in view of the wide application prospects and advantages of oligonucleotide functionalized AuNPs in biological analysis. Naiyu Guan et al. synthesized anti-GLYP antibody and double-stranded oligonucleotide bifunctional AuNP probe and established AuNP biological barcode immuno-PCR (AuNP-BB-iPCR) to detect GLYP (Guan et al. 2021). GLYP and OVA-GLYP coating to compete with functionalized antibodies, thereby releasing signal DNA and detecting by real-time PCR. The detection linear range of GLYP was 61.1 pg g^{-1}–31.3 ng g^{-1}, and LOD was 4.5 pg g^{-1}. This method was seven orders of magnitude lower than the conventional ELISA method (70 μg·g^{-1}) established with the same antibody. The recoveries of soybean, rape, and corn samples were 99.8%, 102.6%, and 103.7%, and the relative standard deviations were all less than 12.9%. The detection time of AuNP-BB-iPCR (including food sample preparation) was 4 h, which can be used for sensitive detection of GLYP in food and environment.

15.5.3 Detection of Veterinary Drug Residues

Residues of veterinary drugs in food are mainly due to the residues in animal foods such as meat, eggs, and milk after the animals are used for drugs (Baynes et al.

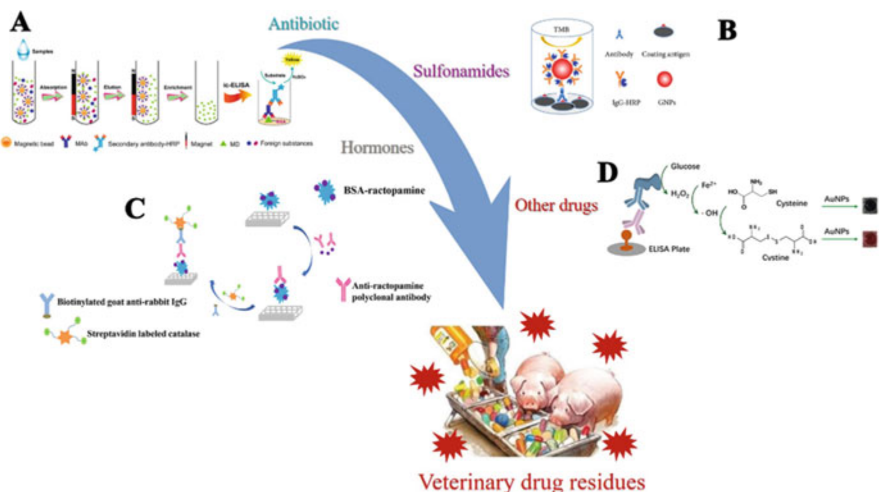

Fig. 15.6 Schematic illustration of nano-ELISA analytical techniques for veterinary drugs residues in food. Reproduced from Refs. with permission from Elsevier (A: Song et al. (2018); C: Han et al. (2018); B: Peng et al. (2013a, b); D: Yu et al. (2018))

2016). Veterinary drug residues in food are mainly antibiotics, sulfonamides, furans, antiparasites, and hormones. Therefore, it is necessary to detect veterinary drug residues in food. Using the enzyme-like activity of nanozymes, a new ELISA based on direct competition, indirect competition, and labeled antigen competition can be used to detect veterinary drug residues in animal foods more sensitively, quickly, and specifically. In this chapter, nano-ELISA detects pesticide residues in food mainly antibiotics, sulfonamides, and hormones (Fig. 15.6).

15.5.3.1 Antibiotics

Antibiotics are used to prevent and treat animal diseases and improve the performance of modern animal husbandry (English and Gaur 2010). Commonly used antibiotics include tetracyclines (TCs), maduramycin (MD), chloramphenicol, macrolides, etc. Illegal or excessive addition of antibiotics may cause animal poisoning or remain in animal muscle tissue (Ben et al. 2019). It is a potential hazard to human and environmental health. Therefore, the development and application of the nano-ELISA method is of great significance in food safety. For example, the use of nanomaterials (MBs and SiO_2) as absorption substrates greatly improves the sensitivity, accuracy, and stability of nano-ELISA. Antibody-functionalized MB (IMB) was synthesized based on the production of a specific anti-MD MAb. Song et al. established an IMBs-based indirect competitive ELISA (ic-ELISA) to detect MD in three chicken tissues (Song et al. 2018). The detection limits of MD in chicken muscle, skin and fat, and liver were 72, 74, and 173 μg kg^{-1}, respectively. The recovery rate was 80.0%–115.8%, and the coefficient of variation was less than 11.3%. Tao et al. proposed a competitive direct chemiluminescence immunoassay

method based on MBs separation and AuNPs labeling technology for the detection of chloramphenicol (CAP) in milk (Tao et al. 2013). In two different extraction methods, the IC50 values of the chemiluminescent magnetic nanoparticle immunoassay (CL-MBs-nano-ELISA) were 0.017 and 0.17 μg L^{-1}, respectively.

Further, by nucleic acid aptamers replace antibodies and nanozymes replace biological enzymes, nano-ELISA improves the selectivity and sensitivity of traditional ELISA. For example, Sheng et al. reported an ultra-sensitive Apt-modified AuNPs (AuNPs-Apt) analysis method to detect tetracycline residues in honey based on the high selectivity of aptamers for analytes and the enhanced catalytic ability of AuNP (Sheng et al. 2020). TCs-BSA were coated on a microplate. Then, the TCs-BSA coating in the microplate competes with the free TCs in the sample for the limited AuNPs-Apt. As a kind of nanoenzyme, AuNPs show peroxidase activity, oxidized 3, 3′, 5, 5′-tetramethylbenzidine (ox-TMB) from colorless to blue, and measured at 652 nm.

15.5.3.2 Sulfonamides

Sulfa drugs are mainly used for antibacterial and anti-inflammatory, such as sulfadimethoxine (SDM), sulfamidine, and sulfadiazine. After long-term intake of animal food containing sulfa drug residues, the drug will continue to accumulate in the body, causing damage to the urinary system, causing crystaluria, hematuria, and tubular urine, etc. (Chang et al. 2020). Therefore, it was clear to regulate and control the useful dose of sulfa drugs at all over the world.

Due to its easy coupling of biomolecules and maintaining the biological activity of labeled molecules (antibodies and DNA), AuNPs make it widely used in the field of biosensors (Chandra et al. 2013; Chandra et al. 2010; Kumar et al. 2020; Mahato et al. 2019). The use of nanozymes to improve the sensitivity of traditional ELISAs brings prospects for the analysis of contaminants in food. For example, Peng et al. developed an ultra-sensitive nano-ELISA to detect SDM in chicken tissue, increasing the sensitivity of traditional ELISA by 20 times (Peng et al. 2013a, b). Using the biological coupling of AuNPs and enzyme-labeled antibodies as signal probes, a simple and sensitive detection of SDM residues in animal tissues is achieved. The sensitivity of nano-ELISA in the buffer was 5 pg·mL^{-1} and the LOD was 0.2 μg·kg^{-1} that can be obtained by simply extracting chicken liver with the buffer. The strategy was convenient and sensitive, which can be applied to improve the performance of the ELISA to detect small molecule contaminants.

15.5.3.3 Hormones

Hormonal drugs are mainly used to improve the reproduction and growth of animals. The hormones suitable for animals are sex hormones and corticosteroids, and sex hormones are the most commonly used. For example, testosterone, progesterone, and ractopamine hydrochloride. Excessive hormone drugs in foods will affect the normal physiological functions of consumers and have certain carcinogenicity, leading to health problems such as precocious puberty and abnormal growth of children (Hoga et al. 2018).

Ractopamine, also known as clenbuterol, is a common hormone veterinary drug residue in animal food. Pingli He et al. developed a colorimetric ELISA based on indirect competition with a linear range of 2–512 ng mL^{-1} and the LOD was 0.35 ng mL^{-1} (Han et al. 2018). Obtaining anti-ractopamine polyclonal antibodies by preparing antigenic ractopamine-glutaric acid-bovine serum albumin antigen. Based on Mn(VII)/Mn(II) conversion-induced change in low-field nuclear magnetic resonance of the transverse relaxation rate, Wang et al. report a magnetic immunosensor for the detection of food-borne pathogen and residue of veterinary drug (Wang et al. 2019a, b, c). This Mn-mediated magnetic immunosensor not only maintains the good stability of the traditional paramagnetic ion-mediated magnetic sensor, but also greatly improves the sensitivity of the sensor. And the LOD improve from ng mL^{-1} to pg mL^{-1}. This method provides a promising platform for sensitive, stable, and convenient biological analysis.

In addition, 19-nortestosterone (19-NT) was also a common hormonal veterinary drug residue in animal food. Peng et al. proposed a nano-ELISA method based on the coupling of AuNPs with goat anti-rabbit IgG and HRP for the highly sensitive detection of 19-NT in beef (Peng et al. 2013a, b). The AuNPs-IgG-HRP conjugate was simple prepare and stable. The sensitivity of this method in buffer was 0.01 ng mL^{-1}, which was 10 times that of c-ELISA. After simple pretreatment of beef samples, the LOD was 0.3 mg·kg^{-1}.

15.5.3.4 Other Drugs

In addition, there are other types of veterinary drugs that are also easy to contaminate animal food, such as antiviral drugs and antiparasitic drugs. Antiviral drugs on the market mainly include amantadine (AMD), rimantadine, ribavirin, and other drugs represented by the symmetrical tricyclic amine structure (De Clercq 2001). Praziquantel (PZQ) is an antiparasitic drug for mammals and fish. Shen et al. developed an ic-ELISA to detect PZQ residues (Shen et al. 2019). The hapten PZQ-HS synthesizes the immunogen and the coating antigen with carrier protein by introducing an amino group into the benzene ring, thereby preparing a highly sensitive monoclonal antibody. Finally, an immunochromatographic test strip (ICS) for rapid detection of PZQ residues in mackerel was developed. Yu et al. developed an ic-ELISA that introduced Fenton reaction and gold nanoparticle aggregation (Yu et al. 2018). By Fenton reaction to form hydroxyl radicals, it significantly accelerates and controls the oxidation of cysteine, and amplifies the signal. At the same time, through the strong Au-S interaction and the AuNPs aggregation, led to a pronounced color change from red to dark purple in the solution, which could be easily distinguished with the naked eye, thereby detecting the residue of amantadine (AMD) in poultry. The detection limit of this method is 0.095 ng mL^{-1}.

15.5.4 Detection of Microorganism

Microbial contamination in food is the most important factor affecting food safety, and pathogenic microorganisms are the food safety issue that has the greatest impact

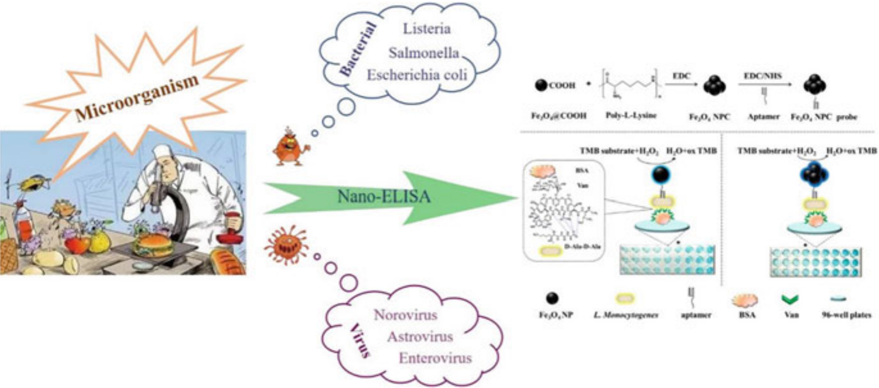

Fig. 15.7 Schematic illustration of nano-ELISA analytical techniques for food-borne pathogenic microorganisms. Reproduced from Refs. with permission from Elsevier (Zhang et al. 2016)

on consumer health. Therefore, microbial contamination in food has caused widespread concern in society. Common food-borne microorganisms are bacteria, fungi, and viruses. In particular, microorganisms that cause food spoilage and decay and food-borne pathogenic microorganisms have attracted great attention from society. They can multiply bacteria in food and even produce toxic metabolites, causing food poisoning or harmful infections. So far, the application of ELISA to detect microorganisms in food is mainly bacteria and viruses. However, traditional ELISA is limited by complicated procedures, relatively low detection limit, and large sample size. The nano-ELISA has been widely developed to enable simple and sensitive detection of microorganisms in food (Fig. 15.7).

15.5.4.1 Bacteria

Bacteria are the main pathogens in food, including *Escherichia coli*, *Salmonella*, *Listeria*, *Staphylococcus aureus Botox, Shigella*, *Streptococcus haemolyticus*, *Vibrio parahaemolyticus*, and so on. Among them, Salmonella has been the major cause of the food-borne contaminants in vegetables, egg, chicken pork, beef, or vegetable row crops (Vinayaka et al. 2019). The World Health Organization (WHO) reported that over 105 million diarrheal cases in the world every year are related to food-borne pathogens (Bull et al. 2020). Among them, Salmonella have drawn the most concerns because it is frequently found in food stuffs and may cause severe diseases (Wang et al. 2019a, b, c).

Based on gold nanoparticle-based enzyme-linked antibody-aptamer sandwich (nano-ELAAS), Wu et al. proposed a immunoassay for robust detection of Salmonella enterica serovar Typhimurium (STM) with high specificity (Wu et al. 2014). The STM in the sample is captured and pre-concentrated from the aptamer-modified magnetic particles, and then combined with the detector antibody. Then, a nanoprobe carrying a large number of reporter antibodies, and HRP is used for colorimetric signal amplification. The quantitative detection range of nano-ELISA was 1×10^3–1×10^8 cfu mL^{-1}, and the detection limit was 1×10^3 cfu mL^{-1}. The

selectivity for STM in high concentration other bacterial samples was more than 10 times, and the detection time was less than 3 h. To achieve immunomagnetic separation and simple target concentration, Herzig et al. described a magnetic bead-based immunoassay for Salmonella with tyramide signal amplification (Herzig et al. 2016). The LOD Salmonella typhimurium and Salmonella enteritidis in beef and poultry samples were increased to 800 and 200 cfu mL^{-1}, respectively. Farka et al. introduced a method by combining Prussian blue nanoparticles (PBNPs) with antibodies and used it to detect microbial contamination of *Salmonella typhimurium* in powdered milk (Farka et al. 2018). Furthermore, the LOD was 6×10^3 CFU mL^{-1} and working range up to 10^6 CFU mL^{-1}. Moreover, Vinayaka et al. proposed a combination of magnetic beads and direct PCR to detect Salmonella vulgaris from food samples without bacterial culturing, DNA isolation, and purification steps (Vinayaka et al. 2019). Based on urease-induced silver metallization on gold nanorods (AuNR), Gao et al. reported an improved ELISA for the determination of Salmonella enterica Choleraesuis (Gao et al. 2019a, b). As aspect ratio (length divided by width) of AuNR decreased, AuNR solution showed a multi-color change, and it also behaved a significant blue shift in the absorption peak ($\Delta\lambda$max) of AuNR. Hence, the LOD of this method were as low as 1.21×10^2 cfu mL^{-1} for qualitative detection with naked eyes, and 1.21×10^1 cfu mL^{-1} for quantitative analysis.

Furthermore, *E. coli* O157:H7 (E. coli) is a highly infectious pathogen that spreads widely in food and water and poses a major challenge to public health (Scallan et al. 2011). Wei et al. synthesized anti-*E. coli* antibody-HRP-$Cu_3(PO_4)_2$ nanocomposites to replace HRP-conjugated antibody, and then the nanozyme label was applied in ELISA for *E. coli* detection (Wei et al. 2016). Based on DNA-based hybridization chain reaction (HCR) and biotin–streptavidin signal amplification, Guo et al. reported on a novel sandwich ELISA for the sensitive determination of E. coli (Guo et al. 2016a, b). The method behaved a detection range of 5×10^2 cfu mL^{-1} to 1×10^7 cfu mL^{-1}; and an LOD of 1.08×10^2 cfu mL^{-1} that is 185 times lower than that of traditional ELISA.

Listeria as a food-borne pathogen has posed great threats on human health all over the world (Li et al. 2018a, b). Based on nucleic acid hybridization reaction and magnetic signal readout, Qi et al. proposed a magnetic DNA sensor for sensitive detection of *Listeria* in ham sausage samples (Qi et al. 2021). The method can be realized in a one-step detection with LOD as low as 50 cfu mL^{-1} within 2 h, and the average recovery can reach 92.6%. Zhou et al. developed a sandwich enzyme-linked immunosorbent method, based on nanoparticle clusters (NPC), using vancomycin (Van) as the loading substrate to capture Listeria, Fe_3O_4 nanoparticle clusters (NPC)-modified aptamers are used as signal amplification nanoprobes to identify Listeria monocytogenes, thereby effectively detecting food-borne pathogens-Listeria monocytogenes in milk and other foods bacteria (Zhang et al. 2016). The linear range of this method was $5.4 \times 10^3 \sim 10^8$ cfu mL^{-1}, and the detection limit was 5.4×10^3 cfu mL^{-1}. Y. Wu et al. developed a sandwich pELISA for Cronobacter detection in powdered infant formula samples by mediating AuNP growth through DNA (Wu et al. 2019a, b). The catalase–hydrogen peroxide (Cat–H_2O_2) system was introduced to bridge the DNA-directed AuNP growth and pELISA, as such DNA

can be cleaved into fragments by the hydroxyl radical generated from oxidation of H_2O_2 through Fenton reagents. The proposed pELISA can qualitatively detect Cronobacter species by the naked eye with a cut-off limit of 3×10^5 cfu mL^{-1}. This method linear range was 3×10^2 to 3×10^7 cfu mL^{-1}.

15.5.4.2 Virus

Viruses are also food-borne pathogenic microorganisms. Currently, common food-borne viruses mainly include hepatitis A and E viruses, rotavirus, astrovirus, enterovirus, and norovirus. Noroviruses is the family of Caliciviridae, which has been identified in human beings and several other animal species (Mauroy et al. 2009). Human norovirus (NoV) may cause viral gastroenteritis for human and viral food-borne outbreaks globally. Weerathunge et al. proposed a colorimetric sensor based on the combination of AuNPs and aptamers, which produces a blue color when norovirus was present. The concentration of the virus was detected by the shade of the color (Weerathunge et al. 2019). Khoris et al. proposed a colorimetric sensor based on peroxidase-like activity of silver ion-incorporated gold nanoparticles, which can make TMB develop color (Khoris et al. 2019). NoV-LPs were assayed with an LOD of 10.8 pg mL^{-1}, corresponding to at least 100-fold higher sensitivity compared to the traditional immunoassay.

15.5.5 Detection of Food Allergens

Food allergens refer to ingredients in food that can cause abnormal reactions in the body's immune system, which can cause a strong allergic reaction in the human body and harm to the body if you consume or contact with them (Sicherer 2001; Sathe et al. 2005; Ekezie et al. 2018). With the occurrence of food allergic diseases increasing year by year, the problem of food allergy has become a more prominent problem in the field of food safety (Monaci et al. 2018; Liu and Sathe 2018). Accurately determining the type and content of allergens in food and providing information about the background level of allergens in food and information about hidden or unlabeled allergens is of great significance for preventing and avoiding food allergies.

Food allergens can be divided into major allergens and minor allergens, which are generally proteins or glycoproteins with a relative molecular mass of 10,000–70,000 (Burgess et al. 2019). Major allergens are derived from peanuts, milk, nuts, crustaceans, and so on (Table 15.1). Those people who are allergic to food allergies can prevent them by avoiding the foods with allergens, which is also the most effective risk management measure (Chan et al. 2018). Therefore, manufacturers are required to correctly label allergic ingredients on food labels to prevent consumers from accidentally eating and causing allergies.

Allergic reactions will not only seriously affect the quality of life of patients, but also life-threatening. For example, more and more studies believe that allergic reactions can cause idiopathic inner ear disease—Meniere's disease (Sicherer and Sampson 2014). Therefore, it is important to analyze the inevitable allergens in food

Table 15.1 The common food sources of food allergens

Food sources	Allergens
Peanuts	Ara h1
	Ara h2
Wheat	Gliadin
Shrimp	Tropomyosin
Eggs	Ovalbumin
	Ovomucin
Milk	Casein
	Lactoglobulin
	Lactalbumin
	BSA

samples. The characteristic fragments present in trace amounts in allergens are usually lost due to pretreatment operations, making the analysis result lower than the actual value. ELISA is still the most extensive detection method at present, and the introduction of nanomaterials enables c-ELISA to overcome their own shortcomings and achieve more sensitive, stable, and low detection limits (Alves et al. 2016). On the other hand, modern analytical techniques have made significant progress in improving the accuracy, sensitivity, and stability of c-ELISA (Andjelkovic et al. 2017). Interestingly, the nano-ELISA has greatly developed the ELISA, which provides more ideas for the detection of food additives (Wu et al. 2019a, b).

15.5.5.1 Peanut Allergies

Peanuts, which are a type of nuts frequently encountered in daily life, are important food allergens and may cause extremely serious allergic reactions (Barnett et al. 1983; Chassaigne et al. 2007). According to British researchers, in the UK, about one in 200 people are sensitive to peanuts. Unfortunately, according to research investigations, peanut allergy is usually caused by childhood and is accompanied by life (Hebling et al. 2013; Turcanu et al. 2003). At the same time, it is also the one with the largest number of food allergy deaths, and 90% of deaths caused by food allergies are caused by peanuts. In the United States, about 100 people die every year from anaphylactic shock caused by peanut allergy (Hua et al. 2016; Vierk et al. 2007). The main peanut allergens are Arah1 (63.5 kDa), Arah2 (17 kDa), other related antigens are Arah3 (60 kDa), Arah4 (14 kDa), and the minor antigens are Arah 6, Arah7, and actin.

Although correct and decisive food labeling will enable consumers to avoid peanut allergens during food processing, cross-contamination, or ingredients containing hidden allergens during food processing will greatly weaken this task (Breiteneder and Radauer 2004). For example, the ELISA inhibition test confirmed that Ara h2 is the main antigen causing the cross-reaction of peanuts, hazelnuts, almonds, and so on. In addition, individuals who are allergic to eggs, milk, or walnuts are also allergic to peanuts, but no cross-reactions with walnuts have been found protein (Pele et al. 2007). Therefore, food manufacturers usually include

precautionary words ("may contain traces of peanuts") on their packaging, not only to protect sensitive individuals, but also to protect themselves. From this perspective, a reliable, accurate, highly sensitive and selective method is needed to safely assess the presence of peanut allergens in food.

At present, the detection of allergens is mainly focused on the traditional ELISA, which is used to quantify the low-level food allergens in food ingredients and the preparation and processing of foods and beverages (Khedri et al. 2018). However, this method has many disadvantages, such as time-consuming and expensive. With the development of nanotechnology, more researchers have applied nanomaterials in ELISA for the detection of allergens, such as gold nanoparticles (AuNPs), grapheme oxide (GO), and even microfluidic technology (Xing et al. 2018; Arya and Estrela 2018; Tan et al. 2018; Cao et al. 2019). Alves et al. developed an electrochemical immunosensor based on AuNPs, using a screen-printed carbon electrode coated with AuNPs as the sensor to detect Ara h1. AuNPs are generated electrochemically on the surface of the electrode, and then two monoclonal antibodies are used to electro-chemical detect the antibody–antigen interaction through the stripping analysis of the deposited silver by alkaline phosphatase (Alves et al. 2015). The immunosensor has been identified to be used in analyzing complex food matrices, with a detection limit of 3.8 ng mL^{-1}, RSD less than 8.7%, and recoveries above 96.6%. Based on the cyclic electrodeposition of alternate monolayers of graphene and AuNPs, Sun et al. reported a biosensor to detection Ara h1 on the surface of a GCE with a multilayer graphene–gold nanocomposite (Sun et al. 2015). Weng et al. applied microfluidic technology to the traditional enzyme-linked immunosorbent platform, combined with a designed light sensor to detect the proteins of Ara h1 and wheat gluten (Weng et al. 2016). Compared with commercial enzyme-linked immunosorbent kits, the developed microfluidic ELISA shortens the total detection time from several hours to 15–20 min, and reduces the sample consumption to 5–10 μL with higher sensitivity. At the same time, it shows that nanotechnology has a broader prospect in c-ELISA.

In addition to the Ara h1 protein, there are other allergen plant proteins that have been quantitatively and qualitatively analyzed by modified ELISA strategies. Glutathione-modified AuNPs (GSH-AuNPs) were used by Liu et al. to develop an electrochemical immunosensor for the detection of Ara h2 antibodies (Liu et al. 2010). The AuNPs were functionalized by 28 amino acid peptide fragments of the main IgE binding epitope of Ara h2 and coated on pyrolytic graphite on the surface of the electrode. Otherwise, Manfredi et al. developed a disposable amperometric biosensor based on AuNP-modified screen-printed carbon electrodes for rapid analysis of cytotoxic gliadin (Manfredi et al. 2016).

15.5.5.2 Crustaceans Allergens

As seafood is favored by more and more people, reports of such food allergies are gradually increasing. Among them, shrimp allergens have attracted much attention (Ho et al. 2014). It is reported that 0.6–2.8% of patients with allergic diseases are allergic to shrimp. People have detected at least 13 IgE-binding proteins in shrimp meat, but tropomyosin (TM) has been identified as the only major allergen with a

relative molecular mass between 34,000 and 39,000. According to reports, TM is an important antigen of invertebrates such as shrimp, crab, oyster, and squid and has a highly conserved amino acid sequence.

TM is extremely stable, not only resistant to the effects of the digestive tract, but also resistant to heat, proteolysis, hydrolysis, and digestion. As a highly conserved muscle protein, TM, accounting for more than 90% of all food allergens, has cross-reactivity in many arthropods; therefore, it is also one of the most dangerous allergens (Moonesinghe et al. 2016; Wong et al. 2019). Angulo-Ibáñez et al. used carboxyl-functionalized magnetic microbeads in sandwich immunoassays, combined with disposable screen-printed electrodes, and developed an electrochemical immunoassay strategy based on H_2O_2 as the enzyme substrate and hydroquinone as the redox mediator to analyze shrimp TM (Angulo-Ibáñez et al. 2019). Specifically, the author used EDC/NHS to chemically activate carboxyl-functionalized MBs, covalently bind to the capture antibody with polyclonal rabbit anti-shrimp TM antibody, and use ethanolamine hydrochloride (ETA) to block unbound active sites to avoid specific adsorption. Next, functionalized MBs are used to specifically capture shrimp TM in all components of food extracts or standard solutions. Finally, a detection antibody and a secondary labeling antibody sandwich the bound TM to form complete sandwich immunity. Not surprisingly, MBs with sandwich immune complexes were brought to the surface of the working electrode, and then Abe was detected in the presence of H_2O_2 enzymatic substrates.

In addition, based on the less conserved sequence of TM in different phylogenetic species, this biosensor is currently being used to identify the adulteration of shellfish products using TM as a biomarker (Wang et al. 2019a, b, c). Jiang et al. embedded fluorescein isothiocyanate (FITC) on the SiO_2 modified Fe_3O_4 core, and then wrapped it in liposomes to form cationic magnetic fluorescent nanoparticles, which were used for electrochemical immunoassay for the detection of shrimp allergen myosin and fish allergen parvalbumin (Jiang et al. 2015). Interestingly, Wang et al. reported a novel immunomagnetic bead-derived ELISA method, which uses antibody-functionalized GO and AuNPs to amplify the detection signal of parvalbumin (Wang et al. 2020a, b).

15.5.5.3 Other Allergens

In addition, there are other types of food allergens that are also easy to hide in food or production lines, such as α-lactoglobulin, ovalbumin, ovomucoid, and casein. These allergens are also not to be ignored. They are easy to be contaminated on the production and processing lines but are most easily ignored. Especially for allergens in eggs, the positive rate is as high as 35% in children with food allergies, and as high as 12% in adults.

Maier et al. reported a sandwich-type immunoassay that can analyze ovalbumin and ovomucin at the same time, using AuNPs as an optical immune chip for signal sensor (Maier et al. 2008). Yang et al. used quantum dots (QDs) to covalently bind anti-α-lactic acid monoclonal antibodies to establish an immunoassay method for the detection of α-lactoglobulin in commercially available dairy products, which they called fluorescence enzyme-linked immunosorbent assay (FELISA). Compared with

traditional competitive ELISA, best detection limit (0.1 ng mL^{-1}) and wide dynamic range (0.1–1000 ng mL^{-1}) of FELISA are better (Yang et al. 2014). He et al. used H_2O_2-mediated cadmium telluride sulfide QDs as an immunosensor for fluorescent signal output to detect β-lactoglobulin (He et al. 2018).

According to the foregoing, it can be seen that the application of nanomaterials such as AuNPs, QDs, and GO, to traditional ELISA has significantly improved the many shortcomings and defects of ELISA, and the accuracy and sensitivity have been greatly improved (Chen et al. 2016; Huang et al. 2016). Therefore, it is of great significance to accelerate the development of the combination of nanotechnology and traditional technology.

15.5.6 Food Additives

Food additives are micro-preparations added to food that can improve the color, flavor, and prevent food spoilage (Martins et al. 2019). It is mainly divided into natural extracts and artificial compounds and is an important part of the food industry. However, most of the food additives currently used is artificial compounds, and excessive use will cause varying degrees of harm to the human body (Carocho et al. 2017; Siegrist and Sütterlin 2017). Since the development of additive residue detection technology lags far behind the development of the food additive industry, illegal and excessive use of additives in the food production process is still very serious.

As necessities of the modern food industry, food additives have as many as 2000 varieties, including antioxidants, colorants, sweeteners, preservatives, and bleaching agents (Young et al. 1987). However, improper use of food additives may cause serious damage to human organs, such as diabetes and heart disease (Corkey 2012; Rangan and Barceloux 2009). Therefore, it is particularly important to construct effective strategies to detect illegal additives in food.

At present, the main analytical methods for food additives are chromatography or other liquid chromatography related to various detectors. Generally, chromatographic methods are a little time-consuming, complex pre-processing, and high cost, so it is particularly important to develop other technologies for additive detection (Zhang et al. 2008). For example, Han et al. reported a combined molecular imprinting technology with high-performance liquid chromatography for the selective detection of Sudan dyes in food (Han et al. 2007). However, the ELISA that has received much attention is rarely used in the detection of food additives. Compared with traditional detection methods, the most significant advantages of enzyme-linked immunosorbent assay are its sensitivity and specificity, simple preparation, and high throughput. Moreover, ELISA has been widely used in biology, agriculture, environment, and other fields, such as detecting proteins, microorganisms, antibiotics, and pesticides (Wu et al. 2019a, b).

In recent years, ELISA has attracted attention in the field of food additives due to the high specificity of the antigen–antibody interaction, making the sample analysis process simple and detecting one or more analytes at the same time. Berlina et al.

used Sudan dyes monoclonal antibody combined with AuNPs to establish a lateral flow immunoassay method to detect the food colorant Sudan (Berlina et al. 2017). The conditions of this experiment are optimized for the qualitative and quantitative control of Sudan in food matrix, and its detection limit is 2.5 ng mL^{-1}. In addition, indirect competitive chemiluminescence enzyme immunoassay (CLEIA) has also been applied to colorant detection. Based on polyclonal antibody and horseradish peroxidase-labeled antibody chemiluminescence system, a CLEIA analysis strategy for malachite green in seafood was established (Zhang et al. 2015). Secondly, the dye FB (Chr FB) can be detected by the horseradish peroxidase-luminol-H_2O_2 system, with p-iodophenol as an enhancer, combined with polyclonal antibodies to establish a chemiluminescence immunoassay method. This method can be used for rapid screening of Chr FB in yogurt candy, vitamin drinks, and bread. Compared with the traditional method, its sensitivity is improved by two orders of magnitude (Xu et al. 2017a, b). It can be seen that the analysis of food additives by ELISA method is very promising. The introduction of nanomaterials can improve the sensitivity of ELISA for trace detection in complex matrices; therefore, nano-ELISA needs to be developed in the field of food additive analysis.

15.6 Advantages and Disadvantages of c-ELISA and Nano-ELISA

Though c-ELISA in immunoassay behaves various disadvantages, it still suffers from many limitations. To improve c-ELISA, a lot of work been done to enhance the detection robustness and make it more convenient in operations. By introducing nanomaterials, c-ELISA has been greatly improved in sensitivity and flexibility, making nano-ELISA a powerful tool in many fields. As mentioned above, tons of functional nanomaterials are developed for ELISA to illustrate their potential application in food safety. The advantages of nano-ELISA are obvious, including higher sensitivity, faster response, richer detection strategies. However, many new problems appear when come to construct all kinds of nano-ELISA.

For instance, for the development of solid substrate, nano-fibers, MIPs, and magnetic nano-beads are commonly used in nano-ELISA, which can provide high specific surface area and easier operations. But their stability and reproducibility may become new problems for nano-ELISA. For the antibody recognition improvement, nanobody and aptamer and are the potential substitutes in ELISA, but the binding force with analytes needs to be further evaluated. For the enzyme labels, nanozymes (CuS nano-sheets, CuO, MnO_2, and CeO_2 nanoparticles) with peroxidase- or oxidase-like activity are widely used to replace natural enzymes, which is a good beginning for the development of enzymes, but how to take count of the advantages of natural enzymes (e.g. selectivity, biocompatibility) is another problem. From this point of view, it seems impossible to put all good things in one method. The detailed advantages and disadvantages of c-ELISA and nano-ELISA are listed in Table 15.2.

Table 15.2 Comparison between c-ELISA and nano-ELISA

c-ELISA		Nano-ELISA
Advantages	High selectivity of enzyme	Controllable catalytic activity of nanozyme
	Good substrate selectivity	Multienzyme mimetic activity
	Excellent biocompatibility	Low cost
	Clear standards	Easy to mass produce
	Mild reaction conditions	Long-term storage
	Stable structure	Robust to harsh environments
	Simple recognition model	Unique physicochemical properties
Disadvantages	High cost	Limited types of nanozymes
	Hard to be purified	Limited substrate selectivity
	Hard to store long term	Limited biocompatibility
	Limited application conditions	Limited stability
	Easy denaturation	Lack of standards

15.7 Perspectives and Challenges

The introduction of nanomaterial into ELISA (nano-ELISA) brings convenience to paper- and fiber-based ELISAs and the miniaturization of ELISA, which also provides a new avenue for other biosensors in electrochemistry, optics, and magnetism. Here, we mainly focused on the development and application of nano-ELISA. As a powerful visual method, ELISA has attracted intensive attention in many fields, and the improvement towards the c-ELISA is becoming more and more popular. Clearly, the performance of c-ELISA has been greatly improved, and the technique is intensively used in food safety. However, concerns still exist the developed ELISA, such as lack of stability and standards, time-consuming, and tedious operations. Hence, it is an alternative to construct facile ELISA by combining new nanomaterials and other techniques.

Up to now, nano-ELISA has been given sufficient attention in food analysis. A large number of studies have been reported on the development of c-ELISA. But most of the ELISA are enhanced in only a part, either the enzyme label or the recognition model. There is no doubt that some properties like sensitivity and simplicity can be greatly enhanced, but in most cases new problems appears. For example, the stability and specificity remain to be explored after applying nanozymes modified detection antibody in ELISA measurements. So, how to evaluate the whole performance of nano-ELISA is a critical issue. It is believed that in the near future the new developed nano-ELISA could have the golden standards like c-ELISA does.

On the other hand, nano-ELISA combined techniques such as electrochemical method, surface-enhanced Raman scattering technique, electrochemiluminescence,

and smartphone are new arising analytical methods, which can realize rapid, sensitive, or even intelligent detection of analytes. Therefore, the combination of nano-ELISA with these techniques is a new trend in future applications. However, the nanomaterials also have problems in real applications due to their relatively low stability and bioconjugate efficiency. Thus, engineering stable and high-performance nanomaterials is still the challenge remained to be addressed. Overall, it remains a great challenge in developing stable and reliable ELISA methods in food safety, and the combination of nanomaterials and methodology can provide a good direction.

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