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## UNIVERSITÀ DEGLI STUDI DI TORINO

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**Non-competitive immunoassay for low-molecular-weight  
contaminants detection in food, feed and agricultural products: a  
minireview**

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**Abstract:**

Background: Immunoassay gains considerable attention in safety assurance of food, feed and agricultural products. Generally, immunoassay is presented either in competitive or non-competitive sandwich-type format, and the former is extensively employed for low-molecular-weight contaminants, which usually bear one accessible epitope. Theoretically, non-competitive sandwich-type immunoassay harbors a higher sensitivity, precision and linearity. However, the analyte to be measured in such format must be large enough to have at least two epitopes to be captured. It is not feasible to detect low-molecular-weight contaminants through conventional non-competitive sandwich-type immunoassay. Consequently, there is a trend to develop new types of sensitive non-competitive immunoassay for low-molecular-weight contaminants.

Scope and approach: This article reviews the progress of non-competitive immunoassay for low-molecular-weight contaminants in food, feed and agricultural products, including the principle and application, and the perspectives of this field are suggested.

Key findings and conclusions: Anti-metatype antibody-based immunoassay was the most promising method, but dissociation of antibody-hapten complex might be a challenge, and therefore more in-depth research should be focused on preparation of new formats of antibody-hapten complex. Meanwhile, strategies for direct detection or aimed at the simultaneous detection of different targets would be especially desirable besides focusing on improving the sensitivity and specificity of the detection.

**Keywords:** anti-metatype antibody, hapten, immunosensor, non-competitive immunoassay, immunocomplex antibody, open sandwich immunoassay.

## 1 Introduction

The low-molecular-weight contaminants (or hapten molecules) with a molecular weight less than 1000 Da, such as pesticides, veterinary drugs and mycotoxins, in food, feed and agricultural products, are great potential risks for food safety (Guo, Feng, Fang, Xu, & Lu, 2015; Malarkodi, Rajeshkumar, & Annadurai, 2017). Therefore, the sensitive detection of these contaminants is of great significance, and a number of methods have been developed for their successful measurement. Thin-layer chromatography (TLC) (Kotzybik, et al., 2016; Li, et al., 2016), gas chromatography-mass spectrometry (GC-MS) (Lee, et al., 2017; Lin, et al., 2015), and high performance liquid chromatography (HPLC) with ultraviolet (de Figueiredo, et al., 2015; de Lima, et al., 2016), fluorescence (Smith, Francis, Johnson, & Gaskill, 2017; Rahman, et al., 2017) or mass spectrometry detection (Santos & Ramos, 2016; Trevisan, Owen, Calatayud-Vernich, Breuer, & Picó, 2017), and immunoassay (Hu, et al., 2017; Spinks, 2000) are widely employed. Among them, immunoassay gains substantial attention due to their rapid, sensitive and high-throughput screening ability (Broto, McCabe, Galve, & Marco, 2017; Yang, Zhang, Chen, He, & Hu, 2017).

As is well known, immunoassay can be categorized into competitive and non-competitive formats. Theoretically, the latter harbors a higher sensitivity, precision and linearity (Islam, et al., 2011). However, the detection of low-molecular-weight contaminants is normally based on the competition principle (Anfossi, Di Nardo, Giovannoli, Passini, & Baggiani, 2015; Liu, Ye, Chen, Wang, & Chen, 2015), as in most other immunochemical detection of small molecules (Suzuki, Munakata, Morita, Shinoda, & Ueda, 2007). A non-competitive immunoassay, such as

conventional sandwich-type immunometric assay, is widely used to determine analyte concentration with high sensitivity and selectivity. However, the analyte to be determined should have at least two accessible epitopes (Janssen, et al., 2015; Kobayashi & Oyama, 2011; Zhu, et al., 2014). Consequently, low-molecular-weight contaminants in food, feed and agricultural products are not suitable for detection through the conventional sandwich-type immunoassay. In the past few years, researchers have proposed several types of non-competitive immunoassay for low-molecular-weight contaminants, and a list of frequently-used formats is shown in Table 1. Existing approaches for non-competitive immunoassay for low-molecular-weight molecules have been previously reviewed (Fan & He, 2012; P. Li & Deng, 2016). This review seeks to present an update of the state-of-the-art and to focus on advances of non-competitive immunoassay for low-molecular-weight contaminants in food, feed and agricultural products.

## **2 Non-competitive immunoassay for low-molecular-weight contaminants in food, feed and agricultural products**

### **2.1 Open sandwich immunoassay**

Open sandwich enzyme-linked immunosorbent assay (OS-ELISA), was firstly proposed by Ueda (Ueda, et al., 1996). OS-ELISA was based on the phenomenon that heavy-chain variable region (VH)/light-chain variable region (VL) association could be enhanced by the presence of the analyte. Since the advent of this method, several studies on detecting low-molecular-weight contaminants in food, feed and agricultural products, such as Gonyautoxin (Hara, Dong, & Ueda, 2013), estradiol (Liu, Eichenberger, Fujioka, Dong, & Ueda, 2012), benzaldehyde (Shirasu, et al., 2009) and zearalenone (Suzuki, et al., 2007), were reported. Generally, VH or VL fragment was immobilized into ELISA plate wells, and the analyte and enzyme-VL or enzyme-VH fusion protein were added, followed by the enzymatic reaction (i.e.: color development) and signal detection. As is true for traditional sandwich-type immunoassay, the detected signal was proportional to the content of the analyte. Fig. 1 illustrated a schematic diagram of OS-ELISA with maltose binding protein as the immobilization ligand and alkaline phosphatase as the signal reporter. The fusion proteins comprised of VH/VL and an immobilization or reporter protein have been used extensively, since such strategy shortened the incubation time and facilitated the detection process.

Apparently, the VH and VL fragments are needed for developing OS-ELISA, and they were originally obtained by cloning and expression of relative genes from monoclonal antibodies. However, whether the antibody used have a suitable property such that the VH/VL interaction would become fairly strong along with the addition of the analyte, can only be decided after measuring the VH/VL interaction and this represents a major limitation for OS-ELISA. Therefore, Aburatani et al. (2003) devised a phage-based “split-Fv system”, a filamentous phage p7-p9 display system individually displaying VH and VL fragments as a functional Fv on the tip of the phage to ensure fast and effective selection of Fv fragment suitable to OS-ELISA. The system was successfully used for selecting VH/VL fragments for bisphenol A and a limit of detection (LOD) of 1 ng/mL was observed. Likewise, by using VH/VL genes cloned from hybridomas, Suzuki et al. (2007) developed an open sandwich phage ELISA to detect the estrogenic mycotoxin zearalenone. In order to confirm the result obtained with the split-Fv phage display, the author performed OS-ELISA with the purified proteins (VH-maltose binding protein for immobilization and VL-alkaline phosphatase for color development) achieving a lower detection limit (approximately 0.1 ng/mL) together with a wider working range compared with a competitive ELISA performed with split-Fv phage or the original monoclonal antibody. Likewise, Dong Ihara, & Ueda, (2009) developed a strategy that based on a phagemid vector in which two identical restriction sites were incorporated into both ends of a human constant region domain. After selection of the M13 phage displaying a Fab fragment, the vector was easily converted to the vector able to produce simultaneously the VH-displaying phage and the light chain in the culture supernatant. Subsequently, they could be directly used for OS-ELISA. By using the strategy proposed by Dong et al. (2009), Hara, Dong, & Ueda (2013) employed an OS-ELISA to detect GTX2/3 (mixture of 114.2  $\mu$ M GTX2 (Gonyautoxin 2) and 43.4  $\mu$ M GTX3 (Gonyautoxin 3)), and the detection limit was 0.5 ng/mL. The analogs of GTX2/3, STX (saxitoxin dihydrochloride) and neoSTX (neosaxitoxin), were checked for cross-reactivity, and reaction was observed for STX in the range of 100–1000 ng/mL.

Recently, other strategies based on OS-ELISA have been described for detecting food, feed and agricultural contaminants. Sakata, Ihara, Makino, Miyahara, & Ueda (2009) proposed an open sandwich-based immuno-field effect transistor (OS-FET) method for label-free and non-competitive detection of bisphenol. The principle of OS-FET was based on the detection of intrinsic molecular charges caused by the small antigen-dependent interchain interaction of separated VL and VH fragments from a single antibody variable region using the field effect. The

LOD of OS-FET for bisphenol was 1 nM; furthermore, the addition of isothiocyanobenzyl-EDTA with negative charges to the VL fragment enhanced the sensitivity of the system pushing down the LOD to 1 pM.

Besides, micro-ELISA system (Ihara, et al., 2010; Islam, et al., 2010), energy transfer-based homogeneous fluoroimmunoassay (open sandwich FIA) (Chung, Makino, Ohmuro-Matsuyama, & Ueda, 2017; Ueda, et al., 1999; Wei, et al., 2006) and open-sandwich molecular imprinting assay (Minami, Ihara, Kuroda, Tsuzuki, & Ueda, 2012) were reported, but were mainly devoted to clinical application.

## **2.2 Anti-metatype antibody-based immunoassay**

An anti-metatype antibody is an immunological reagent specific for the conformation of a usual antibody bound to its antigen and that does not interact with the antigen or the unbound antibody (Voss Jr & Mummert, 1997). After the immunocomplex is formed between an analyte and its antibody, the anti-metatype antibody can be used to capture the immunocomplex, followed by signal development. The detected signal is proportional to the content of the analyte (Fig. 2).

Anti-metatype antibodies were initially obtained by immunization with the antibody-hapten complex (Nagata, Tsutsumi, Yoshida, & Ueno, 1999; Ullman, et al., 1993), allowing to achieve LOD of pg/mL for microcystin (Nagata, Tsutsumi, Yoshida, & Ueno, 1999). However, it is difficult to provide useable anti-metatype antibodies through the immunization strategy (Voss, Dombink-Kurtzman, & Ballard, 1989), because the obtainment of the anti-metatype antibody strictly depends on the stability of the antibody-hapten complex. In addition to the dissociation of the antibody-hapten complex, hapten molecules tend to be almost entirely buried in paratopes of antibodies, which limits the chance of obtaining immunoreagents capable of distinguish between bound and unbound antibodies (Arevalo, et al., 1994; Kobayashi, Oyama, Kato, et al., 2010; Valjakka, et al., 2002).

Thanks to antibody library techniques, antibody or antibody-like molecules can be obtained without immunization of animals. Accordingly, Kim, McCoy, Gee, González-Sapienza, & Hammock (2010) developed a non-competitive phage anti-immunocomplex real-time polymerase chain reaction (RT-PCR) for the detection of 3-phenoxybenzoic acid (3-PBA) and molinate. Firstly, a short peptide loop displayed on the surface of the M13 bacteriophage, which was able to bind specifically to the antibody-hapten complex, was identified through a phage anti-



immunocomplex assay (PHAIA). Then, the anti-3-PBA antibody was immobilized into ELISA plate wells, and 3-PBA as well as the selected phage were added and incubated in the functionalized wells. The bound phages were eluted, and were then used as the DNA template in real time PCR for quantification. For the detection of 3-PBA, the LOD was 20 pg/ mL, while the LOD was 0.2 ng/mL for molinate. Validation of the PHAIA-PCR with real samples was also performed, and results indicated that the developed PHAIA-PCR was suitable for monitoring of water contamination. Dong et al. (2014) selected immunocomplex binding peptides from a circular random eight-amino-acid phage-displayed library to detect leucomalachite green in a similar non-competitive immunoassay. The LOD of the assay was 0.55 ng/mL, with a linear range of 1.35 to 21.56 ng/mL. Arola et al. (2016) screened engineered antibody HT2-10 Fab recognizing both HT-2 and T-2 toxins from a phage display antibody library. Specificity of the immunoassay was introduced by an anti-immune complex scFv binding the primary antibody-HT-2 toxin complex. HT-2 was successfully detected by a time-resolved fluorescence resonance energy transfer (TR-FRET) assay, and the half-maximal effective concentration value and LOD were 9.6 ng/mL and 0.38 ng/mL, respectively. Later, Arola et al. (2017) developed OS-ELISA for HT-2 toxin based on the anti-immunocomplex scFv antibody fragment, which was genetically fused with alkaline phosphatase and recognized the immunocomplex between anti-HT-2 toxin Fab fragment and HT-2 toxin. The LOD values of HT-2 in wheat, barley and oats were 0.3 ng/mL, 0.1 ng/mL and 0.3 ng/mL, respectively. Akter et al., (2016) reported a time-resolved immunofluorometry-based assay for cyanobacterial microcystins (MCs) and nodularins (Nods). The assay was based on the combination of a generic anti-immunocomplex scFv and a monoclonal antibody capable of binding to an Adda-group (3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid) present in all MCs/Nods. The anti-IC scFv was isolated from a synthetic antibody library with phage display. The assay was able to detect eleven commonly occurring hepatotoxins at about 0.1 ng/mL.

The anti-metatype antibody-based strategy promises to be a feasible way for designing non-competitive immunoassays for low-molecular-mass analytes, primary thanks to the availability of screening techniques that permits the easy selection of anti-metatype antibodies from antibody libraries (Gonzalez-Techera, Kim, et al., 2007; Gonzalez-Techera, Vanrell, Last, Hammock, & Gonzalez-Sapienza, 2007). The limiting factors still remain in the dissociation of the antibody-hapten complex, which could occur during the screening, and the masking of hapten into antibody binding sites.

### **2.3 Idiometric assay with anti-idiotypic antibodies**

According to the immune network theory of Niels Jerne (1973), the immune system is a network of interacting idiotypes (Ids) that are involved in the regulation of immune responses, and anti-idiotypic antibodies (Ab2s) can be generated upon immunization with an idiotypic antibody (Ab1s). Generally, Ab2 can be classified into Ab2 $\alpha$ , Ab2 $\beta$ , Ab2 $\gamma$  and Ab2 $\epsilon$  (Dalglish & Kennedy, 1988), among which, Ab2 $\beta$  binds directly to the idiotope within the paratope of Ab1 (Liu, Yang, Wang, & Chen, 2012). Ab2 $\alpha$  recognizes idiotopes that are outside of the hapten binding site, but Ab2 $\alpha$  does not bind to the Ab2 $\beta$ -Ab1 complex because of steric hindrance. So Ab2 $\alpha$  preferentially binds to the hapten-occupied Ab1. Thus, the signal generated by the bound Ab2 $\alpha$  is proportional to the content of the hapten. Based on this theory, a non-competitive immunoassay named as idiometric assay was developed. Barnard et al. (1991) and Barnard & Kohen (1990) firstly reported such non-competitive immunoassay for the detection of estradiol in serum. Fig. 3 illustrates the schematic diagram of the idiometric assay for estradiol. Firstly, a monoclonal antibody against estradiol was immobilized into microtiter wells. Secondly, estradiol was captured, and the unoccupied antibody sites were blocked by excess amount of Ab2 $\beta$ . Lastly, estradiol occupancy was determined by the addition of excess of a europium-labeled Ab2 $\alpha$  through time-resolved fluorometry. Afterward, Mares et al. (1995) developed a similar non-competitive idiometric enzyme immunoassay for estradiol, in which the biotinylated Ab2 $\alpha$  was captured by anti-biotin IgG immobilized into ELISA plate wells. Then, the Ab1 complexed sequentially: an enzyme-labelled secondary antibody, estradiol, and Ab2 $\beta$ . Finally, color development was promoted by the enzyme label.

According to the theory of this approach, a two-step antibody production was needed (Kobayashi & Goto, 2001). Moreover, the preparation and identification of Ab2 $\alpha$  and Ab2 $\beta$  was time-consuming and extremely difficult. Consequently, the application of this strategy was confined to the examples discussed and to date has not been applied for determining low-molecular-weight contaminants in food, feed and agricultural products.

### **2.4 Giraudi's method**

Giraudi et al. (1999) proposed a general method to perform non-competitive immunoassays for small molecules, and herewith, the method was designated as Giraudi's method. Giraudi's method was based on blocking the free sites of a capture antibody by a blocking reagent; this

reagent is a large molecule able to bind to different antibody sites at the same time, so that it is more strongly bound than the hapten to the immobilized antibody. In this way, when an enzyme-labeled hapten is added, competition for the antibody occupancy allows the removal of the analyte molecules, but not of the blocking reagent. Consequently, the measured signal is almost linearly correlated to the hapten concentration. By using cortisol-poly(L-lysine) conjugate as a blocking reagent, a LOD of 0.15 ng/mL was obtained for cortisol detection (Giraudi, et al., 1999). Subsequently, Anfossi et al. (2002) optimized reagents, such as anti-cortisol antibody and cortisol-poly(L-lysine) conjugate dilution, involved in this method to detect cortisol in saliva. And a LOD of 0.2 nmol/L was achieved, which was 10 times lower than previous report from Giraudi, et al (1999). Likewise, total aflatoxins were determined by Acharya & Dhar (2008). In this work, a polyclonal anti-AFB1 antibody was immobilized into ELISA plate wells, and then AF standards (AFB1 + AFB2 + AFG1 + AFG2) and AFB1-protein conjugate were allowed to react. Afterwards, an AFB1-horse radish peroxidase (HRP) conjugate was added, followed by color development. The strategy was based on blocking the free sites of anti-AFB1 capture antibodies by the AFB1-protein conjugate, followed by the replacement of antibody-bound AFs by the enzyme-labeled AFB1. Since the rates of displacement of weakly bound AFB1 congeners by the AFB1-HRP conjugate were faster than that of AFB1, the measured signals from cross-reactants were higher and almost linearly correlated to the AFB1 concentration. The obtained LOD for total AFs was 0.1 µg/L. Meanwhile, spiked and contaminated corn samples were analyzed by this assay without sample cleanup, and the results correlated well ( $R^2 = 0.99$ ) with results obtained by a commercial ELISA kit. Similarly, Lates, Yang, Popescu, & Marty (2012) reported an assay for the detection of ochratoxin A (OTA) based on the use of its dechlorinated analogue, ochratoxin B (OTB), in a displacement immunoassay. OTB was immobilized on controlled-pore glass beads followed by the binding of anti-OTA antibody, with anti-IgG antibody peroxidase conjugate used as a label. In this manner, an original bio-sensing material was obtained. Upon incubation of this material with OTA, the toxin competes with OTB for the binding sites of the anti-OTA antibodies and releases the antibody-tagged peroxidase complex into the solution. The assay was capable of screening OTA at 2 ng/mL.

The critical point for applying this strategy as a general method that could be employed for the detection of other low-molecular-weight contaminants is the identification of the appropriate blocking or displacement reagent. However, the approach is attractive because it does not require the production of extra antibodies, such as VH/VL or anti-immunocomplex antibody.

## 2.5 Non-competitive immunoassay based on special separation

Except the above four strategies, alternative strategies of realizing non-competitive immunoassays for low-molecular-contaminants based on special separation (Fan & He, 2012) were suggested and applied for measuring food, feed and agricultural contaminants.

Anfossi et al. (2004) developed a non-competitive immunoassay for 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), which exploited a polyclonal antibody. 2,2-bis-(4-chlorophenyl)acetic acid (DDA), as an analogue of the analyte, was conjugated to BSA and to a support for affinity chromatography. The assay comprised the following steps: DDT was incubated with excess of the antibody, and then the separation of the DDT-bound antibody from the excess of free antibody was conducted through the DDA-immobilized affinity column, followed by the dissociation of the DDT-bound antibody in DDA-BSA coated wells that captured the antibody previously bound with DDT. Lastly, the captured antibody was quantified. The measured signal was directly proportional to the hapten concentration. The LOD was 8 ng/L, which was better than all reported immunoassays for DDT. Meanwhile, real water samples were validated in a percentage recovery test, and the results highlighted the validity of the proposed method. Likewise, Saha, Roy, & Dhar (2013) developed a non-competitive immunofiltration assay for AFB1. The authors used a device for obtaining the separation of immune complexes of anti-AFB1-antibody and AFB1-HRP from the free AFB1-HRP conjugate by filtration through immunofiltration strips where the anti-AFB1 antibodies were immobilized. The bound AFB1-HRP was visualized by the super-catalyzed reporter deposition signal amplification method, and the measured signal was linearly correlated to the amount of AFB1 added. The LOD was 15 pg/mL, and the detection in wheat and corn was proved to be accurate.

From the foregoing, the performance of these two types of method was great. And the former seemed to be more feasible and convenient for low-molecular-contaminants which had some available analogues. However, optimization of the conditions is needed, such as the choice of the analogue, and the capacity of the affinity column. The latter might become a general method since it was fit for all low-molecular-contaminants.

An extra separation process was needed by both methods which implied concerns about time required for the analysis, its simplicity of use and also of automation that would limit their wide application. In addition to these two types of methods, other types of non-competitive

immunoassay based on special separation for detecting haptens in other research fields can be found in the review of Fan & He (2012).

## **2.6 Method based on direct non-competitive immunosensor**

With the development of high-tech equipment, direct non-competitive immunosensors are an emerging technique used for low-molecular-contaminants detection. Generally, an immunosensor is composed of an immunoreceptor, a transducer, a data recording device, and a display device. The detection relies on measuring changes in signals of optical, mass, electrochemical, etc. Dai, et al. (2017) reported a microcantilever-based immunosensor for carbofuran detection. The surface of gold-coated microcantilever was chemically modified by the crosslinking of l-cysteine/glutaraldehyde. Anti-carbofuran monoclonal antibodies were then immobilized on the side of the microcantilever to fabricate the immunosensor. The immunosensor achieved a detection limit of 0.1 ng/mL and it showed a good linear relationship over the range from  $1.0 \times 10^{-7}$  to  $1.0 \times 10^{-3}$  g/L ( $R = 0.998$ ). Meanwhile, the immunosensor exhibited high sensitivity, specificity and good stability and it was used for carbofuran determination in soil and vegetable samples with satisfactory results. Gan, et al., (2013) developed an electrochemiluminescent immunosensor for aflatoxins M1 (AFM1) in milk using magnetic Fe<sub>3</sub>O<sub>4</sub>-graphene oxides (Fe-GO) as the absorbent and anti-AFM1 antibody-labeled cadmium telluride quantum dots (CdTe QDs) as the signal tag. The working range extended from 1.0 pg/mL to 100 ng/mL, and a LOD of 0.3 pg/mL was reached. Haasnoot & Verheijen, (2001) reported a direct (non-competitive) immunoassay for gentamicin residues with an optical biosensor. In the research, anti-gentamicin monoclonal antibodies were immobilized on sensor chip of an optical biosensor (BIAcore 3000) for direct detection of gentamicin, and a LOD of 10 ng/mL was achieved.

Besides, other immunosensors with the direct non-competitive principle, such as electrochemical impedance immunosensors (Khan, & Dhayal, 2009; Radi, Munoz-Berbel, Lates, & Marty, 2009; Singh, et al., 2013; Zaijun, Zhongyun, Xiulan, Yinjun, & Peipei, 2010), fluorescence resonance energy transfer-based immunosensors (Li, Jeon, Suh, & Kim, 2011; Li, Jo, & Kim, 2012; Li, Byun, Kim, Shin, & Kim, 2013), optical waveguide lightmode spectroscopy-based immunosensors (Adányi, et al., 2007) quartz crystal microbalance (Liao, 2007), were also reported for low-molecular-weight contaminants determination in food, feed and agricultural products. Obviously, methods based on direct non-competitive immunosensors achieve a simple, sensitive, and direct detection. However, the instability of immobilized biomolecules, the

requirement of expensive machinery, and the interference of other components might be challenges.

### 3 Conclusions and Prospective

Because of the rapid, sensitive and high-throughput screening ability of immunoassay, they are preferred for detecting contaminants at trace amounts in food, feed and agricultural products. As is well known, non-competitive immunoassay usually exhibits higher specificity, lower cross-reactivity, and a wider working range compared to competitive immunoassay. However, for low-molecular-weight contaminants with only one accessible epitope, application of competitive immunoassays is dominant. The six types of non-competitive immunoassay discussed in this review provides feasible solutions for the ultrasensitive monitoring of haptens. Open sandwich immunoassay, anti-metatype antibody-based immunoassay, and direct non-competitive immunosensor are more universal, although suffer the difficulty to obtain suitable bioreagents. To the best of our knowledge, most of researches have been focused on detection of clinical markers. Undoubtedly, much effort still need to be put on development of non-competitive immunoassays for low-molecular-weight contaminants in food, feed and agricultural products. ~~Although the six types of non-competitive immunoassay discussed in this review provides feasible solutions for monitoring some haptens, none of them was a versatile strategy. And, to the best of our knowledge, most of researches have been focused on detection of clinical markers. Undoubtedly, much effort still need to be put on development of non-competitive immunoassay for low-molecular-weight contaminants in food, feed and agricultural products.~~

Possibly, future investigations can be directed into three aspects: (a) further development of anti-metatype antibody-based immunoassay. Although anti-metatype antibody-based immunoassay was the most promising, the extensive application remains to be seen. And as mentioned above, dissociation of antibody-hapten complex might be a challenge, so new formats of antibody-hapten complex might help. For example, Kobayashi, Oyama, Suzuki, et al. (2010) developed phage displayed scFv to capture complexes of vitamin D and A derivatives with immobilized  $\beta$ -cyclodextrin or  $\beta$ -maltosyl residues. The scFv enabled novel “semisandwich-type” immunometric assays of haptens with nanomole-range sensitivities; (b) further development of open sandwich immunoassay, OS-ELISA was an elegant alternative to anti-metatype antibody-based immunoassay, however, the principal example was case specific, therefore, much effort has

to be spent to make it as a general strategy; (c) the reported methods were restricted into lab scale, and smart immunosensor, suitable for daily life, of low cost was highly demanded.

It is worthwhile noting that recent findings highlighted new concerns about food, feed and agricultural products contaminants. In particular, these products are usually contaminated by multiple contaminants (Anfossi, Giovannoli, & Baggiani, 2016), so strategies aimed at the simultaneous detection of different targets would be especially desirable besides focusing on improving the sensitivity and specificity of the detection. The direct non-competitive immunosensor might become widely applicable for different multiplexed and high-throughput screening needs. However, as mentioned above, effort has to be put to obtain a portable and cheap immunosensor for daily use.

## **Figure Captions**

**Fig. 1** Schematic diagram of OS-ELISA with maltose binding protein as immobilization ligand and alkaline phosphatase as reporter.

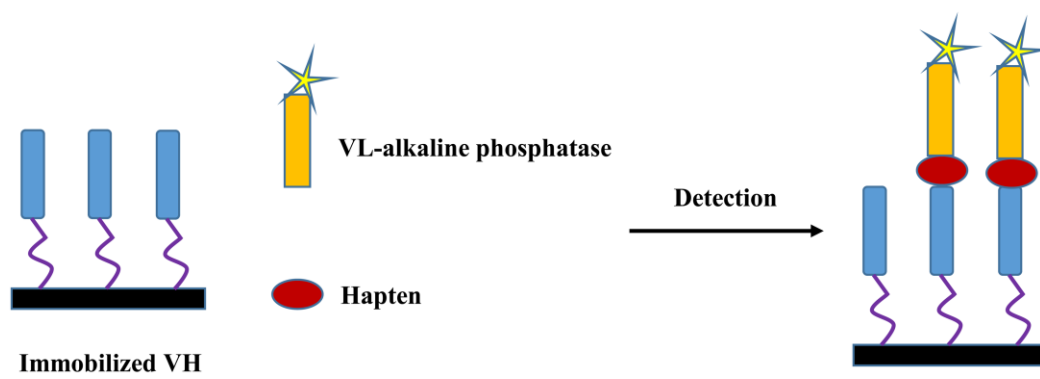
**Fig. 2** Schematic diagram of the non-competitive immunoassay with anti-metatype antibodies.

**Fig. 3** Schematic diagram of the idiometric assay with anti-idiotypic antibodies.

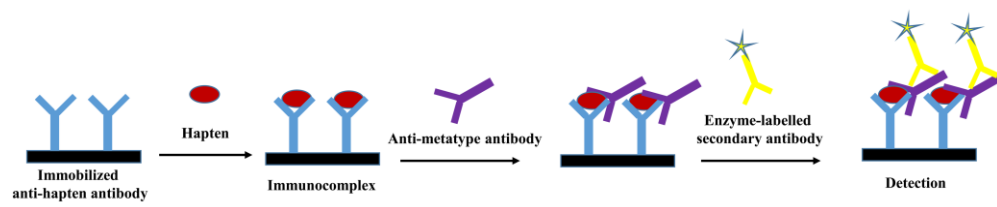


**Figures**

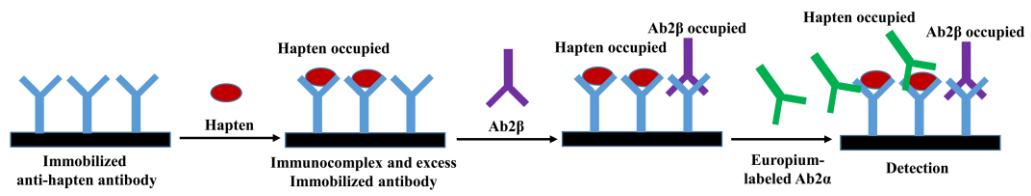
**Fig. 1**



**Fig. 2**



**Fig. 3**



**Table 1** Comparison of non-competitive immunoassays for low-molecular-weight analytes in food, feed and agricultural products

Formats	Methodology	Advantages	Disadvantage	Analyte	References
Open sandwich immunoassay	VH-VL association can be enhanced by the presence of antigen	possibility of obtaining immunoreagents as fusion proteins	Selecting VH/VL pairs for hapten	bisphenol A	Aburatani et al., 2003
				zearalenone	Suzuki et al., 2007
				estradiol	Liu et al., 2012
Anti-metatype antibody-based immunoassay	Anti-metatype antibodies are specific for the conformation of the bound antibody	Selection of suitable immunoreagents is facilitated by the use of libraries	Preparation of anti-metatype antibody	morphine	Pulli et al., 2005
				clomazone	Rossotti et al., 2010
				HT-2 toxin	Arola et al., 2016
Idiometric assay	$\alpha$ -Ald preferentially binds to the hapten-occupied Ab1,	I can't see any advantage in this approach...	Preparation of first antibody and anti-idiotypic antibody	estradiol	Barnard & Kohen, 1990
				estradiol	Barnard et al., 19991
Giraudi's method	Blocking free sites of capture antibody by a "blocking reagent"; which is more strongly bound than the analyte to the immobilized antibody	No needs of producing more than one usual antibody	Preparation of blocking reagent	aflatoxin	Acharya & Dhar, 2008
				cortisol	Anfossi et al., 2002
Special separation	-		Design of separation strategy, no uniform methodology	DDT	Anfossi et al., 2004
				AFB1	Saha et al., 2013
Direct non-competitive immunosensor	At the time of antibody-hapten interaction, measuring changes in signals of optical, mass, electrochemical, etc.	Rapidity and simplicity as no label is required	High-tech instrument and operators are needed	ochratoxin A	Khan & Dhayal, 2009
				ochratoxin A	Li et al., 2012
				carbofuran	Dai et al., 2017

“-”: no uniform methodology.

