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Recent Progress in Noncompetitive Hapten Immunoassays: A Review

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1. Introduction

Detection of small molecules (hapten molecules), such as food contaminants, environmental pollutants, disease factors, drugs and so on, is an area with great significance. Immunoassay, as a simple, rapid and cost-effective detection system, is widely used for the detection of low molecular weight analytes in varied matrix. However, the number of commercial available immunoassay kits for hapten molecules is still limited, this might be partly due to the poor performance such as sensitivity of these immunoassays which can not fulfill the actual requirement. Traditionally, detection of small molecules in solution must employ competitive immunoassay formats, either with immobilized antibody or with immobilized coating conjugate. However, theoretical study has demonstrated that competitive immunoassays are inferior to noncompetitive immunoassays in terms of sensitivity, precision, kinetics and working range of analyte (Jackson and Ekins, 1986). Hence, there is a trend now to develop noncompetitive immunoassay systems for hapten molecules assay.

Although sandwich immunoassay is widely applied to noncompetitive assay of antigen concentration, it has a fundamental limit that the antigen to be measured must be large enough to have at least two epitopes to be captured. Hence, it can not be used to measure low molecular weight compounds. During the last two decades, researchers in related fields have made great efforts to overcome this drawback, and some novel immunoassay systems that can noncompetitively detect small molecules have been established. These new formats are based on chemical modification to the analytes, unconventional antibodies (anti-idiotype antibody, anti-metatype antibody or antibody fragment), special separation steps (capillary electrophoresis, affinity column or membrane), or other elegant tricks. In this paper, an overview of the recent development and application of these elegant approaches was presented.

2. Noncompetitive immunoassays based on chemical modification to the analytes

2.1 Ishikawa's method

As stated, it is due to hapten molecules with just one epitope that can not simultaneously be bound by two antibodies, so traditional sandwich immunoassay can not be implemented. An ingenious scheme, developed by Ishikawa's group in 1990, introduces a molecule of

biotin into the analyte prior to the assay, and then the biotinylated analyte can simultaneously be bound by anti-analyte antibody and avidin. According to this principle, a novel and sensitivity noncompetitive (two-site) enzyme immunoassay for haptens is described and exemplified by L-Thyroxine (T4) detection initially (Tanaka et al. 1990). The principle of this method is outlined in Figure 1. Briefly, samples (or standards) are firstly biotinylated, and then these biotinylated samples are purified by anti-analyte antibody coated polystyrene balls. Secondly, the samples are mixed with labeled anti-analyte antibody and transferred onto a solid phase coated with avidin. Finally, signal can be directly measured after a washing step. The signal strength is directly proportional to the analyte concentration as traditional noncompetitive immunoassay.

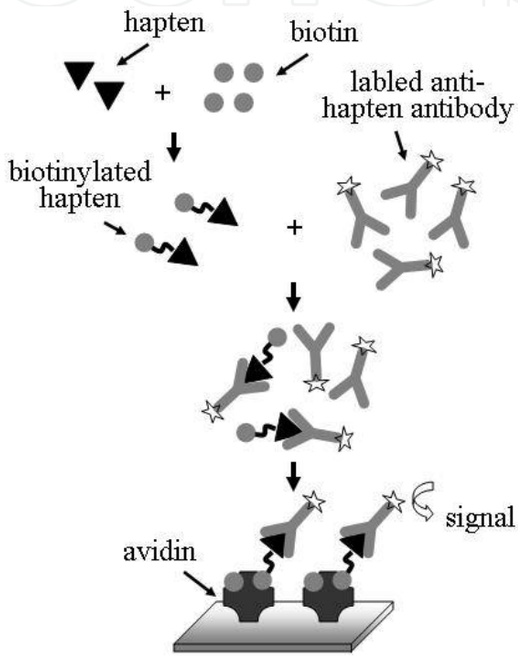

Fig. 1. Principle of Ishikawa's method. Step 1: biotinylation of the analyte and affinity purification; Step 2: formation of antibody/antigen complex; Step 3: captured by immobilized avidin.

This method has been successfully applied to several analytes bearing a primary amino group, which can react with the biotin. The sensitivity of this technique is nearly 10 attomoles for small peptides like angiotensin I (Ishikawa et al. 1990) or arginine vasopressin (Hashida et al. 1991). However, the biotinylated reaction and affinity purification procedures are complicated and time consuming. Due to this weakness, this method is not popularized to the detection of other hapten molecules.

2.2 Solid phase immobilized epitope-immunoassay (SPIE-IA)

The solid phase immobilized epitope-immunoassay (SPIE-IA) method for measuring hapten molecules in noncompetitive format was firstly described by Pradelles et al. in 1994 and its principle is outlined in Figure 2. The method involves a critical cross-linking and epitope release step. Briefly, the analyte is firstly captured by solid phase immobilized antibody, and then covalently cross-linked with corresponding antibody molecules by homobifunctional

reagent (glutaraldehyde or disuccinimidyl suberate). After a dissociating treatment (HCl or methanol) of the analyte/antibody complex to release the epitope from the antibody binding site, the presence of analyte on the solid phase is detected using a labeled antibody. This procedure involves the use of excess reagents (capture and tracer antibodies) to insure the efficiency of analyte/antibody complexes formation and generates calibration curves in which the signal is directly proportional to the analyte concentration, as in conventional sandwich immunoassays (Pradelles et al. 1994).

Like Ishikawa's method, SPIE-IA was firstly applied to the measurement of analytes bearing a primary amino group excluded from the epitope site. But a prederivatization step before performing the SPIE-IA can be applied to introduce amino group to molecules that lack this function group. As in the work of Etienne et al.(1996), in order to develop a SPIE-IA system for thyroliberin, the thytoliberin and biological samples were prederivatized by diazotized 2(4-amino-phenyl)ethyl amine, and then operated as the initial approach. With the aim of extending the procedure to haptens devoid of an amine moiety, new strategies of cross-linking have been developed. For example, in another work of Etienne et al. (1995) the capacity of thyroxine to be photoactivated directly by UV treatment was utilized and resulting in covalent cross-linking with its binding protein. SPIE-IA involving direct cross-linking of hapten by UV irradiation was then named as Photo-SPIE-IA, and it was also successfully applied to the detection of 17ß-estradiol (Buscarlet et al. 1999). More recently, new SPIE-IA procedure (named SPIE-Rad) using the free radical species produced by the Fenton-like reactions during the cross-linking step has been developed and also successfully applied to the detection of 17β-estradiol (Buscarlet et al. 2001)

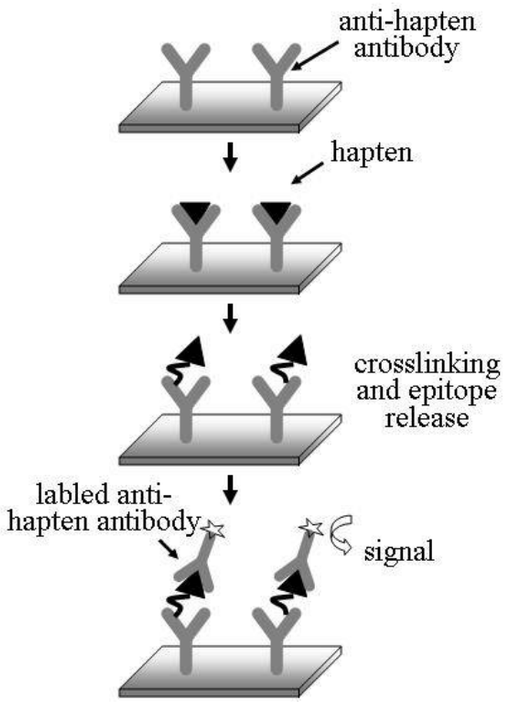

Fig. 2. Principle of SPIE-IA. Step 1: immobilization of analyte on an anti-analyte antibody-coated plate; Step 2: cross-linking of the analyte with solid-phase antibody and then dissociate the analyte/antibody complex; Step 3: analyte is revealed by labeled antibody.

As for the sensitivity, good results have been obtained by this method for various small analytes (<2500 Da) (Pradelles et al., 1994; Volland et al., 1994; Ezan et al., 1995; Volland et al., 1999). In each case, the sensitivity was enhanced (10–300 folds) when compared to the corresponding competitive immunoassay performed using the same antibody. For example, limit of detection of angiotensin II is close to 0.5 and 45pM with SPIE-IA and competitive assay, respectively (Volland et al., 1999). Moreover, the precision of SPIE-IA is equivalent to that of conventional immunoassays. Although, satisfactory performance was obtained from the published SPIE-IA, it is still very difficult to be applied to every hapten molecules.The UV irradiation approach appears limit due to the need for an irradiation device and the limited number of available irradiation wavelengths. In addition, UV irradiation has some deleterious effects by degrading the immunological complex more or less rapidly, depending on the wavelength and the energy used. The SPIE-Rad protocol might work efficiently to assay different molecules, but lots of parameters need to be optimized.

3. Noncompetitive immunoassays based on unconventional antibody

3.1 Anti-idiotype antibody based noncompetitive immunoassay (Idiometric assay)

Anti-idiotype antibody (AId or Ab2), refering to an antibody raised against the variable region of an original antibody (Ab1), is one of the most important concepts on "immune network theory". Jerne et al. (1982) have classified anti-idiotype antibodies according to the location of the idiotype recognization in their early report, and two main types of anti-idiotype antibodies have been distinguished: alphatype (α-AId) and betatype (β-AId). By definition, α-AId recognizes an epitope within the framework of the variable region of the primary antibody, but is not sensitive to the presence or absence of the analyte at the binding site. While, β-AId compete with the antigen for an epitope at the binding site，i.e. the former will bind to the primary antibody in the presence of the antigen, whereas the latter will not. In addition, the α-AId will not bind to the β-AId/Ab1 complex because of steric hindrance. Based on the properties of α-AId and β-AId, Barnard and Kohen (1990) described an original noncompetitive immunoassay (denoted "idiometric assay") for small molecules in 1990, and typified by the measurement of estradiol in serum. The general principle of this method is outlined in Figure 3. After coating wells with anti-hapten antibody (Ab1), test samples (or standard analytes) are added, and then β-AId is used to block the binding sites that are not occupied by hapten molecules on the Ab1. At last, by adding labeled α-AId, the amount of haptens can be detected.

Since the establishment of this method, Barnard's group has applied it to the detection of estradiol (Barnard & Kohen, 1990; Barnard et al., 1991; Mares et al., 1995) and progesterone (Barnard et al., 1995a) in serum and Estrone-3 Glucuronide (Barnard et al., 1995b) in urine， Kobayashi et al. have applied it to the detection of UDCA 7-NAG (a bile acid metabolite) (Kobayashi et al., 2000; Kobayashi et al., 2003 a) and 11-deoxycortisol (Kobayashi et al. 2003 b). More recently, Niwa et al. (2009) established a noncompetitive-type ELISA for cortisol based on idiometric assay model. This assay had an approximately threefold higher sensitivity (detection limit: 90 pg cortisol) than a competitive ELISA using the same anti-cortisol antibody and had practical specificity for providing reasonable determination of normal urinary cortisol levels.

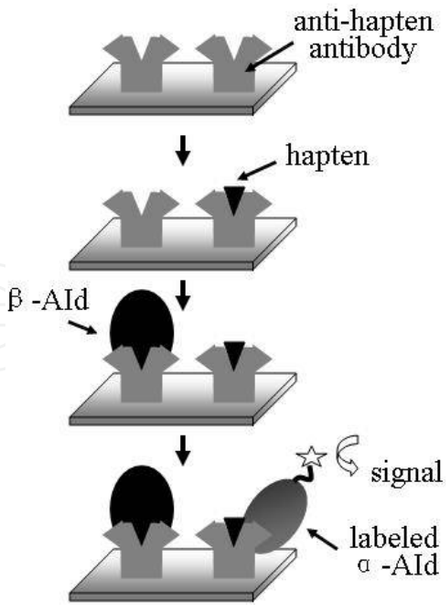

Fig. 3. Principle of idiometric assay. Step 1: capture the analyte by anti-analyte antibody (Ab1)-coated plate; Step 2: blocking the unoccupied binding site on Ab1 by β-AId; Step 3: analyte is revealed labeled α-AId.

Although good performances have been obtained, the idiometric assay requires the sequential addition of the three antibody reagents (capture antibody, α-AId and β-AId). The production of capture antibody is nothing special, but the production and identification of anti-idiotype antibody are very complicated. The difficulties inherent in anti-idiotype antibody generation, especially in producing an array of different AId, from which the most suited ones could be chosen, largely restrict the application of idiometric assay.

3.2 Anti-metatype antibody based noncompetitive immunoassay

The term "metatype" was initially proposed by Voss et al. (1988) for the immunological definition of the liganded active site to distinguish it from idiotype (non-liganded). Therefore, the anti-metatype antibodies recognize the antibody/antigen complex but exhibit very low or no affinity for the antibody or the antigen alone. This remarkable property was cleverly utilized by Self et al. (1994) to develop noncompetitive immunoassay for small molecules, and exemplified by digoxin detection. In this case, the analyte is captured by the specific antibody, and formatted a second epitope for anti-metatype antibody reorganization. If labeled anti-metatype antibody is applied, a detect signal directly proportional to the analyte concentration can be obtained (Figure 4).

Self et al. (1994) showed that this noncompetitive immunoassay system provides a high-performance assay for digoxin in serum samples, being conveniently simple (immobilized primary antibody binds digoxin and then labeled secondary antibody so that when excess unbound label is washed away the immunometric readout reflects the digoxin concentration), rapid (incubation time 1-10 min), sensitive (detection limit 30 ng/L), precise (3-4% within-run CV, 1-8% total CV), and free from interference from digoxin-like immunoreactive factors. In addition, satisfactory results were also obtained by Towbin et al.

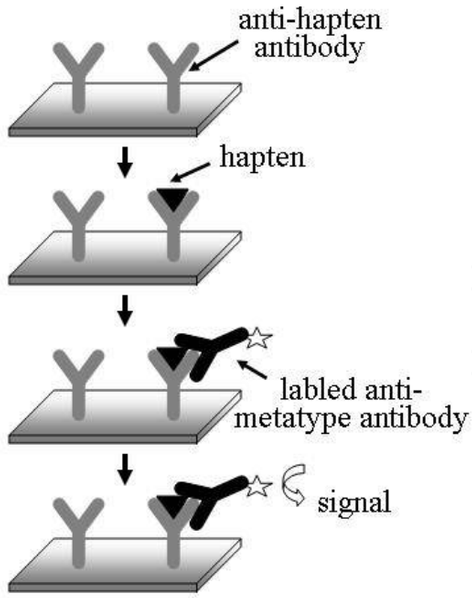

Fig. 4. Principle of anti-metatype antibody based noncompetitive immunoassay. Step 1: capture the analyte by anti-analyte antibody (Ab1)-coated plate; Step 2: labeled anti-metatpye antibody was added for specifically bind to the analyte/antibody complex; Step 3: signal formation.

(1995) and Nagata et al. (1999) for the detection of haptens angiotensin II and microcystin respectively. However, as per the idiometric assay, the difficulties of anti-metatype antibody production are main barriers for the widely application of this method. Such an anti-metatype antibody can seldom be generated by the conventional antibody production methods, consequently, the information now available concerning the strategy for its production is very limited.

3.3 Open sandwich immunoassay (OSIA)

A new kind of noncompetitive immunoassay, named "open sandwich immunoassay (OSIA)", has been described by Ueda et al. in 1996, and successfully applied to detect hen egg lysozyme (Ueda et al. 1996). This assay is based on the observation that for some antibodies, the association of separated VH and VL chains from the variable domain of antibody is strongly favored in the presence of antigen. The general principle of this method is outlined in Figure 5. Briefly, the VL fragment was immobilized on plate, and then the VL plate was incubated with VH fragment together with antigen. If labeled VH fragment is used, the signal can be directly measured after a washing step; otherwise a labeled second antibody is applied. After Ueda's work, this method was extended to hapten molecules detection and new formats such as homogeneous assays were also developed. These homogeneous assay systems are either based on enzymatic complementation (Ueda et al. 2003) or resonance energy transfer (fluorescence (Ueda et al. 1999; Arai et al. 2000; Wei et al. 2006) or bioluminescence (Arai et al. 2001)).

Up to now, the OSIA system has been successfully applied in the detection of hapten molecules such as gibberellin (Lee et al., 2008), benzaldehyde (Shirasu et al., 2009), zearalenone

(Suzuki et al., 2007), 4-hydroxy-3-nitrophenylacetyl (Yokozeki et al. 2002), the carboxyl-terminal peptide of human osteocalcin (BGP) (Lim et al. 2007), bisphenol A (Sakata et al. 2009), and 11-Deoxycortisol (Ihara et al., 2009). This novel immunoassay system could be done in a shorter period of time than using a conventional sandwich assay, due to the omission of an incubation/washing cycle. And in the homogeneous format OSIA, the washing steps can even completely be avoided. Also, the assay was found to be compatible with traditional competitive immunoassay, similar or a lower detection limit as well as wider working range could be attained. However, the preparation of the reagents is complex and time consuming (production and selection of monoclonal antibodies; preparation of DNA fragments encoding VH and VL, construction, production and purification of fusion proteins). Moreover, the principle of this assay, based on differential interactions between separated VH and VL chains in the presence or absence of the antigen, requires a strong antibody selection since only some antibodies meet these criteria. For these reasons, it is still difficult to apply this method widely at present.

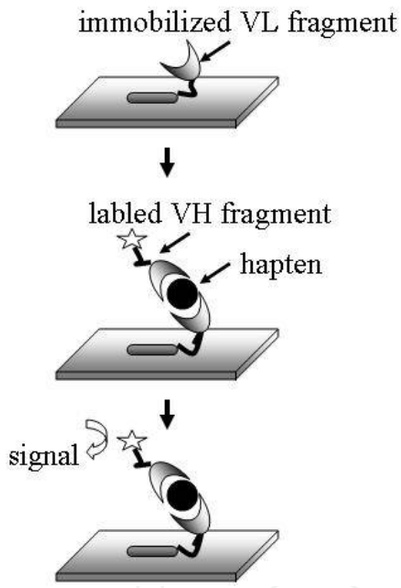

Fig. 5. Principle of the initial OSIA. Step 1: immobilization of VL fragment; Step 2: incubation of labeled VH fragment with analyte; Step 3: measurement of signal.

4. Noncompetitive immunoassay based on special separation steps

4.1 Flow injection immunoassay (FIIA)

Flow injection analysis (FIA) is developed in response to the need for automated analysis, in which a sample is injected into a continuous flow of a carrier solution mixed with other continuously flowing solutions before reaching a detector. FIA-based immunoassays were developed in the early 1980s. After that, Freytag et al. (1984) applied this flow injection immunoassay (FIIA) system to noncompetitive detection of hapten molecules and also exemplified by digoxin detection. This novel approach involves reaction of the sample with an excess of enzyme-labeled antibody. And this mixture is then passed through an affinity column containing immobilized antigen. The excess unreacted antibody is captured in the

column and only Ab-Ag complex is contained in eluate. At last, substrate is added and the concentration of the original antigen is related to the signal produced by the product of the enzymatic reaction (Figure 6). As it is developed, chemiluminescence and fluorescence labels are also applied to the FIIA system for further simplifying its operation. In addition, delicate modifications of this approach have been proposed by other groups. For example, Rabbany et al. used an activated porous membrane to immobilize antibody, then the labeled antigen was applied to saturating the binding sites. After which, target analyte is passed through the membrane, and the displacement of labeled antigen is monitored downstream.

This method has been successfully applied to the detection of numerous hapten molecules and satisfactory sensitivity have been obtained: 10pM for thyroxine (Arefyev et al., 1990); 20 pM for α-(difluoromethyl) ornithine (Gunaratna et al., 1993) and 40pM for digoxigenin (Lovgren et al., 1997). The most inviting advantage of this method is the ability to realize automatic detection. For example, a semi-automated flow-through immunoassay system consisting of an amperometric immunosensor and reagent flow arrangements has been developed by Wilkins's group (Abdel-Hamid et al., 1998). However, during the development of different assays, numerous parameters must be optimized. For instance, the column capacity, related to the immobilized analyte density and column dimensions, must allow removal of all excess free labeled antibody; and the residence time of the sample in the column, related to the flow rate and column dimensions, should be suitable for total binding of free labeled antibody but not so long as to risk the dissociation of the antibody and immobilized analyte (Volland et al., 2004).

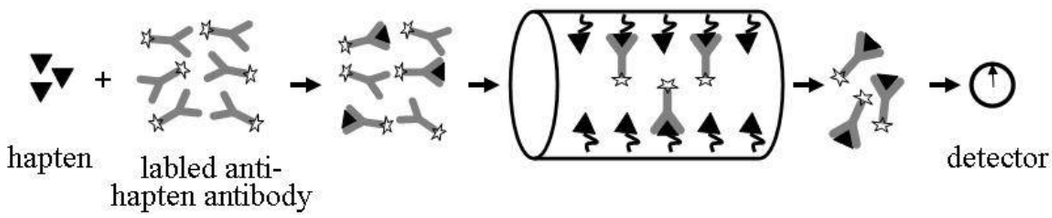

Fig. 6. Principle of FIIA. Step 1: Analyte is mixed with excess labeled antibody; Step 2: Separation of complex antibodies from free antibodies; Step 3: Signal measurement.

4.2 Affinity probe capillary electrophoresis (APCE)

A similar method, which relies on the high separating effect of capillary electrophoresis, was recently emerged for noncompetitive immunoassay of hapten molecules. This method is named as affinity probe capillary electrophoresis (APCE) and first described by Shimura and Karger (1994) using capillary isoelectric focusing for human growth hormone assay and further applied to small analytes by Hafner et al. (2000). In this case, the immune complex is separated from excess labeled antibody since the complexation of the antigen with the antibody induces small changes of electrophoretic behavior of the labeled antibody.

The performances of this method are good, with a limit of detection of 5 pM for human growth hormone (Shimura and Karger, 1994) and 10pM for digoxin (Hafner et al. 2000), respectively. However, the correct separation appears difficult for some neutral analytes and the use of a charged analogue of the analyte is necessary to differentiate the complex from the unbound antibody. In addition, this technique only analyzes one sample at a time, thus limiting its potential application to routine assay of numerous samples (Volland et al., 2004).

5. Noncompetitive immunoassays based on other principles

More recently, Tozzi's group (2002) have invented a new noncompetitive immunoassay system with blocking of unoccupied specific binding sites on sold phase and exemplified this strategy by cortisol detection. This method is based on the use of a "blocking reagent", which is able to bind to antibody sites not occupied by the analyte in a stronger way than the analyte itself. When a labeled analyte is added it substitutes the analyte in the antibody complex, but not the blocking reagent. Generally, this method includes the following steps: initially the analyte is bound to a specific binding partner, after which the unoccupied binding sites of the binding partner are inactivated by a blocker. Then, the bound analyte is dissociated from the binding partner and replaced by a labeled marker, after which the bound labeled marker is determined (Figure 7). The signal from the bound labeled marker is directly proportional to the initial amount of analyte in the sample.

As to the performance of this method, results of Anfossi et al. (2002) showed that the 3σ limit of detection (LOD, 0.2 nmol/L) obtained by the above method was 10 times lower than that obtained by the corresponding ELISA, and recoveries in saliva samples ranged from 80 to 120%. And recently, Acharya and Dhar (2008) have developed a novel broad-specific noncompetitive immunoassay for aflatoxins detection by the blocking & replacement method. In this work, an AFB1-protein conjugate was used as blocking reagent, and the limit of detection was 0.1 μg/L. Generally speaking, the key point of this method is to find an effective blocking reagent.

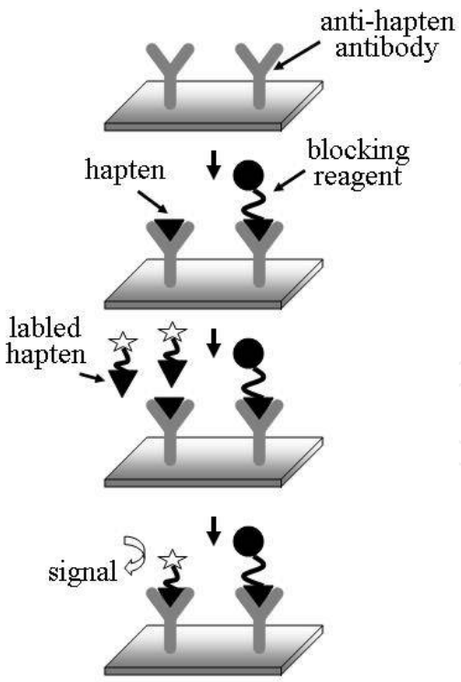

Fig. 7. Principle of the blocking & replacement method. Step 1: the analyte is bound to a immobilized specific binding partner and then blocking reagent is applied to inactivate the unoccupied binding sites; Step 2: labeled analyte is applied to replace the bound analyte; Step 3: signal is developed and measured.

6. Conclusion

From the literature and research finding above and others not concluded in this paper, a question might be put forward, which method is the best one that can be widely applied to most of the hapten molecules, it is not easy to answer this question, the answer may depend on what kind of analyte measured and the purpose of the analysis, but a comparative analysis of these methods could help researchers to choose the most suitable one for their purpose.

The methods based on chemical modification to the analytes need an extra step to immobilize the target, apart from this step, it is very complicated and hard to standardize, whether it will work as we expect is a problem. X-ray analysis of hapten-antibody complexes have indicated that small molecules are buried deep in the binding site with surface areas of 200-400 Å^2 (Arevalo et al., 1993; Xu et al., 1999). Hence, although a model study have indicated that molecules constituting of two epitopes of only 300 Å^2 separated by a spacer as small as 5 Å may be bound by two antibodies (Quinton et al., 2010), a optimization process of the spacer between biotin and analytes in Ishikawa's method or between captured antibody and analytes in SPIE-IA is required. Otherwise, these methods may not work as per the principle.

As to the methods based on unconventional antibody, the difficulties to prepare and identify these unusual antibodies are limitations for their wide application. However, once these specific antibodies have been prepared, these methods can easily be standardized. The recently developed phage display technology is a powerful and reliable technique for generating antibodies, and has successfully been applied to the preparation of anti-idiotype antibody (Goletz et al., 2002; Zhang et al., 2002; Raats et al., 2003;Coelho et al., 2004; Tometta et al., 2007) and anti-metatype antibody (Kim et al., 2009; Kim et al., 2010). Also, a phage display technology-based "split-Fv system" has recently been developed to rapidly evaluate and select antibody variable region (Fv) fragments that are suitable to open sandwich immunoassay (Aburatani et al., 2003). Along with the further improvement of the phage display technology, it will become the conventional method for the preparation of these unconventional antibodies, and then promote the wide application of idiometric assay, anti-metatype antibody based immunoassay and open-sandwich immunoassay in the future.

The methods based on special separation steps need extra equipment, but their greatest strength is the easiness to realize automatic detection. Along with the development of relevant separation technologies, this kind of method will become the choice for developing immunosensor to hapten molecules detection.

As to the method based on "blocking reagent", although various blocking reagents have been utilized in the published work, the available information concerning the strategy for their selection and the principle for their work is very limited. Hence, this type of noncompetitive immunoassay appears hard to wide application, unless a general preparation method and the inherent principle of the blocking reagent was available.

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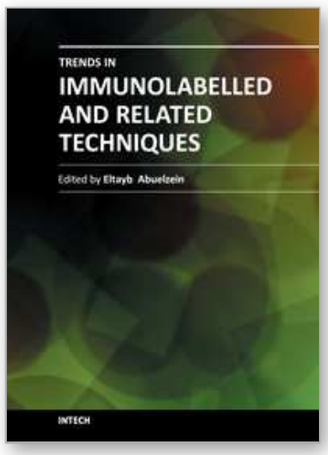

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The book is coined to provide a professional insight into the different trends of immunoassay and related techniques. It encompasses 22 chapters which are grouped into two sections. The first section consists of articles dealing with emerging uni-and-multiplex immunolabelled methods employed in the various areas of research. The second section includes review articles which introduce the researchers to some immunolabelled techniques which are of vital significance such as the use of the conjugates of the Staphylococcus aureus protein "A" and the Streptococcus Spps. protein "G" in immunolabelled assay systems, the use of bead-based assays and an overview on the laboratory assay systems. The book provides technological innovations that are expected to provide an efficient channel for developments in immunolabelled and related techniques. It is also most useful for researchers and post-graduate students, in all fields, where immunolabelled techniques are applicable.

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