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Recent Advances in the Synthesis of Artificial Antigen and Its Application in the Detection of Pesticide Residue

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Abstract: Recent advances in the research of artificial antigen have shown that artificial antigens can be valuable approach for the treatment of some diseases as well as the detection of pesticide residues. By directly/indirectly coupling hapten to an appropriated carrier (macromolecule), artificial antigen can induce animals to produce hapten-specific antibody. Based on this principle, various vaccines have been developed. More impotently, new analytical method, immunological analysis has also been established. Comparing the conventional technologies, such as chromatographic methods, this promising method offers an alternative with high specificity, sensitivity, simplicity and suitability for the analysis of a large number of samples in a short period of time. In this review, we describe the recent advances in the synthesis of artificial antigen and its application in the detection of pesticide residues.

Key words: artificial antigen, synthesis, pesticide residue, analysis

INTRODUCTION

Being a novel and promising analytical technique, immunoassay with high specificity, sensitivity, simplicity and suitability for the analysis of a large number of samples in a short period of time, has exhibited potential usage in the detection of pesticide residues [1,2]. Conventional methods employed to detect/analyze the pesticide residue are chromatographic techniques such as gas chromatography (GC) and high performance liquid chromatography (HPLC), which, however, are time consuming and require sophisticated equipment only available in well-equipped laboratories[3, 4]. In addition, the conventional methods usually require a lot of complex pre-treatment of samples. Immunoassay, however, can *in situ* detect nano-gram scale targeted haptens [5].

The critical component of an immunoassay is the production of antibodies presenting maximum specificity and sensitivity for the targeted hapten. In immunology, haptens do not allow themselves to induce an immune response because of their low molecular weight (≤ 1000 Da). They have to be covalently linked to appropriated carriers, such as protein, to form an artificial immunogenic conjugate to indirectly induce B cell to proliferate, differentiate and produce hapten-specific antibodies [6].

The design, structure-modification of haptens, the length of coupling spacers[7] and the selection of optimized carriers are very important factors in the preparation of hapten-carrier immunoconjugates, which will directly affect the production of high-quality hapten-specific antibodies[8-10]. If haptens contain active groups such as –COOH, -NH_2, -OH, *et al.*, they can directly react with the carrier proteins to form the desired artificial antigens. Or, structural modification, to introduce active groups at appropriate positions in their structures, is required. The carrier proteins [8] such as bovine serum albumin (BSA), ovalbumin (OVA), keyhole limpet hemocyanin (KLH) and human serum albumin (HSA), also, are main component. Different carrier proteins can induce different immune response. Generally, to optimize specificity of immune response, several haptens and several carrier proteins have to be tested. The spacer can be either grafted directly on the target analyte or on a hapten analog. Otherwise a total synthesis of the hapten is necessary. Herein, detailed descriptions on the synthesis of artificial antigen and its application in the detection of pesticide residues are presented.

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Design and structure modification of hapten: The desired haptens should be that hapten-carrier conjugates can induce specific immune response and produce high quality hapten-specific antibodies. While haptens being designed / selected, the final chemical structure and stereochemistry should be identical or similar with the original haptens[8]. If haptens contain active groups such as –COOH, -NH_2, they can be directly coupled with the carrier proteins. Otherwise, derives of the haptens should be prepared to introduce reactive groups into the structure. In addition, the haptens themselves should possess complicated chemical structures[9,10]. Generally, most of these desired haptens are characterized by the following aspects[11]: (1) amino group or carboxyl group or both; (2) aromatic compounds. As reported previously[11], the possibility to produce hapten-specific antibody by the artificial antigen is 1/3, if the hapten composed of aromatic compounds or contain aromatic rings. Or, the possibility is 1/11; (3) high branch; (4) heteroatom rings, since they are all highly immune activity groups. Sometime the metabolic intermediates can also be used as desired haptens which can induce bodies to produce the original hapten-specific antibodies.

The other one very important factor is the length of the spacer. If it is too long, the haptens can overlap along the spacer and change their stereo-structures. If it is too short, the carrier protein can cover the hapten and can not produce specific antibody. In addition, the spacer should be non-polar, or, they can change the distribution of the electric density of the hapten.

Selection of carrier proteins for the synthesis of artificial antigen: The use of carrier protein is not only to simply increase the molecular weight of the hapten-carrier conjugate; they can also affect the quality and quantity of immune responses. In the immunologic memory of secondary immune response they play an important role. In other words, secondary response and recalling response are also determined by the carrier proteins[10].

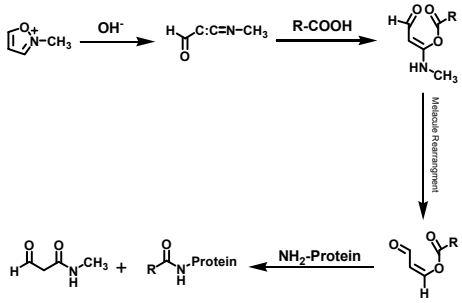
Proteins used as carriers for the preparation of artificial antigen, usually, are bovine serum albumin (BSA), ovalbumin (OVA), keyhole limpet hemocyanin (KLH), and human serum albumin (HSA). Among these proteins, BSA is the popular one because of its physical and chemical stability, not expensive, easily available, more lysine residues and more amino groups. In addition, BSA can also present excellent solubility under various pH value and ionic strength. It can react with the targeted haptens in organic solvents such as pyridine and N, N-dimethylformamide (DMF), and the immune conjugate can well dissolve in the reaction mixture after reaction is done.

Generally, the carrier should be heterogenous with the experimental animals, since it is easier to induce strong immune response and to produce high-titer and hapten-specific antibody. More heterogeneity can produce higher quality antibody. Inspiringly, recent research results exhibited that: (1) homologous protein-carrier could also induce immune response and produce hapten-specific antibody[6]; (2) polypeptide such as poly-L-lysine could also be employed as carrier. Comparing with the conventional protein carrier, poly-L-lysine possesses higher amino density and the coupling reactions carried out more easily. Due to its simply straight chain structure, its immunogenicity is also low[12,13].

Methods for the coupling of haptens to carrier proteins: Binding the desired haptens to the carriers is the critical step in the synthesis of artificial antigen. If the haptens possess active groups such as –COOH, -OH, and -NH_2, as described above, they can directly react with the carrier protein. Or, structural modifications are required[14].

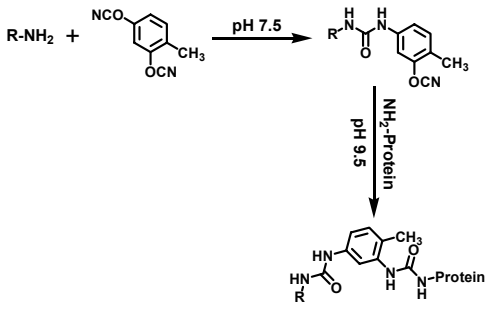
Based on the chemical and stereo-structure of haptens, various synthetic approaches were employed:

(1) Carboxyl-contained haptens, such as fluoroquinolones, polyethers, cephalosporins and peptides, can be coupled with the carrier proteins using N-hydroxysuccinimide active ester/carbon-diimine and Woodward reagent protocol.



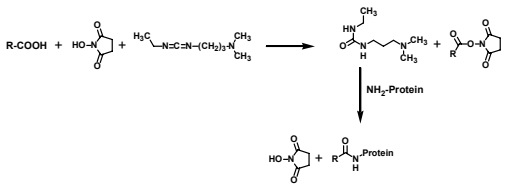
Scheme 1. Woodward Reagent Protocol[15]

(2) Amino-contained haptens, such as sulfanilamides, aminoglycosides, β-lactams, acheomycins, benzenimidazoles, benzenethylamines, fluoroquinolones, can employ glutaraldehyde, diisocyanate, halonitrobenzene, thiophosgenation, diimine ester, and diazotization protocol.



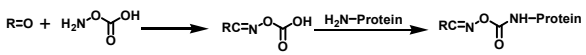
Scheme 2. Diisocyanate Protocol[16]

(3) Hydroxyl-contained haptens, such as chloramphenicols, aminoglycosides, macrolides, avermectins, steroid hormones, and benzenethylamines, can be directly connected to the carrier proteins through succinic anhydride or azobenzoic acid protocol.



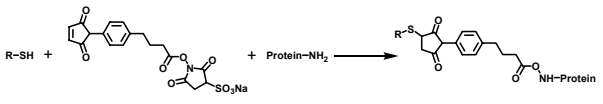
Scheme 3. Succinic Anhydride Protocol[16]

(4) Carbonyl-contained haptens (ketone or aldehyde), such as streptomycins, acheomycins, macrolides, fluoroquinolones, steroid hormones, usually use amino-ox-acetic acid protocol.



Scheme 4. Amino-ox-acetic Acid Protocol[16]

(5)Mercapto-contained haptens can employ homogeneous or heterogeneous difunction reagents to synthesize the immunoconjugates.



Scheme 5. Succinic Anhydride Protocol[17]

Purification of artificial antigens: Before immunizing animals using the artificial hapten-protein conjugate to get the desired antibody, purification is necessary since the unreacted hapten molecules, salts and other impurity will affect the quality of antibody and the research results. Usually, dialysis and chromatography will be employed. Comparing the two techniques, dialysis will take long time (usually 2 days or more). However, it can obtain well purified antigen and the process is simple which suitable for various laboratories. Xu C L *et al*[18] used dialysis to purify their artificial antigens in PBS (pH7.4, 2 d, 4□); Liu Y *et al*[19] replaced the dialyzed solution with DI water and physiological saline, also get desired antigens; Chromatography such as ion-exchange gel chromatography, gel chromatography need sophisticated equipments and the process is complicated. Anyway, how to select the best purification technique and the specific process is depended on the substrates. For examples, Yang Y *et al* [20] employed ion-exchanged gel chromatography and Li L D *et al*[21] used Sephadex G-75 chromatography to purify their artificial antigen, respectively.

Identification of artificial antigen: Prior to further study using artificial antigen, identification is very important. It composes of two aspects [17, 18, 22-25]: the desired haptens have been successfully connected on the carriers; Determination of the binding ratio of desired haptens to carriers. The popular techniques employed to identification of artificial antigen are as followings:

(1) UV spectrometry: UV spectrometry is the common and very useful analytical technique. According to the UV spectral differences of the immune conjugate, hapten and the carrier protein, the binding ratio can be obtained based on the known analytical equations[15].

Binding Ratio = $(\varepsilon_{conjugate} - \varepsilon_{carrier}) / \varepsilon_{hapten}$.

(2) Isotope-labeling: The hapten was labeled with an appropriate isotope when the artificial antigen was synthesized. After the reaction was done, dialysis was employed to remove the un-reacted hapten. Then to determine the difference of radiation intensity between the dialysis sample and un-dialysis sample. The binding ratio can be obtained.

(3) Others: Sometimes, the determining method depends on the samples, e. g. phosphorus method employed in the analysis of phosphorus-contained pesticide.

APPLICATIONS OF ARTIFICIAL ANTIGENS IN THE DETECTION OF PESTICIDE RESIDUES

Immunoassay technique was first employed to determine pesticide residue by Hammock and

Mumma[26]. Comparing the conventional approach, such as high performance liquid chromatogram (HPLC), gas chromatography (GC), mass spectrogram (MS), nuclear magnetic resonance (NMR), immunoassay is a novel and promising analytical technique with high specificity, sensitivity, simplicity and suitability for the analysis of a large number of samples in a short period of time, and can *in situ* detect nano-gram scale targeted compounds[27].

Also, based on antibody technology, a lot of immunoassay approaches have been developed, such as fluorescence immunoassay (FIA), radio immunoassay (RIA), enzyme immunoassay (EIA). According to the applications, fluorescence immunoassay can be divided into two major kinds: (1) fluorescent antibody technique in which antibodies was labeled with fluorescent substances; (2) time-resolved fluorescence immunoassay (TRFIA) and fluorescence polarization immunoassay (FHA), which were used to detect trace liquid substance. Hu X Q *et al*[28] employed radio immunoassay to analysis the carbofuran residue and find that this method has high sensitivity and low cross-reaction. Enzyme-linked immuno-sorbent assay (ELISA) was also widely used in these fields [29-32]. Antonio A [30] utilized this technology to analysis carbamate pesticide: carbofuran, in which monoclonal antibodies (MAbs) from BSA-hapten immunoconjugate immunized mice, was employed. The detection limit can obtain ng/mL. Based on this technology, many immune kits, such as benhexachlor, clofenotane, sumithion, alkron, methylamine, etc., have also been developed and commercial available. The sensitivity can reach ppm-scale. In same instances, it can reach ppb-scale[27]. Table 1 summarized the current applications of this technique.

Table 1: Monoclonal Antibodies Applied in Pesticide Residue immunoassay

Pesticides	Determination methods	Samples	Low limit	Refs.
Diflubenzuron	ElA	Environment	0.05ug/dm^{-3}	[33]
Propoxur	ELISA		0.32ug/kg	[34]
Atrazine	RIA	Water		[35]
Carbaryl	Immune Sensor	Water and syrup	0.029 μg l^{-1}	[36]
Carbofuran	Homogeneous immunoassay	water	□0.1 ug/L	[37]
Permetrin	ELISA	Environment	10ng/ml	[38]
Endosulfan and Endosulfan Sulfate	ELISA	Wheat/Tea	0.4ug/L, 2mg/kg	[39,40]
Ethyl parathion	ELISA	Water and soil	30ng/L	[41]
Polychlorobiphel	ELISA	Soil	265ng/kg	[42]
DDT	Immunosensor	Environment	20 ng L^{-1}	[43]
chlorpyrifos	Immunosensor	Environment	50 ng L^{-1}	[43]
carbaryl	Immunosensor	Environment	0.9μg L^{-1}	[43]
Chlorpyrifos	ELISA	food	0.32ng/ml	[44]
pyrethroid	EMIT	sample	2-5 ng ml^{-1}	[45]

CONCLUSION

Immunoassay is a novel and promising analytical technique with high specificity, sensitivity, simplicity and suitability for the analysis of a large number of samples in a short period of time. Many challenges remain, however, in the development of universal platforms for the analysis of various pesticide residues[46,47]. With new technique such as microfluidics and integrated Microfluidics[48,49] emerge, immunoassay will take on new look in the future. Anyway, significant efforts from various disciplines also need to be devoted to this field[50,51].

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