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Recent Advance of Electrochemical Immunosensor for Pesticide Residues Detection

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Abstract: Electrochemical immunosensors have emerged as a highly sensitive and rapid technique for the detection of specific pesticide based on the immunological interactions between the binding biomolecules (antibody or hapten) onto the transducer interface and the target analyte (hapten or antibody). In terms of the development of electrochemical immunosensors, the antibody (Ab) /hapten immobilization onto a transducer or a support matrice is a crucial step in improving the analytical performance, such as response, reproducibility, stability, selectivity and regeneration. This paper presents an overview of different types of electrochemical immunosensors and their principles and various immobilization protocols used for formation of a biorecognition interface, such as physical adsorption, covalent coupling, entrapment, oriented immobilization, self-assembled monolayer, nanoparticles. In addition, future prospects toward the development of electrochemical immunosensor systems are discussed. *Copyright © 2014 IFSA Publishing, S. L.*

Keywords: Electrochemical immunosensor, Pesticide residues, Immobilization protocols.

1. Introduction

Various pesticides derived from synthetic chemicals are used for crops protection worldwide to enhancement their quality and yield as well as to extend storage lifetime [1, 2]. Among all the pesticides, organochlorine, organophosphorus, carbamate and pyrethroid pesticides are widely used in agriculture due to their high insecticidal activity [3]. Although the use of pesticide s has led to the enhancement of quality and quantity of food worldwide, however, their use in crop production often contaminates food crops [4]. Their indiscriminate use is posing a potential hazard to human health [5, 6]. By transformation through the food chain, their bio-accumulation in animal and human body and eventually show their adverse effects, like: cancer, hormone disruption, birth defect and neurological effects [6, 7]. Thus, the analysis of foods to assess the presence of pesticide residues is crucial importance for ensuring food safety and quality, especially in field of on-line applications.

Traditional analytical methods involving gas chromatography (GC) [8], high performance liquid chromatography (HPLC) [9], capillary electrophoresis (CE) [10] and mass spectrometry (MS) [11] have been developed for detection of pesticides residues. These methods are very sensitive, reliable and standardized techniques, but have drawbacks such as extensive time consumption, expensive instrumentation and complicated pretreatment procedure. Moreover, they can only be performed by highly trained technicians and are not convenient for on-site or in-field

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detection, which limit their application for real-time detection [12-14]. Therefore, the developments of rapid determination and reliable quantification detection methods have become increasingly important for human health and environment protection.

In this respect, biosensors are regarded as suitable complementary tools for the real-time and on-site detection of pesticide residues in real samples and have been an active research area for some years [15]. Biosensor supply advantages such as simplicity, sensitivity, selectivity, high efficiency, miniaturization and fast response times [16-18]. Biosensors have been defined as analytical devices which comprise of two components: biomaterial and transducer. Namely, biosensors combine biorecognition elements with physical transducers for detection of the target compounds [19, 20]. The biological recognition system translates information from the biochemical domain, usually an analyte concentration, into a chemical or physical output signal with a defined sensitivity. The transducer part of the sensor serves to transfer the signal from the output domain of the recognition system, mostly to the electrical domain. Various biological recognition elements involving cofactors, enzymes, antibodies, microorganisms, organelles, tissues, and cells from higher organisms have been used in the fabrication of biosensors [21, 22]. Among the biosensor technology available for the detection of pesticides, enzymatic methods based on acetylcholinesterase (AChE) inhibition have attracted public attention because of their reliability, fast response, high sensitivity and miniature size [23, 24]. Nevertheless, enzyme-based biosensors have a major drawback: different enzyme-inhibiting insecticides cannot be measured selectively [25].

Immunosensors are biosensors that are provided with the selectivity in view of immunological interactions and also being proposed and proved to be efficient analytical devices for monitoring of organic pollutants in food and the environment [26]. Immunosensor, with simple fabrication, easy operation, rapid response and high sensitivity, provides concentration-dependent signals by using antibodies (Ab) or antigens (Ag) as the specific sensing element [27, 28]. The immunosensor's advantage of high selectivity is attributed to the molecular recognition characteristics of an Ab, which binds reversibly with a specific hapten. The measurement of the immunosensor consists of two processes: a molecular recognition process at the surface of receptor and a signal-transfer process. Firstly, in solution phase, Ab molecules interact specifically and reversibly with a hapten to form an immune complex (Ab-hapten) according to the following equilibrium equation:

$$Ab + hapten \underset{Kd}{\overset{Ka}{\rightleftharpoons}} Ab - hapten,$$

where K_a and K_d are the rate constants for association and dissociation, respectively [29, 30]. They are appropriate for identification of a single pesticide or, in some cases, small groups of similar pesticides in environmental monitoring as they are rapid, specific, sensitive and cost-effective analytical devices [31]. And then the transducer changes the detection response into physical or chemical signals. Depending on the method of signal transduction, immunosensors may be divided into four basic groups: electrochemical, optical, piezoelectric and thermometric [32]. Among these different types of immunosensors, electrochemical immunosensors have received increasing attention due to their low cost, high sensitivity, high speed and easy signal amplification [14]. Excellent reviews that focused on electrochemical immunosensors for detection of different pesticide molecules have been reported [29, 33, 34]. However, there is a time gap between current status in the field and the most recent reviews. Therefore, in this review, we provide an overview of the research carried out during the last 7 years relative to electrochemical immunosensor for pesticide residues detection. We will review different types of electrochemical immunosensors and their principles, various immobilization protocols used for formation of a biorecognition interface, and applications of these immunosensors in pesticide analysis. We also will discuss the trends and challenges associated with designing a reliable electrochemical immunosensor for practical applications in detail.

2. Electrochemical Immunosensors and Classification

Electrochemical immunosensor is a kind immunosensor of measuring the electrochemical signal changes before and after the immunological interactions. It serves as a self-contained integrated device which is available to provide specific quantitative or semi-quantitative analysis [35, 36]. The hapten or antibody acted as biorecognition elements contacts directly with electrochemical transducer element. The concentration signals of some or certain kinds of chemical substances are transformed into relevant electrical signal through the transducer element [37]. Electrochemical transducers, classified as amperometric, potentiometric, conductometric, capacitive and impedimetric measure changes in current, potential (voltage), conductance, capacitance and impedance respectively [38-40].

Amperometric immunosensor is based on the measurement of the current resulting from the electrochemical oxidation or reduction of an electroactive species. It is usually performed by maintaining a constant potential at a Pt, Au or C based working electrode or on array of electrodes with respect to a reference electrode, which may also act as the auxiliary electrode, if currents are low (from 10^{-9} to 10^{-6} A) [37].

Potentiometric immunosensor measurements are performed by assaying the potential difference between either an indicator and a reference electrode, or two reference electrodes separated by a permselective membrane. Several ion (F^-, I^-, CN^+, Na^+, K^+, Ca^{2+}, NH_4^+) or gas (CO_2, NH_3) selective electrodes are available to be used as the substrate electrode. Also a biocatalyst layer is often placed adjacent to the potentiometric detector. The response of potentiometric biocatalytic immunosensors is, as for amperometric immunosensors, either steady-state or transient, but it is never an equilibrium response [41].

Conductometric immunosensor is performed by monitoring the changes of conductivity of the solutions or biological membrane receptors caused by immunological interactions. Because the sensitivity of the measurement is hindered by the parallel conductance of the sample solution, usually a differential measurement is performed between a sensor with enzyme and an identical one without enzyme [42, 43].

Capacitive immunosensor is an analysis approach based on the determination of the changes of interface capacitance. When the electrodes are inserted into the solution, the behaviour of the interface between the electrode and the solution is analogous to a parallel-plate capacitor. The movement of the Ab–hapten compounds and the liquids lead to the changes of the dielectric constant between the electric double layers [44].

Recently, impedimetric immunosensors have attained particular interest due to their reduced matrix interferences, relatively simple electrical measurements and possibility of automation. Electrochemical (faradic) impedance spectroscopy (EIS) measurements are often fitted with Randles equivalent electric circuit. Impedimetric signal is based on the change in one of these equivalent electric circuit parameters upon analyte binding [45].

In addition, electrochemical immunosensors are also divided into two categories: labeled type and label-free type. Impedimetric or amperometric immunosensor in label or label-free format and label-free format are often applied for the detection of pesticide residues. Some examples of electrochemical immunosensors for the determination of pesticide residues are presented in Table 1.

3. Immobilization Protocols

In terms of the development of electrochemical immunosensors, the Ab/hapten immobilization onto a transducer or a support matrice is a crucial step in improving the analytical performance, such as response, reproducibility, stability, selectivity and regeneration. Impressive literatures on methods of immobilization and relevant immunosensor development have appeared. The Ab/hapten, with high biological activity, can be immobilized in a thin layer at the transducer surface by using different procedures. A good immobilization method should meet the following requirements: 1) be simple and fast; 2) produce immobilized reagents that are stable and do not leach from the substrate; and 3) maintains its biological integrity flexibility, and proper active site orientation toward the bulk solution. Thereby, Ab/hapten immobilization has been a critical issue in immunosensor technology [29, 63]. Ab/hapten immobilization methods include physical adsorption, covalent bonding, entrapment, oriented immobilization and self-assembled monolayer (SAM).

3.1. Physical Adsorption

Generally, physical adsorption is based on the interactions such as van der Waals forces, electrostatic interactions and hydrophobic interactions between the Ab/hapten and the transducer. It is easy and simple, and it is generally non-destructive for Ab/hapten activity because this technique does not involve any functionalization of the electrode materials or covalent links. Nevertheless, nonspecific attractive forces easily causes Ab/hapten desorption [64]. Besides, the reduction of biological activity of the immobilized Ab/hapten may occur as a result of an inappropriate orientation and weak attachment caused by physical adsorption [65]. This leads to the decrease of the performance of the immunosensor.

Gobi et al., created a functional sensing surface of the immunosensor by immobilizing an ovalbumin conjugate of 2,4-D (2,4-D-OVA) by simple physical adsorption on a surface plasmon resonance (SPR) thin-film gold chip. It has been established that the Au surface of the sensor chip was completely covered by 2,4-D-OVA up to a monomolecular layer and that the 2,4-D-OVA immobilized sensor chip was highly resistive to non-specific binding of proteins [66].

Pividori et al, proposed the immunosensor by immobilizing the tissue transglutaminase (tTG) from guinea pig liver through physical adsorption on graphite-epoxy composite (GEC) electrodes [67]. After the reaction with the human serum (containing the specific antibodies in the case of celiac disease), the electrode was incubated with different kinds of secondary labeled antibodies, namely, horseradish peroxidase (HRP)-conjugated goat antibodies to human whole immunoglobulins (Igs), to human IgG, and finally to human IgA (Fig. 1).

3.2. Covalent Coupling

Covalent coupling method is most widely used for immobilization of Ab/hapten. The specific Ab/hapten can be covalently linked to the surfaces of a transducer through formation of a stable covalent bond between functional groups of Ab/hapten and the

transducer. Covalent modification requires a bifunctional cross-linker, which has one functional group that reacts with a base support, and another group that interacts with an active group of Ab/hapten [68]. However, the immobilization by covalent coupling may result in the random orientation of Ab/hapten, decreasing the activity of Ab/hapten and their binding affinity for target molecules. In addition, blocking steps are usually necessary to limit the non-specific binding site.

Table 1. Some examples of electrochemical immunosensors for the determination of pesticide residues.

Pesticide	Detector	Label	Electrode Modification	Detection limit	Sample	Reference
Chlorpyrifos	Amperometric	free	chlorpyrifos/BSA/anti-chlorpyrifos/GA/MWCNTs-THI-CHIT/GCE	0.046 ng/mL	cabbage, lettuce, Chinese chives	[46]
Chlorpyrifos	Amperometric	free	chlorpyrifos/BSA/anti-chl-orpyrifos/{MWCNTs-COOH-CHIT}$_2$/GNPs/GCE	0.069 ng/mL	cabbage, lettuce, carrot, green peppers	[47]
Carbofuran	Amperometric	free	carbofuran/BSA/Ab/{DpAu/DM DPSE}$_2$/Au electrode	0.06 ng/mL	lettuce, cabbage, pepper, tomato, chive, strawberry	[48]
Carbofuran	Amperometric	free	carbofuran/BSA/Ab/GNPs/ TU/GNPs/GCE	0.11 ng/mL	cabbage	[49]
Carbofuran	Amperometric	free	carbofuran/BSA/Ab/SiSG/GCE	0.33 ng/mL	cabbage lettuce	[14]
Carbofuran	Amperometric	free	carbofuran/BSA/Ab/PA/ DpAu/Au electrode	0.192 ng/mL	Chinese chive, celery, cabbage	[50]
Carbofuran	Amperometric	free	carbofuran/HRP/Ab/GNPs/ L-cysteine/Au electrode	40 ng/mL	-	[51]
Atrazine	Impedimetric	free	interdigitated microelectrodes (IDμE)	0.19μg/L	red wine	[52]
Atrazine	Impedimetric	free	a two coplanar non-passivated interdigitated metallic μ-electrodes	-	wine grapes	[53]
Atrazine	Impedimetric	free	Atrazine/BSA/ histidine-Ab/poly NTA -Cu^{2+}/Au electrode	10 pg/mL	PBS (pH 7)	[54]
Atrazine	Impedimetric	free	atrazine/bio-Fab/ neutravidin/ Gold/MHDA+biotinyl-PE	20 ng/mL	PBS (pH 7)	[55]
Atrazine	Amperometric	HRP	atrazine/atrazine-BSA/Ab/ immobilon membrane/H_2O_2 electrode	5.0×10^{-11}M	buffalo milk, vegetal samples	[56]
Atrazine	Amperometric	HRP	atrazine/atrazine-HRP scAb/PANI/ PVSA/SPE	0.1 ng/mL	-	[57]
Atrazine	Amperometric	HRP	atrazine-HRP/Ab/ ProtA-GEB	6 μg/mL	orange juices	[58]
Atrazine	Conducto-metric	GNPs	Ab_2/Ab_1/atrazine/ GPTS/ N-acetylcysteamine/IDμE	0.1 ng/mL	buffers	[59]
Fenvalerate	Impedimetric	free	fenvalerate/OVA/Ab/ glutaraldehyde/chitosan/GCE	0.80 μg/L	tea	[60]
2,4-D	Potentiometric	HRP	2,4-D-HRP/Ab/GA/Graphite electrode	40 ng/mL	water, serum	[61]
Simazine	Potentiometric	HRP	simazine-HRP/ glycine/Ab/PA/ GA/ISFET	1.25 ng/mL	-	[62]

An example of where this approach has been exploited is that the Ab immobilization was carried out by using carboxylic groups activated with EDC/NHS as a cross-linker to connect the NH_2- group of the antibody with the surface of carboxylized transducer (Fig. 2) [47]. To construct the immunosensor, carboxylated multiwall carbon nanotubes (MWCNTs-COOH) was first dispersed with chitosan (CHIT) to obtain a homogeneous solution and then it was dropped on the surface of glassy carbon electrode (GCE) which was first modified by the electrodeposition of gold nanoparticles (GNPs). And then the modified GCE was immersed in the poly (diallyldimethylammonium chloride) (PDDA) which was positively charged.

Wenbin Liang et al., has developed a highly sensitive, label-free amperometric sensor for immunoassays. They synthesized novel, functionalized gold nanoparticles (SV-GNP) by covalently capping the surface of gold nanoparticles (GNP) with 1,1'-bis-(2-mercapto)-4,4'-bipyridinium dibromide, a kind of sulfhyrdryl viologen (SV) (Fig. 3). After coating the SV-GNP onto a glassy carbon electrode surface; the resulting electrode core could then adsorb a suitable antibody in a second step to afford the desired immunosensor [69].

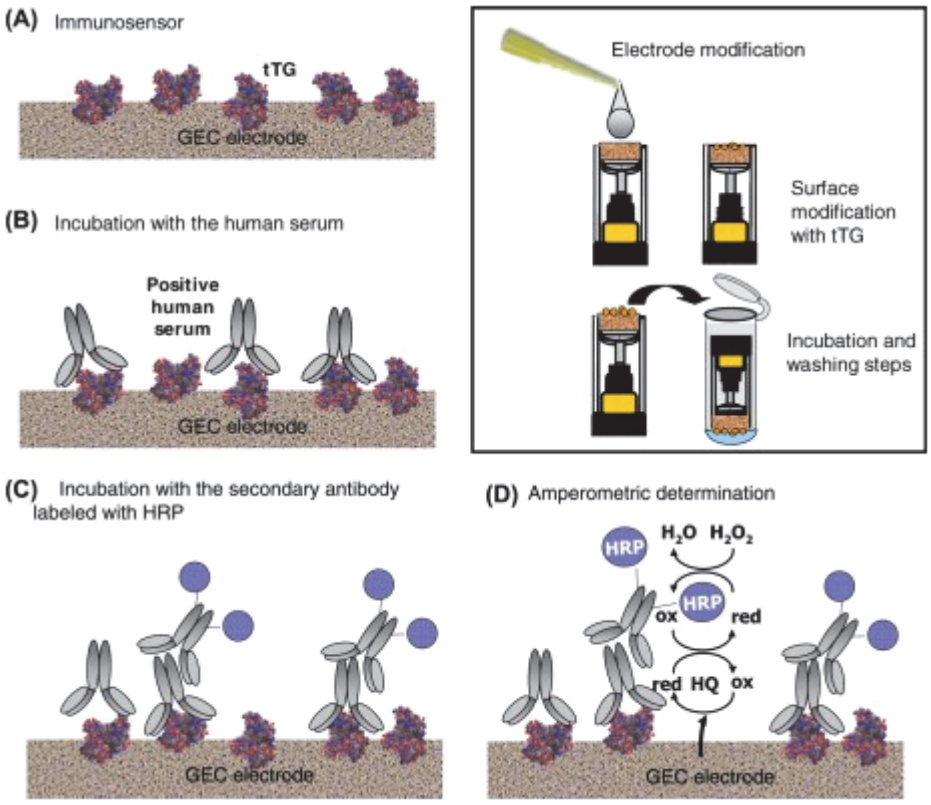

Fig. 1. Schematic representation of GEC material showing the adsorbed tTG (A), immunological reaction with the human serum (B), immunological reaction with the secondary antibody (C), and amperometric determination (D). The inset shows the surface modification for the preparation of the immunosensor as well as the common steps for electrode modification and washing steps.

Another example is that Valera et al., has designed and developed a novel conductometric immunosensor based on antibodies labelled with gold nanoparticles for atrazine detection [59]. The immunosensor consists of an array of two coplanar non-passivated interdigitated metallic μ-electrodes (IDμE) and immunoreagents specifically developed to detect this pesticide. The chemical recognition layer was covalent immobilized on the interdigital space (Fig. 4).

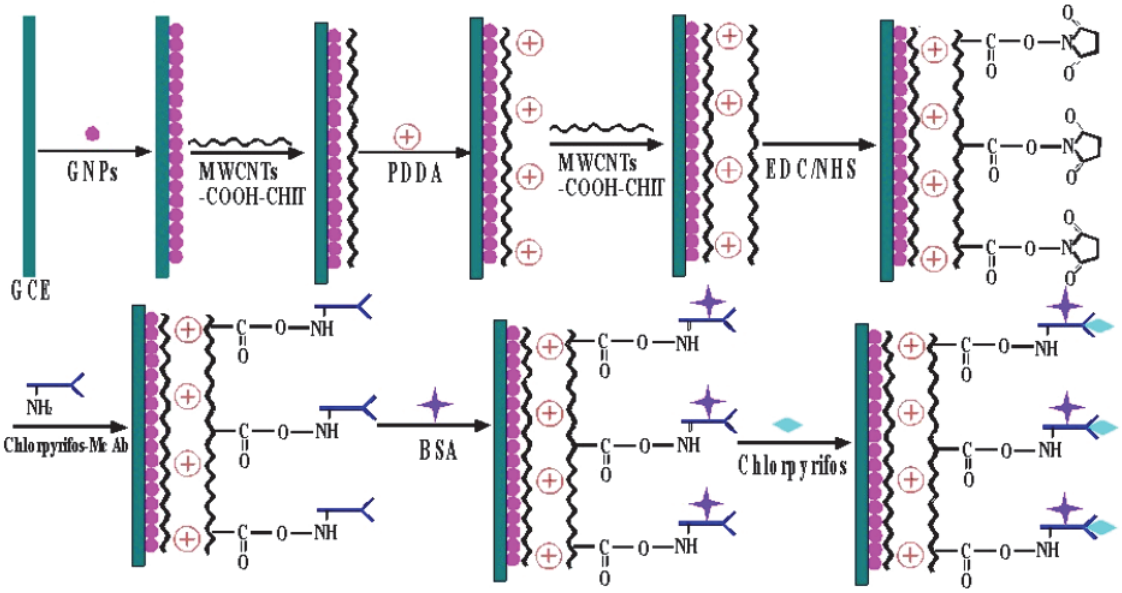

Fig. 2. Schematic illustration of the stepwise immunosensor fabrication process (covalent immoblization of antibody onto carboxylized transducer activated with EDC/NHS as a cross-linker).

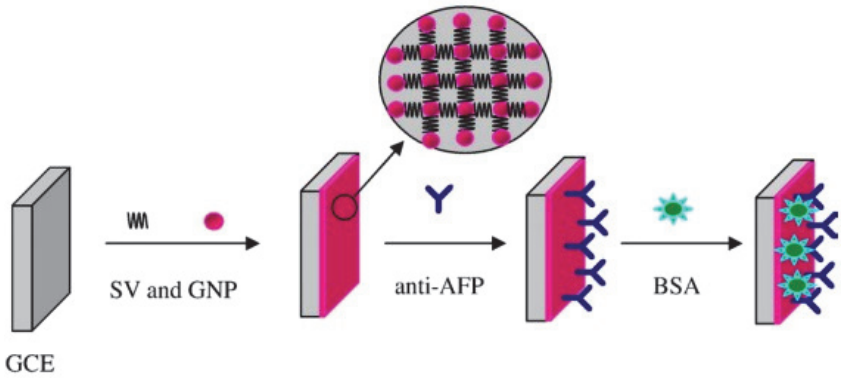

Fig. 3. The schematic diagram of the anti-AFP/SV-GNP modified electrode procedures.

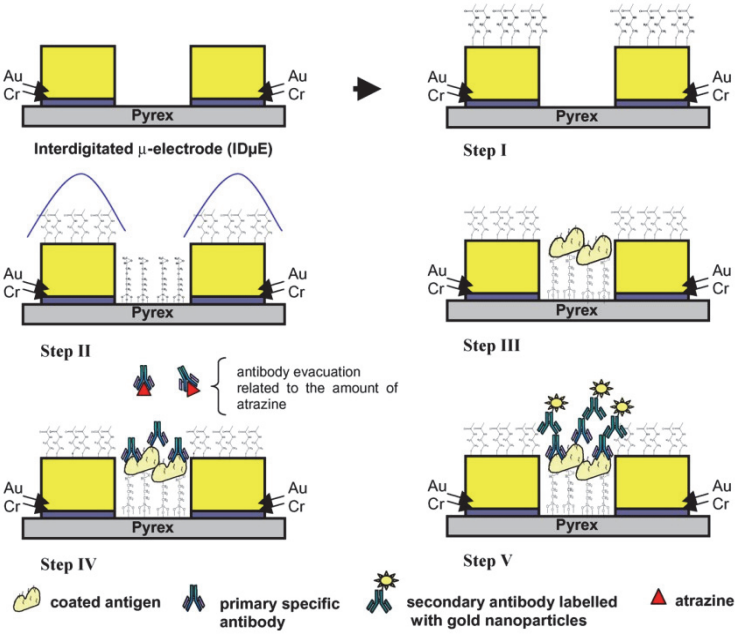

Fig. 4. Schematic diagram of the complete assay system performed on the IDμEs: Step I, protection of interdigitated μ-electrodes with N-acetylcysteamine; Step II, immunosensor surface functionalization with GPTS; Step III, covalent immobilization of the antigen on the IDμE; Step IV, specific primary antibody (Ab_1) capture in the competition step; Step V, secondary labelled with gold antibody (Ab_2) capture. In the Step IV, an amount of the specific antibody (Ab_1) is bounded on the coated antigen layer, whereas other amount is evacuated of the IDμEs, this amount is related to the atrazine concentration. In the Step V, an amount of the secondary antibody (Ab_2) is bounded on the specific antibodies.

3.3. Entrapment

The reagent, physically trapped within a porous matrix, usually do not leach out or leach out very slowly when an appropriate entrapment procedure is applied. Physical immobilization methods such as entrapment in sol-gel matrices and photo-polymerized monomers have been used for electrochemical immunosensors.

3.3.1. Sol-Gel Entrapment

Sol-gel technology provides a unique means to prepare a three-dimensional network suited for the encapsulation of a variety of biomolecules. The sol-gel process is a synthetic inorganic procedure, well known in material chemistry. Recent development in the area of electrochemical immunosensors with sol-gel encapsulation of Ab/hapten as an immobilization matrix is very encouraging and offers potential advantages. The sol-gel forms at low temperatures and offers negligible swelling. It exhibits tunable pore size and pore distribution, which allows small molecules and ions to diffuse into the matrix while larger biomolecules remain trapped in the pores, simplicity of preparation without any kinds of modifications [70]. Nevertheless, the sol-gel method has the drawbacks of low response (as long as several minutes) in aqueous media and slightly change biological activities due to reduced degree of freedom in the pores and/or interactions with the inner surface of the pores [70].

Sun et al., developed a novel label-free impedance immunosensor for the direct detection of carbofuran was developed using silica sol-gel (SiSG) as immobilizing agent. The anti-carbofuran monoclonal antibody was immobilized onto the surface of the glassy carbon electrode (GCE) in aperture of SiSG. The high stability of the silica sol-gel layer provided a congenial microenvironment, increased the surface coverage around antibody, and could accelerate electron transfer [71].

Wei et al., developed a disposable amperometric immunosensor for sensitive detection of chlorpyrifos-methyl (CM) by combining dual signal amplification of platinum colloid with an enzymatic catalytic reaction. The immunosensor was fabricated by modification of the screen-printed carbon electrodes (SPCE) with nanocomposites made by skillful doping of bovine serum albumin conjugated chlorpyrifos-methyl antigen (BSA-Ag) and platinum colloid into silica sol-gel [72].

3.3.2. Electrically Conducting Polymers Entrapment

Taking into account that most of the transducers used for immunosensor construction are constituted of conductive surfaces, the immobilization of biomolecules in or on electrogenerated polymer films is one of the few methods that have aroused a considerable attention because of its electrochemical addressing property [73]. This entrapment process, theoretically, should occur without chemical reaction between the electrically conducting polymer (CP) films and the biomolecules preserving thus their biological activity.

Lin et al., constructed an immunosensor based on an antibody/conducting polymer/TiO_2 nanowire film. TiO_2 nanowires (NWs) were made by the hydrothermal synthesis method and directly spin-coated on a gold microelectrode patterned surface. Conducting polymer polypyrrole propylic acid (PPa) and antibody composite films were electrochemically polymerized on patterned NWs using pyrrole propylic acid (Pa) and anti-rabbit IgG (1 Ab) mixture solutions. The surface properties in the designed immunosensor during the preparation processes of NWs, PPa (PPa/TiO_2 NWs), anti-rabbit IgG (PPa-1Ab/TiO_2 NWs immunosensor), and the measurement of rabbit IgG (2 Ab/PPa-1 Ab/TiO_2 NWs immunosensor) were recorded and expressed by the changes in current-voltage (I-V) (Fig. 5) [74].

A new electropolymerizable monomer, [N-(6-(4-hydroxy-6-isopropylamino-1,3,5-triazin-2-ylamino) hexyl) 5-hydroxy-1,4-naphthoquinone-3-propionamide], has been designed for use in a label-free electrochemical immunosensor when polymerized on an electrode and coupled with a monoclonal anti-atrazine antibody. This monomer contains three functional groups: hydroxyl group for electropolymerization, quinone group for its transduction capability, and hydroxyatrazine as bioreceptor element (Fig. 6) [75].

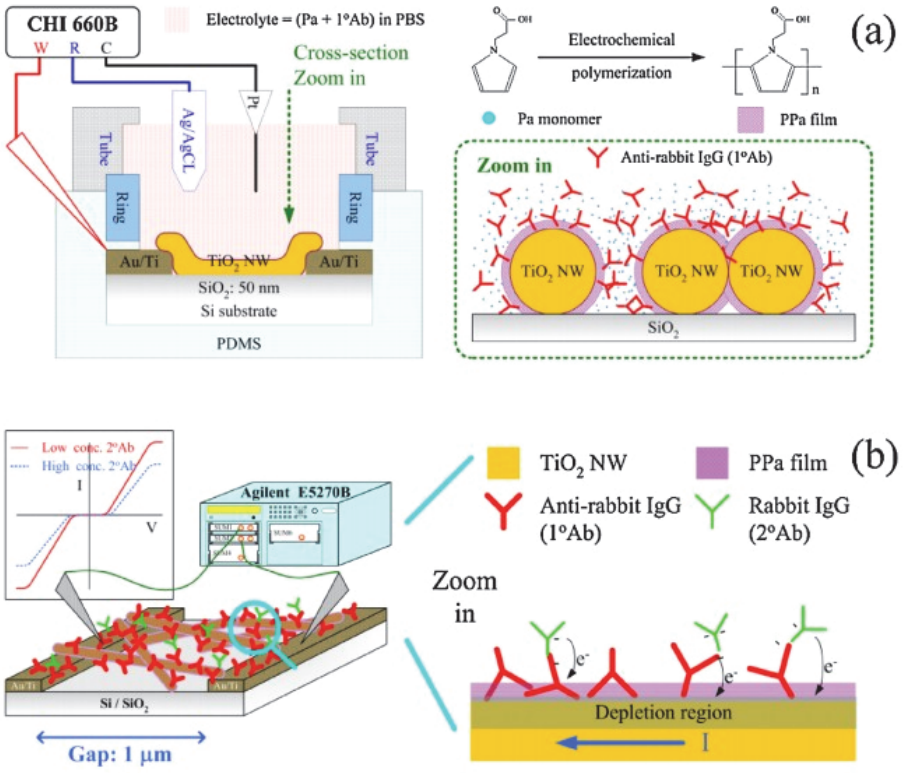

Fig. 5. (a) The experiment setup of the electrochemical polymerization of polypyrrole propylic acid/anti-rabbit IgG immobilized TiO_2 NWs immunosensor system. (b) I-V changes were measured for characterized electron transfer and depletion region difference between the modified layers PPa-1 Ab TiO_2 NW immunosensor.

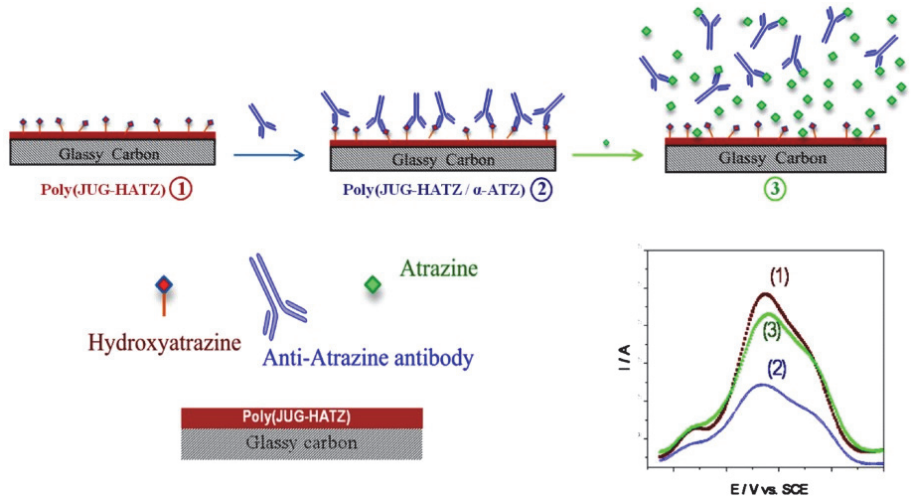

Fig. 6. Strategy for the electrochemical detection of atrazine based on the change in electroactivity of polymer film, poly (JUG-HATZ). SWV recorded with (1) poly(JUG-HATZ)-modified electrode; (2) after complexation with α-ATZ, poly(JUG-HATZ/α-ATZ)-modified electrode; (3) after addition of ATZ in solution.

3.4. Oriented Immobilization

The aforementioned immobilized methods such as adsorption, covalent binding, entrapment within a polymeric matrix and crossing-linking may involve multiple-point attachment, loss of biomolecule activity, biomolecule leaking, random biomolecule orientation and large diffusion barriers, which result in the poorly sensitive, unstable and with long response times of the immunosensor. Antibody-binding proteins (protein A, G, A/G, and L) have been widely used to overcome the above-metioned drawbacks [76]. These proteins specifically bind the Fc region of an Ab and, thus, properly orient the bound Ab for optimal binding of the hapten [77]. The bound antibodies can fully retain their activity and function for the reason that the antibody-binding proteins capture antibodies without any chemical modifications. However, oriented immobilization method has limitations; for example, these proteins are susceptible to denaturation and are difficult to apply in site-specific modifications [78].

In the work of Liu et al., Au nanoparticles and Protein G thiolated with LC-SPDP were immobilized on Au electrodes to act as a composite scaffold for an estradiol electrochemical immunosensor. A large surface area on the immunosensor surface was provided by the deposited Au nanoparticles to enhance the quantity of Protein G-(LC-SPDP) and capture antibody required in developing an immunosensor for estradiol. Compared with passive adsorption, Protein G-(LC-SPDP) facilitated immobilisation of capture antibody with improved orientation [79].

Another example is that: Fowler et al., employed a self-assembled layer of Protein G that was thiolated with succinimidyl-6-[3'-(2-pyridyldithio)-propionamido] hexanoate for the orientation-controlled immobilisation of a capture antibody in a flow-type amperometric immunosensor based on a two-site sandwich immunoassay. The orientation of the analyte-specific capture antibody on the surface of electrochemical immunosensors plays an important role on their overall performance (Fig. 7) [80].

Sun et al., developed an amperometric immunosensor based on protein A/deposited gold nanocrystals (DpAu) for the ultrasensitive detection of carbofuran residues [50]. Firstly, DpAu were electrodeposited onto the Au electrode surface to absorb protein A (PA) and improve the electrode conductivity. Then PA was dropped onto the surface of DpAu film, used for binding antibody Fc fragments. Due to PA's specially binding ability of the Fc fragment of the antibody molecules, the application of PA could improves the capacity of antibody, thus enhance the detection sensitivity (Fig. 8).

3.5. Self-Assembled Monolayer (SAM)

Self-assembled monolayers (SAMs) have drawn public attention because of their potential applications in biosensors, biomolecular electronics and nanotechnology. SAM as a plat form can link biomolecules either using direct chemical linkages or by encapsulation with the help of polymeric supports. SAMs can be formed by chemisorption of organic molecules containing groups like thiols, disulphides, amines, acids or silanes, on desired surfaces to fabricate immunosensors [81]. SAM, with good stability, uniform surface structure and relative ease of varying thickness, is suitable for development of biosensors. The employment of an appropriate SAM contributes to the oriented and controlled immobilization of biomolecules [82, 83]. SAMs can be used to prevent protein denaturation at an electrode surface and for enhancing stability of biomolecules [84].

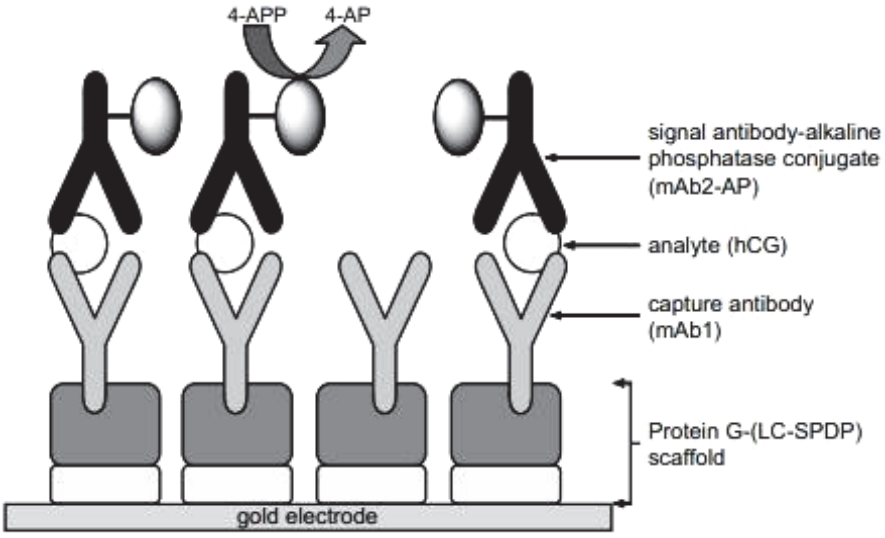

Fig. 7. Schematic representation of immunosensors presented in this work.

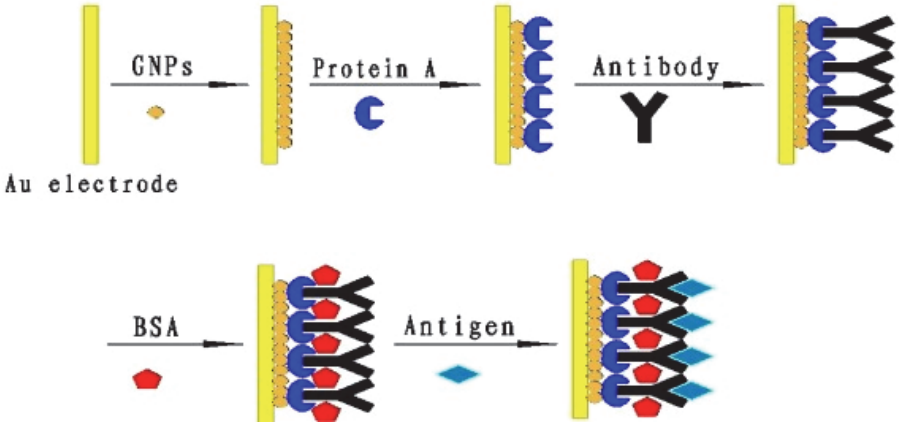

Fig. 8. Schematic illustration of the stepwise immunosensor fabrication process.

Just as Xu et al. presented, a novel amperometric immunosensor for the detection of α-fetoprotein (AFP) based on the integration of microelectronic technology, mixed self-assembled monolayers (mixed SAMs), gold nanoparticles (nanogold) and enzyme amplification was developed [85]. Using mixed SAMs and nanogold, a mixed monolayer comprising cysteamine and 1,6-hexanedithiol was formed on the working electrode surface to assemble nanogold and further to immobilize AFP antibody for detecting AFP in human serum samples (Fig. 9).

Sun et al., proposed a novel label-free amp erometric immunosensor for the detection of carbofuran residues. The deposited gold nanocrystals (DpAu)/4,4'-thiobisbe nzenethiol (DMDPSE) multilayers ({DpAu/DM DPSE}n) membranes were used for modifying Au electrode to fabricate amperometric immunosensor [48]. DpAu/DMDPSE multilayers were modified alternatively to form multiple membranes through Au-S bond by layer-by-layer self-assembly technology. The presence of the multiple membranes not only promoted electron transfer reactions, but also increased the surface area to capture a large amount of antibodies, thus increased detection sensitivity (Fig. 10).

Another example is the development of a new electrochemical immunosensor based on graphene oxide (GO) initiated silver enhancement for the detection of protein biomarker platelet-derived growth factor BB (PDGF-BB) [86]. The immunosensor was fabricated based on the traditional sandwich protocol using secondary anti-PDGF-BB antibody (Ab_2) modified GO as label. Gold electrode was first modified with self-assembled monolayer (SAM) to block the electron transfer between the electrode and $K_3Fe(CN)_6$ solution. After the immobilization of primary anti-PDGF-BB antibody (Ab_1) onto electrode via aminidation to the carboxylic group of SAM and the formation of the sandwich immuno-structure onto electrode surface, the electrode was immersed into silver enhancement solution for silver deposition. The deposited metal silver onto GO then mediated electron transfer across the SAM, producing redox current (Fig. 11).

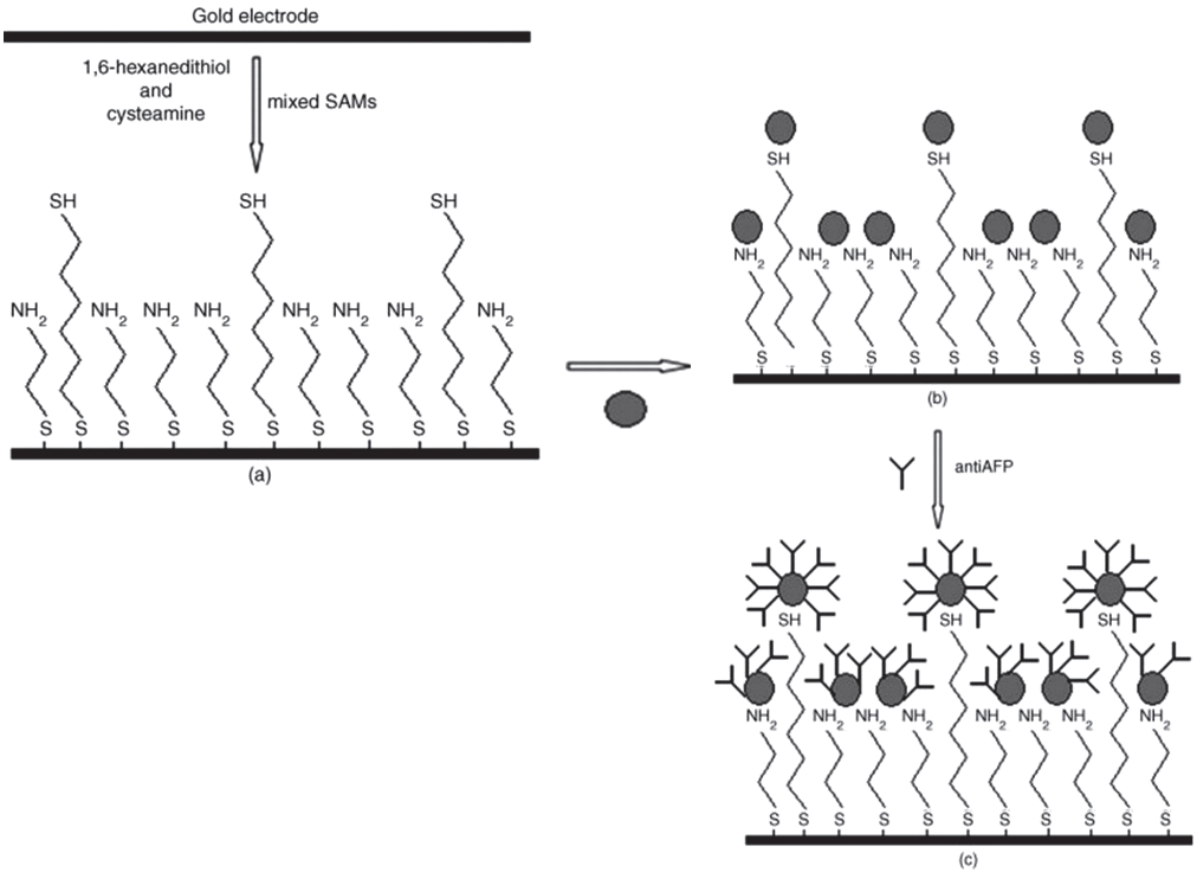

Fig. 9. The schematic illustration of the stepwise antibody immobilization process: (a) formation of mixed SAMs; (b) formation of nanogold monolayer; (c) antiAFP loading.

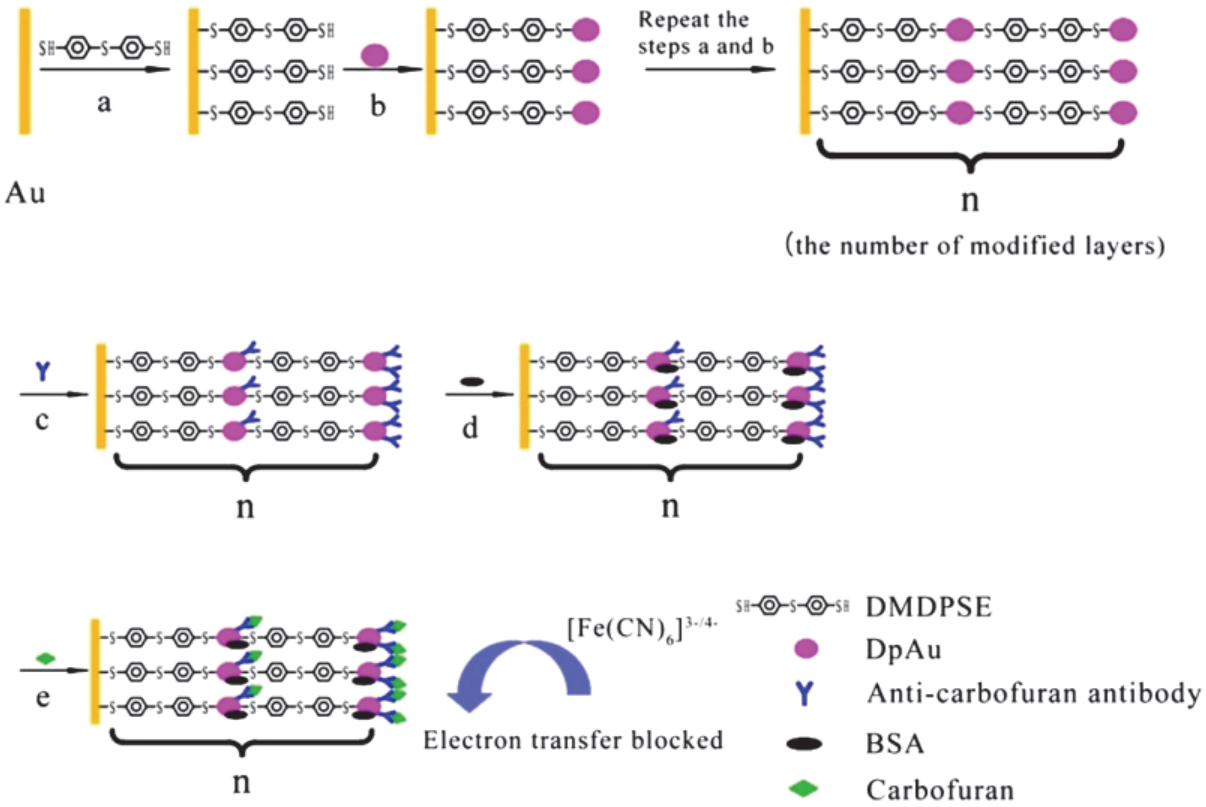

Fig. 10. Fabrication process of the stepwise the stepwise immunosensor based on deposited gold nanocrystals/ 4,4'-thiobisbenzenethiol for determination of carbofuran.

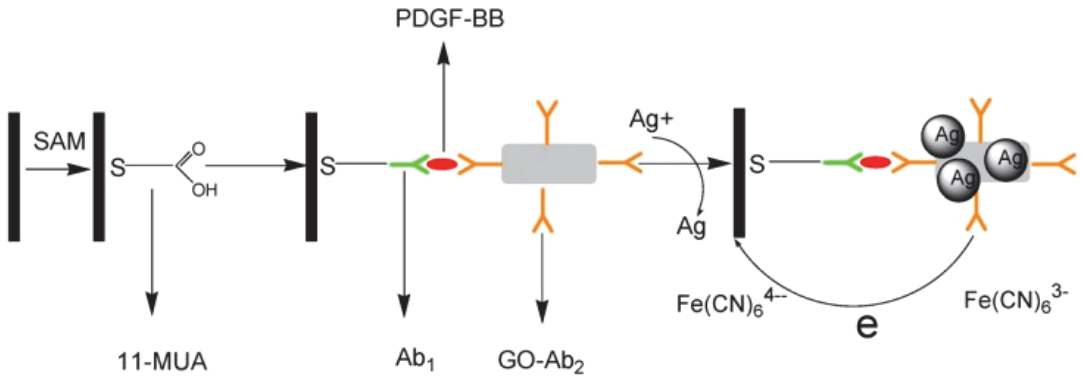

Fig. 11. Schematic representation of the immunosensor preparation procedure.

3.6. Nanoparticles Acted as Immobilized Materials

The immobilization of immunological molecules is the key step of the fabrication of immunosensor. There are different degrees of drawbacks of the common immobilization methods such as physical adsorption, covalent bonding, self-assembled monolayer et al. With the development of nanotechnology, various kinds of nanomaterials have been widely applied in immunosensor fabrication, such as metal nanoparticles [87], multiwall carbon nanotubes (MWCNTs) [88, 89], graphene sheets (GS), and so on. The application of nanomaterials can improve the performance of the immunosensors.

Gold nanoparticles (GNPs) have been widely used in the biosensors for detection of pesticide residues due to their high electron-transfer ability and large specific surface area. GNPs can adsorb biomolecules and play an important role in the immobilization of biomolecules for biosensor construction [90-92]. MWCNTs have received increasing interest due to their great chemical stability, large aspect ratio, excellent electrical conductivity, and extremely high mechanical strength and stiffness and demonstrated to be an excellent material for the development of electrochemical sensors [93-95]. GS, a novel one-atom-thick and two-dimensional graphitic carbon system, has attracted great interests in various fields, including nanoelectronics, sensors, nanocomposites, and energy storage and conversion [96] since its discovery by Geim and coworkers in 2004 [97]. GS has the characteristics including large specific surface area, remarkable mechanical stiffness, high elasticity and excellent conductivity [98].

Sun et al., developed a sensitive, label-free electrochemical immunosensor for the direct determination of carbofuran. A gold electrode was modified with gold nanoparticles (DpAu), 4,4'-Thiobisbenzenethiol-3-Mercaptopropionic acid-gold colloidal nanoparticles (DMDPSE-MPA-GNPs) nanocomposite film and the antibody (Ab) was orientedly immobilized with protein A (SPA). DpAu was used to enhance the electroactivity and stability of the immunosensor. The porous composite nanocomposite film provided many reactive groups to cross-link immobilize SPA. The formation of a self-assembled SPA layer was employed to the electrode surface to increase the binding capacity of antibody (Fig. 12) [25].

Recently, a novel competitive immunosensor was developed as a model system using anti-human serum albumin (HSA)-conjugated gold nanoparticles (AuNPs) as an electrochemical label and mobile crystalline material-41 (MCM-41)-polyvinyl alcohol (PVA) mesoporous nanocomposite as an immobilization platform. MCM-41 was employed for immobilization of HSA, and PVA was used as a sensor platform for better immobilization of MCM-41-HSA on the screen-printed carbon electrode (SPCE) surface. By conjugation of a homemade mouse monoclonal antibody (mAb) against HSA to AuNPs (Ab-AuNPs), a competitive immunoreaction was produced (Fig. 13) [99].

Another example based on nanoparticles technology is reported for sensitive detection of α-fetoprotein (AFP, as a model) using carbon nanoparticles (CNPs)-functionalized biomimetic interface as immunosensing probe and irregular-shaped gold nanoparticles (ISNGs)-labeled horseradish peroxidase- anti-AFP con-jugates (HRP-anti-AFP-ISNG) as trace label [100]. The low-toxic and high-conductive CNPs provided a high capacity nanoparticulate immobilization surface and a facile pathway for electron transfer. In comparison with conventional label methods, i.e. spherical gold nanoparticles-labeled HRP-anti -AFP and HRP-labeled anti-AFP, the electrochemical immunosensor using HRP-anti-AFP-ISNGs as trace labels exhibited high bioelectrocatalytic response toward enzyme substrate and a wide dynamic range from 0.02 to 4.0 ng/mL with a low detection limit of 10 pg/mL toward AFP (at 3σ) (Fig. 14).

In addition, Liu et al., reported an ultrasensitive electrochemical immunosensor based on chitosan-iron oxide-poly(amino-amine) dendrimers-gold nanoparticles ($CS\text{-}Fe_3O_4$-PAMAM-GNPs) nanocomposites and horseradish peroxidase-multiwall carbon nanotubes-antibody (HRP-MWCNTs-Ab) bioconjugates for the detection of

salbutamol (SAL) [101]. CS-Fe_3O_4-PAMAM-GNPs nanocomposites as immobilization matrix were used to enhance the electroactivity and stability of the electrode.

HRP-MWCNTs-Ab bioconjugates as label were used to improve catalytic activity for hydrogen reduction of the electrode. Under the optimized conditions, a calibration plot for SAL was obtained with a linear range between 0.11 ng/mL and 1061 ng/mL (r = 0.9984). The detection limit was 0.06 ng/mL. The immunosensor was examined in real samples for the analysis of SAL (Fig. 15).

A novel electrochemical sensing interface, electrodeposition of gold-platinum alloy nanoparticles (Au-PtNPs) on carbon nanotubes, was proposed and used to fabricate a label-free amperometric immunosensor. On the one hand, the multiwalled carbon nanotubes (MWCNTs) could increase active area of the electrode and enhance the electron transfer ability between the electrode and redox probe; on the other hand, the Au-PtNPs not only could be used to assemble biomolecules with bioactivity kept well, but also could further facilitate the shuttle of electrons. In the meanwhile, horseradish peroxidase (HRP) instead of bovine serum albumin (BSA) was employed to block the possible remaining active sites and avoid the nonspecific adsorption. With the synergetic catalysis effect of Au-PtNPs and HRP towards the reduction of hydrogen peroxide (H_2O_2), the signal could be amplified and the sensitivity could be enhanced (Fig. 16) [102].

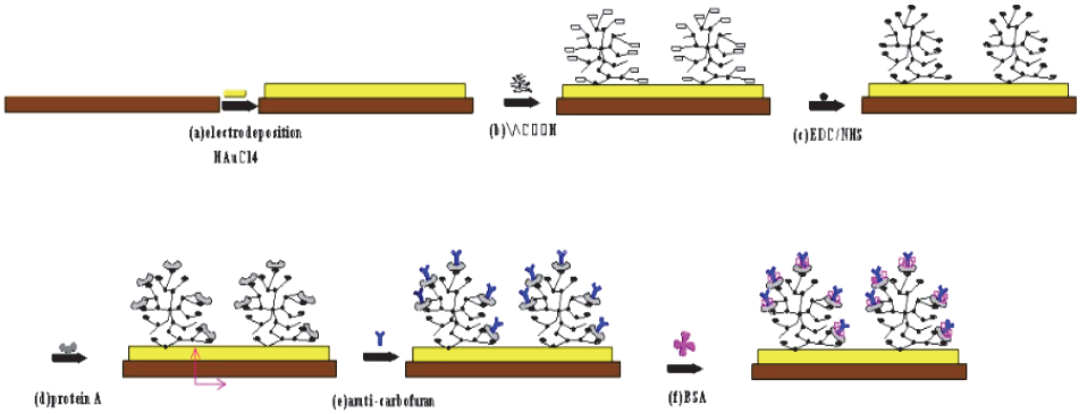

Fig. 12. Schematic illustration of the stepwise procedure of the immunosensor preparation: (a) electrodepositio DpAu, (b) fabrication of DMDPSE-MPA-GNPs, (c) form an NHS ester, (d) the immobilization of SPA, (e) immobilization of anti-carbofuran (f) BSA blocking.

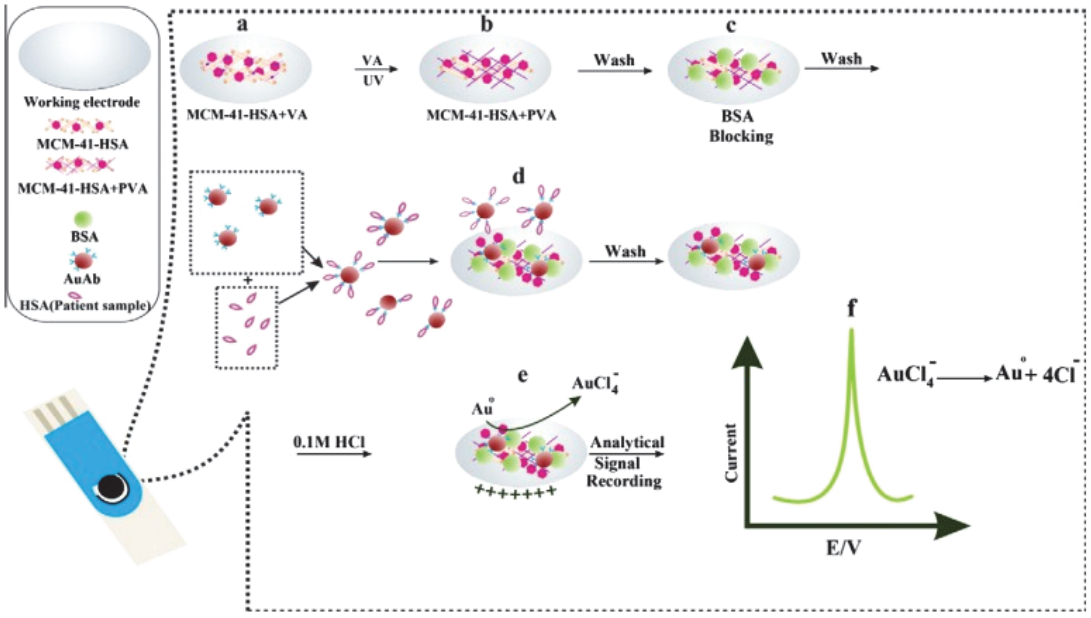

Fig. 13. Schematic illustration of preparation process of the direct competitive assay-based electrochemical immunosensor: (a) dropping of MCM-41-HSA-PVA bionanocomposite on the working electrode of SPCE; (b) photopolymerization of PVA under UV exposure (20 min, 220-250 nm); (c) blocking of uncovered surface by BSA; (d) incubation of the patient/standard samples with Ab-AuNPs; (e) preoxidation of AuNPs at the constant potential of +1.3 V for 80 s in 0.1 M HCl; (f) voltammetric measurement.

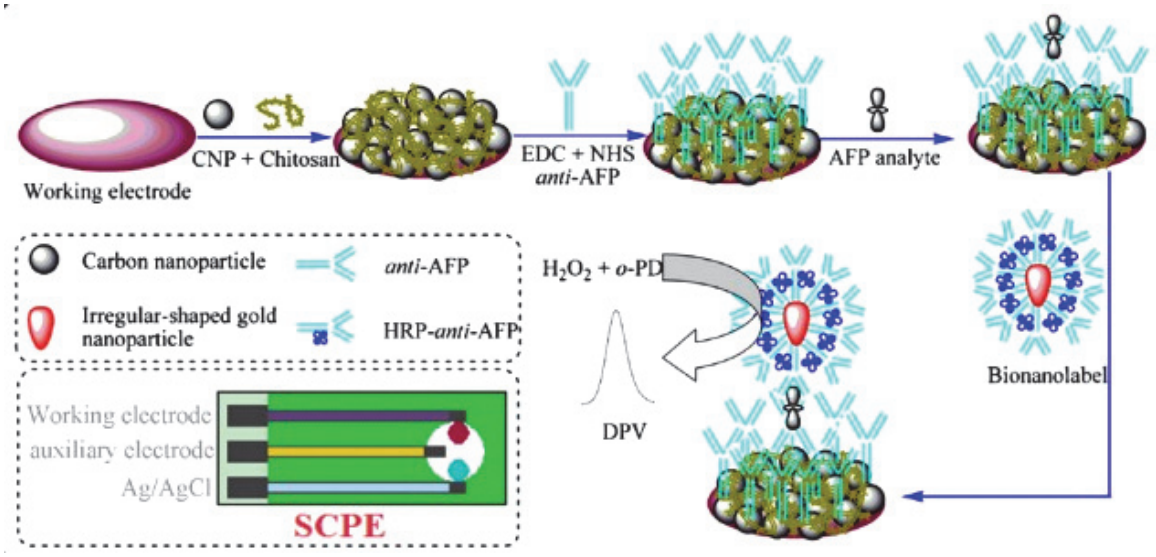

Fig. 14. Fabrication process and detection principle of the sandwich-type electrochemical immunosensor using carbon nanoparticles as matrix and functional irregular-shaped gold nanoparticles as trace label.

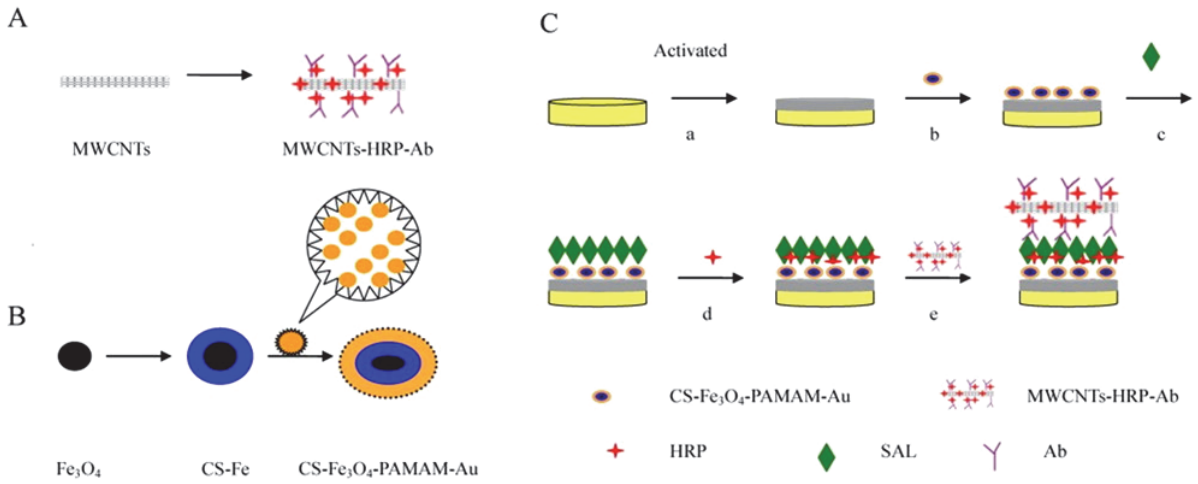

Fig. 15. (a) The synthesis process of the HRP-MWCNTs-Ab bioconjugates; (b) the synthesis process of the CS-Fe_3O_4-PAMAM-GNPs nanocomposites; (c) schematic illustration of the stepwise immunosensor fabrication process.

Yu et al., proposed a novel label-free immunosensor for the sensitive detection of kanamycin using silver hybridized mesoporous ferroferric oxide nanoparticles (Ag@Fe_3O_4 NPs) and thionine mixed graphene sheet (TH-GS) [103]. TH was used as an electron transfer mediator. The electrical signal was greatly improved in the presence of GS due to its good electron-transfer ability. With the advantages of large specific surface area and excellent electrical conductivity, Ag@Fe_3O_4 NPs could immobilize more antibodies of kanamycin and promote the electron transfer (Fig. 17).

Besides, Zhu et al., developed a novel amperometric immunosensor for the detection of carbofuran. Firstly, multiwall carbon nanotubes (MWCNTs) and graphene sheets-ethylene imine polymer-Au (GS-PEI-Au) nanocomposites were modified onto the surface of a glass carbon electrode (GCE) via self-assembly. The nanocomposites can increase the surface area of the GCE to capture a large amount of antibody as well as produce a synergic effect in the electrochemical performance. Then the electrode was coated with gold nanoparticles-antibody conjugation (AuNPs-Ab) and blocked with BSA. The monoclonal antibody against carbofuran was immobilized covalently to AuNPs by glutathione used as a spacer arm (Fig. 18) [104].

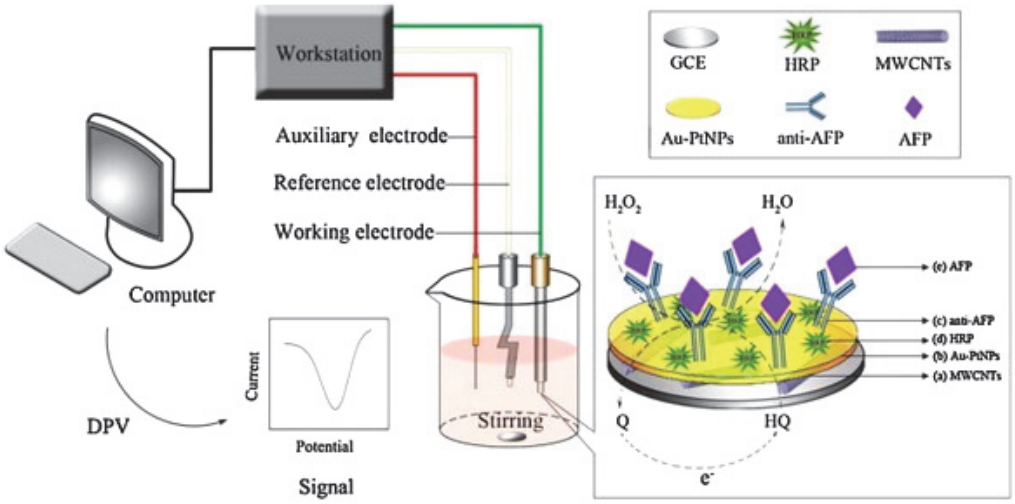

Fig. 16. Schematic illustration of the stepwise immunosensor fabrication process: (a) adsorption of MWCNTs film, (b) electrodeposition of gold-platinum alloy nanoparticles, (c) anti-AFP loading, (d) HRP blocking, (e) AFP loading.

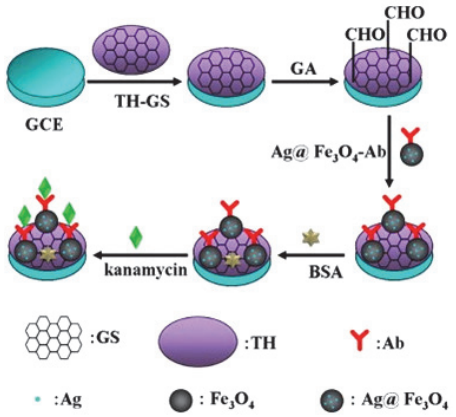

Fig. 17. Schematic illustration of the stepwise procedure for the fabrication of the immunosensor.

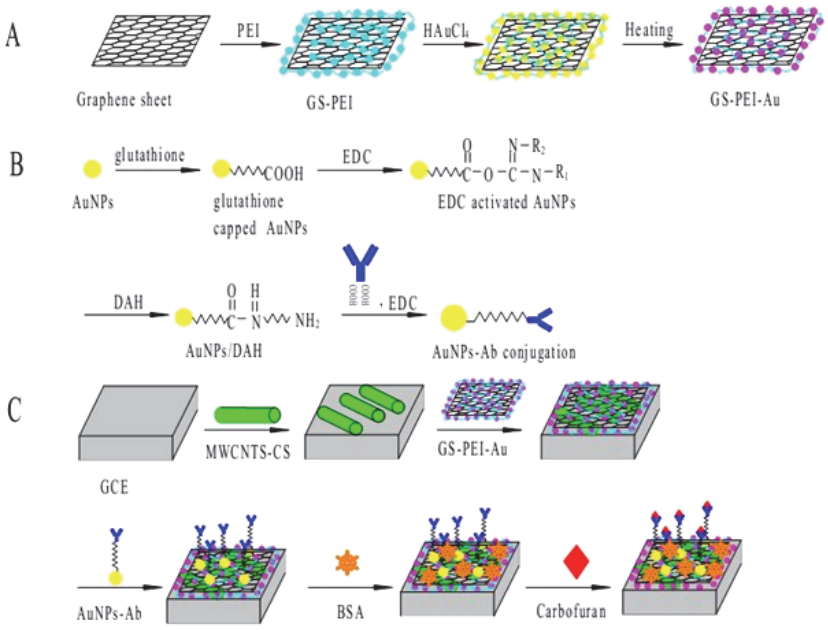

Fig. 18. (A) The experimental procedure in the preparation of GS-PEI-Au nanocomposites; (B) Steps involved in the preparation of AuNPs-Ab conjugation; (C) Construction of the immunosensor.

4. New Trends and Challenges

4.1. Miniaturization

Miniaturization is expected to have a marked impact in the development and applications of the immunosensors. New analytical approaches are oriented to the development of portable systems with high accuracy, low-cost, short-time response, and that can provide qualitative information about the composition of a sample with minimum preparation. Future advances in immobilization will focus on addressable locations of biorecognition elements on micro or nano-sensor arrays. A microelectrode, which dimensions are in the micrometer range, will replace common electrodes due to its miniaturization, faster response, greater sensitivity and increased response per unit electrode surface area (greater current density, increasing the signal-to-noise ratio). Ramón-Azcón et al, have proposed an impedimetric immunosensor based on interdigitated microelectrodes (IDμE) for the determination of atrazine residues in food samples [52].

4.2. High Throughput of Detection Samples

The ability to construct arrays of microelectrode will likely allow current multianalyte detection of several compounds to be expanded to accommodate the analysis of perhaps hundreds or thousands of separate compounds. The combination of microelectrode and microfluidic devices as analytical systems will become a trend to realize high throughput because of their significant reduction of reagent consumption and low operating costs as well as high throughput capability.

4.3. Integration of Detection System

One of the challenges that must be met for this type of system would be the development of parallel computational methods to convert electronic responses for each analyte into meaningful concentration data. In recent years, to improve sample throughput or allow online monitoring of the inhibition processes, silicabased monoliths, coupled with micro-fluidic devices, have been applied as an attractive alternative to packed columns for the analysis of proteins, peptides and nucleic acids with special features of low diffusion resistance during mass transfer, controllable porosity and low back pressure compared to packed columns.

4.4. Real Samples Detections

The majority of immunosensors reported up to now have been designed for detection of pesticides in water samples. Application to other matrices such as food samples (fruits and vegetates) has been restricted due to the problems related to the use of these devices in the presence of organic solvents extracts. Thus, these devices suffered from a low sensitivity and have not been used for the quantification of pesticides in solid samples. So simple and appropriate sample pretreatment methods for immunosensors need further research, for that they hinder the real application of immunosensors.

4.5. Simultaneous Analysis of Multicomponent

Most immunosensors can only detect one kind of analyte, which restricts the promotion and application of the immunosensors. Therefore, multifunctional immunosensor will become a trend for detecting different analytes simultaneously at food samples.

Acknowledgements

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