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Contents

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Research Articles

Research in Nanothermometry. Part 8. Summary
Svyatoslav Yatsyshyn, Bohdan Stadnyk, Yaroslav Lutsyk, Olena Basalkevych 1

Temperature Measurement and Control Based on LabVIEW and SMS
D. Mercy, Ashok M., Karthick N., Rajamanickam M. 16

Theoretical Considerations of Fiber Optic Sensors for Thermal Sensing Under Low and High Temperatures Effects
Ahmed Nabih Zaki Rashed 27

Effect of Firing Temperature on the Micro Structural Parameters of Synthesized Zinc Oxide Thick Film Resistors Deposited by Screen Printing Method
Ratan Y. Borse, Vaishali. T. Salunke and Jalinder Ambekar 45

Design and Analysis of Bulk Micromachined Piezoresistive MEMS Accelerometer for Concrete SHM Applications
S. Kavitha, R. Joseph Daniel, K.Sumangala 62

Lumped Parameter Modeling of Absolute and Differential Micro Pressure Sensors
S. Meenatchisundaram, Ashwin Simha, Mukund Kumar Menon, S. M. Kulkarni and Somashekara Bhat 76

Geometrical Amplification of SMA Actuator Displacement Using Externally Actuated Beam
Elwaleed Awad Khidir, Nik Abdullah Mohamed, Sallehuddin Mohamed Haris 92

High Accuracy Resolver to Digital Converter Based on Modified Angle Tracking Observer Method
Chandra Mohan Reddy Sivappagari, Nagabhushan Raju Konduru 101

Development of Single Place Multiple Obstacle Avoidable System for Guarded Tele-operated Trolley, a Service Robot Using Single Ultrasonic Sensor
Subrata Chottopadhaya and Soumendra Nath Kundu 113

A Real Time Radio Frequency Field Imaging for Detection of Impurities in Liquids
Mohammad Mezaael. 123

Design and Simulation of a Microgripper with the Ability of Releasing Nano Particles by Vibrating End-Effectors
Hamed Demaghsi, Hadi Mirzajani, Ehsan Atashzaban, Habib Badri Ghavifekr 131

Linear Resistivity Response with Relative Humidity of Gd Doped Magnesium Ferrite
Jyoti Shah, Amish G. Joshi and R. K. Kotnala 143

Quartz Crystal Microbalance DNA Based Biosensor for the Detection of *Brugia malayi*
Thongchai Kaewphinit, Somchai Santiwatanakul, Supatra Areekit and Kosum Chansiri. 153
161

Recent Advance in Antibody or Hapten Immobilization Protocols of Electrochemical Immunosensor for Detetion of Pesticide Residues
Ying Zhu, Xia Sun, Xiangyou Wang .....

PSoC Based Blood Coagulation Instrument for the Analysis of PT & APTT
Raghunathan R., Neelamegam P. and Murugananthan K...... 182

L-Asparaginase Extracted From *Capsicum annum L* and Development of Asparagine Biosensor for Leukemia
Kuldeep Kumar and Shefali Walia..... 192

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Digital Sensors and Sensor Systems: Practical Design

Sergey Y. Yurish



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Conference Announcement



Topic E2: Transportation & Mobility

The Euromat conference series, organised by the Federation of European Materials Societies (FEMS), is one of the largest events of its kind in Europe, covering the full width of materials science and technology. We would like to direct your attention to the following Symposia which are focussing specifically on transport applications:

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Recent Advance in Antibody or Hapten Immobilization Protocols of Electrochemical Immunosensor for Detetion of Pesticide Residues

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Abstract: Electrochemical immunosensors have been used to detect or quantify the specific pesticide based on the binding biomolecules (Ab or hapten) onto the transducer surface to interact with the analyte of target (hapten or Ab), resulting in a detectable signal. In terms of the development of electrochemical immunosensor, the Ab/hapten immobilization onto a transducer or a support matrice is a key step in optimizing the analytical performance, such as response, reproducibility, stability, selectivity and regeneration. This paper presents an overview of electrochemical immunosensors for the detection of pesticides residues and various immobilization protocols of Ab or hapten, such as physical adsorption, covalent coupling, entrapment, oriented immobilization, avidin–biotin affinity reaction, self-assembled monolayer, nanoparticles. Future prospects toward the immobilization protocols for the development of electrochemical immunosensor are discussed. *Copyright © 2012 IFSA.*

Keywords: Electrochemical immunosensor, Pesticide residues, Immobilization protocols.

1. Introduction

Pesticides derived from synthetic chemicals are essential inputs in increasing agricultural production by preventing control pest and crop losses before and after harvesting. One-third reduction in crop yield would be happened if pesticides are not used against pest [1, 2].

Depending upon the species of pest, these chemicals have been divided into groups (e.g., herbicides, insecticides, fungicides, rodenticides, and nematocides). However, their indiscriminate use, apart from being an operational hazard, is posing a serious threat to human health [3]. 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 [4]. Therefore, there is a growing need to introduce and develop new, sensitive, reproducible and rapid methods for monitoring of pesticide residues in agricultural products at trace levels.

Numerous analysis methods such as gas chromatography [5], high-performance liquid chromatography [6], capillary electrophoresis [7], flow injection immunoanalysis [8-10] and fluorimetry [11] have been developed for detection of pesticides residues. However, these methods have some drawbacks such as poor selectivity, high cost, slow response, poor stability and time-consuming [12]. Moreover, they can only be performed by highly trained technicians and are not convenient for on-site or in-field detection, which limit their application for real-time detection.

In this respect, biosensors are potentially useful as suitable complementary tools for the real-time detection of pesticides residues and have been an active research area for some years [13]. Enzyme-based biosensors for pesticide determination have caused public interest due to their reliability, fast response, high sensitivity and selectivity. In recent years, enzyme-linked immunosorbent assays (ELISA) have grown rapidly as tools for pesticide measurement [14-19]. However, false positive may easily appear and this method also need some improvements (e.g. for continuous detection).

Many biosensors which are used for pesticide detection are based on the inhibition reaction or catalytic activity of several enzymes in the presence of pesticides [20-22]. Enzyme-based biosensors (e.g. acetylcholinesterase biosensor) for pesticide determination have been widely reported in the literature [23-27]. Since a number of pesticides have a similar mode of action affecting the activity of the same enzyme, most of enzyme-based biosensors are used for screening purposes and are unspecific for individual pesticides. They can only detect total pesticide content and do not provide specific information about a particular pesticide [28].

Immunosensors have been used to detect or quantify the specific pesticide based on the binding interactions between immobilized biomolecules (Ab or hapten) on the transducer surface with the analyte of interest (hapten or Ab), resulting in a detectable signal. The sensor system takes advantage of the high selectivity provided by the molecular recognition characteristics of an Ab, which binds reversibly with a specific hapten. 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]. They appear to be 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 [30]. Currently, many electrochemical, optical and piezoelectric immunosensors have been developed for pesticides detection [28]. Among them, electrochemical immunosensors have received increasing attention due to their lower cost, high sensitivity, simple instrumentation, and easy signal amplification [31]. Excellent reviews that focused on electrochemical immunosensors for detection of different pesticide molecules have been reported [32, 33].

However, there is a time gap between current status in the field and the most recent reviews. Thus, in this review, we specifically provide an overview of the research carried out during the last 5 years relative to electrocheimical immunosensor for pesticide residues detection. We will review several types of

electrochemical immunosensors developed for their applications in pesticide analysis, various immobilization protocols used for formation of a biorecognition interface. We also will discuss the trends and challenges associated with designing a reliable immunosensor for practical applications in detail.

2. Electrochemical Immunosensors

Formation of Ab–Ag complex in electrochemical transducers alters the change in ion concentration or electron density on the electrode surface, which, in turn, is measured by electrodes. Electrochemical transducers, classified as amperometric, potentiometric, conductimetric, capacitative and Impedimetric measure changes in current, potential (voltage), conductance, capacitance and impedance respectively [34-36]. Depending on if labels are used or not, immunosensors are divided into two categories: labeled type and label-free type.

Electrochemical immunosensors could be competitive and revolutionize analysis, because of their simplicity, rapidity and cheap technology. For pesticide detection, most of them use impedance or amperometry in label or label-free format and label-free format is a tendency. Some examples of electrochemical immunosensors for the detection of pesticides residues are presented in Table 1.

3. Immobilization Protocols

In terms of the development of electrochemical immunosensor, the Ab/hapten immobilization onto a transducer or a support matrice is a key step in optimizing the analytical performance, such as response, reproducibility, stability, selectivity and regeneration. 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 [54-58].

Ab/hapten immobilization consists of physical adsorption and chemical binding, which depends on the driving force [59]. In general, they mostly fall into following methodologies.

3.1. Physical Adsorption

Physical adsorption is generally based on interactions such as van der Waals forces, electrostatic interactions and hydrophobic interactions between the Ab/hapten and the transducer. Physical adsorption is simple and easy, but nonspecific attractive forces easily causes Ab/hapten desorption [59]. In addition, the immobilized Ab/hapten can be susceptible to the reduction of biological activity by an inappropriate orientation caused by physical adsorption [60]. This leads to the limitation that the sensing elements have decreasing response with time and, thus, short life-times.

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 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 [61].

3.2. Covalent Coupling

More specific and stronger attachment of Ab/hapten can be obtained by covalent modification 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 [62, 63, 57]. The procedure provides increased stability of the Ab. However, the immobilization by covalent coupling may results in the random orientation of Ab/hapten, decreases the activity of Ab/hapten and is generally poorly reproducible due to the chemical modification of critical residues and random protein orientations [64]. In addition, blocking steps are usually necessary to limit the nonspecific binding. BSA

GNPs: colloidal gold nanoparticles; TU: thiourea; GCE: glassy carbon electrode; SiSG: silica sol-gel; HRP: Horseradish peroxidase; DpAu: deposited gold nanocrystals; PA: staphylo-coccal protein A; DMDPSE: 4,4'-thiobisbenzenethiol; GA: glutaraldehyde; ISFET: ion-selective field effect transistor; SPE: screen-printed electrodes; PB: Prussian blue; IDμE: interdigitated microelectrodes; PANI: polymer polyaniline; PVSA: poly(vinylsulphonic acid); biotinyl-PE: phospholipid dipalmitoyl-sn-glycero-3-phospho-ethanolamine-N-(biotinyl); ProtA-GEB: Protien A-graphite-epoxy biocomposite; Poly(JUG-HATZ): poly[N-(6-(4-hydroxy-6-isopropylamino-1,3,5-triazin-2 -ylamino)hexyl)5-hydroxy-1,4-naphthoquinone-3-propionamide] and tween is usually used as the appropriate material to block nonspecific binding sites.

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 NH2- group of the antibody with the surface of carboxylized transducer (Fig. 1).

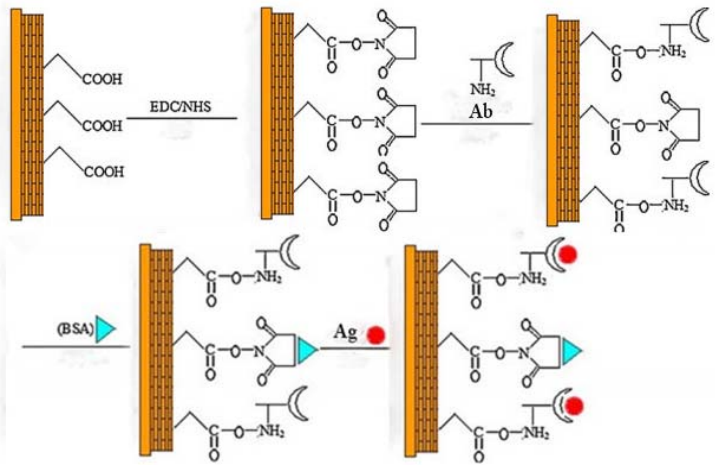


Fig. 1. Covalent immoblization of antibody onto carboxylized transducer activated with EDC/NHS as a cross-linker.

By using 2,4-D immobilized through its carboxylic group covalently to the silanized surface of the gold working electrode, Kalib et al., developed a disposable immunochemical biosensor for the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), with a detection limit close to 0.1 μg/L of free 2,4-D. For covanlent immobilizations, the 2,4-D molecule was activated by isobutyl chloroformate and then it was linked to the free amino group, which was obtained: (1) directly from the APTS moledule, (2) using gultaraldehyde/hexametyhlenediamine spacer, (3) using glutaraldehyde/albumin spacer [65].

Table 1. Some examples of electrochemical immunosensors for the detection of pesticides residues.

Pesticide	Detector	Label	Electrode Modification	Detection limit	Sample	Assay time	Reference
Carbofuran	Amperometric	free	carbofuran/BSA/Ab/GNPs/ TU/GNPs/GCE	0.11 ng/mL	cabbage	15 min	[37]
Carbofuran	Amperometric	free	carbofuran/BSA/Ab/SiSG/GC E	0.33 ng/mL	cabbage lettuce	20 min	[38]
Carbofuran	Amperometric	free	carbofuran/HRP/Ab/GNPs/ L-cysteine/Au electrode	40 ng/mL	-	12 min	[39]
Carbofuran	Amperometric	free	carbofuran/BSA/Ab/PA/DpA u/Au electrode	0.192ng/mL	Chinese chive, celery, cabbage	15 min	[31]
Carbofuran	Amperometric	free	carbofuran/BSA/Ab/ $\{DpAu/DMDPSE\}_2$/Au electrode	0.06 ng/mL	Lettuce, cabbage, pepper, tomatoe, chive, strawberry	40 min	[40]
2,4-D	Potentiometric	HRP	2,4-D-HRP/Ab/GA/Graphite electrode	40 ng/mL	water, serum	12 min	[41]
Simazine	Potentiometric	HRP	simazine-HRP/ glycine/Ab/PA/ GA/ISFET	1.25 ng/mL	-	50 min	[42]
Diuron	Impedimetric	free	diuron/Ab/GNPs/SPE	5.46 ng/mL	water	-	[43]
Diuron	Amperometric	free	Ab/DCPU-BSA/PB-GNP/LC-LAGE	1 ppt	-	-	[33]
Paraoxon	Amperometric	free	paraoxon/Ab/Nafion-GNPs/GCE	12 ng/mL	aqueous samples	20 min	[44]
Picloram	Amperometric	HRP	HRP-G, anti-RIgG/picloram/ Ab/BSA-picloram/GNPs/ GCE	0.5 ng/mL	peach	-	[45]
Atrazine	Conducti-metric	GNPs	Ab_2/Ab_1/atrazine/ GPTS/ N-acetylcysteamine/IDμE	0.1 ng/mL	buffers	-	[46]
Atrazine	Amperometric	HRP	atrazine/atrazine–BSA/Ab/ immobilon membrane/H_2O_2 electrode	5.0×10^{-11}M	buffalo milk, vegetal samples	15 min	[47]
Atrazine	Amperometric	HRP	atrazine/atrazine-HRP scAb/PANI/ PVSA/SPE	0.1 ng/mL	-	-	[48]
Atrazine	Impedimetric	free	atrazine/bio-Fab/ neutravidin/ Gold/MHDA+biotinyl-PE	20 ng/mL	PBS (pH 7)	-	[49]
Atrazine	Impedimetric	free	atrazine/bio-Fab K47/ BSA/PPy/ neutravidin/Au electrode	0.1 ng/mL	PBS (pH 7)	-	[50]
Atrazine	Amperometric	HRP	atrazine-HRP/Ab/ ProtA-GEB	6 μg/mL	orange juices	-	[51]
Atrazine	Impedimetric	free	Atrazine/BSA/ histidine-Ab/poly NTA -Cu^{2+}/Au electrode	10 pg/mL	PBS (pH 7)	-	[52]
Atrazine	Impedimetric	free	Ab_{11}/antigen2d-BSA/3-(glyci doxypropy)trimethoxysilane/ N-acetylcysteamine/IDμE	0.19 μg/mL	red wine	-	[32]
Atrazine	Impedimetric	free	Atrazine/BSA/ histidine-Ab/poly NTA -Cu^{2+}/Au electrode	10 pg/mL	PBS (pH 7)	-	[52]
Atrazine	Amperometric	free	ATZ/α-ATZ/poly (JUG-HATZ)/GCE	0.2 ng/L	-	-	[53]

Another example is that Ramon-Azcon et al., developed a novel impedimetric immunosensor based on an array of interdigitated μ-electrodes (IDμE) and immunoreagents specifically developed to detect atrazin. In this study, an atrazine-haptenized protein was covalently immobilized on the surface of the IDμE area (interdigits space) previously activated with (3-glycidoxypropyl)trimethoxysilane. With this configuration, the immunosensor detects atrazine with a limit of 0.19 μ/gL in red wine, far below the Maximum Residue Level (MRL) established by EC for residues of this herbicide in wine (Fig. 2) [32].

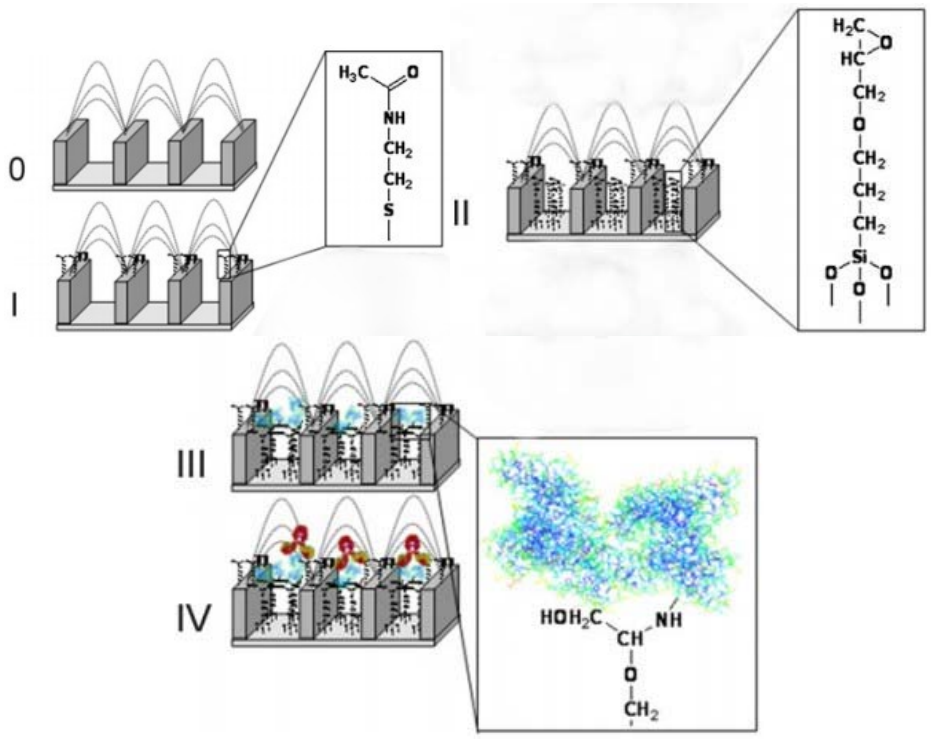


Fig. 2. Scheme showing steps used to prepare the immunosensor surfaces and antibody binding: (a) IDμE, (b) step I: N-acetylcysteamine, gold protection, (c) step II: functionalization of Pyrex substrate with (3-glycidoxypropyl)trimethoxy-silane, (d) step III: coating antigen 2d-BSA, covalent immobilization and (e) step IV: antibody Ab11.

Valera et al., has designed and developed a novel conductimetric immunosensor for atrazine detection using covalent immobilization of the competitor antigen which was performed on the interdigitated μ-electrodes surface via the side chain amino groups of lysines or arginines with the epoxy groups on the device surface. The immunosensor developed detects atrazine with limits of detection in the order of 0.1–1 μg/mL (Fig. 3) [46].

Using this technique, Valera et al., have developed a simple and low-cost method for the fabrication of mechanically flexible interdigitated μ-electrodes (FIDμEs) for the development of a conductimetric immunosensor for atrazine detection recently [66].

3.3. Entrapment

In an encapsulation method, the reagent is physically trapped within a porous matrix. It is simple and compatible with various reagents. The reagents trapped in the matrix usually do not leach out or leach out very slowly when an appropriate entrapment procedure is used. It appears that the encapsulation method avoids the disadvantages and combines the advantages of the first two methods. Organic polymeric matrices have been widely used for entrapment of sensing agents [67].

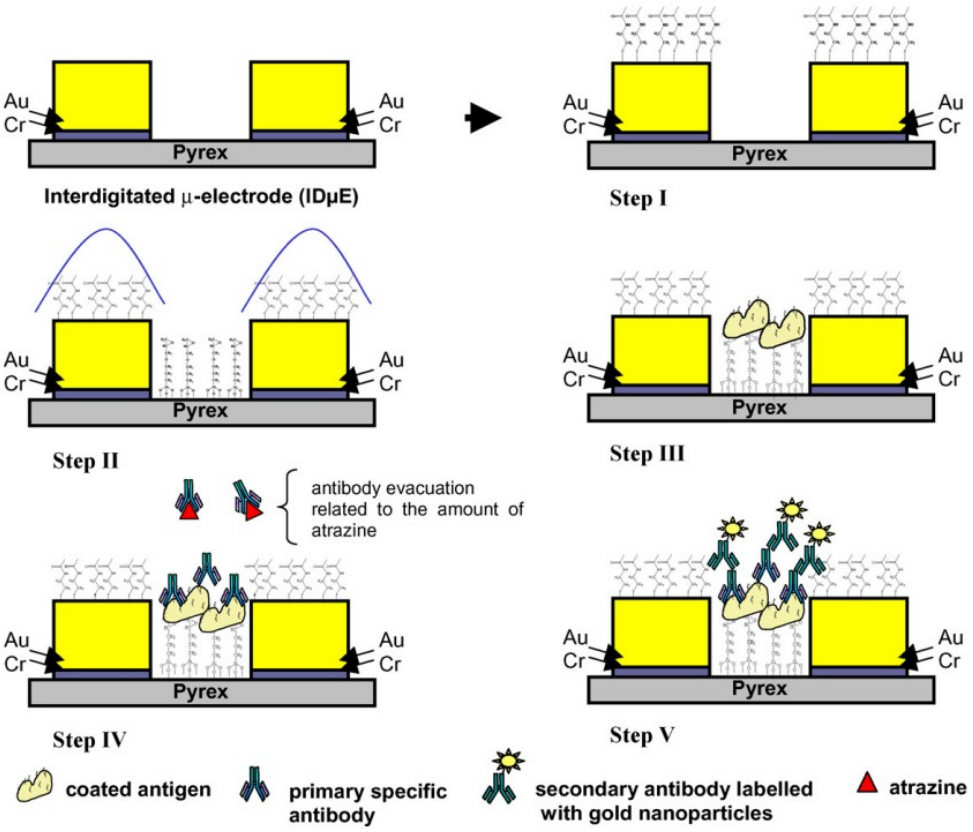


Fig. 3. Schematic diagram of the complete assay system performed on the IDμEs: Step I, protection of interdigitated μ-electrodes with N-acetylcysteamine; Step II, immunosensor surface functionalization with GPTS; Step III, covalent immobilization of the antigen on the IDμE; Step IV, specific primary 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.

3.3.1. Sol-gel Entrapment

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. These advantages include the ability of sol-gel (1) to form at low temperatures and under chemical, mechanical stability and offers negligible swelling, (2) open to a wide variety of chemical modifications based on the inclusion of various polymer additives, redox modifiers and organically modified silanes, resulting in electrically conducting materials and (3) to exhibit 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 [54].

Although it has many advantages over other methods, the sol-gel method has some disadvantages: 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 [54].

Turniansky et al., report the successful entrapment of an anti-atrazine antibody in a SiO2 sol-gel matrix, retaining its ability to bind antigen from aqueous solutions based methods for monitoring pesticide residues and other organo-synthetic environmental contaminants. Under appropriate sol-gel-forming conditions, high amounts of atrazine were bound to the sol-gels, ranging between 60 % and 91 % of the amount applied to the column. The combination of the properties of the sol-gel matrix (e.g., stability,

inertness, high porosity, high surface area and optical clarity), together with the selectivity and sensitivity of the antibodies, enable extension of this feasibility study to development of a novel group of immunosensors which could be used for purification, concentration and monitoring of a variety of residues from different sources [68].

Sun et al., developed a novel label-free impedance immunosensor for the direct detection of carbofuran using silica sol–gel (SiSG) as immobilizing agent. Sol–gel technology provides a unique means to prepare a three-dimensional network suited for the encapsulation of a variety of Ab [38].

3.3.2. Electrically Conducting Polymers Entrapment

The electrically conducting polymers (CP) are known to possess numerous features, which allow them to act as excellent materials for immobilization of biomolecules and rapid electron transfer for the fabrication of efficient biosensors [69].

Recently, CP, such as polyaniline(PANI) etc, has captured attention of scientific community due to its applications including those in biosensors because of a number of useful features such as 1) direct and easy deposition on the sensor electrode, 2) control of thickness, 3) redox conductivity and polyelectrolyte characteristics, 4) high surface area, 5) chemical specificities, 6) long term environmental stability and 7) tuneable properties. Fig. 4 is the 3D and 2D structure of PANI [70].

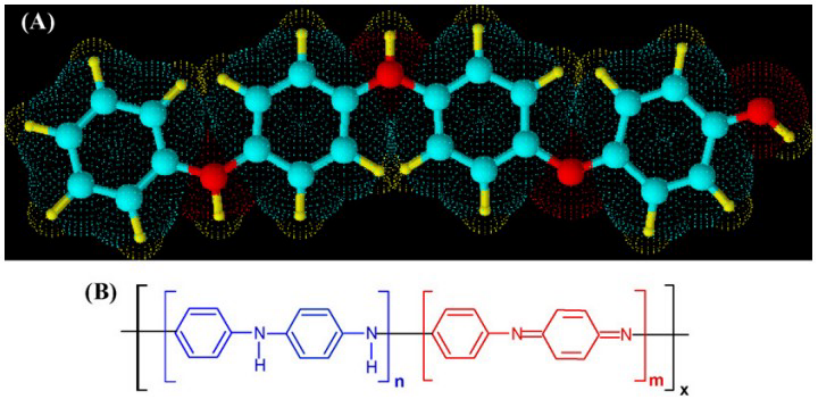


Fig. 4. (A) 3D and (B) 2D structure polyaniline.

A new electropolymerizable monomer, [N-(6-(4-hydroxy-6-isopropyl-amino-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 for the detection of atrazine (Fig. 5). This monomer contains three functional groups: hydroxyl group for electropolymerization, quinone group for its transduction capability, and hydroxyatrazine as bio-receptor element. This constitutes a direct, label-free and signal-on electro-chemical immunosensorwith a very low detection limit of 0.2 ng/L, one of the lowest reported for such immunosensors [53].

Ionescu et al., reported a label-free impedimetric immunosensor for the determination of atrazine, based on a poly(pyrrole-nitrilotriacetic acid) (poly NTA) film and combined with an impedimetric detection of atrazine without reagent and label. The poly NTA film constituted a convenient tool for the easy anchoring of histidine-labelled antibody directed against atrazine, allowing the detection of extremely low atrazine concentration namely 10 pg/mL [52].

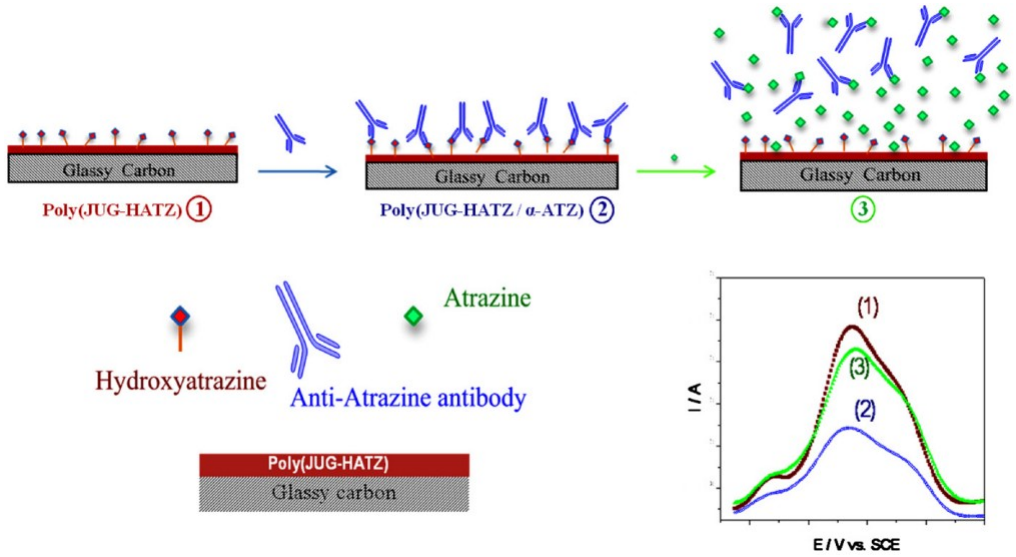


Fig. 5. Strategy for the electrochemical detection of atrazine based on the change in electroactivity of polymer film, poly(JUG-HATZ). SWV recorded with (1) poly(JUG-HATZ)-modified electrode; (2) after complexation with α-ATZ, poly(JUG-HATZ/α-ATZ)-modified electrode; (3) after addition of ATZ in solution.

Grennan et al., described the development of an electrochemical immunosensor for the analysis of atrazine using recombinant single-chain antibody (scAb) fragments. The sensors are based on carbon paste screen-printed electrodes incorporating the conducting polymer polyaniline (PANI)/poly(vinylsulphonic acid) (PVSA), which enables direct mediatorless coupling to take place between the redox centres of antigen-labelled horseradish peroxidase (HRP) and the electrode surface (Fig. 6) [48].

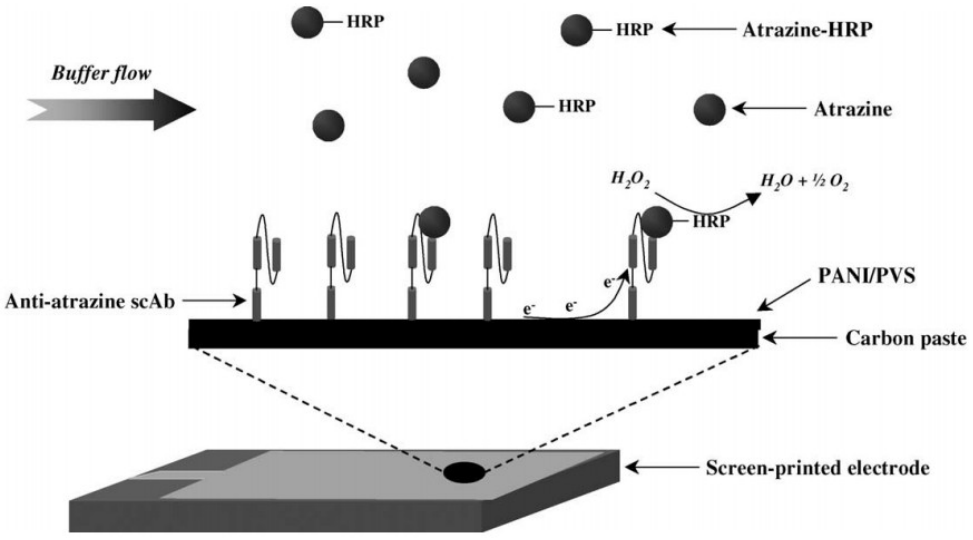


Fig. 6. Schematic diagram of the electrochemical real-time sensing process for atrazine detection.

3.4. Oriented Immobilization

Antibodies immobilized by these methods such as physical adsorption or covalent coupling, however, often suffer from reduced hapten binding ability due to a combination of denaturation, random orientation, and chemical modification of the antibodies [71]. Antibody-binding proteins (protein A, G,

A/G, and L) have been widely used to overcome the aforementioned drawbacks [72, 73]. These proteins specifically bind the Fc region of an Ab and, thus, properly orient the bound Ab for optimal hapten binding [74]. Moreover, because the antibody-binding proteins capture antibodies without any chemical modifications, bound antibodies fully retain their function. Improved surface orientation of these engineered antibody-binding proteins enhanced the subsequent Ab/hapten immobilization. Despite the evident advantages of using antibody-binding proteins for Ab immobilization, these proteins have limitations; for example, they are susceptible to denaturation and are difficult to use in site-specific modifications [75]. This technology has been widely used in electrochemical immunosensors through the antibody's oriented immobilization for pesticides detection.

An immunological reaction for the detection of atrazine performed on the Protien A (2%)-graphite-epoxy biocomposite (ProtA-GEB) biosensors is based on the antibody bonding through Fc fragment to Protein A and a direct competitive assay using atrazine-HRP tracer as the enzymatic label (Fig. 7). The electrochemical detection is thus achieved through a suitable substrate and a mediator for the enzyme HRP. The detection limit for atrazine in orange juices was found to be 6μg/mL [51].

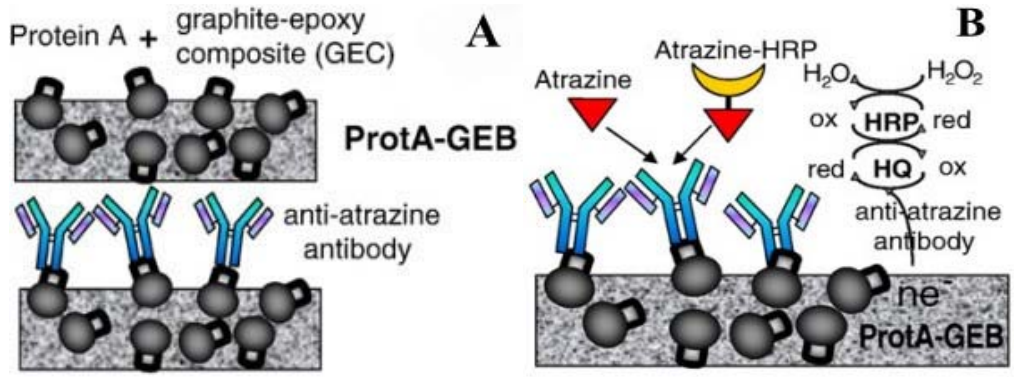


Fig. 7. The immobilization of anti-atrazine antibodies on the surface of the electrochemical transducer for the detection of atrazine in orange juice with ProtA-GEB-based electrochemical immunosensors (A) and the competitive immunological reaction (B).

Recently, Sun et al., introduced a strategy for preparing a new label-free amperometric immunosensor, which successfully immobilized the anti-carbofuran antibody on the PA/DpAu modified electrode surface for the detection of carbofuran. Due to PA's specially binding ability of the Fc fragment of the antibody molecules, the application of PA improves the capacity of antibody, thus enhance the detection sensitivity. With this strategy, a detection limit of 0.1924 ng/mL was achieved for carbofuran (Fig. 8) [31].

3.5. Avidin–biotin Affinity Reaction

One of the most valuable strategies for t he effective immobilization of biomaterial on different substrate s is based on the avidin–biotin affinity reaction [76].This interaction is highly resistant to a wide range of chemical (detergents, protein denaturants), pH range variations and high temperatures [77]. In addition, the avidin–biotin based immobilization method maintains the biological activity of the biomolecule being immobilized more successfully than other commonly used methods [78]. Ab/hapten can be readily linked to biotin without serious effects on their biological, chemical or physical properties. In particular, the extremely specific and high affinity interaction between the biotinylated antibodies and avidin (Ka ~$10^{15}M^{-1}$) leads to strong associations similar to the formation of a covalent bonding.

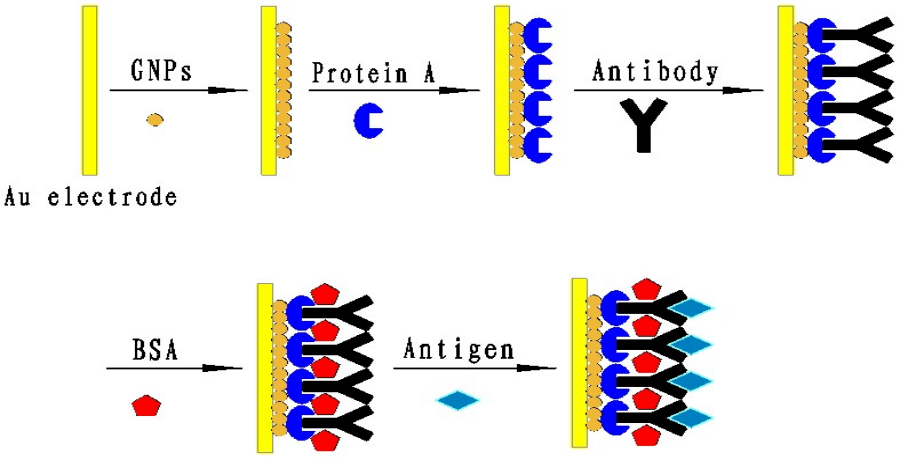


Fig. 8. Fabrication process of the stepwise Amperometric immunosensor based on a protein A/deposited gold nanocrystals modified electrode for carbofuran detection.

This technique was used to immobilize anti-atrazine antibodies on the surface of avidin-graphite-epoxy biocomposite based (Av-GEB-based) electrochemical transducer for the detection of atrazine in orange juice (Fig. 9) [79].

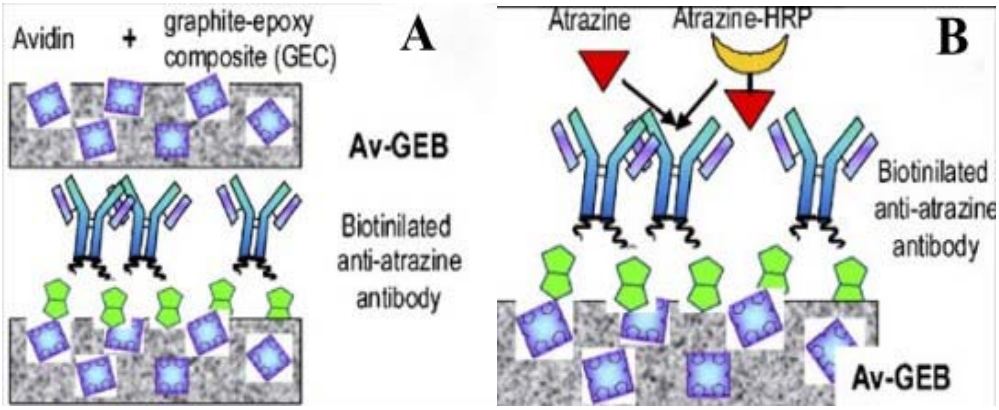


Fig. 9. The immobilization of anti-atrazine antibodies on the surface of the electrochemical transducer for the detection of atrazine in orange juice with Av-GEB-based electrochemical immunosensors (A) and the competitive immunological reaction (B).

Another example of this technique is to attach the biotinylated anti-triazine Fab fragment to the polypyrrole (PPy)/ neutravidin modified electrode throughout the well-studied biotin–neutravidin interaction for the detection of atrazine. The immunosensor was very sensitive to atrazine antigen in the range of 0.1–200 ng/ml and the detection limit attained 0.1 ng/ml (Fig. 10) [50].

3.6. Self-assembled Monolayer (SAM)

Self-assembled monolayers (SAMs) have aroused much interest due to their potential applications in biosensors, biomolecular electronics and nanotechnology. This has been largely attributed to their inherent ordered arrangement and controllable properties. SAMs can be formed by chemisorption of organic molecules containing groups like thiols, disulphides, amines, acids or silanes, on desired surfaces to fabricate immunosensors [80].

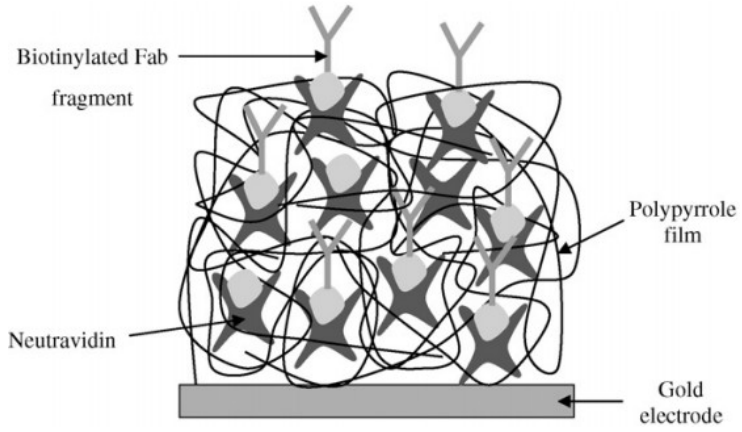


Fig. 10. Schematic representation of the immunosensor architecture based on the immobilization of biotinylated Fab fragment through the interaction biotin–neutravidin within the electro-generated polypyrrole for the detection of atrazine.

The stability, uniform surface structure and relative ease of varying thickness of a SAM make it suitable for development of biosensors. And the immobilization of biomolecules on a SAM requires very small amount and desired analytes can be easily detected via various transduction modes. The use of an appropriate SAM helps in oriented and controlled immobilization of biomolecules [81-83]. SAMs can be used to prevent protein denaturation at an electrode surface and for enhancing stability of biomolecules[84, 85].

A novel label-free amperometric immunosensor for the detection of carbofuran residues was developed based on immobilization anti-carbofuran antibody on deposited gold nanocrystals (DpAu)/4,4'-thiobisbenzenethiol (DMDPSE) multilayers ($\{DpAu/DMDPSE\}_n$) through Au-S bond by layer-by-layer self-assembly technology. Compared with a separate layer of DpAu/DMDPSE, 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 with a detection limit of 0.06 ng/mL (Fig. 11) [40].

Recently, describes the development of an electrochemical immunosensor for the analysis of atrazine associated to biotinylated-Fab fragment K47 antibody. The sensors are based on mixed self-assembled monolayer consisting of 1,2 dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl) (biotinyl-PE) and 16-mercapto-hexadecanoic acid (MHDA). The tethered neutravidin was used the biotin sites present in the mixed monolayer, with those associated to the biotinyl-Fab fragment K47 antibody (Fig. 12) [49].

3.7. Nanoparticles

In addition to these conventional methods, new materials such as nanoparticles have been employed in immobilizing Ab/ hapten when constructing immunosensors. Gold nanoparticles (GNPs) have been widely used for immobilization of biomolecules due to their large specific surface area, high surface free energy and biocompatibility. GNPs can adsorb biomolecules and play an important role in the immobilization of biomolecules for biosensor construction [86]. So far, GNPs have been widely applied in the biosensors for detection of pesticide residues [87-89]. Biological interactions, such as biotin/streptavidin interactions can be used to easily immobilize the Ab on the surface of nanoparticales. Combining the catalytic and protein-adsorptive characteristics of gold nanoparticles, Hu et al., prepared a label-free electrochemical immunosensor with paraoxon antibodies loaded on the gold nanoparticles to monitor the concentration of paraoxon in aqueous samples with a detection limit of 12 μg/L. TEM experiment of colloidal gold indicated an average diameter to be 10±0.5 nm (Fig. 13) [44].

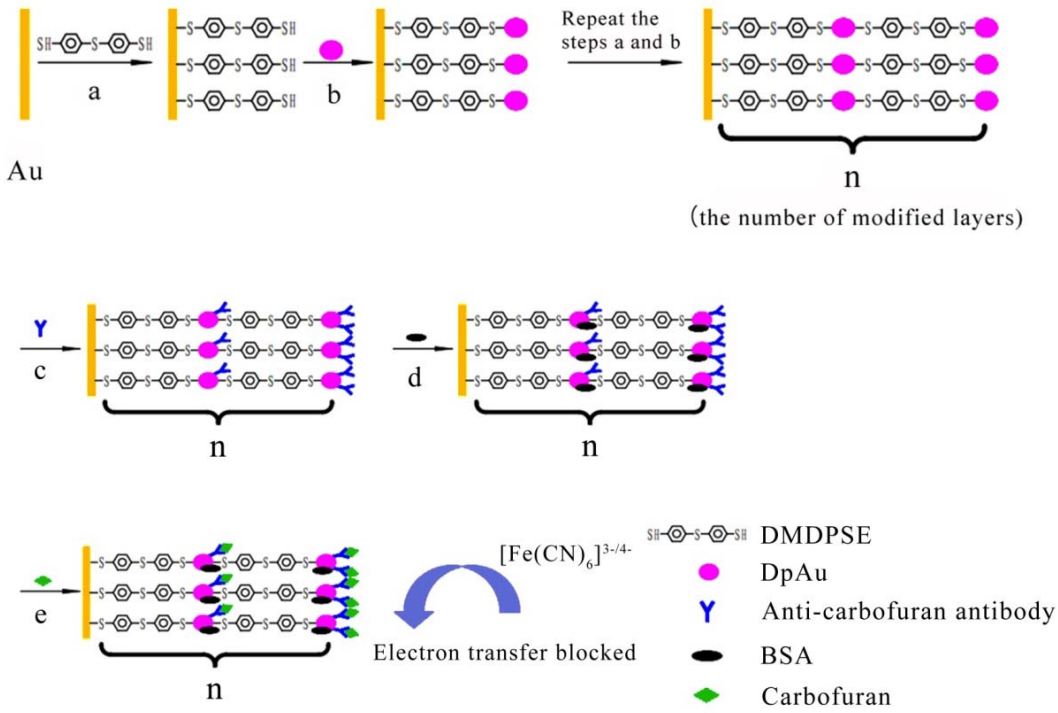


Fig. 11. Fabrication process of the stepwise the stepwise immunosensor based on deposited gold nanocrystals/ 4,4'-thiobisbenzenethiol for determination of carbofuran.

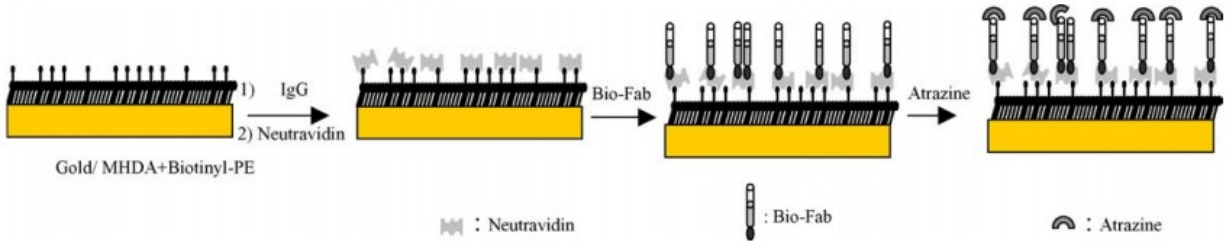


Fig. 12. Schematic showing the assembly of a mixed SAM based immunosensor.

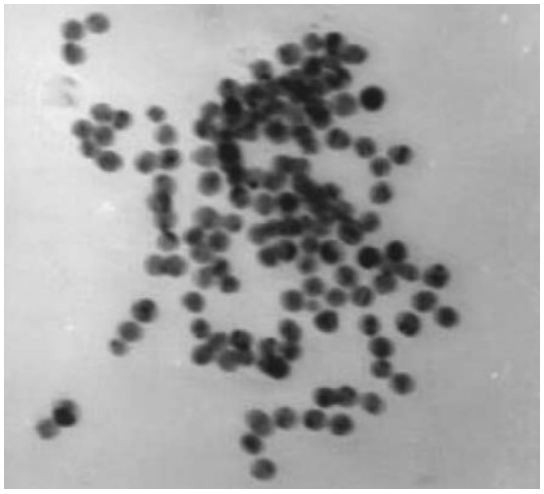


Fig. 13. TEM of gold nanoparticles.

Recently, a type of ordered three-dimensional (3D) gold (Au) nanoclusters obtained by two-step electrodeposition using the spatial obstruction/direction of the polycarbonate membrane was reported. The electrodeposited Au nanoclusters built direct electrical contact and immobilization interface with protein molecules without post-modification and positioning (Fig. 14) [45].

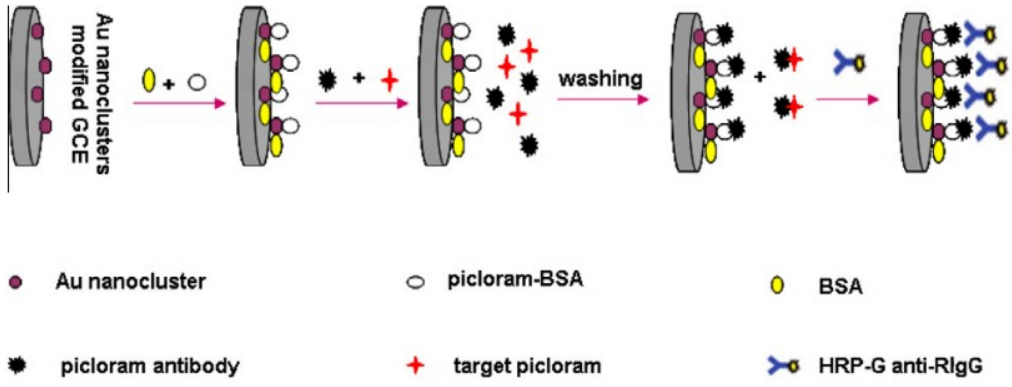


Fig. 14. Schematic diagram of the immunosensor based on 3D gold (Au) nanoclusters and competitive immunoreaction.

Recently, Sun et al., developed a novel immunosensor for direct determination of carbofuran concentration by immobilizing anti-carbofuran antibody on the gold nanoparticles(GNPs)/Thiourea (TU)/GNPs composite film with the detection limit 0.11 ng/mL. The presence of GNPs can enhance electron transfer between Ab and electrode surface and provide a favorable microenvironment for immunoreaction. In addition, GNPs on the composite film had a profound influence on enhancing the conductivity and biocompatibility (Fig. 15) [37].

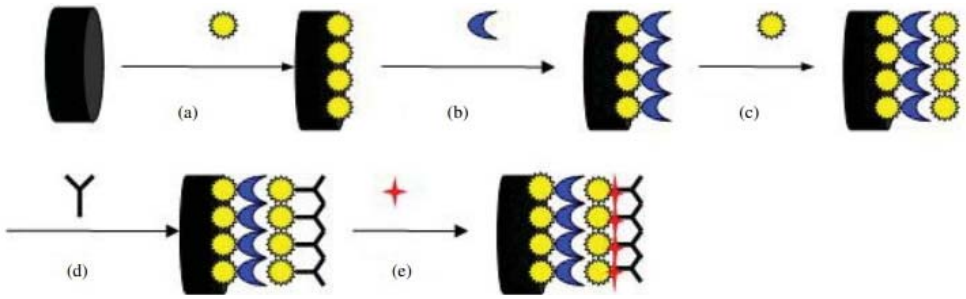


Fig. 15. Schematic illustration of the stepwise procedure of the immunosensor preparation: (a) electrodeposited GNPs; (b) modified TU; (c) electrodeposited the second layer of GNPs; (d) adsorption o f anti-carbofuran and (e) BSA blockin.

In addition, Bhalla et al., reported a label-free detection of phenylurea herbicides by impedance spectroscopy based on immobilization class specific anti-diuron antibodies on gold nanoparticles (~20 nm). Gold nanoparticles, used as signal enhancers cum immobilization matrix, were electrodeposited on carbon screen-printed electrodes (SPE) and functionalized with specific anti-diuron antibodies for the development of bio-interface (Fig. 16) [43].

Another example based on nanoparticles technology is reported for sensitive atrazine determination based on magnetic beads. The immuno-method is a competitive solid-phase immunoassay where the anti-atrazine antibody is immobilized on the magnetic beads surface and fixed at the reaction cell bottom using a simple magnet, which generates a magnetic field. The performance of magnetic beads-based

immunoassay for atrazine determination was evaluated demonstrating that the magnetic beads-based immunoassay is one of the most sensitive methods for atrazine determination (Fig. 17) [90].

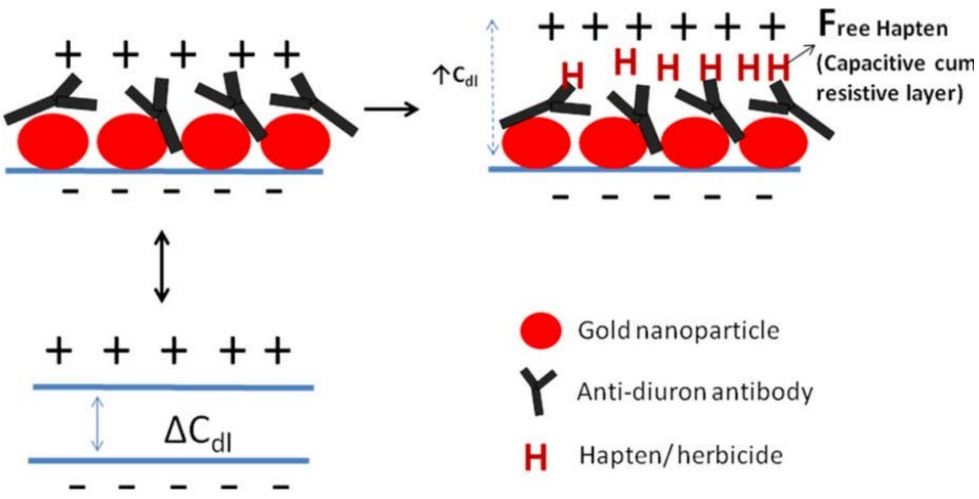


Fig. 16. Schematic illustration of the stepwise procedure of the immunosensor preparation.

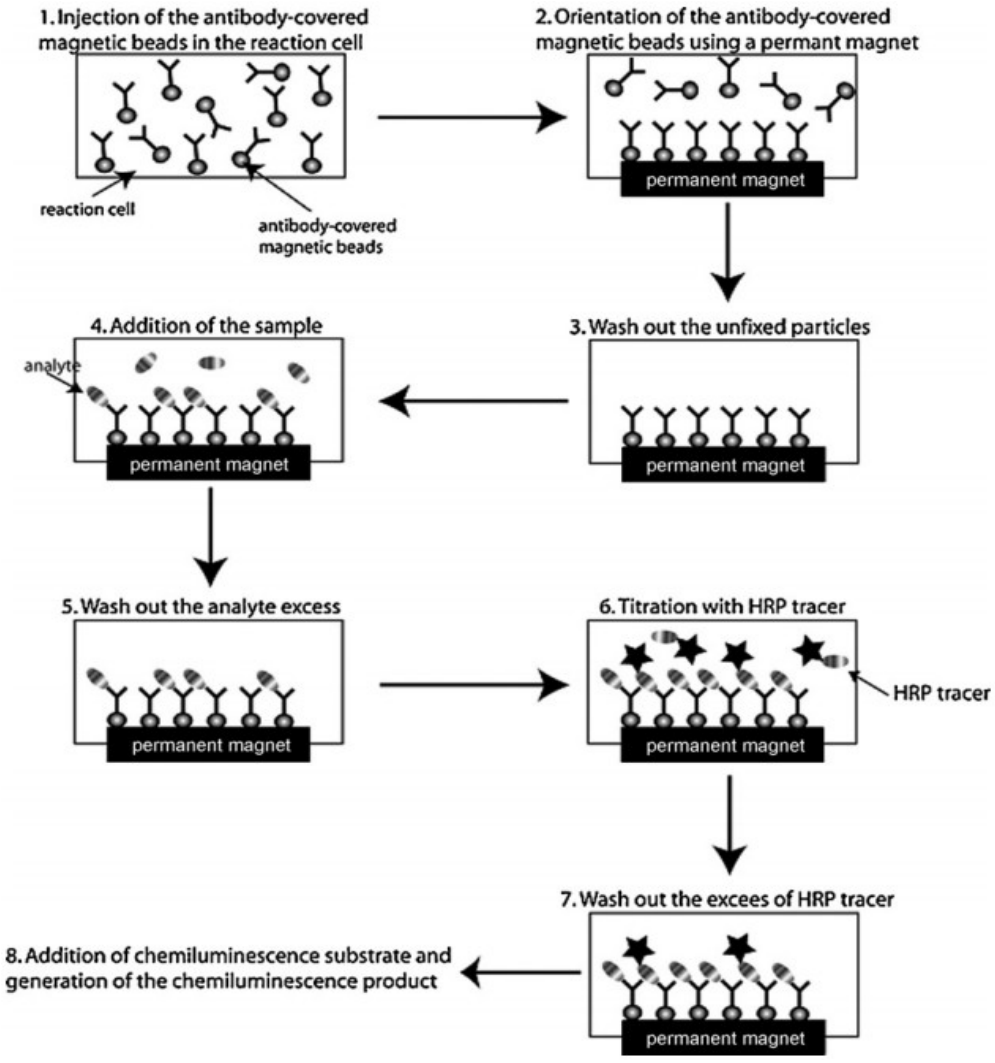


Fig. 18. Principle steps for performing the magnetic beads-based immunoassay.

4. New Trends and Challenges

4.1. Miniaturization

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 likely focus on directing biorecognition elements to addressable locations on micro or nano-sensor arrays. A microelectrode, its dimensions are in the micrometer range, which become a trend to replace common electrod 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). Ramon-Azcon et al., have reported an array of interdigitated μ-electrodes (IDμE) for atrazine detection (32).

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 microelectrod and microfluidic devices as analytical systems will become a trend to realized high throughput due to 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. Recently, silicabased monoliths, coupled with micro-fluidic devices, have been used 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

Despite the promise of immunosensors, they do have certain limitations. For example, few immunosensors are commercially available at the present time and are yet to be established as research or routine tools, due to a lack of validated protocols for a wide range of sample matrices.

4.5. Using Aptamer to Replace Antibody

Immunosensor, itself still has several problems, such as biomolecule deactivation or leaking and high diffusion resistance of the substrate/biocomponent, which are also key factors in the development of immunosensors that can be successfully applied to pesticide detection. Aptamers are short, single-stranded, functional DNA or RNA molecules selected from random-sequencenucleic acid combina-torial libraries by Systematic Evolution of Ligandsby Exponential Enrichment (SELEX).The aptamers are more chemically stable, smaller in size, cheaper and can bind nearly any target with high afnity and specicity compared to antibody [91].

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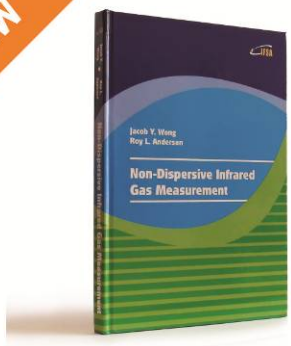
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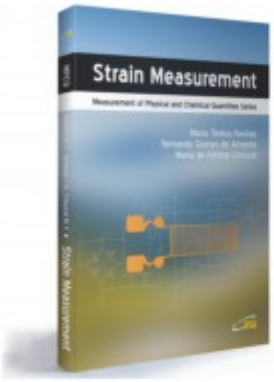
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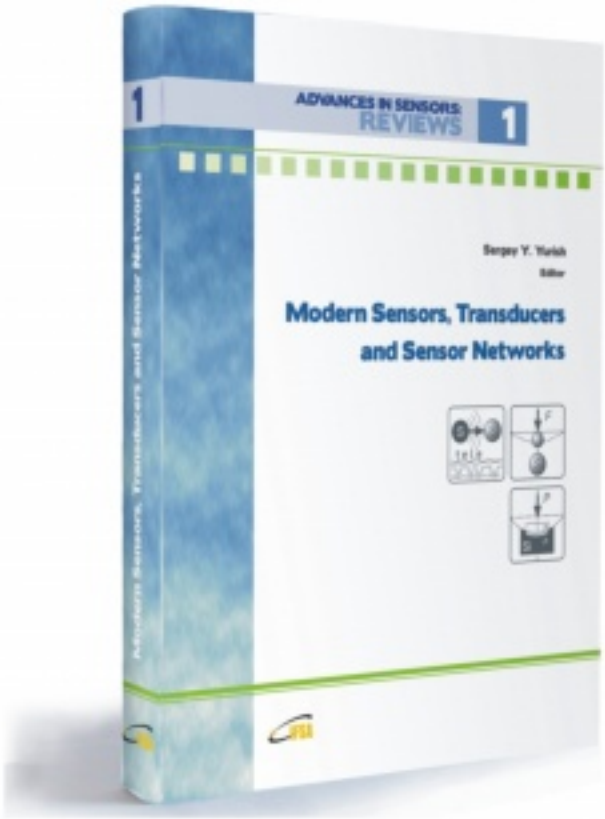
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