

Impedance Immunosensor Based on Interdigitated Array Microelectrode and its Experiment Parameter Optimization

^{1,2} Wen Ping Zhao, ² Hui Ying Jia, ² Xia Sun, ^{1,*} Zhi Huai Mao,
and ² Xiang You Wang

¹ College of Engineering, China Agricultural University, P. O. Box 50, 17 Qinghua Donglu,
Beijing 100083, P. R. China

² School of Agriculture and Food Engineering, Shandong University of Technology, No. 12,
Zhangzhou Road, Zibo 255049, P. R. China

* Tel.: +86-533-2786558, fax: +86-533-2786558

* E-mail: maozh@cau.edu.cn, wxy@sdut.edu.cn, 187286528zwp@163.com, sunxia2151@sina.com

Received: 4 March 2014 / Accepted: 30 April 2014 / Published: 31 May 2014

Abstract: This article accounts for a novel impedance immunosensor based on the specificity reaction of the antigen-antibody and the sensitivity of the interdigitated array microelectrode (IDAM) for the determination of chlorpyrifos residues. The basic knowledge of immunoassay was in relation to its IDAM electrode surface modification, antibody immobilization, bovine serum albumin (BSA) blocking and sample detection. The SPA was dropped onto the surface of IDAM electrode, used for binding antibody Fc fragments. Next, antibody was immobilized on the SPA modified electrode. Finally, BSA was employed to block the possible remaining active sites avoiding any nonspecific adsorption. Target chlorpyrifos was then captured by the immobilized antibody, resulting in a change in the impedance of the IDAM microelectrode surface. The fabrication procedure of the immunosensor and the sample detection were characterized by electrochemical impedance spectroscopy (EIS). The influences of the experiment parameters were investigated. Under optimized conditions, an excellent biosensor was fabricated. Many of the antibodies, enzymes and other reagents integral to immunoassays were very expensive, often hundreds of dollars per milligram, therefore miniaturization reduces reagent costs drastically. In this article the volume of the reagents was micro upgrade, the antibodies, SPA and BSA were 30 μL , the chlorpyrifos sample and detection solution were 50 μL . The advantages of the immunosensor were exhibited in its better specificity, stability, selectivity and regeneration. The proposed method was proven to be a feasible quantitative method for chlorpyrifos analysis in vegetables and fruits. *Copyright © 2014 IFSA Publishing, S. L.*

Keywords: Immunosensor, Interdigitated array microelectrode, Electrochemical impedance spectroscopy, Parameter optimization, Chlorpyrifos immunosensor.

1. Introduction

Chlorpyrifos (O, O-diethyl-O- (3, 5, 6-trichloro-2-pyridyl)-phosphorothioate), a broad spectrum OP insecticide, is one of the most widely used

organophosphate as a very potent insecticide and acaricide in agriculture to control pests and enhance production [1].

Chlorpyrifos is moderately toxic to humans [2]. Poisoning from chlorpyrifos may affect the

central nervous system, the cardiovascular system, and the respiratory system. It is also a skin and eye irritant [3]

They are a potential threat for the human health safety and environmental protection purposes. Therefore, it is necessary to develop sensitive and fast detection technology for pesticide residues. Considerable effort has been directed towards the development of electrochemical biosensors can substitute the current analytical methods such as liquid chromatography [4], gas chromatography [5] enzyme-linked immunoabsorbant assays [6] by simplifying or eliminating sample preparation, thus decreasing the analysis time and cost [7].

Electrochemical biosensors can be divided into amperometric, potentiometric and conductimetric (impedimetric) biosensor, they are highly sensitive, rapid, inexpensive, and are suitable for designing integrated microsystems [8, 9].

In recent years the electrochemical impedance spectroscopy has gained widespread use in developing biosensors for monitoring formation of antibody-antigen [10, 11]. Impedance detection works by measuring the impedance change caused by binding of target molecules to receptors (proteins, DNA, antibodies, and other bio-recognition elements) immobilized on the surface of the electrodes [8, 12-15].

In order to miniaturize the sensor and improve the sensitivity, microelectrode has been considered as a potential candidate to combine with traditional detection methods. Microelectrodes favor a greater rate of reactant supply (while macroelectrodes cause greater depletion of reactants) and can perform impedance measurement even in low conductivity solution, where macroelectrodes may not be sensitive. [16,17]. Among microelectrodes, interdigitated array microelectrodes (IDAM) present promising advantages such as low ohmic drop, fast establishment of steady-state, rapid reaction kinetics, and increased signal-to-noise ratio.

IDAM consist of a series of parallel microband electrodes in which alternating microbands are connected together, forming a set of interdigitating electrode fingers. The typical dimensions of an individual micro-band "finger" are 0.1-0.2 μm in height, 1-20 μm in width; 2-10 mm in length, with a gap of 1-20 μm between the fingers [18].

Fig. 1 shows the schematic design of a typical planar interdigitated array microelectrode chip that metal interconnects or bonding pads are for connecting electrodes to the impedance analyzer and the interdigitated microelectrodes are for sensing.

Additionally, IDAM eliminates the need for a reference electrode which is comparatively simpler to detect as compared to three or four electrode systems and provides simple means for obtaining a steady-state current response [19, 20]. Their low response time also favors rapid detection.

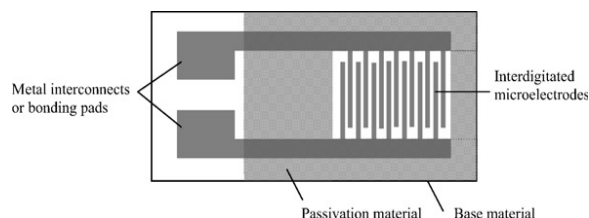


Fig. 1. The Schematic representation of interdigitated array microelectrodes.

2. Experimental and Methods

2.1. Reagents and Materials

Anti-chlorpyrifos monoclonal antibody, chlorpyrifos, protein A and bovine serum albumin (BSA, 96–99 %) were all purchased from Sigma (St. Louis, MO, USA). Different concentration of anti-chlorpyrifos monoclonal antibody was prepared by dissolving with 0.01 M phosphate buffer solution (PBS, pH 7.5) processed by high pressure sterilization and stored at 4°C. Ethanol and other chemicals were used of analytical grade.

PBS (10 mM, pH 5.5-8.5) containing 5mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (1:1 mixture) and 0.1 M KCL were used as the detection solution. Protein A and BSA were both reconstituted in PBS. Bovine serum albumin (BSA, w/v) was prepared in PBS as a blocking solution. All other solutions were prepared with deionized water from Millipore (Milli-Q, 18.2 $\text{M}\Omega\text{-cm}$). The rest of the reagents are analytically pure or above.

2.2. Apparatus

Electrochemical impedance spectroscopy (EIS) was performed with a CHI 660D electrochemical workstation (Shanghai Chenhua Co., Shanghai, China).

The working electrode, reference and auxiliary electrodes were integrated on gold interdigitated array microelectrodes (IDAM, IME AU-1550.5), and it was purchased from AB-tech Scientific Inc. (Richmond, VA). Each electrode had 50 digital pairs with 15 μm digit width, 15 μm interdigitated spaces, and a digit length of 4985 μm . The electrode area was calculated as 14.88 mm^2 .

2.3. Preparation of the Biosensor

The bare IDAM electrode was cleaning according to the reference literature [21], which we had mentioned in our previous literatures, and a little change was made: the IDAM electrode was cleaned by immersing in 1 M NaOH for 15 min, rinsed with deionized water, immersing in 1 M HCL for 15 min, rinsed with deionized water, and then wiped gently with alcohol wetted lens paper for 5 min. After final

rinsing with deionized water, the electrode was dried with a stream of nitrogen after each pretreatment. After above cleaning procedure, the IDAM electrode was ready for surface modification and antibody immobilization.

Then, it was observed under microscope (40*40) to check for irregular features, damaged microelectrode, or foreign objects on the microelectrode. If no irregular feature was observed, impedance could be measured for the microelectrode in the detection solution which the pH was optimized. This impedance value would be compared with previous impedance value measured for a bare electrode. If a similar value was obtained, new antibodies can be immobilized, whereas if the value is different, the cleaning step should be repeated.

30 μL of protein A solution (10 $\mu\text{g}/\text{mL}$, SPA) was dropped onto the surface of the cleaning IDAM electrode at room temperature (22-25 $^{\circ}\text{C}$) for 50 min. Following the incubation, the microelectrode was then rinsed with deionized water to remove loosely adsorbed SPA and dried with nitrogen stream and then incubated with antibody (100 $\mu\text{g}/\text{mL}$, 30 μL) for further 1 h at 4 $^{\circ}\text{C}$. After rinsing with deionized water and drying with nitrogen, the microelectrode was incubated with 5% BSA (30 μL) as a blocking solution at room temperature for 1 h. After another rinsing with deionized water and drying with nitrogen stream, the microelectrode was ready for use in detection tests. All impedance test were performed in 50 μL $[\text{Fe}(\text{CN})_6]^{3-/4-}$ dropped on the electrode surface.

The mechanism diagram of impedance biosensor by EIS detection on IDAM electrode was illustrated in Fig. 2 as follows.

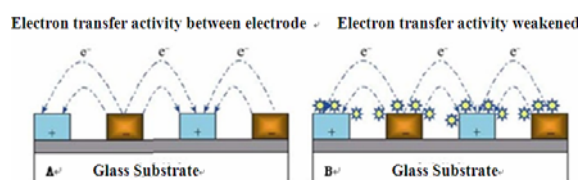


Fig. 2. Impedance biosensor detection mechanism diagram on IDAM electrode.

2.4. Parameter of the Immunosensor Optimization

The experimental parameters, including the antibodies concentration, the pH of the detection solution, BSA concentration and chlorpyrifos sample incubation time can affect the current response of the immunosensors and were thus investigated in 50 μL $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as the redox probe by EIS.

The effect of the concentration of antibodies on the current response was investigated with the concentration from 10 $\mu\text{g}/\text{mL}$ to 500 $\mu\text{g}/\text{mL}$ (10 $\mu\text{g}/\text{mL}$, 20 $\mu\text{g}/\text{mL}$, 30 $\mu\text{g}/\text{mL}$, 50 $\mu\text{g}/\text{mL}$, 80 $\mu\text{g}/\text{mL}$, 100 $\mu\text{g}/\text{mL}$, 120 $\mu\text{g}/\text{mL}$, 150 $\mu\text{g}/\text{mL}$, 200 $\mu\text{g}/\text{mL}$, 300 $\mu\text{g}/\text{mL}$, 500 $\mu\text{g}/\text{mL}$) by EIS.

The pH of the detection phosphate buffer is an important influencing factor for the sensitivity of immunosensors. The effect of pH of the detection solution on the impedance response of the proposed immunosensor was studied in series of 0.1 M PBS containing 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl with the pH from 5.5 to 8.5 (5.5, 6, 6.5, 7, 7.5, 8, 8.5) by EIS.

The effect of the concentration of BSA on the current response was investigated with the concentration from 1 mg/mL to 10 mg/mL (1 mg/mL, 2 mg/mL, 3 mg/mL, 4 mg/mL, 4.5 mg/mL, 5 mg/mL, 5.5 mg/mL, 6 mg/mL, 6.5 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL, 10 mg/mL) by EIS.

The incubation time was an important parameter before and after capturing chlorpyrifos by immunosensor. It was studied from 0 to 160 min (0, 20 min, 40 min, 60 min, 80 min, 120 min, 160 min).

The tested frequency range was from 1 Hz to 1 MHz with amplitude of 5 mV. Bode (impedance and phase vs. frequency) and Nyquist (imaginary impedance vs. real impedance) diagrams were recorded.

3. Results and Discussion

3.1. Electrochemical Characterization of the Modifying Process

Impedance spectroscopy was a powerful tool for analysis surface-modified electrodes and theoretical analysis of impedance properties [22-25].

Characterization of stepwise changes in electrochemical properties upon the formation of each layer were carried out using electrochemical impedance spectroscopy in the presence of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as a redox couple.

Fig. 3 presents the impedance spectra of (a) the bare electrode, (b) the protein A modified electrode, (c) the antibody immobilized electrode, and (d) parathion bound electrode in the presence of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as the redox probe in PBS. The frequency range is from 1 Hz to 1000 kHz. Each step was measured three times. As shown in Fig. 3, in the frequency range from 1 to 1000 Hz, the impedance value at a given frequency 100 Hz increased upon the addition of (b) protein A, (c) the antibody layer, and (d) the BSA blocking electrode and (e) chlorpyrifos to the electrode surface compared to (a) the bare electrode. At frequencies higher than 10 kHz, no significant difference is observed between spectra a, b, c, d and e.

Impedance changes of the open IAM sensor as a function of frequency for different chlorpyrifos samples are investigated. The detection signals for different concentrations (0.001 $\mu\text{g}/\text{mL}$ to 100 $\mu\text{g}/\text{mL}$) were distinguishable from each other. The quantitative detection could be achieved in this study. So we could use this biosensor in subsequent quantitative detection.

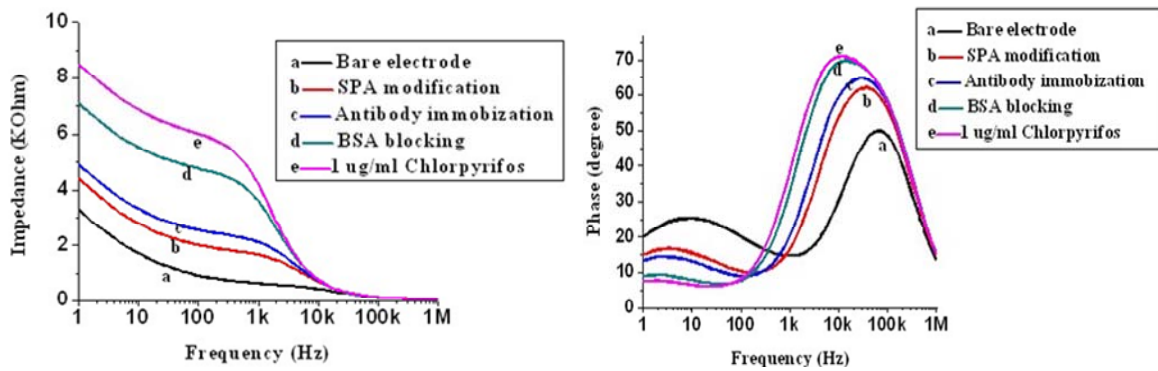


Fig. 3. Impedance-frequency and phase-frequency in bode diagrams of 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ with 0.1 M KCl at different electrodes (a) the bare electrode, (b) the protein A modified electrode, (c) the antibody immobilized electrode, (d) the BSA blocking electrode and (e) chlorpyrifos bound electrode in the presence of $[\text{Fe}(\text{CN})_6]^{3-/4-}$.

3.2. Optimization of the Experimental Parameter

The experimental parameters included the antibodies concentration, the pH of the detection solution, BSA concentration and antigen incubation time. As shown in Fig. 4.

Fig. 4a shows the effect of the concentration of antibody (Ab) captured by SPA on the IDAM electrode. The impedance change (ΔR) curve of Ab/SPA attaching on the surface of IDAM electrode before and after was recorded with a different

Ab concentration. The ΔR increased with the increasing of the Ab concentration ranges from 0 to 100 $\mu\text{g}/\text{mL}$, and then a little change took place as the Ab concentration increased further ranges from 100 to 500 $\mu\text{g}/\text{mL}$. In the other hand, the biospecific antibody binded onto the sensor surface existed competition, which led to a reduction of the number of available antibody binding sites for capturing the chlorpyrifos. Therefore, antibody loading of 100 $\mu\text{g}/\text{mL}$ was chosen in all following experiments.

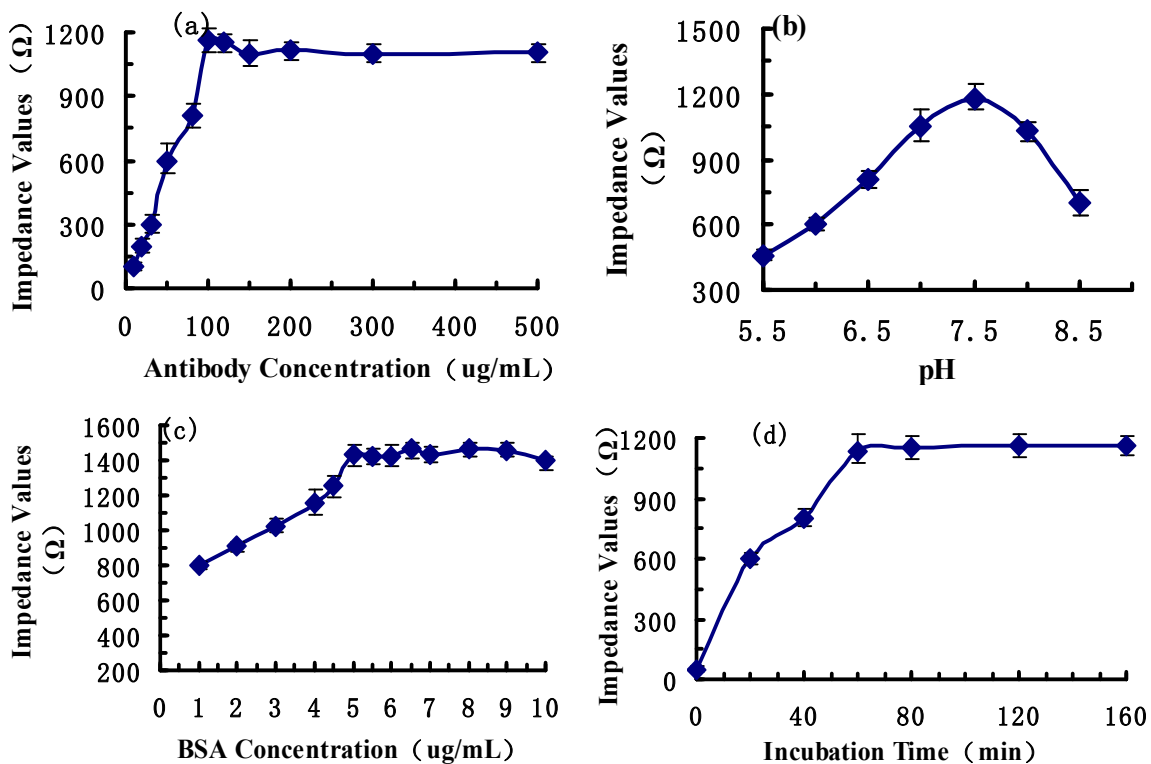


Fig. 4. Optimization of experimental parameters: (a) Influence of anti-chlorpyrifos antibody concentration in the range from 0 to 500 $\mu\text{g}/\text{mL}$ on impedance signal; (b) Influence of working buffer pH range from 5.5 to 8.5 on impedance signal; (c) Influence of BSA concentration in the range from 1 to 10 $\mu\text{g}/\text{mL}$ on impedance signal; (d) Influence of incubation time for different times from 0 to 160 min on impedance signal.

Fig. 4b illustrates ΔR of chlorpyrifos/BSA/Ab/SPA/IDAM in detection solutions of different pH in the range from 5.5 to 8.5. The impedance responses increased with the increase of pH from 5.5 to 7.5, and then decreased as pH increased further. The reason for that was the antigen-antibody complex could easily be dissociated in a detection solution with unsuitable pH. It was easy to lose the activity of Ab irreversibly at the lower or higher pH values. Obviously, in order to get the highest sensitivity, suitable pH value should be chosen on the Ab/SPA/IDAM sensor. The pH 7.5 of PBS was used as working solution in the subsequent experiment.

Fig. 4c indicates that ΔR of Ab/SPA/IDAM in different BSA concentration range from 1 to 10 $\mu\text{g/mL}$. The ΔR increased with the increasing of the BSA concentration ranges from 1 $\mu\text{g/mL}$ to 5 $\mu\text{g/mL}$, and then slight changes happen as the BSA concentration increased further range from 5 to 10 $\mu\text{g/mL}$. So the BSA concentration of 5 $\mu\text{g/mL}$ was used as best choice in the subsequent experiment.

The incubation time was an important parameter for capturing chlorpyrifos. The immunosensor was incubated in a concentration of 10 $\mu\text{g/mL}$ chlorpyrifos standard solution for different time. The influence of incubation time on response signals was also investigated. The immunosensor was incubated with a standard chlorpyrifos solution (10 $\mu\text{g/mL}$) for different times from 0 to 160 min, and then tested in 0.01 M PBS (pH 7.5). Because the immunoreaction of the antigens with Ab and immunocomplexes formation needs some time, as shown in Fig. 4d, the ΔR increased with increasing incubation time and reached a maximum value at 60 min and after that the immunoreaction variation was slowed, indicating immunoreaction Ab with chlorpyrifos was saturated. Thus, the incubation time of 60 min was selected.

3.3. Specificity of the Detection and Regeneration Performance

The IDAMs based impedance immunosensor were evaluated for specificity by testing other non-target small molecule pesticides such as methomyl, dichlorophos, Carbofuran, phoxim and mixture as shown in Fig. 5. The specificity of the immunosensor was mainly dependent upon the antibodies that were immobilized on the electrode surface.

The results indicated the proposed immunosensor had a high degree of selectivity for chlorpyrifos detection.

Good regeneration performance is an important index for the popularization and application of the immunosensor used common electrode. The generating process was tedious, regenerating steps was very complex and regenerating time was too long.

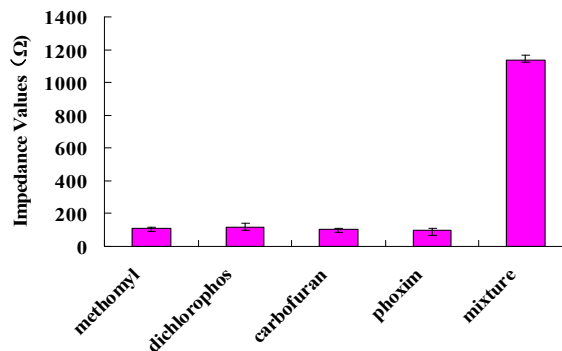


Fig. 5. Immunosensor specificity analysis.

Many bioreagent integral to immunoassays was very expensive, such as the antibodies, enzymes and other reagents often hundreds of dollars per milligram, while miniaturization reduces reagent costs drastically. In our article the volume of the reagents were micro upgrade, the antibodies, SPA and BSA were 30 μL , the chlorpyrifos sample and detection solution were also only 50 μL .

Now the reality is that microelectrode is too expensive. Low cost, mass production technology is not yet mature. In the near future, if we could buy disposable microelectrode at a very low price, the repeatability and regeneration problem based on microelectrode biosensor would be solved.

4. Conclusions

In this work, a sensitive and stable label-free impedance immunosensor has been developed for the fast and direct detection of chlorpyrifos residues based on the immobilization of chlorpyrifos antibody on IDAM electrode by SPA. In order to improve the capacity of antibody and enhance the detection sensitivity, the experimental parameters were optimized.

The presence of the SPA not only promoted impedance signal, but also increased the surface area to capture a large amount of antibodies, thus increased detection sensitivity. This result demonstrated that SPA was suitable bio-receptor for the selective detection of small molecules.

The constructed immunosensor processing prominent characteristic and performance such as better specificity, reproducibility and economical efficiency have potential application for the detection of other pesticides or compounds. This biosensor system could be implemented for detection of chlorpyrifos analysis in real filed sample such as contaminated food products after further optimized.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 30972055, 31101286), Agricultural Science and Technology Achievements Transformation Fund Projects of the

Ministry of Science and Technology of China (No. 2011GB2C60020) and Shandong Provincial Natural Science Foundation, China (No. Q2008D03).

References

- [1]. X. Guardino, J. Obiols, M. G. Rosell, A. Farran, C. Serra, Determination of chlorpyrifos in air, leaves and soil from a greenhouse by gas-chromatography with nitrogen-phosphorus detection, high-performance liquid chromatography and capillary electrophoresis, *Journal of Chromatography A*, 823, 1-2, 1998, pp. 91-96.
- [2]. Registration Standard (Second Round Review) for the Reregistration of Pesticide Products Containing Chlorpyrifos, U. S. Environmental Protection Agency, Washington, DC, 1989, pp. 5-44.
- [3]. M. A. Gallo, N. J. Lawryk, Organic phosphorus pesticides, In Handbook of Pesticide Toxicology, Hayes, W. J., Jr. and Laws, E. R. Jr., Eds., Academic Press, New York, NY, 1991, pp. 5-3.
- [4]. J. Ye, J. Wu, W. P. Liu, Enantioselective separation and analysis of chiral pesticides by high-performance liquid chromatography, *Trends in Analytical Chemistry*, 28, 10, 2009, pp. 1148-1163.
- [5]. H. X. Guan, W. E. Brewer, S. T. Garris, S. L. Morgan, Disposable pipette extraction for the analysis of fruit and vegetables using gas chromatography/mass spectrometry, *Journal of Chromatography A*, 1217, 12, 2010, pp. 1867-1874.
- [6]. K. Rekha, M. S. Thakur, N. G. Karanth, Biosensors for the Detection of Organophosphorous Pesticides, *Critical Reviews in Biotechnology*, 20, 3, 2000, pp. 213-235.
- [7]. D. D. Tuoro, M. Portaccio, M. Lepore, F. Arduini, D. Moscone, U. Bencivenga, D. G. Mita, An acetylcholinesterase biosensor for determination of low concentrations of Paraoxon and Dichlorvos, *New Biotechnology*, 29, 1, 2011, pp. 132-138.
- [8]. S. M. Radke, E. C. Alocilja, A high density microelectrode array biosensor for detection of E. coli O157:H7, *Biosens. Bioelectron.*, 20, 8, 2005, pp. 1662-1667.
- [9]. E. Bakkar, Y. Qin, Electrochemical Sensors, *Anal. Chem.*, 78, 12, 2006, pp. 3965-3984.
- [10]. F. Darain, D. S. Park, J. S. Park, Y. B. Shima, Development of an immunosensor for the detection of vitellogenin using impedance spectroscopy, *Biosensors and Bioelectronics*, 19, 10, 2004, pp. 1245-1252.
- [11]. G. Chen, Y. Lin, J. Wang, Monitoring environmental pollutants by microchip capillary electrophoresis with electrochemical detection, *Talanta*, 68, 2006, pp. 497-503.
- [12]. L. J. Yang, Y. B. Li, G. F. Erf, Interdigitated Array Microelectrode-Based Electrochemical Impedance Immunosensor for Detection of Escherichia coli O157:H7, *Anal. Chem.*, 76, 4, 2004, pp. 1107-1113.
- [13]. S. M. Radke, E. C. Alocilja, Design and fabrication of a micrompedance biosensor for bacterial detection, *IEEE Sens. J.*, 4, 4, 2004, pp. 434-440.
- [14]. S. M. Radke, E. C. Alocilja, A Microfabricated Biosensor for Detecting Foodborne Bioterrorism Agents, *IEEE Sens. J.*, 5, 4, 2005, pp. 744-750.
- [15]. L. J. Yang, Y. B. Li, AFM and impedance spectroscopy characterization of the immobilization of antibodies on indium-tin oxide electrode through self-assembled monolayer of epoxysilane and their capture of Escherichia coli O157:H7, *Biosens. Bioelectron.*, 20, 7, 2005, pp. 1407-1416.
- [16]. M. Ciszowska, Z. Stojek, Voltammetry in solutions of low ionic strength. Electrochemical and analytical aspects, *J. Electroanal. Chem.*, 466, 2, 1999, pp. 129-143.
- [17]. J. Min, A. Baeumner, Characterization and Optimization of Interdigitated Ultramicroelectrode Arrays as Electrochemical Biosensor Transducers, *Electroanalysis*, 16, 9, 2004, pp. 724-729.
- [18]. M. Varshney, Y. B. Li, Interdigitated array microelectrodes based impedance biosensors for detection of bacterial cells, *Biosensors and Bioelectronics*, 24, 10, 2009, pp. 2951-2960.
- [19]. D. J. Liu, R. K. Perdue, L. Sun, M. Crooks, Immobilization of DNA onto Poly (dimethylsiloxane) Surfaces and Application to a Microelectrochemical Enzyme-Amplified DNA Hybridization Assay, *Langmuir*, 20, 14, 2004, pp. 5905-5910.
- [20]. E. Nebling, T. Grunwald, J. Albers, P. Schafer, R. Hintsche, Electrical Detection of Viral DNA Using Ultramicroelectrode Arrays, *Anal. Chem.*, 76, 3, 2004, pp. 689-696.
- [21]. R. H. Wang, Y. Wang, K. Lassiter, Y. B. Li, B. Hargis, S. Tung, L. Berghmand, W. Bottje, Interdigitated array microelectrode based impedance immunosensor for detection of avian influenza virus H5N1, *Talanta*, 79, 2, 2009, pp. 159-164.
- [22]. Prodromidis M. I., Impedimetric immunosensors, *Electrochim. Acta.*, 55, 2010, pp. 4227-4233.
- [23]. Berggren C., Bjarnason B., Johansson G., Capacitive biosensors, *Electroanalysis*, 13, 2001, pp. 173-180.
- [24]. Janek R. P., Fawcett W. R., Ulman A. J., 1997 Impedance spectroscopy of self-assembled monolayers on Au(111): evidence for complex double-layer structure in aqueous NaClO₄ at the potential of zero charge, *J. Phys. Chem. B*, 101, 1997, pp. 8550-8558.
- [25]. Hubrecht J., Electrochemical impedance spectroscopy as a surface analytical technique for biomaterials, in Metals as Biomaterials, J. A. Helsen and J. Breme (eds.), John Wiley & Sons, New York, 1988, pp. 405-466.