IMMUNOSENSORS FOR ATRAZINE DETECTION IN RED WINE SAMPLES

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Keywords: Immunosensor; Interdigitated μ-electrodes, Atrazine; Impedance spectroscopy, Conductive measurements, Wine matrix effect, Food safety.

Abstract: Two novel immunosensors, one impedimetric and other one conductimetric, for atrazine detection in red wine samples have been developed. Impedimetric immunosensor is based on an array of interdigitated μ-electrodes (IDμEs) and bioreagents specifically developed to detect this pesticide. Conductimetric immunosensor incorporates additionally gold nanoparticles. Bioreagents were covalently immobilized on the surface of the electrodes (interdigital space). In both cases the biochemical determination of atrazine is possible without any redox mediator. For the case of the impedimetric immunosensor, the detection method is based on impedimetric measurements (in a wide range of frequencies), whereas in the case of the conductimetric immunosensor the detection method is based on conductimetric measurements (DC measurements).The potential of the impedimetric immunosensor to analyze atrazine in complex sample matrices, such as red wine, have been evaluated. This immunosensor can detect atrazine with limits of detection in the order sub-ppb, far below the maximum residue level (MRL) (50 μg L^{-1}) established by European Union (EU) for residues of this herbicide in the wine grapes.

1 INTRODUCTION

In the recent years, modern chemical analysis has been revolutionized by the electrochemical biosensors because of their accuracy, technical simplicity, high efficiency, possibility of portability and miniaturization, and because they offer fast (instantaneous) response times, allow a rapid and permanent control and a direct transduction of the biomolecular recognition event into electronic signals (Murphy, 2006; Pumera, 2007; Wang, 2006).

An important disadvantage of the electrochemical sensors is that the impedance changes due to biomolecular recognition are generally very small and it can not reach the necessary detection limits required by the legislation. However, this disadvantage can be solved by applying techniques such as electrochemical impedance spectroscopy (EIS) or including labels that amplify the signal.

By means of EIS is possible to record information on biorecognition events, occurring at the electrode surfaces, inducing impedance changes (Guan et al., 2004; Katz and Willner, 2003), that can be directly measure, allowing the development of label-free biosensing devices.

On the other hand, many types of labels are used to amplify biorecognition events. Between the labels, the gold nanoparticles are some of the most recently used (Pumera, 2007; Zhang, 2007), because its unique properties at nanoscale dimension.

In this work we report the description of two immunosensors for atrazine detection. One immunosensor is label-free, whereas the other one is labelled with gold particles (40 nm). In the case of the label-free immunosensor, impedimetric measurements (impedance spectroscopy) are used as detection method. In the second case, due the presence of the gold particles, conductimetric measurements (DC measurements) could be used.

Valera E., Rodríguez Á., Ramón-Azcón J., J. Sanchez F. and Marco M. (2009).
IMMUNOSENSORS FOR ATRAZINE DETECTION IN RED WINE SAMPLES .
In *Proceedings of the International Conference on Biomedical Electronics and Devices*, pages 226-230
DOI: 10.5220/0001542002260230

In order to demonstrate the applicability of the devices developed, wine production has been selected as scenario for the proof-of-concept study. Reasons for this selection are because wine is a high value product (with a great economic relevance in EU) and because their strong matrix effect. In fact, if the sensors are demonstrated to red wine samples, their use can be extrapolated to many other matrices. Likewise, we will prove that the impedimetric immunosensor can respect the Maximum Residue Level (MRL) established by EU (European Union) for residues of this herbicide in wine grapes (50 µg L^{-1}).

2 EXPERIMENTAL

2.1 Instrumentation

Impedimetric and conductive measurements were carried out at room temperature in a probe station (Faraday cage) KARL SUSS. Impedance analyses were performed using an Agilent 4294A Precision Impedance Analyzer and conductive measurements were performed using an Agilent 4156C Semiconductor Parameter Analyzer. The competitive curves were analyzed with a four-parameter logistic equation using the software SoftmaxPro v2.6 (Molecular Devices) and GraphPad Prism version 4.00 for Windows (GraphPad Sofware, San Diego California USA). Data shown correspond to the average of at least three replicates per concentration of atrazine.

2.2 Arrays of Interdigitated µ-electrodes

The immunosensors developed are based on a two coplanar non-passivated interdigitated metallic µ-electrodes. Thin Au/Cr (200 nm thickness) interdigitated µ–electrodes with 10 µm pitch were patterned on a Pyrex 7740 glass substrate (purchased from Präzisions Glas&Optik GmbH, 0.7 mm (±0.05) thickness). For the immunosensor measurements, electrode arrays were constructed consisting on six IDµEs organized on a 0.99 cm^2 area. The Pyrex substrate was first cleaned using absolute ethanol. Following metal deposition was performed by sputtering and the interdigitated µ-electrodes were patterned by a photolithographic metal etching process. The chromium layer, much thinner than the gold layer, was deposited prior the gold to improve adhesion to the Pyrex substrate.

2.3 Immunosensors Functionalization

Biofunctionalization with 2d-BSA was done selectively on the surfaces of the gold electrodes. The chemical recognition layer was covalent immobilized on the interdigital space. For this purpose the array of IDµEs were treated as follows.

2.3.1 Surface Cleaning

Before functionalization, the IDµEs samples were first cleaned with a combination of ethanol:Mili-Q water 70:30, absolute ethanol absolute, piranha solution and absolute ethanol.

2.3.2 Surface Activation

After the pre-treatment explained above, surface activation took place in two steps to modify selectively the gold electrodes and the Pyrex substrate.

Activation of gold surfaces was readily and specifically performed using thiol-chemistry. N-acetylcysteamine was used to cover the gold electrodes and to protect the sensor from undesired non-specific absorptions. Thus, the surface texture of the IDµE defines the template for deposition of layers, since the gold fingers have been deposited on a solid support such as glass with the necessary controlled geometry. In a second step, the Pyrex was derivatized with 3-glycidoxypropyl trimethoxysilane (GPTS). The epoxy group provided the necessary reactivity for further attachment of the bioreagents through a nuchelophylic attack of functional groups of the biomolecule such as the amino groups of the lysine residues (Luzinov, 2000). The immunosensor surface functionalization is schematically shown in Figure 1.

2.3.3 Antigen Immobilization

Covalent immobilization of the pesticide antigen 2d-BSA was performed on the interdigitated µ-electrodes surface via the amino groups of the lysine residues by reaction with the epoxy groups of the surface.

2.4 Competitive Assay

The assay of detection, common for both immunosensors, relies on the immunochemical competitive reaction between the atrazine residues and the immobilized antigen on IDµEs for a small amount of the specific antibody (primary antibody). The detection of a small number of molecules of

atrazine is performed under competitive conditions involving the competition between the free pesticide (analyte) and a fixed amount of coated antigen for a limited amount (low concentration) of primary antibody (Ab_1). At the end of the reaction the amount of Ab_1 captured on the IDμE surface and hence the free antigen (analyte) is determined.

Finally, and only in the case of the conductimetric immunosensor, a secondary labelled with gold antibody (Ab_2) is captured in order to amplify the conductive signal. The immunosensor assay is schematically shown in Figure 2.

2.5 Impedance Measurements

Impedance measurements were carried out at room temperature. No redox mediator was used in the devices presented in this work. The two electrodes were covered by a diluted PBS solution with a conductivity of 1.6 μS cm^{-1} and connected to the input of an Agilent 4294A Precision Impedance Analyzer by means of standard probe tips.

Measurements were taken in the 40 Hz to 1MHz frequency range using 0V of polarization potential and a modulation voltage of 25mV amplitude. All impedance measurements were performed in a Faraday cage.

2.6 Conductance Measurements

As for impedance measurements, the conductance measurements were also carried out at room temperature without any redox mediator and in a Faraday cage. The two electrodes were covered by a diluted PBS solution with a conductivity of 1.6 μS cm^{-1} and connected to the input of an Agilent 4156C Semiconductor Parameter Analyzer by means of standard probe tips. Conductivity was measured to +25 mV, from +22.5 to +27.5 mV sweep bias. These conductive measurements were performed after the incubation step of the secondary antibody labelled with gold.

3 ATRAZINE DETECTION

In the case of the impedimetric immunosensor, the quantitative tool that seems adequate to provide sensitivity graphs is the impedance measurement in a wide frequency range and the fitting of the Nyquist plots of impedance spectra to an equivalent circuit.

Then, the atrazine concentration should finally be related to the values of at least some of the parameters of the equivalent circuit. The equivalent circuit used was previously reported (Valera, 2007) and the resistance of the solution (Rs) was chosen as parameter of analysis.

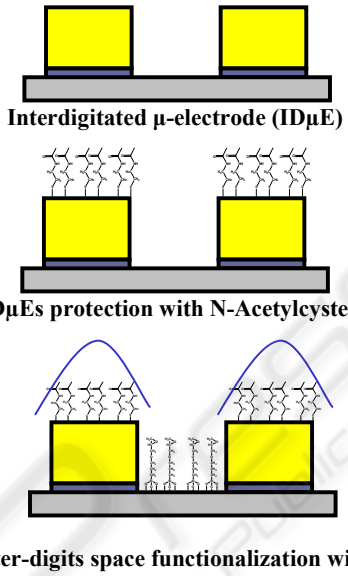

Figure 1: Schematic diagram of: i) protection of interdigitated μ-electrodes with N-acetylcysteamine; and ii) immunosensor surface functionalization with GPTS.

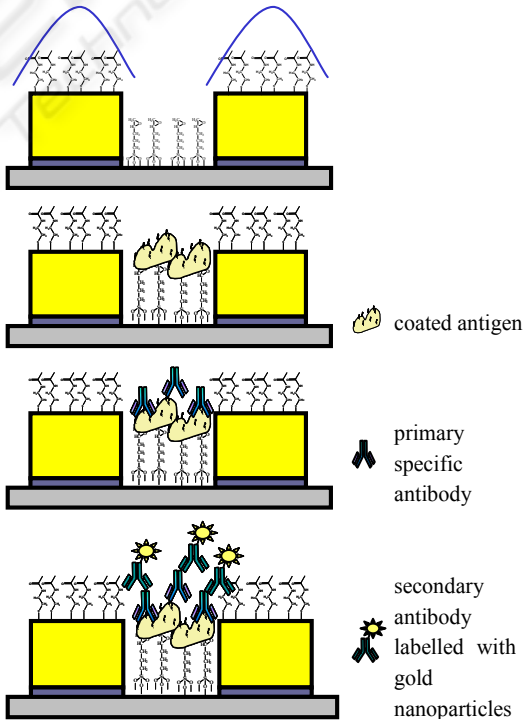

Figure 2: Schematic diagram of the complete assay system performed on the IDμEs for the immunosensors developed.

For the conductimetric immunosensor, the measurement of the conductance after the capture of the secondary labelled with gold antibody could be used as detection method, as it was previously demonstrated using PBS in the competitive assay (Valera, 2008). Then, the atrazine concentration would be related to the amount of gold nanoparticles present on the immunosensor.

The impedimetric response of the immunosensor in red wine is shown in Figure 3.

To validate the sensing approach, our immunosensors were also characterized by means of chemical affinity methods. In Figure 4 the results obtained from the immunosensor are compared with the results of an ELISA assay on the same IDμEs devices but using a chemically raised colorimetric signal. In order to comparatively show the immunosensor performance, in Figure 4 the normalized values of the change in the Rs as a function of the atrazine concentration as well as the normalized results of the ELISA assay are plotted.

It is important to remark that the ELISA assay performed on the IDμE device show a similar analytical profile that the obtained using microtitrer plates. Therefore, we could be confident that our immunosensor really reflects the selective binding event. The more important analytical features of the atrazine assays (immunosensors and ELISA) are shown in Table 1.

As it can be seen in Table 1, using the impedimetric immunosensor is possible to detect atrazine in sub-ppb concentrations (0.19 μg L^{-1}). These good results are directly related to the advantages of the impedimetric device presented such as the use of IDμEs and the competitive assay based on the antibodies variation.

Comparing these results with the recent literature, the impedimetric immunosensor presented in this work have been demonstrated to be more sensitive that other biosensors based on impedimetric methods for the atrazine detection (Helali, 2006; Hleli, 2006; Fredj, 2008), specially taking into account that our immunosensor have been tested using red wine samples.

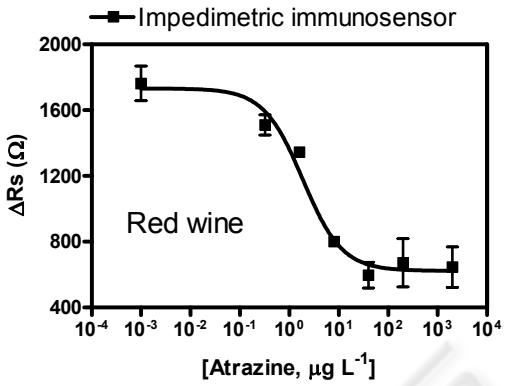

Figure 3: Impedimetric response when the immunosensor are used to detect atrazine. Reprinted from Biosensors and Bioelectronics, 23, J. Ramón-Azcón et al., An impedimetric immunosensor based on interdigitated microelectrodes (IDμE) for the determination of atrazine residues in food samples, 1367–1373, (2008), with permission from Elsevier.

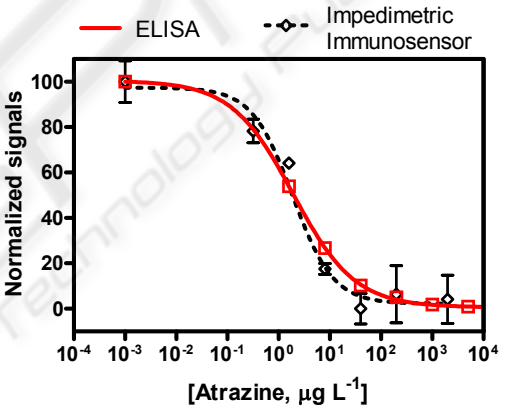

Figure 4: Normalized calibration curves of the optimized atrazine immunoassay and the impedimetric immunosensor presented. Measures were taken in diluted PBS solution.

Table 1: Features of the atrazine assays in red wine samples.

	Impedimetric Immunosensor	ELISA
IC_{50}, μg L^{-1}	1.876±0.23	1.93±0.02
LOD, μg L^{-1}	0.19	0.09
R^2	0.86	0.99

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4 CONCLUSIONS

Two immunosensors, one impedimetric and other one conductimetric, for the atrazine detection in red wine samples have been developed. Both devices are based on an array of IDμEs and in bioreagents specifically developed. Impedimetric immunosensor is able to detect atrazine in red wine at sub-ppb concentrations, far below the Maximum Residue Level (MRL, 50 μg L^{-1}) required by EC. However, this result could be improved using the conductimetric device, which includes secondary antibodies labelled with gold particles that increase the conductive signal.

ACKNOWLEDGEMENTS

This work has been partially supported by the Ministry of Science and Technology (Contract number TEC2007-67081) and FEDER funds. The MNT group is a consolidated *Grup de Recerca de la Generalitat de Catalunya* since the year 2001 (expedient 00329). The AMR group is a consolidated *Grup de Recerca de la Generalitat de Catalunya* and has support from the Departament d'Universitats, Recerca i Societat de la Informació la Generalitat de Catalunya (expedient 2005SGR 00207).

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