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Chapter 10

Immunosensors: Concepts and Structures for Fast and Accurate Sensing

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/55969

1. Introduction

Biosensors based in interdigitated transducers have demonstrated the capability to fulfil the requirements of the market in a broad range of applications: their combination with biomolecules such as antibodies allows the development of high selectivity sensors. Their electrical excitation and readout makes easy to develop electronic equipment that perform automatically the operations required for the measurement and the fluidics manipulation involved in it. The sensitivity levels required for many applications can be reached using low cost interdigitated electrodes with finger sizes of several microns, thus eliminating the need for expensive submicron technologies. In another hand the use of plastic substrates helps further reducing the costs of the sensors. All this together makes these sensors excellent candidates to satisfy the demands of potentials users in terms of quality, cost, suitability of apparatus that can be developed, automatization, etc.

One of the important issues that arise when potential users are consulted is the time required to perform a test. The total time since the sample is introduced in the sensor until the measurement is ready, thus involving fluidic manipulation, functionalization, and the measurement itself often is desirable to be in the order of several (few) minutes or lower. While different schemes can be applied to optimize the duration of the fluidic manipulation to adapt it to the time per test required in each case, the time in which the electrical measurement is done may be important: A very common detection method is the impedance spectroscopy sweeping a wide frequency range. This is a powerful, but cumbersome tool to study the sensor performance providing trustworthy results. Performing impedance measurements in a broad range of frequencies is time consuming, especially if a high number of tests are to be done and when these tests involve low frequencies.

Markets demands faster alternatives capable of providing measurements in shorter times without compromising the requirements of sensitivity of the measurements.

In this chapter three strategies for the fast quantification are explained:

- Single Frequency measurements
- DC measurements
- Multi-sine burst signal

These techniques are analyzed and their advantages in comparison with the classical wide range impedance spectroscopy method are commented.

The technique has been applied to the detection of atrazine in foodstuff products. Though in this contribution atrazine has been chosen as the analytical target, this immunosensor technology can be used for a wide variety of compounds using the appropriate selective antibodies.

Many types of sensors have been developed for the detection and quantification of pesticides and traces of them. The MRL of a given pesticide, which often lies in the order of few tens of ppbs, determines the minimum sensitivity of these sensors. Also the quantification, or the detection, of one substance in the complex chemical matrix of some foods as wine, milk or juices also poses important requirements to their selectivity.

Currently, in the literature, good examples of impedimetric immunosensors devoted to food safety can be found [1]. Although these devices have demonstrated to be efficient, typically the impedance analysis in a wide frequency range is not fast, requires expensive equipments and qualified personnel. These features imply a great difficulty to obtain a commercial product. Therefore, if commercial devices are desired from this technology, some variations should be including in order accomplishing the market requirements.

In this chapter two cases of immunosensors are analysed. Firstly, an impedimetric immunosensor based on the analysis of only one frequency, instead of the broad frequency spectrum is studied. By means of this technique, the data treatment related to the analysis of the broad frequency spectrum is avoided. Alternatively, another measurement technique based on the application of a multi-sine burst signal is also explored, as a method to hardly reduce the cost related to the expensive equipments. Then, the advantages of a second device, the conductimetric immunosensor are also exanimate. In this device a secondary antibody labelled with gold nanoparticles is included in order to produce a conductive label on the electrodes that opens the possibility of applied simple and inexpensive DC measurements. In addition, but within the same device, an interesting change of substrate is studied.

2. Description of the immunosensors

In this section, structure, functionalization, working mechanisms, as well as the contribution of the immunosensors treated to obtain a commercial product are described.

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2.1. Interdigitated μ-electrodes (IDμE's)

Interdigitated μ-electrodes (IDμE's) were used as transducers for the immunosensors presented in this chapter. Interdigitated μ-electrodes are two coplanar electrodes (that works as counter and working electrodes) which have equal surface areas and each is presumed to contribute equally to the measured network impedance. The standard procedure of the electrodes fabrication, which can be made in large quantities and at low cost, is as follows:

Thin Au/Cr (~ 150 nm thickness) interdigitated μ-electrodes (IDμE's, ~ 5 μm thick and electrode gap) were patterned on a Pyrex 7740 glass substrate. The objective of the chromium layer is just to improve the adhesion of the gold to the Pyrex substrate, thus, the Cr layer is much thinner than the Au layer (aprox. 1:10 ratio).

Before metal deposition, a washing step of the substrate, using absolute ethanol, is required. The metal deposition was performed by means of sputtering deposition and the interdigitated μ-electrodes were then patterned on the Pyrex substrate by a photolithographic metal etch process. For the immunosensor measurements, arrays consisting on six IDμE's organized on a 1 cm^2 area were constructed.

Before functionalization, the samples were first cleaned in a solution of ethanol absolute 70% and Milli-Q water 30%. Then, the samples were plunged for 12 h in a solution of NaOH 2.5% in Milli-Q water. Afterwards, the 12 h the samples were rinsed in 100mL of Milli-Q water in order to neutralize the action of the NaOH. Finally, the arrays of IDμE's were dried with ethanol and N_2.

2.2. Impedimetric immunosensor based on singly frequency impedance measurements

The impedimetric immunosensor presented, whose detection method is based on single frequency impedance measurements, is a robust and *label-free device*. This device is based on the use IDμE's arrays, bioreagents specifically developed against the desired compound and on the *impedimetric change* that occurs when the immunoassay is performed on the electrodes surface.

An immunochemical competitive reaction (Figure 1), between the pesticide residues and the immobilized antigen on IDμE's for a small amount of the specific antibody, is performed on the electrodes. This reaction involves the competition between the free pesticide and a fixed amount of coated antigen for a limited amount of antibody (Ab). The amount of Ab captured on the IDμE surface, and hence the free antigen, is determined by means of impedimetric analysis.

As the immunosensor does not measure an amount of pesticide, because it measures an amount of antibody, the change in the impedance is due to the addition of antibody in the sensor surface, and not to the addition of molecules of pesticides. This approach has an important effect on the sensitivity of this immunosensor, because the molecules of antibody are much bigger than the molecules of pesticide and their effect on the impedance of the device is much higher. This feature represents an important advantage in comparison with other impedimetric immunosensors reported previously [2-4]. As a consequence, authors of these

works must reduce the electrode size to nanometer scale [2], or otherwise their limits of detection can only achieve tens of ppbs [3, 4].

Immunosensor functionalization consists on two main steps: i) the coating antigen (CA) immobilization; and ii) the specific antibody capture.

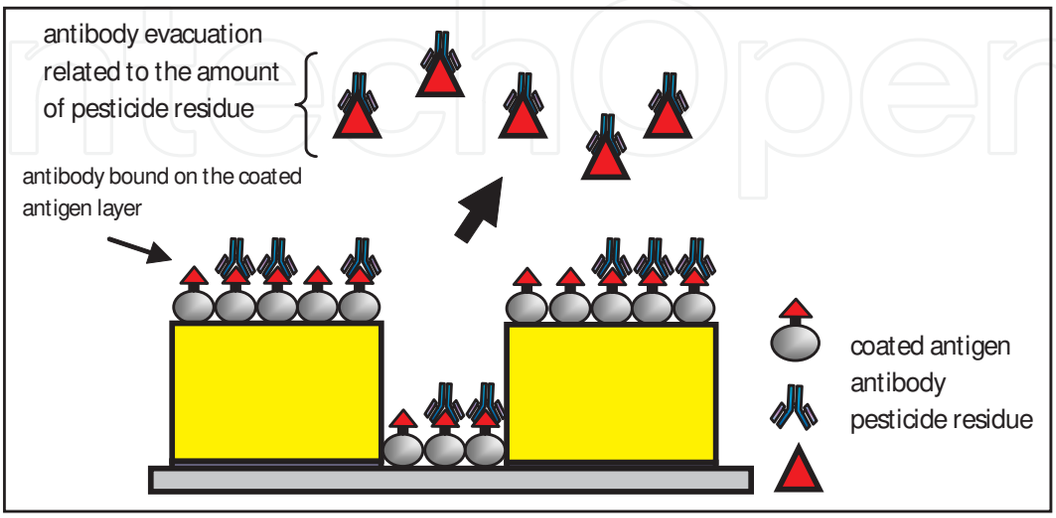

Figure 1. Immunosensor reaction. An amount of the specific antibody is bound on the coated antigen layer. Other quantity is evacuated of the IDμE's; this amount is related to the pesticide concentration.

As it has been demonstrated in the literature [1], the detection method based on impedance measurement in a wide frequency range, works correctly and accurate results can be obtained from it. Nevertheless, this method can be tedious and time consuming because computer processing is required to extract the values of all parameters involved. For this reason, an alternative method for the detection of pesticide based on measurements at single frequencies was raised.

By this method, single frequencies are applied, instead of the wide frequency range, for the atrazine detection. In the case the computer processing related to the fitting of the Nyquist plots of impedance spectra to the equivalent circuit is completely avoided because this detection method is based on the measurement of just the module (|Z|), the real part (Zre) or the imaginary part (Zim) of the impedance at a single frequency.

As it is well known, the inclusion of antibody on the immunosensor (antibody capture step) implies a variation in the immunosensor impedance (real and imaginary part of the impedance). This variation can be represented for the values of the equivalent circuit elements. Therefore, the changes of the values of the elements of the equivalent circuit are due to variations of immunosensor impedance. Then, the changes of the elements of the equivalent circuit are strongly related to the variation of |Z|, Zre and Zim.

This detection method consists on the measurements of the module, the real part or the imaginary part of the impedance of the immunosensor at a single frequency for the analysis. For this reason, both |Z|, Zre and Zim have been studied in order to analyze which of them

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have better performance for the different range of frequencies. Their performance is determined by comparing the limit of detection (LOD) for their response curves. The variation of the |Z|, Zre or Zim of the IDμE's, between the antigen incubation and the competition step is plotted against atrazine concentration to obtain the response curve. In order to find the best immunosensor response and improvements in the LOD, two kind of variation of the impedance have been taking into account:

i. Absolute difference:

$$\Delta Zabs = |Z(Ab) - Z(AT)|, \quad (1)$$

and

ii. Relative difference:

$$\Delta Zrel = \left| \frac{Z(Ab) - Z(AT)}{Z(AT)} \right| \quad (2)$$

where Z(Ab) is the impedance (module, real part or imaginary part) of the antibody captured and Z(AT) is the impedance (module, real part or imaginary part) of the antigen previously immobilized.

The impedance measurements were executed at room temperature, applying an AC signal of 25 mV of amplitude in the frequency range from 40 Hz to 1 MHz and using 0 Vdc bias potential, in diluted PBS solution (1.6 μS cm^{-1}). Likewise, all impedance measurements were done in a Faraday cage.

This method has been demonstrated using several, representing the ranges of the middle (30 kHz to 150 kHz) and high (150 kHz to 1 MHz) frequencies. The bandwidth was chosen in order to taking into account all regions of the impedance spectrum between the middle and high frequencies.

2.3. Conductimetric immunosensor based on DC measurements

Although this device is also based on the use IDμE's arrays, and bioreagents specifically developed against atrazine, this immunosensor includes a *secondary antibodies labelled with gold nanoparticles*. The deposition of this secondary antibody on the electrodes surface produces a detectable *conductimetric change*.

Again, an immunochemical competitive reaction is used for reach the pesticide detection. However in this case, a secondary antibody (Ab_2) is included (Figure 2). These secondary antibodies, linked to the gold particles, constitute a conductive film between the electrodes. Thus, the conductance of this film will depend on the concentration of gold labelled antibodies.

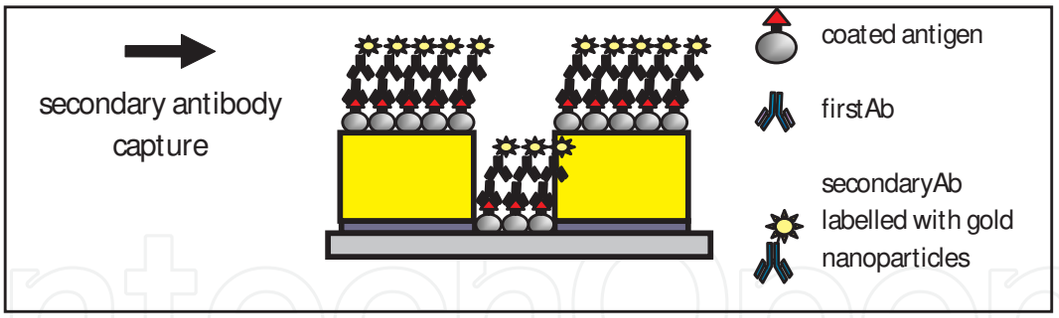

Figure 2. Schematic diagram of the complete assay system performed on the IDμE's for the conductimetric immunosensor.

The functionalization of this immunosensor consists in three main steps: i) the coating antigen (CA) immobilization; ii) the specific antibody capture (Ab_1); and iii) the capture of a non-specific antibody (Ab_2) labelled with gold nanoparticles.

The competition reaction is performed during step i and ii. During step iii, Ab_2 is linked to Ab_1, and then the Ab_2 concentration is related to the Ab_1 concentration included. Therefore, the concentration of the free pesticide tested is related to the amount of gold nanoparticles, which is measured by DC measurements. In this new structure, gold particles act as new *small fingers*, reducing the gap of the interdigitated μ-electrodes [5].

Using this conductimetric immunosensor has been demonstrated that pesticides residues can be detected by means of simple and inexpensive DC measurements, when gold nanoparticles are included as labels in the immunosensor [6, 7]. When a DC voltage is applied to an interdigitated μ-electrode with gold particles attached to it, the DC current which passed through the electrodes grows clearly in relation to the concentration of pesticide included.

As well as the impedimetric case, the conductive measurements were also carried out at room temperature, in a Faraday cage and without the use of any redox mediator. The two interdigitated 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.

In comparison with the impedimetric measurements performed for the typical impedimetric immunosensors, the conductive measurements are easier to execute, because impedance spectroscopy is not required and the fitting procedure to the equivalent circuit model is also completely avoided.

3. Functionalization of the immunosensors

For the immunosensors development, the biofunctionalization (immobilization) of the biological element onto the transducer surface is required

Over the coated layer of antigen, the free specific antibody is captured by affinity. In the case of the conductimetric immunosensor, a secondary antibody labelled with a gold nanoparticle

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is attached to the primary specific antibody in order to amplify the affinity event and obtain a good conductive response.

As the dielectric properties of the biological systems are very remarkable, the developed devices can exhibit a good impedimetric response.

3.1. Functionalization of the impedimetric immunosensor

In the case of the impedimetric immunosensor, the passive adsorption technique was used. By means of this method the chemical changes on the impedimetric immunosensor surface follow basically two steps:

i. Step I, antigen immobilization on the IDμE;

ii. Step II, specific antibody capture in the competition step.

The chemical recognition layer was deposited on top of the interdigitated μ-electrodes area (fingers and inter-digits space). These chemical procedures are schematically shown in Figure 3.

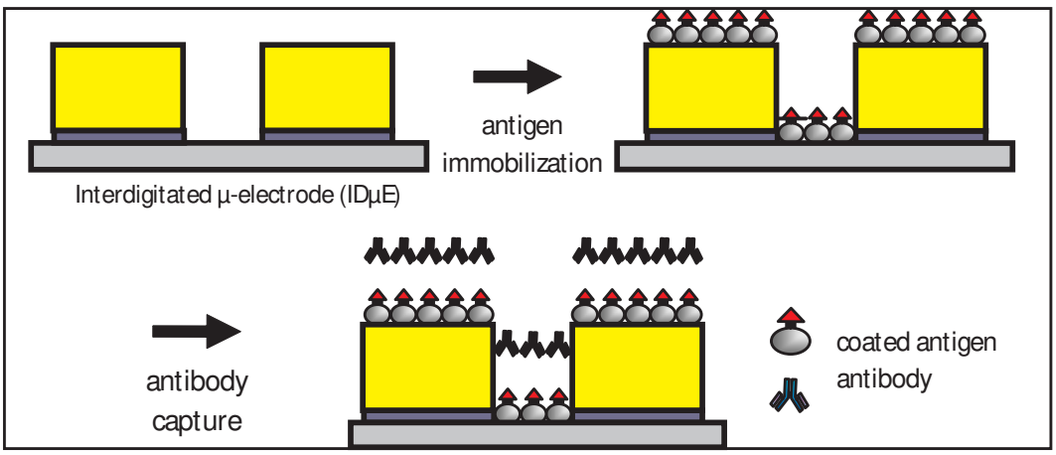

Figure 3. Schematic diagram of the complete assay system performed on the IDμE's for the impedimetric immunosensor.

3.2. Functionalization of the conductimetric immunosensor

In this case, a covalent immobilization technique was applied. Then, the chemical changes on the conductimetric immunosensor surface follow five steps, two previous steps for the immunosensor surface functionalization and other three steps for the immunosensor reaction:

i. Step I, protection of interdigitated μ-electrodes with N-acetylcysteamine;

ii. Step II, immunosensor surface functionalization with GPTS;

iii. Step III, covalent immobilization of the antigen on the IDμE;

iv. Step IV, specific primary antibody (Ab_1) capture in the competition step;

v. Step V, secondary labelled with gold antibody (Ab_2) capture.

Due to the covalent immobilization, the chemical recognition layer was deposited only on the gap of the interdigitated μ-electrodes. The complete functionalization procedures of the conductimetric immunosensor are schematically shown in Figure 4.

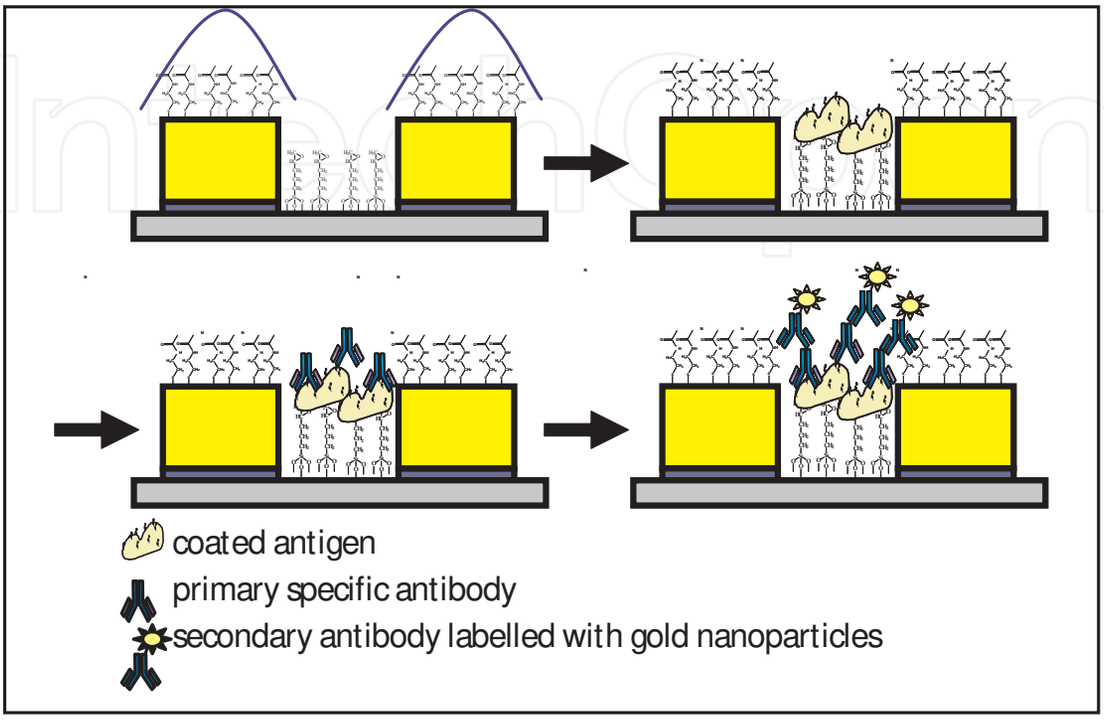

Figure 4. Schematic diagram of the complete assay system performed on the IDμE's for the conductimetric immunosensor.

4. Pesticide residues detection

Both types of immunosensors described in previous sections (impedimetric and conductimetric) have been tested for the detection of free pesticide samples. For that, *atrazine*, a widely used pesticide in the wine industry, as well as for the test of novel biosensors [8-12], has been used as pesticide of test.

Atrazine, a widely used selective herbicide for the control of annual grasses and broadleaved weeds, has often been found in drinking water, and therefore, they are a potential threat for the public health [13-16]. The European Community has established maximum residue level for residues of this herbicide in wine grapes in 50 $\mu g\ L^{-1}$.

The competitive reaction carried out on the interdigitated μ-electrodes has been performed in buffer solution (impedimetric and conductimetric immunosensor) and in wine samples (conductimetric immunosensor). The performance details of both types of devices as atrazine detectors are detailed below.

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4.1. Impedimetric immunosensor based on singly frequency impedance measurements

In this case, experiments were carried out with different atrazine concentrations (1.6 – 1000 μg L^{-1}) during the competition step in order to qualitatively show how the impedimetric immunosensor is sensitive to the atrazine concentration using the detection method of single frequencies. These experiments were performed using different ID's samples for every concentration.

Several frequencies of work between 35 kHz to 1 MHz were chosen in order to take into account all regions of the impedance spectrum between the middle and high frequencies. Frequencies below 30 kHz were excluded from the sensor characterization because their unstable response. As it is well known, these frequencies are related to the Warburg impedance and to the double-layer capacitance. Typically, in the impedimetric immunosensors, some scattering was found in the impedance variations at these frequencies due to the influence of these parameters. Therefore, these frequencies were found inadequate to obtain confident results.

Measurements were also differential in order to suppress the non-ideal effects related to the geometry or technology of the IDμE's. Due to that, the change in the impedance (ΔZ_{abs} and ΔZ_{rel}) was calculated between the Step I and the Step II of the impedimetric immunosensor functionalization. The impedance variations, in relation with different atrazine concentrations and with the frequency of work, were analyzed and the results are shown below. Several detection curves at different frequencies of work have been executed using the variation of the module of impedance (|Z|), the variation of the real part of the impedance (Zre) and the variation of the imaginary part of the impedance (Zim). These changes have been obtained taking into account the absolute and relative differences described above. In order to analyze the impedimetric immunosensor response, detection curves obtained at these frequencies were compared and studied separately for each frequency range, as it can be seen in Figures 5, 6 and 7.

These figures only show some examples of the detection curves obtained by means of the relative difference of impedance. Detection curves using the absolute difference of impedance are not included in this manuscript because their shape is very similar to the detection curves using the relative difference of impedance and because, as it will be noticed, important improvements in the features of the atrazine assay (LOD) have been found, at middle frequencies (35.37 kHz to 131.95 kHz), when the relative difference is used, in comparison to the absolute difference in all cases.

Figure 5 shows the response of a typical impedimetric immunosensor using the relative difference of |Z| at three different frequency ranges: from 35.37 kHz to 131.95 kHz (curves 5a); from 401.96 kHz to 602.70 kHz (curves 5b); from 738.01 kHz to 1 MHz (curves 5c). As additional examples, Figure 6 and 7 show the response of a typical impedimetric immunosensor using the relative difference of Zre and Zim respectively, for the frequency range from 738.01 kHz to 1 MHz. As it can be seen in the figures, the inclusion of the antibody in the Step II implies a higher increase in the absolute value of the impedance when low atrazine concentrations are included in the competition step at all the frequency ranges under study.

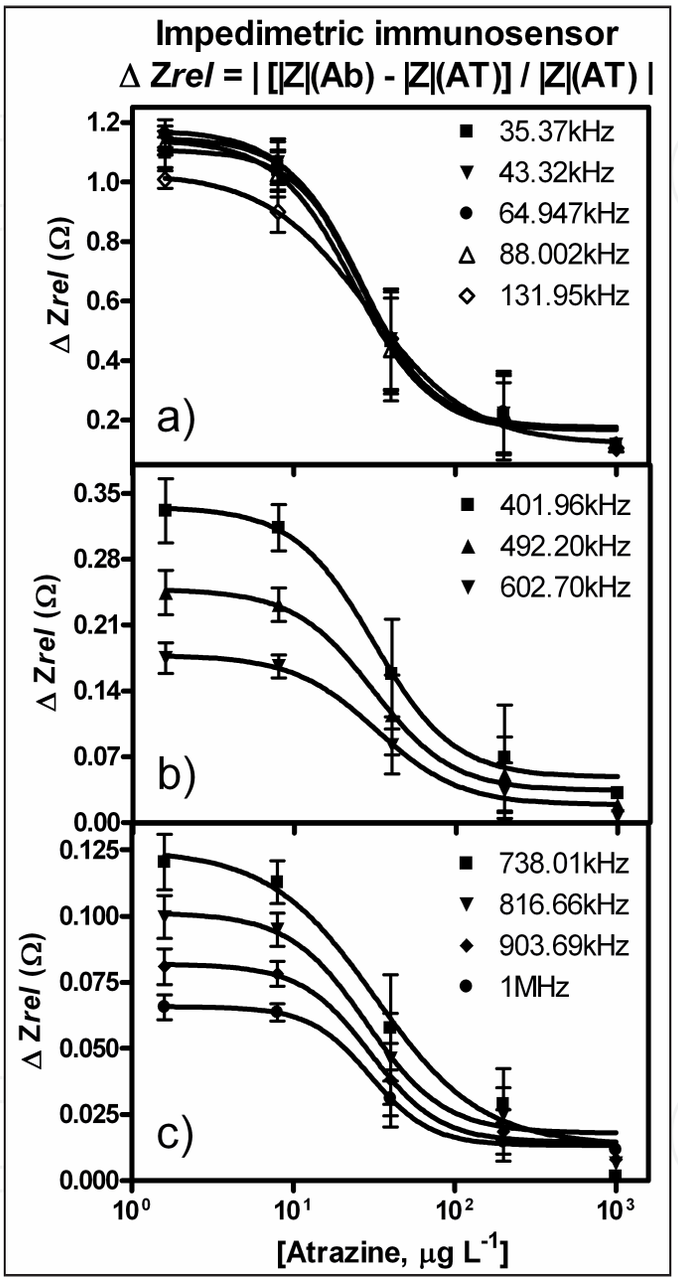

Figure 5. Impedimetric immunosensor responses using the relative difference of Zre at high frequencies from 738.01 kHz to 1 MHz.Impedimetric immunosensor responses using the relative difference of |Z| at: a) middle frequencies from 35.37 kHz to 131.95 kHz; b) high frequencies from 401.96 kHz to 602.70 kHz; c) high frequencies from 738.01 kHz to 1 MHz. Reprinted from Sensors and Actuators B, 129/2, Ángel Rodríguez, Enrique Valera, Javier Ramón-Azcón, F.-J. Sanchez, M.-P. Marco, Luis M. Castañer, Single frequency impedimetric immunosensor for atrazine detection, 921-928, Copyright (2008), with permission from Elsevier.

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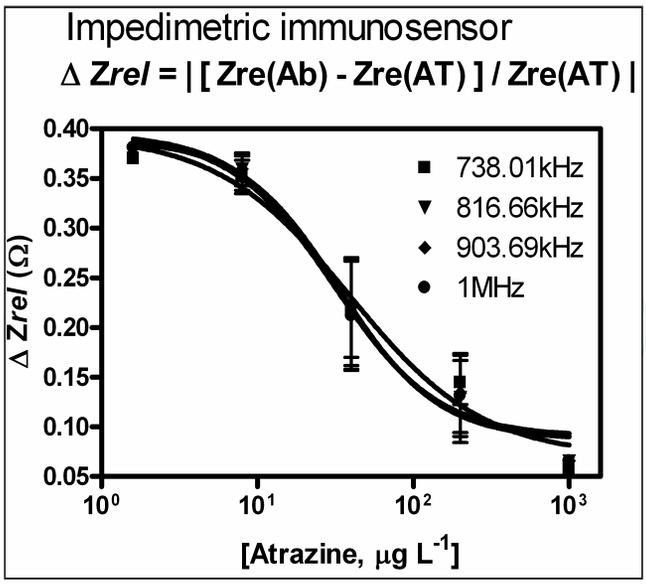

Figure 6. Impedimetric immunosensor responses using the relative difference of Zre at high frequencies from 738.01 kHz to 1 MHz.Reprinted from Sensors and Actuators B, 129/2, Ángel Rodríguez, Enrique Valera, Javier Ramón-Azcón, F.-J. Sanchez, M.-P. Marco, Luis M. Castañer, Single frequency impedimetric immunosensor for atrazine detection, 921-928, Copyright (2008), with permission from Elsevier.

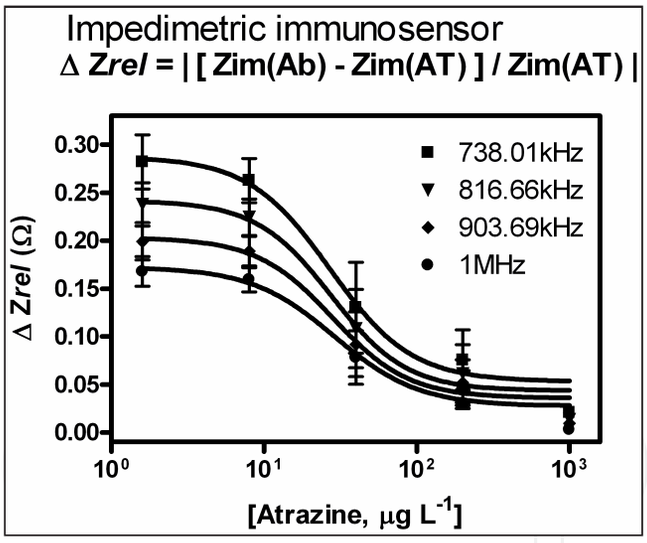

Figure 7. Impedimetric immunosensor responses using the relative difference of Zim at high frequencies from 738.01 kHz to 1 MHz. Reprinted from Sensors and Actuators B, 129/2, Ángel Rodríguez, Enrique Valera, Javier Ramón-Azcón, F.-J. Sanchez, M.-P. Marco, Luis M. Castañer, Single frequency impedimetric immunosensor for atrazine detection, 921-928, Copyright (2008), with permission from Elsevier.

A characteristic of the behaviour of the impedimetric immunosensor and the detection method to single frequencies is that the difference between the response curves is very small, almost imperceptible. This can be appreciated in Figures 5a and 6. This could be used for the auto-tuning of the sensor and to reduce the effect of the dispersion of the initial capacitance of the

devices in the measurement, by using two or more frequencies in the measurement instead of only one.

The main influence of choosing the absolute or the relative difference is observed in the values of the features assays (LOD) obtained for the different ranges of frequency of work. This table summarizes the values of the LOD in μg L^{-1} for some frequencies of work used to represent each part of the spectrum and for the three kinds of impedance considered.

In Table 1 an important improvement in the LOD at middle frequencies (35.37 kHz to 131.95 kHz) is found when the relative difference is used, in comparison to the absolute difference in all cases. On the other hand, at high frequencies, from 401.96 kHz, the differences in LOD between the absolute difference and relative difference are small in comparison with the differences obtained at middle frequencies. In some cases the LOD of the absolute difference becomes the minimum.

Features of the atrazine assays	ΔZ	frequency of work (kHz)					
		35.37	64.95	131.95	401.86	738.01	1000
LOD, μg L^{-1}	$\lvert\lvert Z\rvert(Ab)-\lvert Z\rvert(AT)\rvert$	13.77±0.16	13.34±0.16	11.95±0.18	11.93±0.17	9.79±0.19	9.57±0.17
	$\left\lvert\frac{\lvert Z\rvert(Ab)-\lvert Z\rvert(AT)}{\lvert Z\rvert(AT)}\right\rvert$	10.02±0.21	8.32±0.22	6.87±0.23	9.87±0.20	8.56±0.23	12.06±0.17
	$\lvert Zre(Ab)-Zre(AT)\rvert$	14.30±0.13	13.63±0.16	15.96±0.17	11.03±0.39	9.83±0.23	8.41±0.22
	$\left\lvert\frac{Zre(Ab)-Zre(AT)}{Zre(AT)}\right\rvert$	8.95±0.22	7.62±0.23	5.76±0.25	19.16±0.37	8.25±0.30	8.01±0.25
	$\lvert Zim(Ab)-Zim(AT)\rvert$	28.61±0.29	19.60±0.25	9.55±0.36	7.43±0.28	7.19±0.23	7.81±0.23
	$\left\lvert\frac{Zim(Ab)-Zim(AT)}{Zim(AT)}\right\rvert$	12.52±0.21	11.95±0.17	7.51±0.21	9.10±0.23	8.61±0.22	9.46±0.21

[a] Parameters extracted from the four-parameter equation used to fit the standard curve.

Table 1. Features of the atrazine assays [a]. Reprinted from Sensors and Actuators B, 129/2, Ángel Rodríguez, Enrique Valera, Javier Ramón-Azcón, F.-J. Sanchez, M.-P. Marco, Luis M. Castañer, Single frequency impedimetric immunosensor for atrazine detection, 921-928, Copyright (2008), with permission from Elsevier.

Comparing directly the three cases, it is clear that the best response is obtained using the Zre variation by means of relative difference at middle frequencies (*5.76 – 8.95 μg L^{-1}*), and that the worst response is obtained using the Zim variation by means of absolute difference also at middle frequencies (*19.60 - 28.61 μg L^{-1}*), although in all cases the limits of detection obtained are still lower than the MRL (50 μg L^{-1}) established by European Union.

These results can be seen more clearly in Figure 8. This figure shows the evolution of the LOD in the frequency ranges analyzed, for the variation of |Z| (curve 8a), Zre (curve 8b) and Zim (curve 8c). In Figure 8b is clear the improvement in the limit of detection at middle frequencies, when the relative difference of the Zre is used instead of the other impedance variation, as it was observed above.

Although the best responses were obtained using the real part of the impedance, when the module of the impedance is used to find the features of the assay, measurements show lower

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dispersion between different devices, what means a better compensation of the effects of geometry or technology of the IDμE's. As consequence the immunosensor resolution (LOD) is almost constant in the whole range of frequencies under study, both in the case of the absolute difference or relative difference, as it can be comprehended in Figure 8a. This is important because, the frequency of work can be chosen independently of the immunosensor resolution, therefore providing a degree of freedom to the design of the circuitry of control.

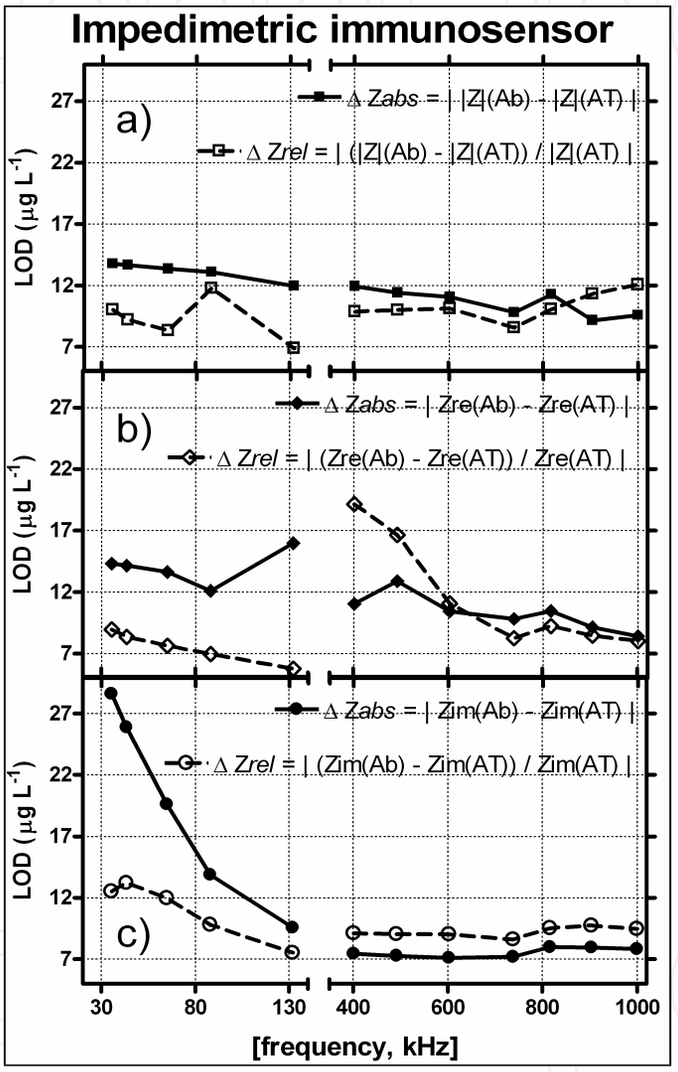

Figure 8. Evolution of the limit of detection by means of absolute and relative variation of: a) module of impedance; b) real part of impedance; c) imaginary part of impedance Reprinted from Sensors and Actuators B, 129/2, Ángel Rodríguez, Enrique Valera, Javier Ramón-Azcón, F.-J. Sanchez, M.-P. Marco, Luis M. Castañer, Single frequency impedimetric immunosensor for atrazine detection, 921-928,

4.1.1. Multi-sine burst signal

The characterization technique previously shown is based on the monitoring of the electrical response of the device under test after application of only one AC signal in a single frequency.

By the burst technique, a multi-sine signal is used in order to perform simultaneous measurements at different frequencies at the same time. These signals are useful for fast measuring biological objects which electrical properties changes quickly with time, in order to obtain the frequency transfer function free of modulation.

Mathematically, a multi-sine signal is formed by the sum of N tones, each one with its own phase. To be precise, in bio-impedance measurement scenario is just necessary a signal burst and not a continuous time signal. An example of a multi-sine burst signal can be seen in Figure 9.

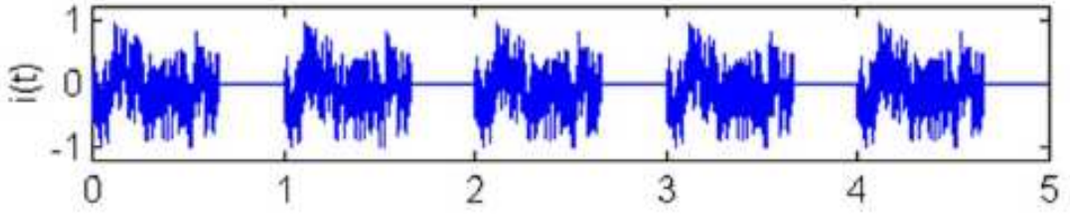

Figure 9. Five multi-sine burst signal with 1ms of period.

Signal generation and acquisition is a crucial part in the hardware implementation since they are the connector between the analog world and the discrete world of digital signal processing algorithms. Multi-sine signal is based on Bilateral Quasi-Logarithmic (BQL) [17] frequency distribution and is implemented on a custom arbitrary signal waveform generator based on a Field Programmable Gate Array (FPGA). Acquisition is done with a specific front-end connected to a fast A/D board which transfers the data to the Digital Signal Processor (DSP) silicon for real time processing. Thus, it is possible to make simultaneous measurements in a wide spectrum in a short time. The main idea is to use broadband signals because the energy is spread over the desired frequency range. A photo of the waveform generator can be seen in Figure 10, whereas Figure 11 shows a schematic of the signal generation and acquisition configuration.

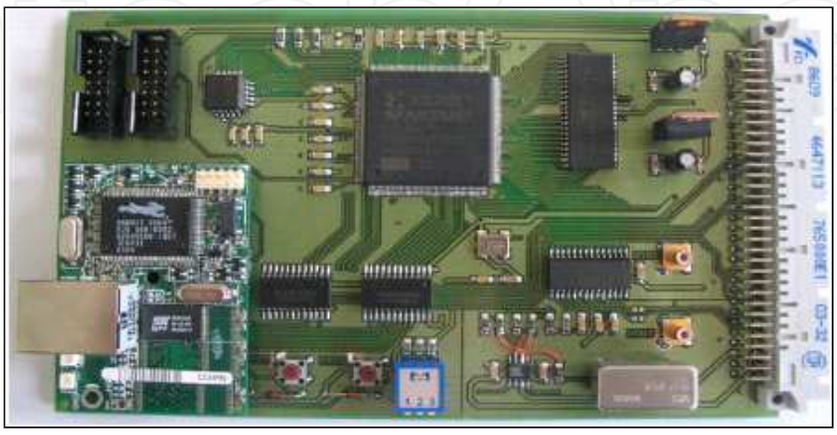

Figure 10. Multi-sine generator board.

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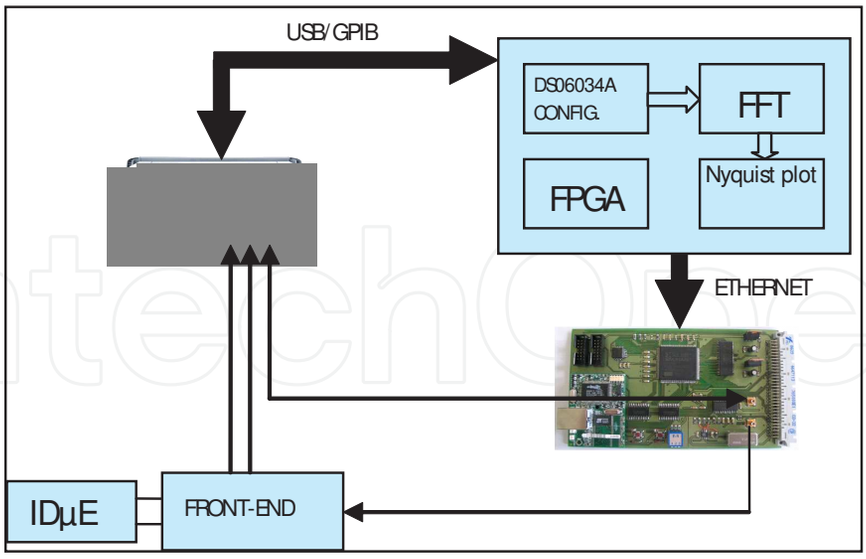

Figure 11. Signal generation and acquisition configuration.

In this case the data acquisition system was designed to only interpret the region semicircle and not the area of diffusion (Nyquist plot). Likewise, the number of points (fundamental frequencies) was chosen in 21 because some hardware limitations related to the memory, such as capacity and velocity (reading and writing) and to avoid difficulties in the design and benefits worsen. An example of the plots obtained by this method is shown in Figure 12.

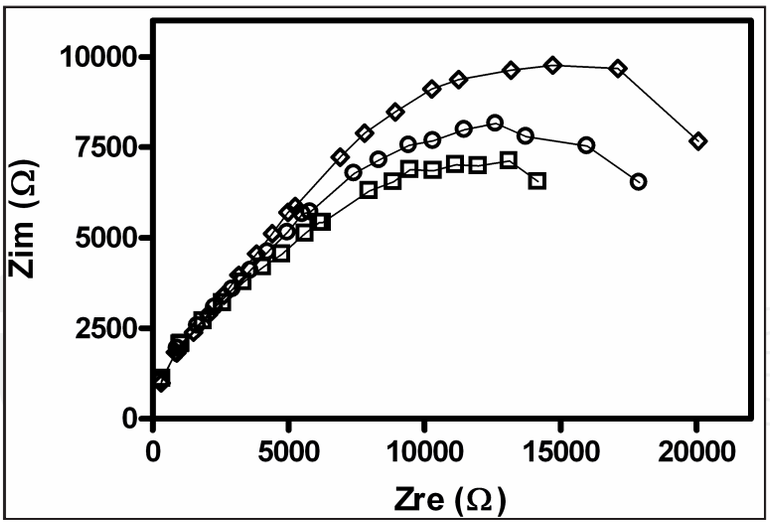

Figure 12. Immunosensor response obtained from a multi-sine burst signal.

In this figure we can see how the system is able to represent different curves that are related to the antibody concentration. Although in these curves the bandwidth is smaller in comparison with the previous case, the curves obtained are enough to appreciate differences in the impedance contribution. Then, these differences could be also related to differences in parameters of the equivalent circuit, and thus, this system could be used as detection system.

The work related to the multi-sine burst signal was performed in collaboration with Dr. R. Bragós and co-workers, members of the *Divisió Intrumentació i Bioenginyeria (UPC)*.

4.2. Conductimetric immunosensor

In the case of the conductimetric immunosensor, impedimetric measurements were discarded as detection method, because the inclusion of the gold particles (conductive elements) *subtracts* the contribution of the secondary antibody (resistive element). Therefore, only DC measurements were applied in this case.

4.2.1. Atrazine detection in buffer samples

Atrazine concentrations between 0.32 to 2000 μg L^{-1} during the competition step (Step IV) were included. The electrodes were covered by a diluted PBS solution and the measurements were executed to +25 and +100 mVdc bias. The results obtained by this method are represented in Figure 13.

The limits of detection obtained for the atrazine residues detection using the conductimetric immunosensors, when the competitive assay was performed in buffer solution, were *0.446 μg L^{-1}* (100 mVdc bias) and *1.217 μg L^{-1}* (25 mVdc bias), both far below the MRL.

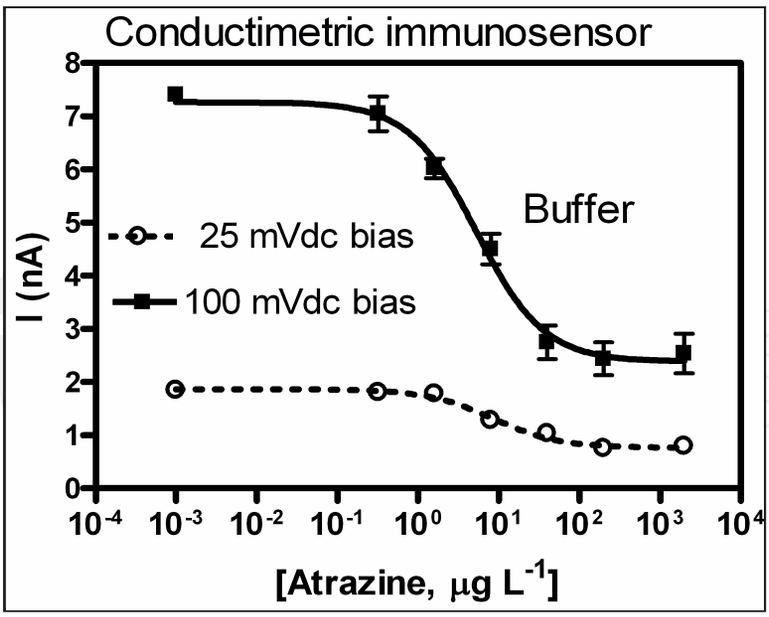

Figure 13. Response curve of the conductimetric immunosensor, using the covalent immobilization technique, for the atrazine detection in relation with the presence of gold particles (40 nm). Buffer solution was used for the competitive reaction. Measures were taken in diluted PBS solution. See Table 2 for the features of the atrazine assay.

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Features of the atrazine assays [a]	Conductimetric immunosensor (buffer)	
	25 mV	100 mV
IC_{50}, µg L^{-1}	8.47±0.19	5.29±0.14
LOD, µg L^{-1}	**1.217**	**0.466**
R^2	0.89	0.91

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve.

Table 2. Features of the atrazine assays [a]

4.2.2. Atrazine detection in red wine samples

After the demonstration of both types of immunosensors for the detection of free pesticide in buffer samples, the conductimetric immunosensor was also studied using a complex matrix such as red wine samples. Red wine was chosen instead of other matrixes such as white wine, water or grape juice, because its strong matrix effect. Therefore, if the red wine matrix effect can be measured, the other matrix effects will be easier. Again, atrazine was used as pesticide of test.

Red wine samples were obtained from a local retail store and used, on a first instance, to evaluate the extension of the potential non-specific interferences. Prior measurements with the immunosensors, the wine samples were purified by solid-phase extraction (SPE) using LiChrolut RP-18 (500 mg, 6 mL) sorbent (Merck, Darmstadt, Germany) pre-conditioned with MeOH (3 mL), and MeOH:Mili-Q water (15:85, v/v, 3 mL) at a flow rate of 3 mL min^{-1}. The wine samples (3 mL) were loaded at 5mL min^{-1}, and the SPE cartridges washed with of MeOH:Mili-Q water (70:30, v/v, 1 mL), dried, and finally eluted with of MeOH:Mili-Q water (80:20, v/v, 1 mL). The fractions collected were diluted 1:50 in PBST and used for the impedimetric measurements [1].

Again, the experiments carried out in this section included atrazine concentrations (0.32 – 2000 µg L^{-1}) during the competition step (Step IV), the electrodes were covered by a diluted PBS solution, and the measurements were performed to +25 and +100 mVdc bias.

The results obtained by this way are shown in Figure 14. The limits of detection obtained for the detection of residues of atrazine, when the competitive assay was performed in red wine samples, were *0.489 µg L^{-1}* (100 mVdc bias) and *0.034 µg L^{-1}* (25 mVdc bias). As in the previous cases, the MRL required by EC was largely reduced.

4.2.3. New approach: A flexible device

As it was proven in the previous sections, both types of immunosensors described in this chapter have been able to detect residues of atrazine when buffer samples were used for the competitive assay. In both cases, the transducer was supported by a PYREX substrate. In this section a new approach, flexible plastic substrates, is introduced, in order to hardly reduce the cost of the device.

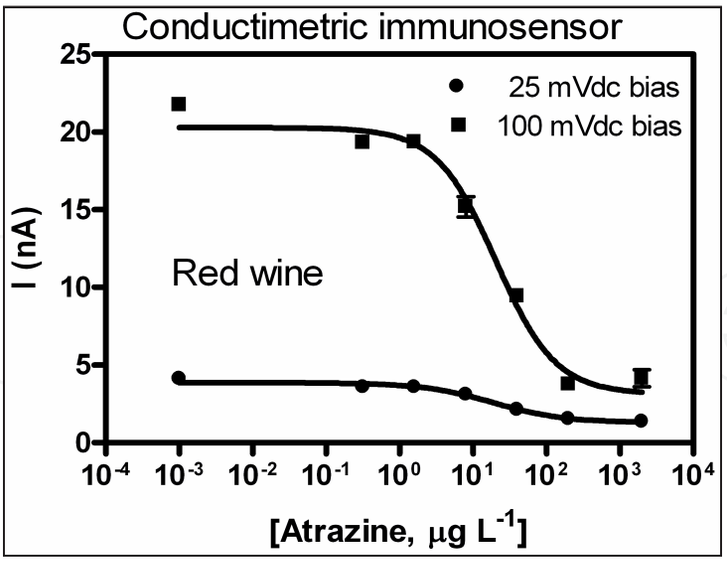

Figure 14. Response curve of the conductimetric immunosensor, using the covalent immobilization technique, for atrazine detection in relation with the presence of gold particles (40 nm). Red wine samples were used for the competitive assay. Measures were taken in diluted PBS solution. See Table 3 for the features of the atrazine assay.

Features of the atrazine assays [a]	Conductimetric immunosensor (red wine)	
	25 mV	100 mV
IC_{50}, μg L^{-1}	19.05±0.10	20.54±0.07
LOD, μg L^{-1}	**0.034**	**0.489**
R^2	0.96	0.98

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve.

Table 3. Features of the atrazine assays [a]

4.2.3.1. Flexible interdigitated μ-electrode (FIDμE)

Although Pyrex properly complies with the conditions of isolation and compatibility necessary, the possibility of a flexible sensor was also explored. Therefore, flexible interdigitated μ-electrodes (FIDμE) for biosensor applications were fabricated. A sample of the FIDμE's developed can be seen in Figure 15.

The flexibility of the FIDμE's was reached using a plastic substrate. The plastic chosen as new substrate was polyethylene naphthalate (PEN), 0.075 mm, purchased from Goodfellow Cambridge Limited.

The fabrication procedure of the FIDμE's is as follows: Thin Au (150 - 200 nm thickness) interdigitated μ-electrodes (IDμE's) with, 30 μm thick with electrode gap of 30 μm were patterned on a PEN substrate. As a good adhesion between gold and the PEN substrate exist, the chromium layer was avoided. Before metal deposition (performed by sputtering), the PEN substrate was cleaned using absolute ethanol. Then, the interdigitated μ-electrodes were then patterned by a photolithographic metal etch process.

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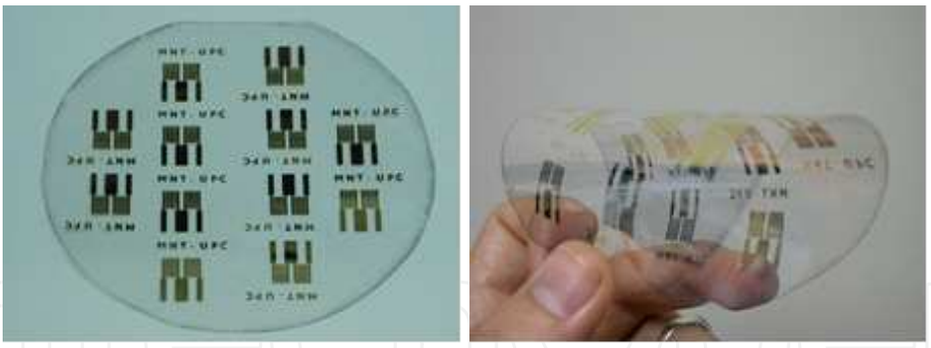

Figure 15. Flexible interdigitated μ-electrodes fabricated: a) top view; b) demonstration of flexibility. Reprinted from Microelectronic Engineering, 87/2, Enrique Valera, David Muñiz, Ángel Rodríguez,, Fabrication of flexible interdigitated μ-electrodes (FIDμEs) for the development of a conductimetric immunosensor for atrazine detection based on antibodies labelled with gold nanoparticles, 167–173, Copyright (2010), with permission from Elsevier

In order to apply FIDμE's as biosensor, the electrodes surface must be activated. For this reason, SiO_2 was deposited by sputtering on the electrodes surface, because silicon oxide surface contains reactive SiOH groups, which can be used for covalent attachment of organic molecules and polymers [18].

4.2.3.2. Conductimetric immunosensor using FIDμE's

The functionalization performed in this case followed the covalent immobilization technique previously explained. Atrazine concentrations between 0.32 – 2000 μg L^{-1} were included. The electrodes were covered by a diluted PBS solution and the measurements were performed with a bias of +100 mVdc. The response curve obtained can be seen in Figure 16. In this case, the curve is based on the current through the electrodes, related to amount of gold particles.

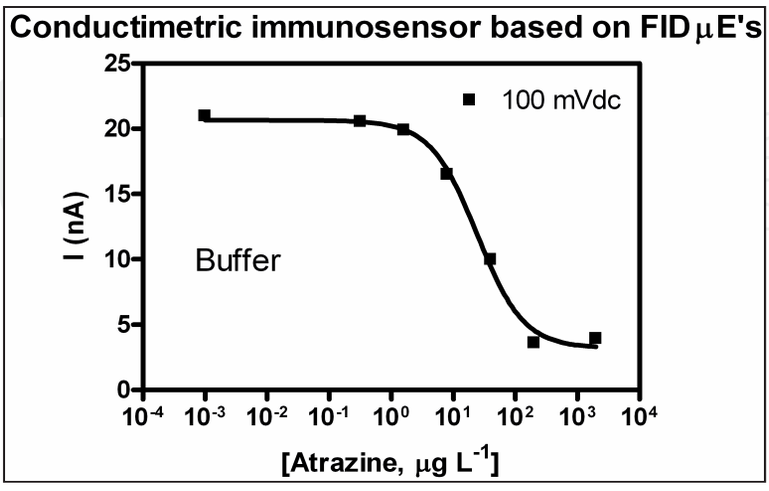

Figure 16. Response curve of the conductimetric immunosensor, using the covalent immobilization technique, for the atrazine detection in relation with the presence of gold particles (40 nm). Buffer solution was used for the competitive reaction. Measures were taken in diluted PBS solution. See Table 4 for the features of the atrazine assay.

In this case, the LOD is *2.975 μg L^{-1}* and the R^2 was 0.9922. Nevertheless, another important conclusion is that in this case, a very small gap is not needed to achieve the atrazine detection in low concentrations.

The most relevant analytical features of the conductimetric atrazine assay are summarized in Table 4.

Features of the atrazine assays [a]	Conductimetric immunosensor (buffer)
IC_{50}, μg L^{-1}	24.17±0.03
LOD, μg L^{-1}	**2.975**
R^2	0.99

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve.

Table 4. Features of the atrazine assays [a]

5. Conclusions

In this chapter, two immunosensors based on interdigitated μ-electrodes together with three strategies for the excitation and readout of their response are described. The goal of the readout methods was to reduce the time of measurement. They have been implemented and characterized in depth.

It can be concluded that each of the methods represent an important reduction of the time of measurement and a reduction of the complexity, and cost of the electronics required for its implementation compared to broadband spectroscopy. Each of the proposed methods has particularities: Using the method of single frequency measurements, the use of non labelled antibodies is allowed, and the complexity of the electronics required is low. The maximum simplicity in the electronics circuitry is probably achieved with DC measurements but this makes necessary the use of antibodies labelled with conductive particles. In these two cases the measurements can be done in seconds. The expense in terms of sensitivity is low as it can be seen from the LOD obtained with each of them: in both cases the LOD is well below the MRL established for the detection of atrazine by the EC.

The third method, based in burst signals, allows for the fast measurement of the impedance of the devices, also in a few seconds, in the broad band from low to high frequencies used in the conventional impedance spectroscopy. Its advantage comes from the fact that it uses a broad band measurement and therefore it is expected to provide LOD similar to the conventional broadband spectroscopy. This method requires more complexity in the electronics to be used for the excitation of the sensors and also a higher complexity in the analysis of the readout signals of the devices. Nevertheless can been readily programmed in common portable devices with little extra electronics.

http://dx.doi.org/10.5772/55969

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