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QCM Technology in Biosensors

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1. Introduction

In the fields of analytical and physical chemistry, medical diagnostics and biotechnology there is an increasing demand of highly selective and sensitive analytical techniques which, optimally, allow an in real-time direct monitoring with easy to use, reliable and miniaturized devices. Biomolecular interactions such as: antigen-antibody, pathogen detection, cell adhesion, adsorption and hybridization of oligonucleotides, characterization of adsorbed proteins, DNA & RNA interactions with complementary strands and detection of bacteria and viruses, among others, are typical applications in these areas.
Conventionally, analytical methods include different techniques depending on the application. For instance, for low molecular weight pollutants detection, gas and liquid chromatography are classical techniques. These techniques precise of sophisticated sample pre-treatment: extraction of crude sample with large amounts of organic solvent, which is expensive and needs to be discarded; precolumn filtration and extensive purification (De Kok et al., 1992). Due to these shortcomings the analysis of a large number of samples may be both cost and time prohibitive (Ahmad et al., 1986).
Immunoassays for low molecular weight compounds (pesticides, industrial chemical pollutants, etc.) have already gained a place in the analytical benchtop as alternative or complementary methods for routine classical analysis as they are simple, fast, inexpensive, and selective as well as highly sensitive although, in general, not as much as chromatographic techniques. Immunoassays are able to detect specifically one target analyte in a complex sample. Moreover, immunoassays can be performed on portable devices, irrespective of centralized laboratories, which turn them into a suitable tool for quantification analysis in on-line applications. These techniques are based on the interaction of one antigen (analyte) with an antibody which recognizes it in a specific way. Currently, Enzyme Linked ImmunoSorbent Assay (ELISA) and Immunosensors are the most popular immunoassays. In ELISAs the detection of the analyte is always indirect because one of the immunoreagents is labeled. In immunosensors, or immunological biosensors, the detection is direct, one of the immunoreagents is immobilized on the surface of the transducer, and a direct physical signal is produced when interaction occurs (Marty et al, 1998; Byfield et al, 1994; Montoya et al, 2008). In those techniques where labels are necessary, the actual

quantitative measurement is only done after the biochemical recognition step. Moreover, label can compromise the biochemical activity (Hawkins et al., 2006). This label-free direct detection represents an essential advantage of immunosensors as compared to label-dependent immunoassays (Janshoff et al., 2000).

Immunosensors combine the selectivity provided by immunological interactions with the high sensitivity achieved by the signal transducers and are being proposed and proving to be powerful analytical devices for the monitoring of low molecular weight compounds such as organic pollutants in food and the environment (Su, et al., 2000; Fung et al., 2001).

Different sensing technologies are being used for biochemical sensors. Categorized by the transducer mechanism, electrochemical, optical and acoustic wave sensing techniques have emerged as the most promising biochemical sensor technologies (Coté et al., 2003). Common to most optical and electrochemical principles popular exceptions are Surface Plasmon Resonance (SPR) or electrochemical impedance spectroscopy, is the requirement of a label, as in the case of ELISAs, equipped with the physical information to stimulate the transducer, but increasing the complexity and thus the cost for analysis. Examples of labels are the coupling with an enzyme, a fluorescent molecule, a magnetic bead or a radioactive element (Asch et al., 1999).

Acoustic sensing has taken advantage of the progress made in the last decades in piezoelectric resonators for radio-frequency (RF) telecommunication technologies. The piezoelectric elements used in: radars, cellular phones or electronic watches for the implementation of filters, oscillators, etc., have been applied to sensors (Lec, 2001). The so-called gravimetric technique is based on the change in the resonance frequency experimented by the resonator due to a mass attached on the sensor surface (Sauerbrey, 1959); it has opened a great deal of applications in bio-chemical sensing in both gaseous and liquid media.

Most of the biochemical interactions described above are susceptible of being evaluated and monitored in terms of mass transfer over the appropriate interface. This characteristic allows using the gravimetric techniques based on acoustic sensors for a label-free and a quantitative time-dependent detection. Acoustic sensor based techniques combine their direct detection, real-time monitoring, high sensitivity and selectivity capabilities with a reduced cost in relation to other techniques. As mentioned previously, optical techniques, like Surface Plasmon Resonance (SPR), depend on the optical properties of the materials used; on the contrary, the most applied principle of detection in acoustic sensing for biochemical applications is based on mass (gravimetric) properties and it is, therefore, independent of the optical properties of the materials, allowing to perform studies over a great variety of surfaces and suitable for direct measurement on crude, unpurified samples. This eliminates the need for sample preparation and therefore reduces the number of steps involved in the process – bringing many benefits, including significant time and cost-savings. Additionally, acoustic systems provide information on the real binding to a receptor and not simply proximity to a receptor, as could be the case with SPR techniques. Furthermore, the key measuring magnitude of acoustic wave devices is the frequency of a signal which can be processed easily and precisely, unlike other devices.

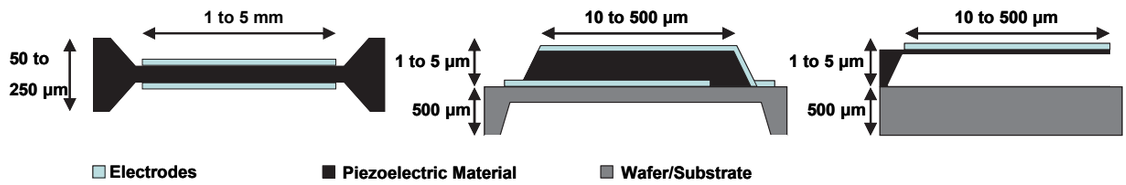
The classical quartz crystal microbalance (QCM) has been the most used acoustic device for sensor applications; however, other acoustic devices have been, and are being used, for the implementation of nano-gravimetric techniques in biosensor applications. Although this chapter is focused on QCM technology, a broader view of the different techniques used in the implementation of acoustic biosensors could be very useful for three reasons: first because it gives a complete updated sight of the acoustic techniques currently used in

biosensors, second because some of the challenges remaining for QCM can be applied to other acoustic devices, and third because the new aspects presented in this chapter, mainly in relation to the new sensor characterization interfaces, can be considered for the other devices as well. With this purpose, a brief description of the state of the art of the different acoustic techniques used in biosensors is included next.

Different types of acoustic sensing elements exist, varying in wave propagation and deflection type, and in the way they are excited (Ferrari & Lucklum, 2008). They can be classified into two categories: bulk acoustic waves (BAW) and surface-generated acoustic waves (SGAW). Moreover they may work with longitudinal waves (with the deflection in the direction of propagation) or shear waves (with the deflection perpendicular to the direction of propagation). The number of biochemical applications is extended for in-liquid applications; in these cases it is necessary to minimize the acoustic radiation into the medium of interest and the shear wave is mostly used.

1.1 Bulk acoustic wave devices (BAW)

Bulk acoustic wave (BAW) devices utilize waves travelling or standing in the bulk of the material. They are mostly excited through the piezoelectric or capacitive effects by using electrodes on which an alternative voltage is applied. The three important BAW devices are: quartz crystal microbalances (QCM), film bulk acoustic resonators (FBAR) and cantilevers. Figure 1 shows their basic structure and typical dimensions. Because the vibrating mode of cantilevers is not suited for operation in liquids due to the high damping we will focus our discussion on QCM and FBAR devices.



a) b) c)

Fig. 1. Bulk acoustic devices: a) QCM, b) FBAR and c) Cantilevers

1.1.1 QCM for biosensing applications

The classical QCM is formed by a thin slice of AT-cut quartz crystal. Acoustic waves are excited by a voltage applied to an electrode structure where the quartz crystal is sandwiched (see Figure 1a). Shear waves are excited which makes the operation in liquids viable (Kanazawa & Gordon, 1985). QCM has been the most used acoustic device for sensor applications since 1959, when Sauerbrey established the relation between the change in the resonance frequency and the surface mass density deposited on the sensor face. The theoretical absolute mass sensitivity for this shift is proportional to the square of the resonant frequency, according to the following expression (Sauerbrey, 1959):

$$S_a = \frac{\Delta f}{\Delta m} = -\frac{2}{\rho v}\frac{f_n^2}{n} \text{ (Hz cm}^2\text{ ng}^{-1}\text{)} \quad (1)$$

where Δf is the frequency shift, Δm is the surface mass density change on the active sensor's surface, ρ is the quartz density, v the propagation velocity of the wave in the AT cut crystal,

f_n is the frequency of the selected harmonic resonant mode and n is the harmonic number (n=1 for the fundamental mode). Theoretical mass sensitivity, i.e., the lineal relationship between the frequency variation and the mass surface density change so obtained in Sauerbrey's equation, is right only on ideal conditions, where only inertial mass effects contribute on the resonant frequency shift of the QCM sensor (Voinova et al., 2002; Kankare, 2002; Jiménez et al., 2008; Jiménez et al., 2006). For AT cut quartz crystals, the limit of detection (LOD) or surface mass resolution for a minimum detectable frequency shift $\Delta f_{\min}$ will be given by:

$$\Delta m_{\min} = \frac{\Delta f_{\min}}{S_a} \tag{2}$$

Many commercial systems are already on the market (Coté et al., 2003). Absolute sensitivities of a 30 MHz QCM reach 2 Hz cm^2 ng^{-1}, with typical mass resolutions around 10 ng cm^{-2} (Lin et al., 1993). Lower mass resolutions down to 1 ng cm^{-2} seem possible by improving the characterization electronic interface as well as the fluidic system.

This technique has extensively been employed in the literature just for the monitoring of many substance absorption and detection processes (Janshoff et al., 2000). QCM technology has a huge field of applications in biochemistry and biotechnology. The availability for QCM to operate in liquid has extended the number of applications including the characterization of different type of molecular interactions such as: peptides (Furtado et al., 1999), proteins (BenDov et al., 1997), oligonucleotides (Hook et al., 2001), bacteriophages (Hengerer et al., 1999), viruses (Zhou et al., 2002), bacteria (Fung & Wong, 2001) and cells (Richert et al., 2002); recently it has been applied for detection of DNA strands and genetically modified organisms (GMOs) (Stobiecka et al., 2007).

Despite of the extensive use of QCM technology, some challenges such as the improvement of the sensitivity and the limit of detection in high fundamental frequency QCM, remain unsolved; recently, an electrodeless QCM biosensor for 170MHz fundamental frequency, with a sensitivity of 67 Hz cm^{-2} ng^{-1}, has been reported (Ogi et al, 2009); this shows that the classical QCM technique still remains as a promising technique. Once these aspects are solved the next challenge would be the integration; in this sense, commercial QCM systems are mostly based on single element sensors, or on multi-channel systems composed of several single element sensors (Tatsuma et al., 1999). They are to date expensive, mainly because currently their manufacturing is complex, especially for high frequencies, and their application for sensor arrays is difficult due to lack of integration capability. Most of these shortcomings could be overcome with the appearing of film bulk acoustic resonators (FBAR).

1.1.2 FBAR devices for biosensing applications

A typical film bulk acoustic resonator (FBAR) consists of a piezoelectric thin film (such as ZnO or AlN) sandwiched between two metal layers. A membrane FBAR is shown in Figure 1b. In the past few years, FBARs on silicon substrates have been considered for filter applications in RF devices (Vale et al., 1990). Gabl et al. were the first to considerer FBARs for gravimetric bio-chemical sensing applications (Gabl et al., 2003). They basically function like QCMs; however, unlike QCMs, typical thicknesses for the piezoelectric thin film are between 100 nm and a few μm, allowing FBARs to easily attain resonance frequencies in the GHz range. The main advantage of FBAR technology is its integration compatibility with CMOS technologies, which is a prerequisite for fabrication of sensors and sensor arrays

integrated with the electronics, and hence low cost mass fabrication of miniature sensor systems. However, the miniaturization of sensor devices should go in parallel with the miniaturization and optimization of the microfluidic system which is of extreme importance for reducing the noise and increasing the stability of the complete system; the main problems of the microfluidics are the complexity of integration and the cost. Moreover, due to higher resonance frequency of these devices and according to (1), higher sensitivities than for QCMs could be reached; however, the higher sensitivity does not mean necessarily that a higher LOD or mass resolution is achieved. Effectively, thin film electroacoustic technology has made possible to fabricate quasi-shear mode thin film bulk acoustic resonators (FBAR), operating with a sufficient electromechanical coupling for use in liquid media at 1-2 GHz (Bjurstrom et al., 2006; Gabl et al., 2004); however, the higher frequency and the smaller size of the resonator result in that the boundary conditions have a much stronger effect on the FBAR performance than on the QCM response. This will result in a higher mass sensitivity, but in an increased noise level as well, thus moderating the gain in resolution (Wingqvist 2007, 2008). So far only publications of network analyzer based FBAR sensor measurements have been published in the literature, which show that the FBAR mass resolution is very similar if not better than for oscillator based QCM sensors (Weber et al., 2006; Wingqvist 2007, 2008, 2009). The first shear mode FBAR biosensor system working in liquid environment was reported in 2006 (Weber et al., 2006). The device had a mass sensitivity of 585 Hz cm^2 ng^{-1} and a limit of detection of 2.3 ng cm^{-2}, already better than that obtained with QCM (5.0 ng cm^{-2}) for the same antigen/antibody recognition measurements. However, these results have been compared with typical 10MHz QCM sensors; therefore high fundamental frequency QCM sensors working, for instance, at 150MHz could have much higher resolution than the reported FBAR sensors. In 2009 a FBAR for the label-free biosensing of DNA attached on functionalized gold surfaces was reported (Nirsch et al., 2009). The sensor operated at about 800 MHz, had a mass sensitivity of about 2000 Hz cm^2 ng^{-1} and a minimum detectable mass of about 1ng cm^{-2}. However, studies of the mass sensitivity only do not provide a comprehensive view of the major factors influencing the mass resolution. For instance in FBAR sensors, in contrast to the conventional QCM, the thickness of the electrodes is comparable to that of the piezoelectric film and hence cannot be neglected. The FBAR must, therefore, be considered like a multilayer structure, where the acoustic path includes the piezoelectric film as well as an acoustically "dead" material, e.g. electrodes and additional layers such as for instance Au, which is commonly used as a suitable surface for various biochemical applications, or SiO_2 which also is used for temperature compensation (Bjurstrom et al., 2007). In general there is a set of factors which must be considered and affects the quality factor of a FBAR sensor such as: loss mechanisms, multilayer effects, lateral structure, spurious modes, etc.
Another approach used to get higher mass sensitivities by increasing the frequency is by using surface generated acoustic wave devices (SGAW)

1.2 Surface generated acoustic wave devices (SGAW)

SGAW devices have been used as chemical sensors in both gaseous and liquid media. The input port of a SGAW sensor is comprised of metal interdigital electrodes (IDTs), with alternative electrical polarity, deposited or photodesigned on an optically polished surface of a piezoelectric crystal. Applying a RF signal, a mechanical acoustic wave is launched into the piezoelectric material due to the inverse piezoelectric phenomenon. The generated acoustic wave propagates through the substrate arriving at an output IDT. The separation

between the IDTs defines the sensing area where biochemical interactions at the sensor surface cause changes in the properties of the acoustic wave (wave propagation velocity, amplitude or resonant frequency) (Ballantine et al., 1997). Thus, at the output IDT the electrical signal can be monitored after a delay in an open loop configuration. Figure 2, shows a schematic view of different SGAW devices

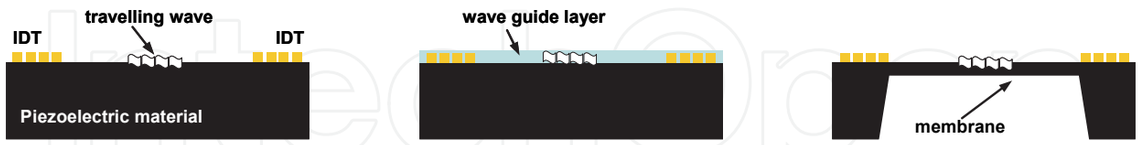


Fig. 2. Different types of SGAW devices: a) typical SAW configuration, b) Love-wave SGAW device and c) flexural plate SGAW device

In SGAW devices the acoustic wave propagates, guided or unguided, along a single surface of the substrate. SGAW devices are able to operate, without compromising the fragility of the device, at higher frequencies than QCMs (Länge et al., 2008) and the acoustic energy of these devices is confined in the surface layer of about one wave length, therefore, the base-mass of the active layer is about one order of magnitude smaller than that of the QCM, increasing dramatically the sensitivity (Gronewold, 2007; Francis 2006; Fu et al., 2010). The longitudinal or Rayleigh mode SAW device has a substantial surface-normal displacement that easily dissipates the acoustic wave energy into the liquid, leading to excessive damping, and hence poor sensitivity and noise. Waves in a shear horizontal SH-SAW device propagate in a shear horizontal mode, and do not easily radiate acoustic energy into the liquid and thus maintain a high sensitivity in liquids (Barie & Rapp, 2001). Shear Horizontal Surface Acoustic Wave (SH-SAW), Surface Transverse Wave (STW), Love Wave (LW), Shear Horizontal Acoustic Plate Mode (SH-APM) and Layered Guided Acoustic Plate Mode (LG-APM), have recently been reported as more sensitive than the typical QCM-based devices (Rocha-Gaso et al., 2009).

In most cases, Love-wave devices operate in the SH wave mode with the acoustic energy trapped within a thin guiding layer (typically submicrometer). This enhances the detection sensitivity by more than one order of magnitude as compared with a different SAW device owing to a much-reduced base-mass (Josse et al., 2001; McHale, 2003). In addition, the wave guide layer in the Love mode biosensor could, in principle, also protect and insulate the IDT from the liquid media which might otherwise be detrimental to the electrode. Therefore, they are frequently utilized to perform bio-sensing in liquid conditions (Lindner, 2008; Jacoby & Vellekoop, 1997; Bisoffi et al., 2008; Andrä et al., 2008; Moll et al., 2007, 2008; Branch & Brozik, 2004; Tamarin et al., 2003; Howe & Harding, 2000), arising as the most promising SGAW device for this purpose due to its high mass sensitivity and electrode isolation characteristics from liquid media (Rocha-Gaso et al., 2009; Francis et al., 2005).

The mass sensitivity of LW sensors can be evaluated by different techniques based on incremental modifications of the surface density on the sensing area of the device (Francis et al., 2004). Experimental and theoretical techniques to evaluate mass sensitivity of Love Wave sensors are reported in literature (Francis et al., 2004; Harding, 2001; Wang et al., 1994). Kalantar and coworkers reported a sensitivity of 95 Hz cm^2ng^{-1} for a 100MHz Love mode sensor, which is much better than the values reported for QCM technology (Kalatar et al., 2003); however, Moll and coworkers reported a LOD for a Love sensor of 400 ng cm^{-2},

this reveals once again that an increase in the sensitivity does not mean, necessarily, an increase in the LOD (Moll et al., 2008). Moreover, in spite of the initial advantage of the guiding layer for isolating the IDTs, in real practice the capacitive coupling between the IDTs due to the higher permittivity of the liquid makes necessary to avoid the contact of the liquid with the guiding layer just over IDTs, at the same time that it is necessary to allow the contact of the central area between the IDTs with the liquid medium. This increases the complexity of the design and practical implementation of the flow cell for LW acoustic devices; this is one of the reasons why there are very few commercial microgravimetric systems based on LW-devices for in-liquid applications.

Consequently, although acoustic techniques have been improved in terms of robustness and reliability and allow measuring molecular interactions in real time, the main challenges remain on the improvement of the sensitivity, but with the aim of getting a higher mass resolution, multi-analysis and integration capabilities and reliability, as well as the availability of a functional system, specifically designed for each application, which permits the use of acoustic based techniques in a flexible and reliable way.

This chapter is focused on QCM technology applied to Biosensors. The main aspect of improving the sensitivity and the limit of detection is treated in detail and can be mostly applied to other type of acoustic devices. A new concept for the sensor characterization along with its electronic implementation is included and compared with an improved oscillator configuration. The different biochemical steps included in a typical biosensor application are covered as well in this chapter, through a case study of a QCM immunosensor for the detection of low molecular weight pollutants. The obtained results validate the new sensor characterization concept and system as a new QCM characterization technique. Moreover, this technique offers the opportunity of undertaking the remaining challenges in the acoustic biosensor technologies: 1) improvement in the sensitivity and limit of detection by working with very high frequency QCM sensors; and 2) the possibility to easily implement a QCM sensor array system with integration capabilities.

2. Fundamentals of QCM: physical bases and instrumentation techniques

2.1 Physical bases

The use of the AT-cut quartz crystal resonator as the so-called QCM (quartz-crystal microbalance) sensor has been based on the Sauerbrey equation (Sauerbrey, 1959), generalized in (1) for harmonic resonant frequencies. When a Newtonian semi-infinite liquid medium is in contact with the resonator surface, Kanazawa equation provides the associated frequency shift due to the contacting fluid (Kanazawa & Gordon, 1985). For a QCM sensor one face in contact with an "acoustically thin layer" contacting a semi-infinite fluid medium, as it is the normal case in biosensor applications, the contribution of the coating and the liquid properties can be considered additive and Martin's equation (3) can be applied (Martin et al., 1991), which combines both effects on the frequency shift, the mass effect of the coating (Sauerbrey effect) and the mass effect of the liquid (Kanazawa effect)

$$\Delta f = -\frac{2 f_o^2}{Z_{cq}} \left(m_c + m_L \right) \tag{3}$$

In the former equation, written for fundamental resonant frequencies f_o, the first term of the second member corresponds to the Sauerbrey effect and the second to the Kanazawa effect,

where Z_{cq} is the characteristic acoustic impedance of the quartz, m_c is the surface mass density of the coating and $m_L=\rho_L\delta_L/2$ where ρ_L and δ_L are, respectively, the liquid density and the wave penetration depth of the acoustic wave in the liquid: m_L is, in fact, the equivalent surface mass density of the liquid, which moves in an exponentially damped sinusoidal profile, due to the oscillatory movement of the surface of the sensor. Assuming constant properties of the liquid medium, which can be accepted in most of QCM biosensing applications, the frequency shift provides a measuring parameter to monitor the interactions occurring at the coating interface and which can be evaluated in terms of surface mass changes.

According to (2), for a certain surface mass density of the coating, the associated frequency shift increases directly proportional to the square of the resonance frequency – only for fundamental frequencies (1). Consequently, it seems logic to think that the higher the resonance frequency the higher the sensitivity. In fact the resonance frequency of the resonator has been always the main parameter for sensor characterization.

2.2 Instrumentation techniques

In practice, all the QCM sensor characterization techniques provide, among other relevant parameters, the resonance frequency shift of the sensor (Arnau et al., 2008; Eichelbaum et al., 1999): network or impedance analysis is used to sweep the resonance frequency range of the resonator and determine the maximum conductance frequency (Schröder et al., 2001; Doerner et al., 2003), which is almost equivalent to the motional series resonance frequency of the resonator-sensor; impulse excitation and decay method techniques are used to determine the series-resonance or the parallel-resonance frequency depending on the measuring set-up (Rodahl & Kasemo, 1996); oscillator techniques are used for a continuous monitoring of a frequency which corresponds to a specific phase shift of the sensor in the resonance bandwidth (Ehahoun et al., 2002; Barnes, 1992; Wessendorf, 1993; Borngräber et al., 2002; Martin et al., 1997), this frequency can be used, in many applications, as reference of the resonance frequency of the sensor; and the lock-in techniques, which can be considered as sophisticated oscillators, are designed for a continuous monitoring of the motional series resonance frequency or the maximum conductance frequency of the resonator-sensor (Arnau et al., 2002, 2007; Ferrari et al., 2001, 2006; Jakoby et al., 2005; Riesch & Jakoby 2007). In order to assure that the frequency shift is the only parameter of interest, a second parameter providing information of the constancy of the properties of liquid medium is of interest, mainly in piezoelectric biosensors; this parameter depends on the characterization system being: the maximum conductance or the conductance bandwidth in impedance analysis, the dissipation factor in decay methods and a voltage associated with the sensor damping in oscillator techniques

The different characterization methods mentioned can be classified in two types: 1) those which passively interrogate the sensor, and 2) those in which the sensor forms part of the characterization system. In the first group impedance or network analyzers and decay methods are included. Advantages of impedance analyzers are mainly related to the fact that the sensor is almost characterized in isolation and no external circuitry influences its electrical behaviour; additionally, electrical external influences can be excluded by calibration. The accuracy of decay methods is high provided that the accuracy in the data acquisition is high as well, both in phase and amplitude, which becomes very complicated for high resonance frequencies; therefore, for high frequency resonators only impedance analysis provides accurate results, but its high cost and large dimensions, prevent its use for

sensor applications. Consequently, oscillators are taken as alternative for sensor resonance frequency monitoring; the low cost of their circuitry as well as the integration capability and continuous monitoring are some features which make the oscillators to be the most common alternative for high resonance frequency QCM sensors. However, in spite of the efforts carried out to build oscillator configurations suitable for in-liquid applications (Barnes, 1991; Auge et al., 1994, 1995; Chagnard et al., 1996; Paul & Beeler, 1998; Rodríguez-Pardo, 2004, 2006; Wessendorf, 2001; Benes et al., 1999) the poor stability of high frequency QCM systems based on oscillators has prevented increasing the limit of detection despite the higher sensitivity reported (Rabe et al., 2000; Uttenthaler et al., 2000; Zimmermann et al., 2001; Sagmeister et al., 2009).

3. A new concept for sensor characterization

In QCM based biosensors, in which this chapter is focused, the experimental frequency shifts expected are usually small, in the order of tens of Hertz. Therefore, the great efforts performed to improve the sensitivity of the sensor are useless if they are not accompanied with an increase in the limit of detection. As mentioned, increasing the sensor frequency has not carried a parallel improve in the resolution; this suggests that the resonant frequency is not the only parameter to take into account to get our purposes.
Effectively, the sensitivity will not be improved if the frequency stability is not improved as well. Two aspects should be distinguished: on one hand on the experimental set-up which must be designed to minimize the disturbances or interferences which can affect the resonance frequency of the resonator such as: temperature, vibrations, pressure changes due to the fluid pumps, etc.; and on the other hand on the ability of the characterization system for an accurate measuring of the parameter of interest, in this case the appropriate resonance frequency of the resonator-sensor. Assuming that the experimental set-up is maintained under maximum control, the frequency stability depends on the measuring system.

3.1 Problem outline

The measuring systems used for high fundamental frequency QCM applications, apart from routing impedance analysis, have been oscillators for the reasons mentioned above. It is important to realize that the role of crystal resonators in radio-frequency oscillators is to improve the frequency stability. The oscillation frequency in an oscillator is the result of a delicate balance among the phase responses of each one of the elements in the oscillator (Arnau et al., 2008, 2009); if the phase response in one of the elements changes, the oscillation frequency shifts to find the new balance point. Therefore the origin of the frequency instability is the phase instability and a direct relationship exists between a phase shift and the corresponding frequency shift. This relationship can be easily obtained through the definition of the stability factor S_F of a crystal resonator operating at its series resonance frequency f_o:

$$S_F = \frac{\Delta\phi}{\Delta f} f_o = 2Q \tag{4}$$

where Δf is the frequency shift necessary to provide a phase shift $\Delta\varphi$ in the phase-frequency response of the resonator, around f_o, and Q is the series quality factor of the resonator.
According to (4) the frequency noise Δf_n associated to a phase noise in the circuitry $\Delta\varphi_n$ is:

$$\Delta f_n = \frac{f_o}{2Q}\Delta\varphi_n \tag{5}$$

Consequently, because the quality factor is normally reduced proportionally to $1/f_o$, the frequency instability is increased in relation to the square of frequency. Moreover, the phase response of the electronic components of an oscillator gets worse with increasing the frequency, which increases, even more, the noise. Furthermore, if the limit of the detection is assumed to be three times the level of noise ($\Delta\varphi_{min}=3\Delta\varphi_n$), the minimum detectable surface mass density change of a QCM, according to (2) and (5) will be:

$$\Delta m_{\min} = \frac{f_o}{2QS_a}\Delta\varphi_{\min} \tag{6}$$

The former equation seems to indicate that for a given minimum detectable phase of the measuring system, the surface mass limit of detection does not depend on the frequency. Fortunately this is not completely true; the liquid medium has not been taken into account in the obtaining of the previous equation. Recently, the following phase-mass relationship has been obtained for a QCM in contact with a liquid medium (Arnau et al., 2009):

$$\Delta m_{\min} \approx -m_L\Delta\varphi_{\min} \tag{7}$$

Therefore, because $m_L=\rho_L\delta_L/2$ and $\delta_L=(\eta_L/\pi f\rho_L)^{1/2}$, where η_L is the liquid viscosity, is reduced proportionally to $1/f^{1/2}$, so does m_L and then the resolution of the surface mass density Δm_{min} increases with $f^{1/2}$ for a given $\Delta\varphi_{min}$.

Effectively, the ratio between the limits of detection of surface mass density at two different frequencies, $f_2 > f_1$, for a given phase limit of detection of the monitoring system, according to (7), is:

$$\frac{\Delta m_{\min}(f_2)}{\Delta m_{\min}(f_1)} = \sqrt{\frac{f_1}{f_2}} \tag{8}$$

Therefore, the surface mass limit of detection, for a constant phase limit of detection of the measuring system, reduces proportionally to $f^{1/2}$ and so the resolution increases correspondingly. This is not in contradiction with (6), simply the effective reduction of the quality factor of the sensor in liquid, is proportional to $1/f^{1/2}$ instead to $1/f$ when the contacting liquid is considered. This is not true in air because the approximation given in (7) is not acceptable. In air, an increase in frequency does not improve the limit of detection unless the stability and the phase limit of detection of the measuring system are improved.

The previous analysis allows concluding the following important remarks: 1) The sensitivity of a QCM always increases with increasing the frequency; however, the mass resolution, which is the parameter of interest, only increases with the frequency if the noise is, at least, maintained constant or reduced. Moreover, this increase in the mass resolution is only valid for in-liquid QCM and not for in-gas QCM; and 2) Once all the cares have been taken into account to reduce the perturbations on the resonator-sensor such as: temperature and pressure fluctuations, etc., the mass resolution is only depending on the interface system, its stability and its phase detection limit.

Consequently, unlike in RF-oscillators, in QCM sensor oscillators the quality factor of the resonator is strongly reduced, and any phase instability in the rest of the elements of the oscillator is compensated with a much larger frequency-shift of the sensor, which contributes in a frequency noise increase. Therefore for high frequency QCM sensor applications in liquid, the components which from part of the oscillator, apart from the resonator-sensor, should be selected as ideal as possible to avoid the phase noise which is transferred into frequency noise. Unfortunately to design and implement an ideal oscillator for high frequency QCM sensors in liquid is not an easy task as mentioned.

3.2 Concept description

The great sensitivity of the QCM sensors is due to the great acceleration suffered by the mass layer deposited on the sensor surface (for 10MHz sensors in air, it is around 10^7 times the gravity). This big acceleration is due to two parameters: frequency and displacement amplitude of sensor surface; therefore, it is very important to work at maximum displacements and this occurs at resonance. However, the important part of this argument is that we have a resonance bandwidth in which the amplitude of displacement is reasonably big. Therefore, taking into account that the expected frequency shifts in QCM biosensors are very small, it could be possible to interrogate the sensor at an appropriate fixed frequency in the resonance bandwidth and then measure the change in the phase response of the sensor, due to the experimental process to be monitoring, without losing the resonance; Fig.3a depicts this idea. The advantage of this approach is that the sensor is interrogated with an external source which can be designed to be very stable and with extremely low phase and frequency noises. A similar approach has been already applied by some authors (Dress et al., 1999; Pax et al., 2005), but recently a simple relationship between the surface mass change and the corresponding sensor phase shift, for a sensor operating at its motional series resonant frequency, has been already obtained as follows (Arnau et al., 2009):

$$\Delta\varphi(\text{rad}) = -\frac{\Delta m_c}{m_q + m_L} \tag{9}$$

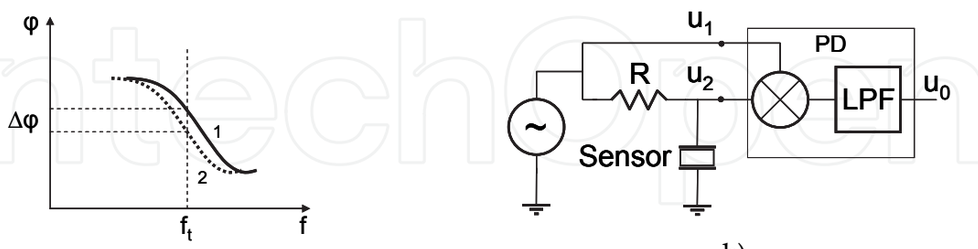
where $m_q=\eta_q\pi/2v_q$, being η_q the effective quartz viscosity and v_q the wave propagation speed in the quartz. In liquid applications $m_q << m_L$ and (9) reduces into (7).

The former equation is very simple but, apart from introducing the mathematical quantification of the phase-mass approach, makes clear a very important aspect: in contrast with Sauerbrey equation in which the frequency shift associated with a change in the surface mass density of the coating does not depend on the medium, (9) includes the additional effect of the medium. From the previous equation it is clear that the bigger m_L the bigger Δm_c for a given phase-shift detection limit. In other words, Sauerbrey equation predicts the same shift in the resonant frequency for a sensor in vacuum or in liquid for a given change in the surface mass of the coating; however the corresponding phase-shift is much smaller for the sensor in liquid than in vacuum. Therefore, although the Sauerbrey equation predicts the same frequency-mass sensitivity in both cases, much higher phase stability of the system is necessary for the case of the sensor in liquid than in vacuum to have, in practice, the same mass resolution.

In principle, the new method based on monitoring the phase shift of the sensor at an appropriate fixed frequency in the resonance bandwidth, allows characterizing the sensor almost in isolation with a RF signal of lowest phase and frequency noises, even at very high frequencies, in a simple way.

3.3 System description

A simple circuit to implement the phase-mass characterization approach is depicted in Fig.3b, where a mixer is used as a phase detector. A more specific circuit has been recently proposed (Fig.4) (Arnau et al., 2009) and a practical implementation of the sensor circuit part is shown in Fig.5.



a) b)

Fig. 3. a) Description of the phase approach and b) Simple implementation

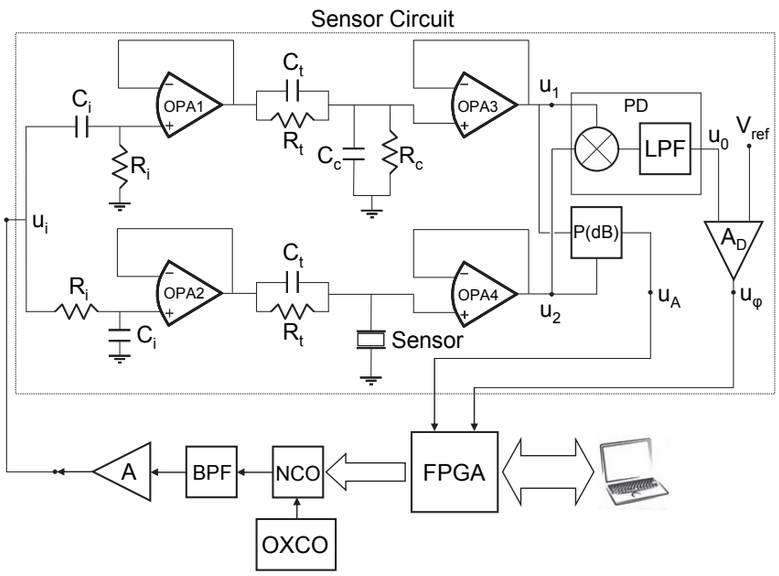
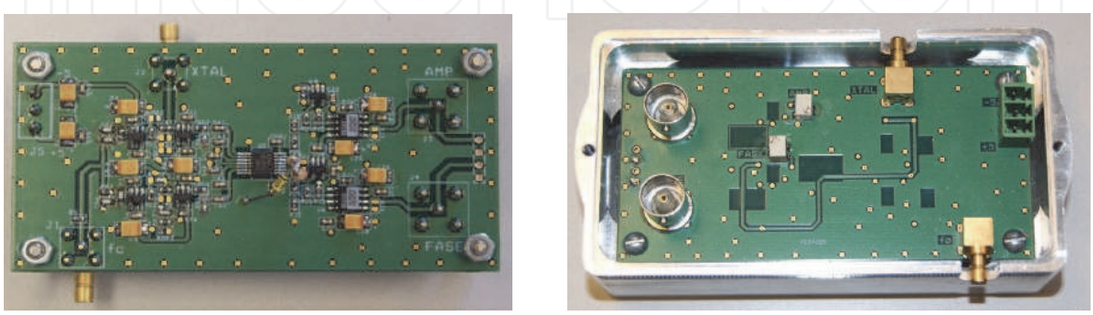


Fig. 4. Proposed system (Arnau et al. 2009)



a) b)

Fig. 5. Implemented system: a) bottom b) top

4. Case study: QCM immunosensor for carbaryl detection. Concept validation

A biosensor can be defined as an analytical device in which a biological receptor, such as: an enzyme, an antibody, a tissue portion, a whole cell, etc., is immobilized onto the surface of an electronic, optic or optoelectronic transducer. When a target analyte, from a complex mixture, is recognized by the immobilized biological material, a biochemical interaction is produced and transformed into a quantifiable signal by means of the transducer.
An immunosensor is a particular type of a biosensor in which the biological component and the target analyte are immunoreagents involved in an immunoassay. The term "immunoassay" refers to and comprises all the analytical procedures based on the specific antigen-antibody recognition. With regards to the immunoreagents, several antigens (free analytes, protein-hapten conjugates) can be involved in the reaction, whereas usually only one antibody takes part in the immunoassay (Montoya et al, 2008).

4.1 Piezoelectric immunosensors for low molecular weight pollutants

An antibody is a protein produced by the immune system of mammals as a natural defence reaction against the exposure to an external agent (an antigen). Antibodies can be obtained in the laboratory in order to be used in immunoassays for analytical purposes. For antibody production against low-molecular weight compunds, these analytes must be chemically modified (haptens), and covalently bound to proteins. Subsequently, the hapten-protein conjugates obtained are used both as antigens for mammal immunization and as assay conjugates in immunoassays.
In the most popular immunoassay configuration one of the immunoreagents (the antigen or the antibody) is immobilized on a solid support. Depending on the immobilized molecule, two main solid-phase immunoassay formats can be defined: the conjugate-coated; and the antibody-coated formats. With low-molecular weight compounds, the conjugate coated format (when the immobilized immunoreagent is the hapten-protein conjugate) must preferably be chosen (Montoya et al, 2008; March et al, 2009). In this type of assay, the detection of the analyte is based on a binding inhibition test and thus a competitive assay is performed; the free analyte competes with the immobilized conjugate for binding to a fixed, limited amount of the antibody. As in any competitive assay, the signal decreases as the analyte concentration increases. This inverse relationship allows us to obtain the typical dose-response curves of a competitive immunoassay.
In QCM piezoelectric immunosensors the transducer is a piezoelectric acoustic device, usually a quartz crystal resonator, although other acoustic sensing technologies are used as mentioned. The most common electrode-configuration of quartz resonators for biosensor applications implements gold electrodes which can be used as the support for immobilization of immunoreagents (antibodies, antigens, or hapten-conjugates), in such a way that a subsequent immunoreaction (antigen-antibody binding) could be detected as a mass variation.

4.1.1 Immunoreagent immobilization

The immobilization of biomolecules on the transducer surface is essential to ensure sensor's performance, playing an important role on the specificity, sensitivity, reproducibility and recycling ability of the immunosensor. As a consequence of that some of the requirements that should be fulfilled by an immobilization process include: (1) retention of biological activity of biomolecules; (2) achievement of reproducible and stable attachment with the

substrate against variations of pH, temperature, ionic strength, and chemical nature of the microenvironment; and (3) uniform, dense, and oriented localization of the biomolecules.
Among all the immobilization methods reported in the literature (Bizet et al., 1998; Su et al., 2000; Tombelli & Mascini, 2000), covalent binding (Pribyl et al., 2003; Prohanka & Skládal, 2005) is the most promising technique because it fulfils most of the requirements mentioned above.
Great effort has been devoted to achieve and optimize the conditions for covalent binding. Self-assembled monolayer (SAM) technology has been providing the best results (Vaughan et al., 1999; Ferreti et al., 2000; Mauriz et al., 2006; Briand et al., 2006). SAM is the generic name given to the methodologies and technologies that allow the generation of monomolecular layers, also called monolayers, of biological molecules on a variety of substrates. This technique allows a reliable control over both the packing density and the environment of an immobilized recognition centre or multiple centres at a substrate surface.
Many organic compounds are adequate to self-assemble: long chain carboxylic acids or alcohols (RCOOH, ROH), where R is an alkyl chain, reacting with metal oxide substrates; organosilane species ($RSiX_3$, R_2SiX_2 or R_3SiX), where X is a chlorine atom or an alkoxy group, reacting with hydroxylated substrates (glass, silicon and aluminium oxide, etc.); and organosulfur-based species reacting with noble metal (gold, silver) surfaces. Up to date, the latest system has been the most widely studied being the best characterized in terms of stability and physicochemical properties. Moreover, sulfur-containing compounds (alkanethiols, dialkyl disulfides and dialkyl sulfides) have a strong affinity for noble metal surfaces as they are spontaneously chemisorbed, with a regular organisation and high thermal, mechanical and chemical stability, on perfectly cleaned gold surfaces (Ferreti et al., 2000). Their adsorption to the surface has been shown to proceed by two methods: by ionic dissociation (10a) and, more favourably, by radical formation (10b) (Vaughan et al., 1999).

$$RSH + Au \rightarrow AuRS^- + H^+ \quad (10a)$$

$$RSH + Au \rightarrow AuRS\cdot + H\cdot \quad (10b)$$

Because of its stability, orientation and ability to functionalize the terminal groups on the molecules, SAMs can offer a very convenient and versatile method for covalent immobilization of biomolecules on gold surfaces for biosensor development. Being in intimate contact with the support surface, SAMs do not have the problems associated with mass transport, thus providing the advantage of a faster and potentially more intense response when exposed to external stimuli (Vaughan et al., 1999; Ferreti et al., 2000).
The covalent binding of a protein to a gold surface by means of SAM formation, basically consists on the following stages: (1) SAM formation with an ethanolic solution of a long chain thiolated acid which is adsorbed onto the gold sensor surface; (2) activation of the terminal carboxylic groups of the thiolated acid, to an intermediate reagent (N-hydroxy-succinimide ester), which takes place by means of an ethanolic or aqueous mixture of N-hydroxi-succinimide (NHS) and carboxi-diimide (EDC); (3) covalent attachment of the active intermediate, thus obtained, to the amine groups of the hapten-protein conjugates; and (4) addition of ethanolamine to deactivate all the unreacted intermediate NHS-esters remaining on the sensor surface. This procedure ensures that only covalently bound analyte

derivatives (hapten-conjugates) remain on the sensor surface (Duan & Meyerhoff, 1995; Disley et al., 1998; Mauriz et al., 2006; Briand et al., 2006; March et al., 2009).
The process described can be done with simple or mixed SAMs. Mixed SAMs are generally formed by co-adsorption of mixtures of two thiols, one of them providing a functional terminal group (like a carboxylic acid, COOH) at a low molar fraction, and the other one being the "diluting" thiol (with, for example, CH_3 or OH terminal groups) at a high molar fraction. The second thiol reduces the surface concentration of functional groups, thus minimizing steric hindrance, partial denaturation of the potential immobilized protein and non-specific interactions that could produce interference signals. Also the diluting thiol can be used to tailor the overall physico-chemical properties of the interface (such as its hydrophobic/hydrophilic character). Consequently, the use of mixed SAMs of alkanethiols (long chain thiols) on gold is particularly recommended in order to minimize steric hindrances, to prevent denaturation, and hence to improve the activity of immobilized proteins (Subramanian & Irudayarj, 2006; Bonroy et al., 2006; Briand et al., 2006, 2007).

4.2 Characterization of a piezoelectric immunosensor

The resonance frequency shift is usually handled as monitoring parameter in piezoelectric immunosensors; however the phase-shift monitoring has been proposed above as a new QCM monitoring parameter for high resolution QCM applications. A comparison between the classical technique based on frequency shift monitoring and the new one based on phase shift monitoring, under the same experimental conditions, is presented next to validate the proposed technique. Only with this purpose, a piezoelectric immunosensor for the detection of the pesticide carbaryl, as a validation model, has been developed.

4.2.1 Experimental set-up and methodology

AT-cut quartz crystals with gold electrodes (10 MHz, International Crystal Manufacturing) were functionalized by immobilizing BSA-CNH carbaryl hapten conjugate on the sensor surface through the formation of a thioctic acid self-assembled monolayer (March et.al.,2009). The crystal was placed in a custom-made flow cell and included in a flow-through setup, controlled by a peristaltic pump (Minipuls 3, Gilson), with the injection loop and solutions at the input of the flow cell exchanged by manual Rheodyne valves (models 5020 and 5011, Supelco). The whole fluidic system was placed inside a custom made thermostatic chamber and all the experiments were performed at 25ºC ±0.1ºC. To avoid unwanted disturbances the chamber was placed on an anti-vibration table. The sensor characterization circuit, shown in the previous section, was connected to the piezoelectric sensor and it was also placed in the thermostatic chamber. A RF signal generator model HP8664A (Hewlet Packard) generated the signal applied to the circuit and the voltage variations related to the phase shift and attenuation were measured with a digital multimeter HP 34401A (Agilent) and sent to a PC via GPIB bus. The experimental set-up is presented in figure 6.
The immunoassay developed to determine carbaryl was an inhibition test based on the conjugate coated format, in which the hapten-conjugate was immobilized on the sensor surface. A fixed amount of the respective monoclonal antibody was mixed with standard solutions of the analyte and pumped over the sensor surface. Since the analyte inhibits antibody binding to the respective immobilized conjugate, increasing concentrations of analyte will reduce the phase shift induced on the piezoelectric sensor and the corresponding demodulated voltage.

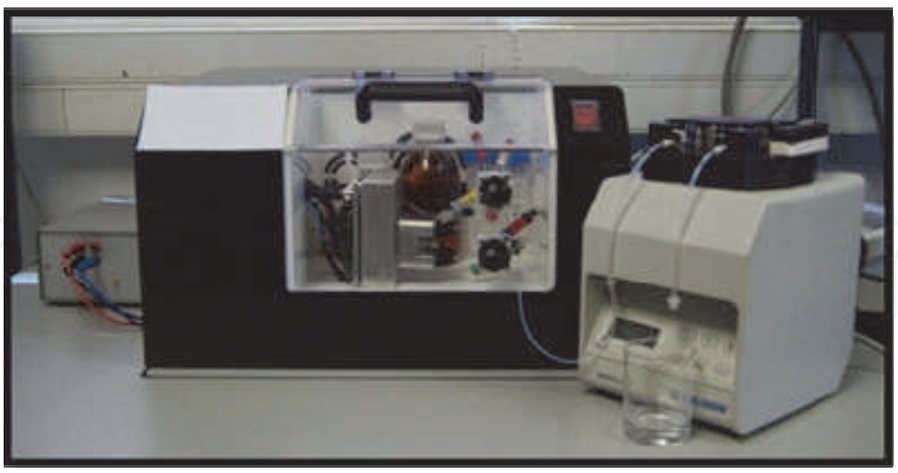


Fig. 6. Experimental set-up

Different standard concentrations of carbaryl were prepared by serial dilutions in PBS, from a 1 mM stock solution in dimetylformamide at -20ºC. The standards were mixed with a fixed concentration of the monoclonal antibody LIB-CNH45 (from I3BH-UPV, Abad et al., 1997) in PBS. Analyte-antibody solutions were incubated for one hour at chamber temperature and then injected onto the sensor surface. The phase-shift was monitored in real-time for each analyte concentration, as the binding between free antibody and the immobilized conjugate took place. For each assay, after stabilization of the initial signal at a flow rate of 30 µL/min for 2 min, the sample (250 µL) was injected for 12 min to measure the immunoreaction. Once each assay was finished, regeneration of the sensing surface was performed using diluted hydrochloric acid, HCl, 0.1M at a flow rate of 280 µL/min for 4 min to break the antibody-hapten linkage. After the regeneration, buffer solution was again flown-through for 2 min at the same flow rate.

4.2.2 Results and discussion

Figure 7 shows the typical real-time signals obtained in the immunoassay developed for the detection of carbaryl with the phase shift concept. As it can be seen on the figure, the typical inverse relationship for a competitive assay is obtained between the phase-shift voltage (ΔV_φ) and the pesticide concentration in the sample. Only a representative part of the signals obtained in the immunoassay, corresponding to concentrations of antibody-analyte of 10, 20, 100 and 500µg/L are shown in Fig. 7.

A representative standard curve (Fig. 8) was finally obtained by averaging three individual standard curves starting from samples that were run at least in duplicate. In Fig. 8 the decrement of the phase voltage has been normalized and represented as a percentage of the maximum decrement obtained ($100x\Delta V_\varphi/\Delta V_{\varphi 0}$, being ΔV_φ the voltage variation of each sample and $\Delta V_{\varphi 0}$ the variation for the zero analyte concentration sample, which provides maximum signal). The experimental points were fitted to a four-parameter logistic equation, then showing the typical decreasing sigmoidal shape of binding inhibition immunoassays.

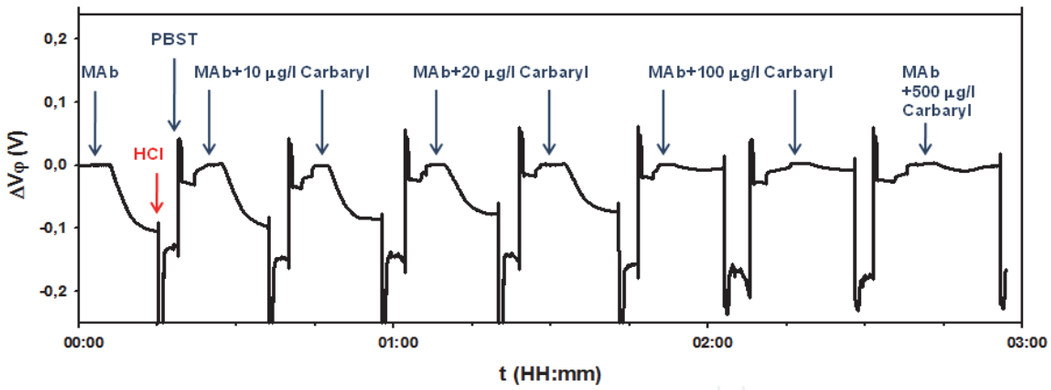


Fig. 7. Real time piezoelectric immunosensor response to different concentrations of analyte

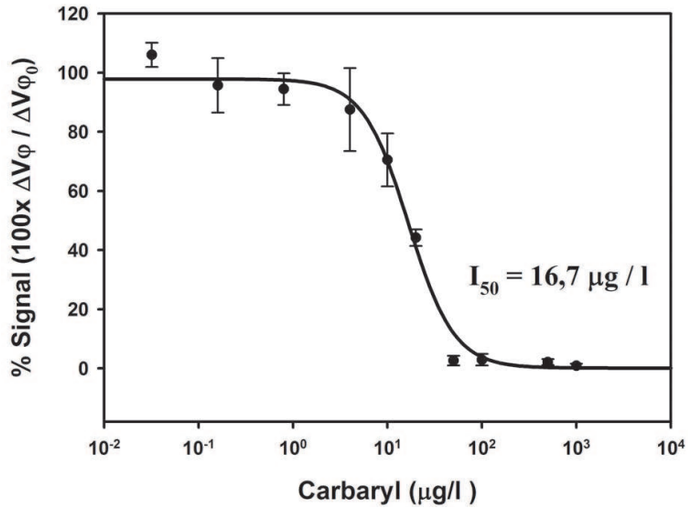


Fig. 8. Average standard curve for the carbaryl piezoelectric immunosensor based on phase-shift characterization method

One of the parameters of interest of the immunosensor, generally accepted as a good approach of the immmunosensor sensitivity, is the I_{50} value. This point is related to the analyte concentration giving 50% inhibition of the maximum signal. In this case, the I_{50} value obtained was 16.7 µg/L. The limit of detection (LOD), another parameter of interest calculated as the pesticide concentration that provides 90% of the maximum signal (I_{90} value) was 4 µg/L. The quantification range, this is, the working range in which the signal inhibition is linear (between 20% and 80% of the maximum), covered concentrations of analyte between 7 and 35 µg/L.

These results were compared with those obtained in the same immunoassay format and conditions for carbaryl detection but using a different characterization circuit (Table 1).

As it can be observed, both the sensitivity and limit of detection of the developed immunosensor were of the same order of magnitude as compared to previously reported results (March et al., 2009; Montagut et al., 2011). These results validate the new characterization concept and the developed interface. An improvement trend of the analytical parameters (I_{50} and LOD), due to the reduction of the noise in the new system, is

	Phase Shift Method	Oscillator (Montagut, 2011)	(March et al, 2009)
Sensitivity I_{50} (µg/L)	16.7	24.0	30.0
L.O.D. I_{90} (µg/L)	4.0	6.5	11.0
Linear Range (µg/L)	7 – 35	11 – 42	15 - 53

Table 1. Comparative results obtained for the QCM immunosensor using different electronic characterization techniques

observed as well. Effectively, the noise level in the oscillator technique was of 2Hz for a maximum signal of 137Hz, while for the phase-shift interface was of 1mV for a maximum signal of 200mV, this indicates an improvement of three times the noise to maximum signal, which could provide a better improvement of the immunosensor sensitivity and limit of detection by optimizing the biochemical parameters, although this is not the main purpose of this work. Moreover, it is important to notice that the improvement trend has been got even with relative low frequency sensors (10MHz), where electronic components and circuits have a very good performance. Recent preliminary results, not shown, using the new concept with high fundamental frequency resonator sensors seem to indicate that a significant improvement, both in sensitivity and limit of detection, could be found with very high fundamental frequency sensors.

5. Conclusions and future lines

The new method for QCM biosensors characterization, based on the monitoring of the phase-shift experimented by a signal of constant frequency in the resonant bandwidth of the sensor, has been validated under real-experimental conditions, and compared with classical interface techniques. An improvement trend, both in sensitivity and limit of detection, is observed, even for relative low frequency resonators (10MHz), due to the signal to noise ratio improvement. Moreover, the new characterization system, particularly useful for biosensor applications, has special advantages which make it ideal for addressing the remaining challenges in high resolution QCM applications: a) the sensor is passively interrogated by an external source, which can be designed with high frequency stability an very low phase noise, even at very high frequencies, b) the sensor circuit can be made very simple with high integration capabilities, and c) sensors working at the same fundamental resonance frequency could be characterized, in principle, with only one source, opening the possibility of working with sensor arrays for multianalysis detection.
Following the results presented here, the next step is to perform experiments with high fundamental frequency BAW resonators based on inverted mesa technology.

6. Acknowledgment

The authors are grateful to the Spanish Ministry of Science and Technology the financial support to this research under contract reference AGL2009-13511, and to the company Advanced Wave Sensors S.L. (www.awsensors.com) for the help provided in the development of some parts of this work.

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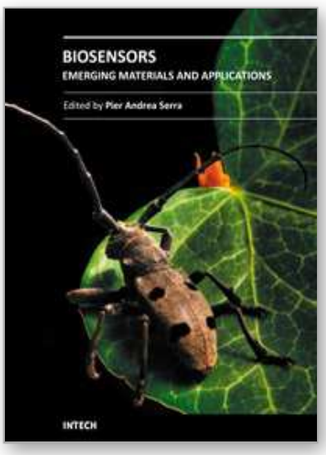

Biosensors - Emerging Materials and Applications

Edited by Prof. Pier Andrea Serra

ISBN 978-953-307-328-6
Hard cover, 630 pages
Publisher InTech
Published online 18, July, 2011
Published in print edition July, 2011

A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 19 different countries. The book consists of 27 chapters written by 106 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

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