

Integration of bio, nano, micro and cogno in biosensor devices for human health applications

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The design, fabrication and testing of micro/nanobiosensor devices based on optoelectronics and nanomechanical highly sensitive transducers is shown. Most of the devices are fabricated by standard silicon CMOS microelectronics technology after a precise design for achieving a high sensitivity for biosensing applications. Three biosensors have been developed: (a) a Surface Plasmon Resonance biosensor (b) an integrated Mach-Zehnder interferometer micro/nanodevice based on optical waveguides and (c) nanomechanical biosensors based on microcantilevers. Direct biosensing with all the sensors has been tested, after a specific receptor coupling to the surface device using nanometer scale immobilization techniques. Further integration of the micro/nano sensors, the microfluidics, the sources, the photodetectors and the CMOS electronics will render in complete lab-on-a-chip microsystems which could be used in field applications.

Keywords: MEMS; Biosensor; Silicon DMOS

1. Introduction

The research and technological development of biosensors have experimented an exponential growth during the last decade because this technology has a great potential for the direct, real-time and label-free detection of many chemical and biological substances [1,2]. The success of the biosensor technology can be deduced for the increasing number of commercially available instruments. But there is still a long way to replace completely the conventional analysis by the biosensor technology in many fields and, specially, in clinical diagnostics. To achieve such objective, we still need to develop biosensors able of detect, in a direct way, very low levels (picomolar to femtomolar) of a great number of substances in the areas of environmental monitoring, industrial and food process, health care, biomedical technology, clinical analysis, etc.

For that reason, there is an increasing interest in systems based on micro/nanotechnologies for the development of ultrasensitive and miniaturized biosensors [1] which can be easy integrated in Microsystems. A highly multidisciplinary approach including microelectronics, MEMS, micro/ nanotechnologies, molecular biology, nanobiotechnology and chemistry are needed for the implementation of such new analytical devices [2]. Biosensing devices fabricated with optoelectronics and MEMS micro/nanotechnologies are powerful devices which can fulfill these requirements. In this line, our group is working in two different approaches [3]:

(A) A platform based on optoelectronics biosensors (evanescent wave detection). Two optical biosensors have been already developed: a portable Surface Plasmon Resonance Sensor (actually in commercialization) and an integrated Mach-Zehnder interferometer device. For the last one, the use of standard Si microelectronics technology allow the possibility for integration of optical, fluidics and electrical function on one optical sensing circuit in order to obtain a complete lab-on-a-chip. A limit of detection close

to femtomolar is achievable with this sensor in a direct format [4].

(B) A platform based on nanomechanical biosensors. Microcantilever biosensors are a new class of high sensitivity biosensors able of performing local, high resolution and label-free molecular recognition measurements [5]. Moreover, nanomechanical biosensors based on microcantilevers have been recently reported as a promisingly alternative to current DNA-chips allowing real-time monitoring of DNA without need of labeling. For that reason, we are working in the development of a portable multibiosensor microsystem based on an array of microcantilevers [6] able to detect analytes with femtomolar sensitivity and ability for discerning single base variations in DNA strands.

2. Optoelectronics biosensors platform

Optical biosensors are providing an increasingly impact analytical technology for the detection of biological and chemical species. Most of the integrated optical sensors make use of the evanescent field detection principle for sensing [7]. In an optical waveguide the light travels inside the waveguide, confined within the structure by Total Internal Reflection (TIR). The light is transmitted through a model of the electromagnetic field called "guided modes" [7]. Although light is confined inside those modes, there is a part of it (*evanescent field, EW*) that travels through a region that extends outward, around a hundred of nanometers, into the medium surrounding the waveguide (see Fig. 1).

This EW field can be used for sensing purposes. When a receptor layer is immobilized onto the waveguide, as it is shown in Figure 1, exposure of such a surface to the partner analyte molecules produces a (bio) chemical interaction, that takes place into the surface of the waveguide and induces a change in its optical properties that is detected by

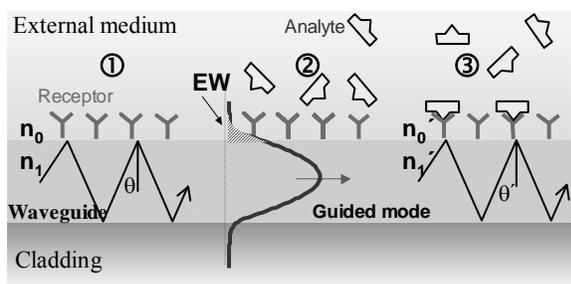


Figure 1. Biomolecular interaction sensing by the evanescent wave detection principle in an optical waveguide sensor

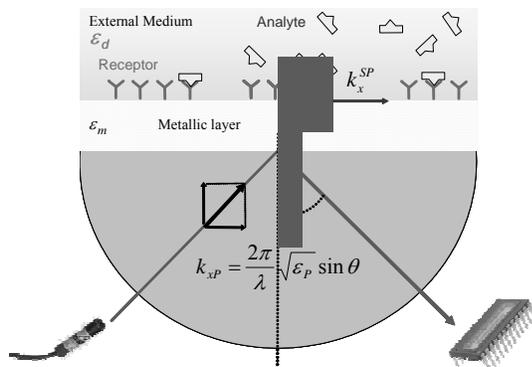


Figure 2. Scheme of the working principle of a Surface Plasmon Resonance Biosensor.



Figure 3. Portable SPR sensor prototype system including sensor, optics, electronics and flow delivery system. Commercialized by Sensia,S.L.

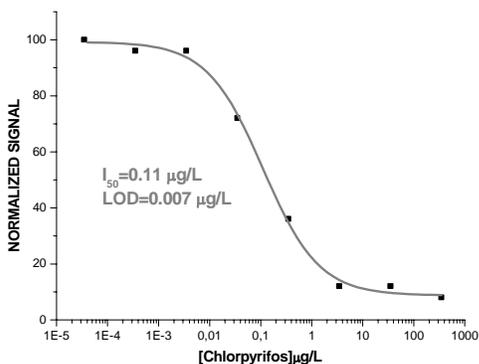


Figure 4. Calibration curve for the immunoassays determination of Chlorpyrifos by SPR.

the evanescent wave. The extent of the optical change will depend on the concentration of the analyte and on the affinity constant of the interaction, obtaining, in this way, a quantitative sensor of such interaction. The evanescent wave decays exponentially as it penetrates the outer medium and, therefore, only detects changes that take place on the surface of the waveguide, because the intensity of the evanescent field is much higher in this region. For that reason it is not necessary to carry out a prior separation of non-specific components (which is necessary in conventional analysis) because any change in the bulk solution will hardly affect the sensor response. In this way, evanescent wave sensors are selective and sensitive devices for the detection of very low levels of chemicals and biological substances and for the measurement of molecular interactions in-situ and in real time [4,8]. The advantages of the optical sensing are significantly improved when this approach is used in an integration schema [7]. The technology of integrated optics allows the integration of many passive and active optical components (including fibres, emitters, detectors, waveguides and related devices, etc...) onto the same substrate, allowing the flexible development of miniaturised compact sensing devices, with the additional possibility of fabrication of multiple sensors on one chip.

2.1. Surface Plasmon Resonance Biosensor

One of the best known and more developed EW biosensor is the Surface Plasmon Resonance (SPR) sensor [8,9,10], because of its sensibility and simplicity as can be seen in Figure 2. Surface plasmons are elementary excitations, which result from a collective oscillation of the free-electron plasma at a metal-dielectric film interface. In a SPR sensor a thin metal film (usually Au) is evaporated on the dielectric material surface. The sensing mechanism is based on variations of the refractive index of the medium adjacent to the metal sensor surface during the interaction of an analyte to its corresponding receptor, previously immobilized at the sensor surface in the region of the evanescent field.

The recognition of the complementary molecule by the receptor causes a change in the refractive index and the SPR sensor monitors that change. After the molecular interaction, the surface can be regenerated using a suitable reagent to remove the bound analyte without denaturing the immobilized receptor.

We have developed a portable SPR sensor prototype (see Fig. 3) as a highly sensitive field analytical method for environmental monitoring. As a proof of its utility towards detection of pathogens, we have determined several pesticides, as the chlorinated compound DDT, and the neurotoxins of carbamate type (carbaryl) and organophosphorus type (chlorpyrifos). For the determination of these compounds a binding inhibition immunoassay, consisting of the competitive immunoreaction of the unbound antibody present in an analyte-antibody mixture with the hapten derivative immobilized at the sensor surface, has been applied. With

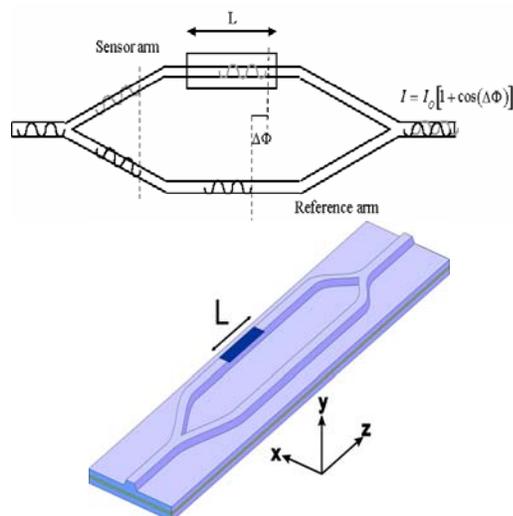


Figure 5. Mach-Zehnder interferometer configuration and working principle.

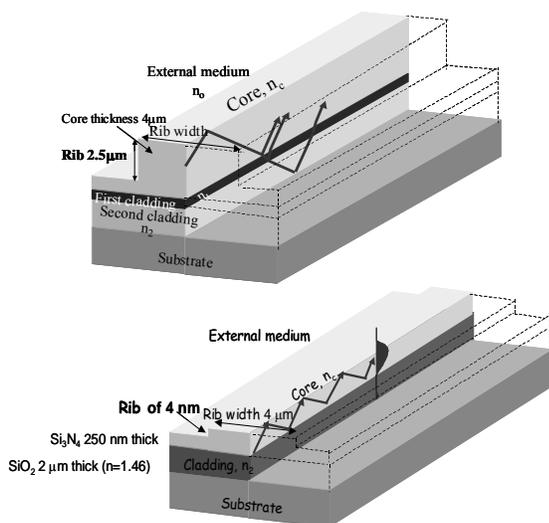


Figure 6. Structure of the MZI devices (top) ARROW-MZI (down) TIR-MZI.

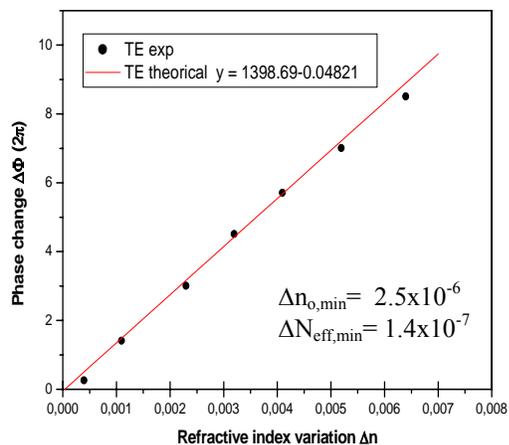


Figure 7. Sensitivity evaluation of a TIR-MZI nanodevice by using glucose solutions of varying refractive indices.

the aim of assuring the regeneration and reusability of the surface without denaturation of the immobilized molecule, the formation of an alkanethiol monolayer was carried out to provide covalent attachment of the ligand to the functionalized carbodiimide surface in a highly controlled way.

For DDT, the assay sensitivity was evaluated in the 0.004-3545 μg/l range of pesticide concentration by the determination of the limit of detection (0.3 μg/l) and the I₅₀ value (4.2 μg/l). For carbaryl, the dynamic range of the sensor is 0.12-2 μg/l, with an I₅₀ value for standards in buffer of 0.38 μg/l and a detection limit of 0.06 μg/l. Likewise the immunoassay for chlorpyrifos determination, afforded a high sensitivity (I₅₀ = 0.11 μg/l) working in the 0.02-1.3 μg/l range. As an example, Figure 4 shows the calibration curve obtained from the pesticide chlorpyrifos.

The performance of the inhibition immunoassay enables the SPR biosensor to monitor the immunoreaction between the hapten immobilized on the sensor surface and the monoclonal antibody, from the incubation of a mixed antibody-analyte solution. In addition, the reusability of the sensor was demonstrated after 250 assay cycles, without significant variations of the average maximum signal. The reusability of the sensor combined with the small time of response (approximately 15 min), makes the SPR immunosensing a valuable method for real-time and label-free analysis of environmental samples. This immunosensing technique together with the portable surface plasmon resonance sensor developed can be applied as a fast and cost-effective field-analytical method for the monitoring of any chemical and biological compound if the corresponding receptor is available.

2.2. Integrated Mach-Zehnder Interferometers

In an interferometer (see Fig. 5) two light beams of equal intensity are made to travel across two areas of a waveguide (one is the sensor and the other is the reference) and finally they are combined, creating an interference pattern. When a biochemical reaction takes place in the sensor area, only the light that travels through this arm will experience a change in its effective refractive index. At the sensor output, the intensity of the light shows a sinusoidal variation that depends on the difference of the effective refractive indexes of the sensor and reference arms (ΔN) and on the interaction length (L) and can directly related to the concentration of the analyte to be measured [11].

The interferometric sensor platform is highly sensitive and is the only one that provides with an internal reference for compensation of refractive-index fluctuations and unspecific adsorption. Interferometric sensors have a broader dynamic range than most other types of sensors and show higher sensitivity as compared to other integrated optical biosensors. Due to the high sensitivity of the interferometer sensor the direct detection of small molecules (as for example environmental pollutants where concentrations down to 0.1 ng/ml must be detected) would be possible with this device. Detection limit is generally

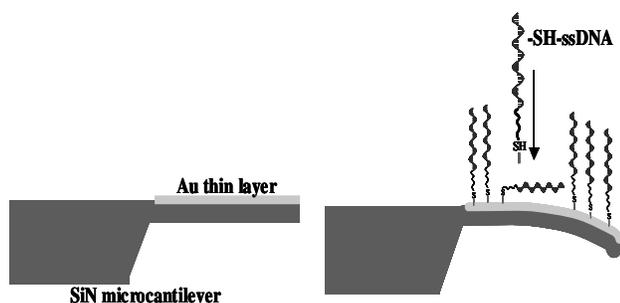


Figure 9. Working principle in nanomechanical cantilevers: (left) cantilever with a thin gold layer for covalent attachment of the receptor (right) bending of the cantilever after the adsorption or biomolecular interaction of molecules due to a change on the surface stress.

limited by electronic and mechanical noise, thermal drift, light source instabilities and chemical noise.

But the intrinsic reference channel of the interferometric devices offers the possibility of reducing common mode effects like temperature drifts and non-specific adsorptions. Detection limit of 10^{-7} in refractive index (or better) can be achieved with these devices which opens the possibility of development of highly sensitive devices,

for example, for extreme protein concentration determination (femtomolar) in a direct way [11].

We have fabricated two integrated Mach-Zehnder interferometric devices using two technologies: (a) a MZI Microdevice based on ARROW waveguide [12] (b) a MZI Nanodevice based on TIR waveguides [13].

MZI Microdevice based on ARROW waveguide

For the development of a highly sensitive integrated optical sensor based on the Mach-Zehnder interferometer configuration it is necessary to design optical waveguides that verify two conditions: monomode behaviour and high surface sensitivity. ARROW (Anti Resonant Reflecting Optical Waveguides) structures based on Silicon technology meet these requirements. This optimised waveguide consist on a rib-ARROW structure (see Fig. 6) with a silicon oxide core layer ($n_{\text{core}}=1.485$) and thickness higher than $2 \mu\text{m}$; a silicon oxide second cladding layer with a refractive index of 1.46 and a fixed thickness of $2 \mu\text{m}$ and a silicon nitride first cladding layer, $0.12 \mu\text{m}$ thick, with a refractive index of 2.00. The rib depth is 60% of the core thickness and the rib width should be lower than $8 \mu\text{m}$ to obtain single-mode behaviour [12].

MZI Nanodevice based on TIR waveguide

The basis of the TIR structure is (see Fig. 6): (i) a Si wafer, (ii) a $2 \mu\text{m}$ thick thermal Silicon-Oxide layer ($n=1.46$), (iii) a LPCVD Silicon Nitride layer of 100 nm thickness ($n=2.00$), which is used as a guiding layer. To achieve monomode behavior is needed to define a rib structure, with a depth of only 4 nm by a lithographic RIE step. Finally, a Silicon-Oxide protective layer is deposited by LPCVD over the structure with a $2 \mu\text{m}$ thickness

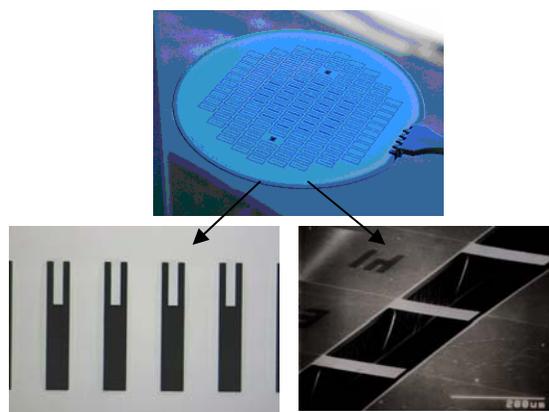


Figure 10. Photographs of cantilevers array fabricated by surface micromachining. Arrays of up to 20 have been fabricated with different lengths (from 100 to $500 \mu\text{m}$) and widths (from 20 to $40 \mu\text{m}$). Cantilever thickness was 300 nm .

($n=1.46$), which is patterning and etching by RIE to define the sensing arm of the interferometer.

For evaluating the sensor sensitivity a calibrating curve was recorded using solutions with different refractive indexes, as is depicted in Fig. 7 for a TIR-MZI device. For the TIR device [13], the lower detection limit measured was $\Delta n_{o,\text{min}} = 2.5 \cdot 10^{-6}$ that means an effective refractive index of $\Delta N = 1.4 \cdot 10^{-7}$. For the ARROW-MZI devices⁵, a minimum detectable refractive index variation of $\Delta n_{o,\text{min}} = 2 \cdot 10^{-5}$ was obtained.

We have applied the MZI biosensors (ARROW and TIR) for the detection of the insecticide carbaryl [2] and detection of DNA hybridisation. Some of the results can be observed in Figure 8

3. Nanomechanical biosensor platform

Nanomechanical biosensors is an excellent example of the application of micro- and nanotechnologies in the development of a new type of biosensors. Microcantilevers, such as those used in Atomic Force Microscopes, have been recently employed as this new class of biosensors [14]. The so-called nanomechanical biosensors have demonstrated that they are capable of detecting single-base mismatches in oligonucleotide hybridization without labelling as well as performing protein recognition [15] with extreme sensitivity. Among the advantages of nanomechanical biosensors are the potential for performing local, high resolution and label-free molecular recognition measurements on a portable device. Also, the reduced sensor area allows drastic decrease of the reagent consumption.

The working principle for nanomechanical biosensors relies on the induced surface stress that arises when molecules bind to a surface. When a monolayer of receptor molecules is immobilized on one side of the cantilever, a cantilever deflection results from the differential surface stress between opposite sides of the cantilever. Molecular recognition produces also a change of the surface stress of the sensitised cantilever side with respect to the other side,

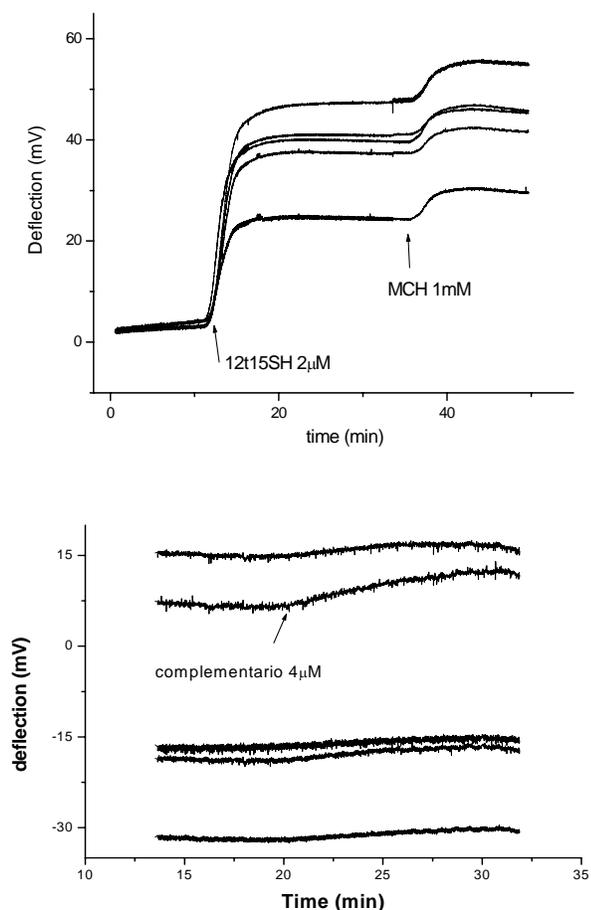


Figure 11. Real time simultaneously monitoring of five microcantilevers. (top) Immobilization and blocking treatment, and (down) hybridisation bending signal.

giving a cantilever deflection of few nanometers (see Fig. 9). Surface stress mainly arises from electrostatic, van der Waals, configurational and steric interactions between the adsorbed molecules. This method, which is often called DC, has allowed the measurement of submonolayer adsorption of different molecular species, changes of pH and salt concentration, and biomolecular interactions.

Cantilever bending (deflection) measurements are carried out by using the well-known optical beam deflection method employed in most of the atomic force microscopes. A laser beam is focused on the free end of the cantilever and the deflection of the reflected beam, which is proportional to that of the cantilever, is measured with a four-segment photodetector. For example, for the DNA hybridization detection, nucleic acids are immobilized on one side of the micromachined lever. Exposure of the cantilever to a sample containing complementary nucleic acid gives rise a cantilever bending (deflection) of a few nanometers. The nanomechanical response is due to the surface stress change of the active side with respect to the other side, in which DNA is not immobilized. The deflection is measured with sub-nanometer resolution by the optical system, in which the

laser beam reflects off the back of the cantilever to the position sensitive photo-detector.

Normally, micrometer-sized cantilevers are designed for atomic force microscopy and are fabricated using Silicon technology. But for application as highly sensitive biosensors the cantilevers have to be re-designed carefully according with the dimensions and the mechanical material properties. For the design and fabrication we have established a 100% yield technology based on standard Si fabrication [6]. Fig.10 shows some photographs of the fabricated cantilevers.

Nanomechanical biosensors based on microcantilevers have been reported as a promisingly alternative to current DNA-chips, allowing real-time monitoring of DNA without need of labeling [14]. The nanomechanical biosensing technology readily lends itself to fabrication of microarrays using well-known and standard microfabrication techniques, offering the promising prospect of multiple protein or DNA analysis and allowing the simultaneous detection of pathogens. To proof the reliability of such approach, we have used the arrays of Si cantilevers shown above for the real-time detection of (a) immobilization of DNA strands and (b) hybridization with the corresponding complementary DNA strands, as it is shown in Fig. 11.

A 12-mer ssDNA was derivatized with the alkylthiol SH-(CH₂)₆ in terminal 5'. Thiols spontaneously form self-assembled ordered monolayers onto the cantilevers array surface giving a pronounced deflection of about 20 nm. After -SH-ssDNA immobilization, the array of cantilevers was exposed to 6-mercapto-1-hexanol (MCH), a 6-carbon chain molecule terminated with thiol (-SH) and hydroxyl (-OH) groups on each of the extremes, respectively.

Thiol group of MCH rapidly displaces the possible weaker adsorptive contacts between the nucleotide chain and gold. Since hydroxyl group negligibly interacts with the nucleotide chain, MCH treatment assures that the immobilized ssDNA is only attached to the gold surface through the sulphur atom. This treatment enhances the accessibility of gold-tethered ssDNA molecules for base pairing with complementary nucleic acids, increasing the hybridisation efficiency from less than 10% to 80% approximately. MCH adsorption produces a cantilever deflection of about 10 nm. The array of cantilevers is then exposed to the complementary DNA sequence and the corresponding hybridisation signals were recorded, showing the feasibility of the cantilever technology for the real-time detection of DNA detection.

4. Conclusions

We have presented the development of different micro and nano biosensor platforms in which we integrate the bio, nano, micro and cogno aspects in order to achieve highly sensitive and minaturised devices. We have shown the development of: a portable Surface Plasmon Resonance Sensor (actually in commercialization), an integrated Mach-Zehnder interferometer device made on Si technology and a nanomechanical biosensor based on

MEMS technology. The feasibility of the different biosensors platforms have been proved by the immunological recognition of several pesticides, as the chlorinated compound DDT, and the neurotoxins of carbamate type (carbaryl) and organophosphorus type (chlorpyrifos) and the real-time detection of DNA hybridization. These results open the way for further development of portable and multianalyte platform for the detection of several biological molecules of interest in-situ and in real-time.

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