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From Nanobiotechnology to Organic and Biological Monitoring of Health and Environment for Biosafety

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Abstract

This overview describes the optimal implementation and utilization of different, newly conceived nanosensors for human biosafety purposes, exploiting a variety of methods (amperometric, conductometric, spectrometric and nanogravimetric), and a wide range of nanocomposites, genes and recombinant enzymes. Namely, while biological nanosensors were designed based on Nucleic Acid Programmable Protein Arrays (NAPPA), with or without SNAP-tag, and on Langmuir-Blodgett (LB) thin films of recombinant laccase (*Rigidoporus lignosus*, formerly known as *Rigidoporus microporus*), organic nanosensors were based on matrices of calcium oxide (CaO) and on carbon nanotubes–either multi-walled (MWNTs) or single-walled (SWNTs)– embedded in poly(o-methylaniline) (POTO). Special attention was paid both to detecting useful and relevant substances (such as carbon dioxide, phenols and phenolic derivatives and compounds) and designing devices and molecules for human biosafety like vaccines and others, by means of amperometry, conductimetry, mass spectrometry (MS) and other label-free technologies, such as quartz crystal microbalance with dissipation monitoring (QCM_D).

Keywords: Calcium oxide (CaO) Matrices; Carbon dioxide (CO_2); Carbon Nanotube Composites (CNTs); Laccase; Mass Spectrometry (MS); Nanosensors for biosafety; Nucleic Acid Programmable Protein Arrays (NAPPA); Phenols and phenolic derivatives and compounds; Protein synthesis Using Recombinant Elements (PURE) technology; Quartz Crystal Microbalance with Dissipation monitoring (QCM_D); SNAP-tag; Vaccines

Introduction

According to the Cartagena Protocol [1], biosafety (known also as biosecurity or biocontainment) is defined as a discipline dealing with the prevention of the loss of the biological integrity, and with its safeguard and preservation from natural and anthropogenic hazards.

It is definitely a multidisciplinary science, combining the expertise of different fields, ranging from biomedicine and agriculture, to chemistry and ecology.

In order to unravel the key elements of both biomedical and environmental sciences, it is mandatory to exploit a complex strategy, which unifies many different technologies [2-7]. Bioinformatics tools play a role in identifying the most important genes, and/or molecules involved in key biological processes [8]. Our previously described Leader Genes algorithm [9,10] enables to find the so-called "hub genes", that, being highly interconnected, form an important sub-network within the complex gene-gene interactions map useful to design and investigate with the help of DNA analyzer (DNASER) [11] ad hoc targeted micro-arrays. The selected genes of high interest can be subsequently expressed and investigated using label free technologies [12-14], based on NAPPA with or without the SNAP-tag [15,16] (Figure 1). This new approach overcomes indeed the limits of correlated fluorescence detection plagued by the background still present after extensive washes. This is being accomplished in conjunction with an homogeneous and well defined human cell free expression capable to achieve the above goals making realistic the ambitious objective to quantify the regulatory protein networks in humans, a task being fundamental for nanomedicine, and in the fields of biosafety and natural disasters, through the development of a quartz crystal microbalance with dissipation monitoring (QCM_D) sensor for health and environment of new conception [17,18], which can be utilized along with mass spectrometry (MS) and Nucleic Acid Programmable Protein Arrays (NAPPA)-SNAP arrays [19].

NAPPA arrays may play a key role, being able to detect bacterial and viral agents. Molecular epidemiology and surveillance have been found to be useful in monitoring natural disasters, as proven by scholars after the Katrina hurricane in Louisiana (US) with enterococcosis [20], gonorrhea [21] and other sexually transmitted diseases (STDs), and after an earthquake in Japan with influenza [22] and tuberculosis [23]. Molecular biotechnologies are recommended by international organisms and health authorities during Public Health emergencies [24], and thus, the role of NAPPA is emerging.

A second concern with profound implication on biosafety is the environment, where nanotechnology did play a role since the onset with the implementation of effective gas sensors based on the fact that the conformation of the polymer backbone seems also to affect the molecular rearrangement occurring during the doping process, and in some cases, due to the sterical hindrance generated by "too close" substituents to the aromatic ring, is responsible of the spontaneous undoping process [6,7]. Sensors for the detection and monitoring of carbon dioxide (CO_2) [5], an important greenhouse gas, can be extremely helpful in revealing the quality of indoor air and the increased emissions of gases from fossil fuels and coal combustion, industrial processes (such as the manipulation of hydrocarbons), and agriculture associated to deforestation and habitat destruction. This can results in

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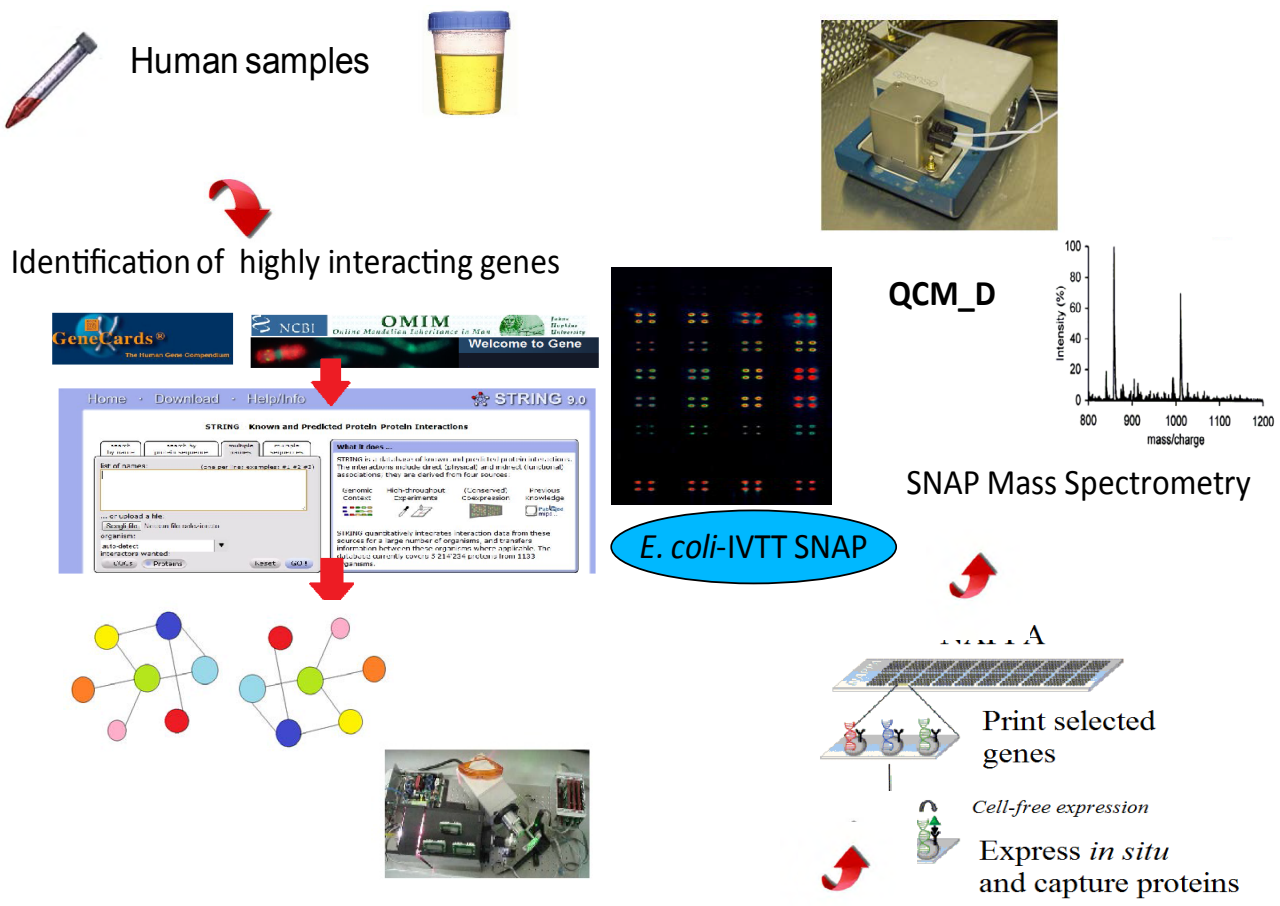

Figure 1: Unified Strategy to Nanosensors for Biosafety. From human samples to leading genes identification and to NAPPA array printing with SNAP Pure Express , characterized subsequently by fluorescence , Mass Spectrometry and QCM_D.

consequent changes in the atmospheric chemical composition with direct biological effects and negative impact on the Earth's climate, such as the global warming issue, considering that fossil fuel CO_2 emissions can remain high in the atmosphere for long periods (also up to tens of thousands of years).

Moreover, CO_2 has been involved in sea and ocean acidification and other environmental hazards. The instruments generally employed in these determinations basically consist of infrared (IR) detectors performing a continuous monitoring and plotting in the site with a good degree of accuracy, but unfortunately, they cannot be used for extensive mapping work because of the large number of expensive devices and specialized people that would have to be involved. Long-term sampling devices, such as diffusive sampling techniques are, therefore, the cheapest and easiest way. Environment plays also a key role in shaping human diseases and for this reason, ecological factors cannot be neglected. Climate change, wild urbanization together with deforestation and globalization, are having indeed a tremendous impact on human health.

Natural disasters, such as hurricanes and earthquakes are environmental drivers of diseases, since they favor the dispersal and dissemination of vectors and pathogenic microbes. Environmental drivers are considered both precursors and determinants of diseases, such as air pollution as a driver of influenza [25], heat waves as a driver of West Nile fever, Dengue, Chikungunya and other tropical diseases [26], tsunami as a driver for Mycobacteriosis [27], and El Nino Southern Oscillation (ENSO), the Indian Ocean Dipole (IOD) as determinants of cholera epidemics [28]. Even though the increase of natural hazards is only apparent and is considered controversial by some scholars [29], its consequences, in term of damages and crop and resources losses are extremely relevant.

Our third goal was finally to build the prototype of an enzyme-based biosensor for biomedical and environmental purposes, in which the immobilization procedure was carried out *via* Langmuir-Blodgett (LB) films. The enzyme implemented in our device [30] was laccase [31], which is a blue oxidase capable of oxidizing phenols and aromatic amines and their derivatives by reducing molecular oxygen to water by means of a cluster of copper atoms. Laccase belong to a large group of the multicopper enzymes, which catalyze the oxidation of highly diverse and heterogeneous compounds.

Materials and Methods

Taking into account all the considerations so far discussed and the sensors recently introduced [32], here in this section, we summarize the techniques and procedures utilized in the construction of the above-mentioned distinct matrices.

NAPPA matrices

In order to prove the feasibility of the NAPPA arrays [12-16], we have used a matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF MS) and a model protein system, where the known query proteins immobilized in a blind order were successfully identified (Figures 2 and 3) [18]. Thus, we have recently shown that is possible to utilize label free techniques coupled with MS for the detection of NAPPA slides containing Jun, p53, Cdk2 and CdkN1A genes spotted, immobilized and later expressed [17], as shown in Figures 2 and 3. The scheme of the experimental set up for the MS is shown in Figure 2 below and in reference [13]. In alternative to fluorescent staining and labeling approaches, the new label free NAPPA method emerging from the combined utilization of independent and complementary nanotechnological approaches finds applications in analyzing protein function and protein-protein interaction both in basic and applied studies ideal for nanomedicine [2,18], namely QCM_D and QCM_F-based nanogravimetry [17,18], MS [13], atomic force microscopy (AFM) [14] and anodic porous alumina (APA) [14]. QCM_D, measured in a temperature chamber where the quartz was positioned and was monitored at the same time for frequency and dissipation factor variations, is discussed later in the "Results" section; it basically detects the normalized dissipation factor of the quartz crystal by means of the "half-width half-height" principle of its impedance curve. In our case, the quartz was connected to an RF gain-phase detector (Analog Devices, Inc., Norwood, MA, USA), and was driven by a precision Direct Digital Synthesizer (DDS) (Analog Devices, Inc., Norwood, MA, USA) around its resonance frequency, thus acquiring conductance vs. frequency curve, which showed a typical Gaussian behavior.

For what concerns the health prototypes, one biosensor of new conception was realized coupling the traditional QCM_F and QCM_D, using an innovative protein cell-free expression system named NAPPA that allowed immobilizing on the quartz surface as sensing molecule any kind of proteins. Standard nanogravimetry exploited the piezoelectric quartz crystals properties to vary the resonance frequency, f, when a mass, Δm, was adsorbed to or desorbed from their surface according to Sauerbrey equation [17]. Quartz resonators used in fluids are more than mere mass or thickness sensors: sensor response depends also on viscoelastic properties of the adhered biomaterial, surface charges of adsorbed molecules and surface roughness. The modern QCM_D technology utilizing impedance measurement offers access to the resonance bandwidth, in addition to resonance frequency, which is strictly connected with the viscoelastic properties of the sample. Building upon the successful use of in vitro NAPPA translated protein (IVTT), Ramachandran et al. [15,16] substituted the use of purified proteins with the use of cDNAs encoding the target proteins at each feature of the microarray. The proteins were translated using a T7-coupled rabbit Reticulocyte Lysate IVTT system. Mammalian proteins can be expressed in a mammalian milieu, providing access to vast collections of cloned cDNAs. The addition of a C-terminal glutathione S-transferase (GST) tag to each protein enabled its capture to the array through an antibody to GST printed simultaneously with the expression plasmid. We manage to couple QCM_D and QCM_F with NAPPA technology, optimizing the monitoring in real time of the kinetics of the reaction to obtain information not only on the mass, but also on the viscosity of the sample and sensing the interaction among a query protein and the expressed protein.

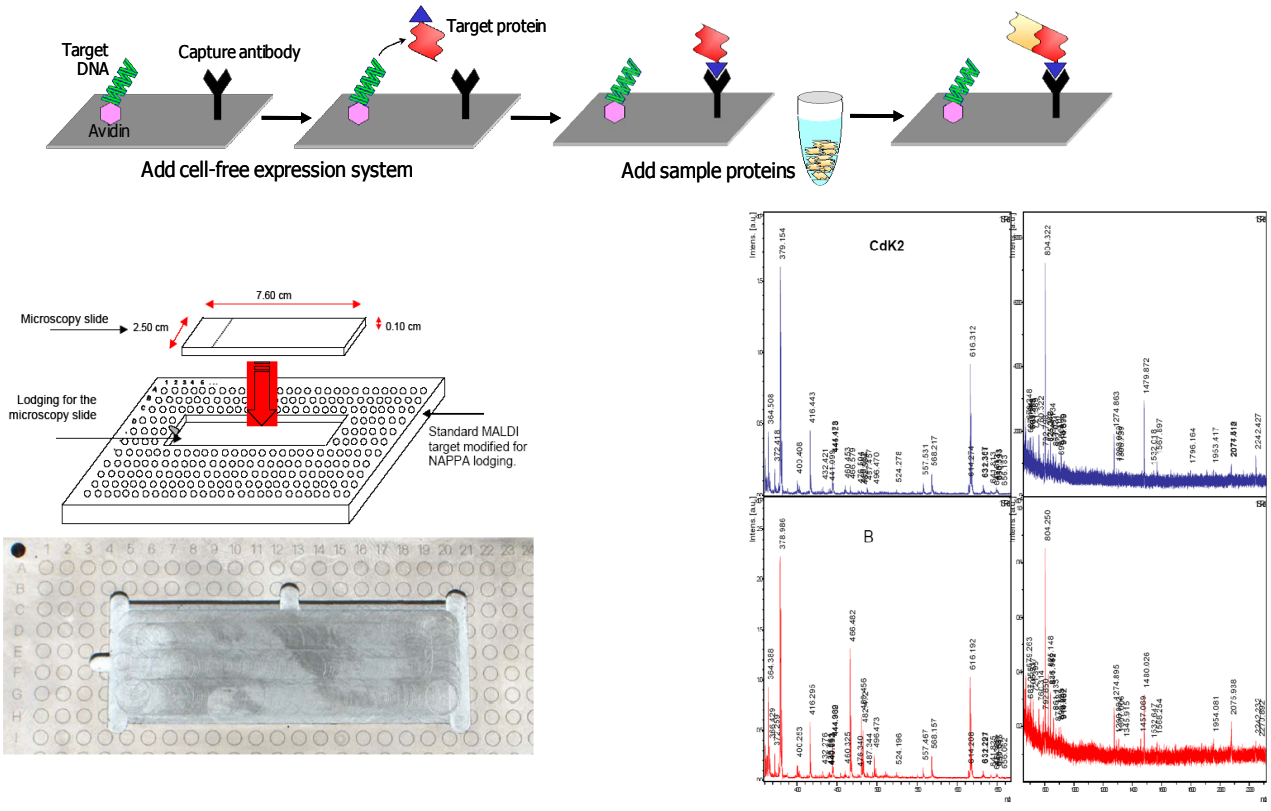

Figure 2: NAPPA technology (above) and MS MALDI standard target adapted to carry NAPPA (below,left) was utilized (below,right) to carry MALDI TOF Spectra of NAPPA after protein triptych digestion, 5–20 kDa range, for optimal identification of single unknown genes.

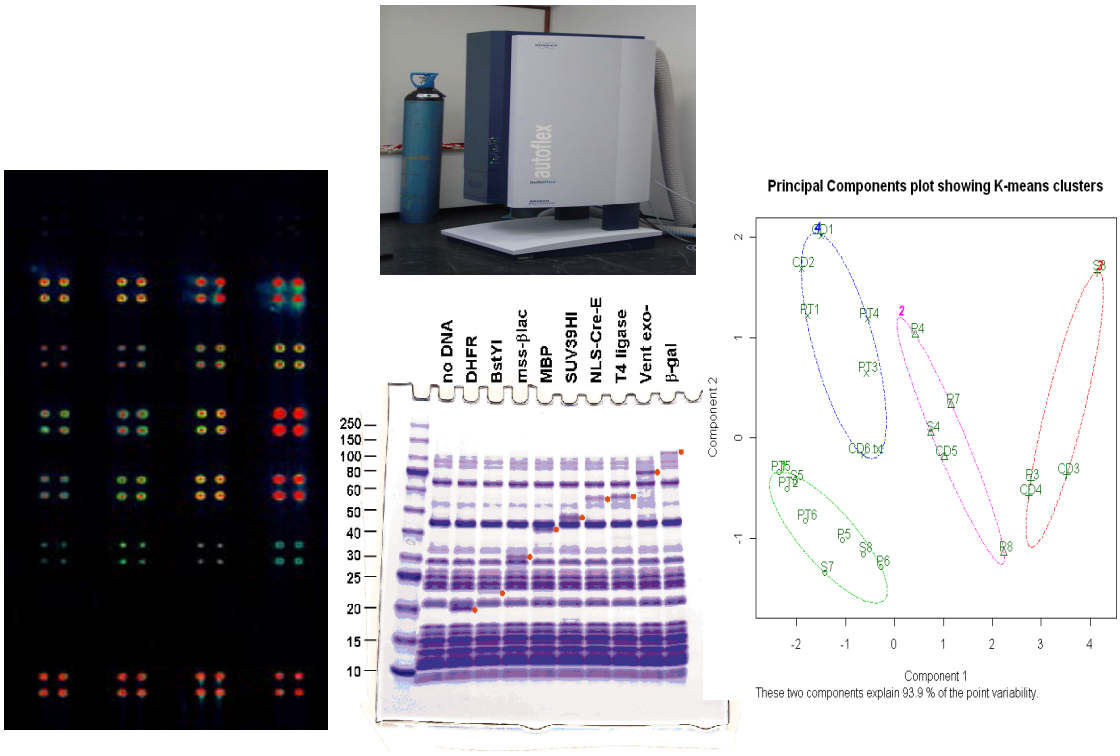

Figure 3: Amplification of the NAPPA signal with SNAP for MS identification of multiple protein-protein interaction. (left) Fluorescence analysis of SNAP Arrays; (middle, below) *In vitro* translation of a diverse set of target proteins using PURExpress system, improving by 20 fold the yield; (middle,above) MS Autoflex Bruker; (right) SpADS and Clustering solution for a specimen of 23 protein samples of raw data. Binning preprocessing function was performed for optimizing conclusive cluster analysis run on the ROI 1000/1200 spectra obtained by Autoflex Bruker MS (43).

NAPPA-SNAP matrices

As a follow-up in cooperation with the New England Biolabs (NEB) and the Biodesign Institute at Arizona State University (ASU), we have used an innovative cell-free expression NEB SNAP system based on bacteria recently proposed [33], in which all protein species and concentrations are well defined. The combination of NAPPA and SNAP [19] is emerging as a promising tool, since utilizes a complex mammalian cell free expression system to produce proteins in situ (33), shown in Figure 3. On the whole, considering the abundance of protein material different from target and query protein, the results are very encouraging, showing the potential to couple the NAPPA and SNAP technologies with the MALDI-TOF MS for a label free investigation of protein samples, in combination with sophisticated bioinformatics tools and software [19]. The chemistry and the algorithms progressively implemented prove for the first time that MS can characterize proteins immobilized on NAPPA, pointing that with further developments, this label-free procedure will prove to be fully effective when compared to the fluorescence NAPPA (Figure 3, left), that has already seen significant clinical applications in the last decades [15,16]. To verify the proper protein expression and capture on SNAP-NAPPA, a preliminary test has been carried out using fluorescence analysis. The same SNAP-NAPPA samples employed for MS analysis (namely p53, CDK2, SH2-Src and SH2-PTPN11) were spotted on microscope glass in a 2×2 spots per box configuration using increasing SNAP concentrations. As negative control on the gold slides was printed a box only with master mix (Figure 3, left), while the positive controls mouse IgG and rabbit IgG were added in a printing mix, instead of DNA.

Proteins were synthesized by two different IVTT systems, a new system extracted from human cells (1-Step Human Coupled IVTT, HCIVTT, Thermo Scientific) and *E. coli* IVTT. It is known that HCIVTT performs better than RRLIVTT. The yield of protein synthesized in HCIVTT is more than 10 times higher than RRL. Moreover, HCIVTT showed a robust lot-to-lot reproducibility. In immune assays, the signals of many antigens were detected only in HCIVTT-expressed arrays, mainly due to the reduction in the background signal and the increased levels of protein on the array [19]. The protein yields obtained through the process "Protein synthesis Using Recombinant Elements" (PURE) system. PURE system [33] has then been matched with that obtained with this innovative cell free IVTT system. In Figure 3 are reported the images of three SNAP-NAPPA slides after proteins expression fluorescence acquired. Two slides were expressed with HCIVTT and a third with *E. coli* IVTT; the level of protein displayed on the array was measured using respectively anti-SNAP antibody or anti-p53 antibody followed by a Cy3-labeled secondary antibody. The results obtained not only confirmed the proper protein expression and capture on the array surface, but, moreover, demonstrated that *E. coli* IVTT systems ensured a protein yield form 2 to 8 times higher respect to HCIVTT, considering the higher SNAP concentration. The gain respect RRL is, therefore, more than twenty times. For the first time an improved version of NAPPA, that allows for functional proteins to be synthesized *in situ*-with a SNAP tag-directly from printed cDNAs just in time for assay, has been expressed with a novel cell-free transcription/translation system reconstituted from the purified components necessary for *Escherichia coli* translation-the PURE system–and analyzed both in fluorescence and in a label free manner by four different mass spectrometry techniques, namely three MALDI-TOF, a Voyager, a Bruker Autoflex and a Bruker Ultraflex, and a liquid

chromatography-electrospray ionization MS (LC-ESI-MS/MS). Due to the high complexity of the system, an ad hoc bioinformatic tool has been needed and has been developed for their successful analysis. The contemporary fluorescence analysis of NAPPA, expressed by means of PURE system, has been performed to confirm the improved characterization of this new NAPPA-SNAP system [19].

Calcium oxide (CaO)

An innovative long-term sampling method for the determination of environmental CO_2 accumulation takes advantages of the properties of CaO to be carbonated by this gas, according to the following equation:

$$CaO+CO_2 \leftrightarrow CaCO_3 \quad \text{(Equation 1)}$$

For this purpose, we studied here the variation of mass connected to the carbonation process in order to assess the quantity of gas absorbed by a fixed and known amount of composite in relation to the concentration of environmental CO_2 [5]. This long-term sampling method for the determination of environmental CO_2 accumulation takes advantages of the properties of CaO to be carbonated by this gas. We carried out preliminary tests to assess the best concentration of CaO in the composite, individuated in the CaO/PEG weight ratio of ¼, and we tested the sensing properties of the composite materials *via* a nanogravimetric method by using a home-made glass chamber of 340 ml in volume. The home-made chamber (illustrated in Figure 4) was provided with four input sockets, able to arrange up to four quartzes at the same time, besides inlet and outlet valves to feed and empty the gas. As transducers, AT-cut quartz crystals were used with a native frequency equal to 9.5 MHz, a blank diameter equal to 0.550", an etched surface, an electrode diameter equal to 0.295" with 100 Å Cr and 1000 Å Au as electrode materials (International Crystal Manufacturing Co, Inc., Oklahoma City, USA). The preliminary experimental data highlighted that the composite was able to detect selectively CO_2 *via* a nanogravimetric method by performing the experiments inside an atmosphere-controlled chamber filled with CO_2. Furthermore, the composite material showed a linear absorption of CO_2 as a function of the gas concentration inside the atmosphere-controlled chamber, thus paving the way for the possible use of these matrices for applications in the field of sensor devices for long-term evaluation of accumulated environmental CO_2.

The previous reported considerations allowed us to design and realize a dosimeter for the long-term analysis of the carbon dioxide. We used the same transducers, but they were inserted in a home-made and ad hoc designed and built Plexiglas measuring chamber, divided into two parts: the upper one, with a funnel opening, allowed the exchange of the sensing matrix with the environment and the lower one that allowed the housing of the transducer.

The nanogravimetric instrument used for relating CO_2 concentrations with the mass variations consisted of a base unit, interfaced to a PC *via* USB port and able to drive up to four oscillator units. The base unit embedded the interface circuitry to/from the USB port, a digital signal controller and a fast programmable logic device containing an accurate four-channel counter, plus the four interfaces to the oscillator units. The counter logic was fully parallel, this meaning that the four input signals were acquired and counted-up simultaneously at a gate interval selectable from fractions of a second to 10 seconds. The base unit was powered by means of an external pluggable +12 V power supply, which sustained input AC voltages from 90 to 240 VAC.

The choice to have the oscillators outside the base unit allows the

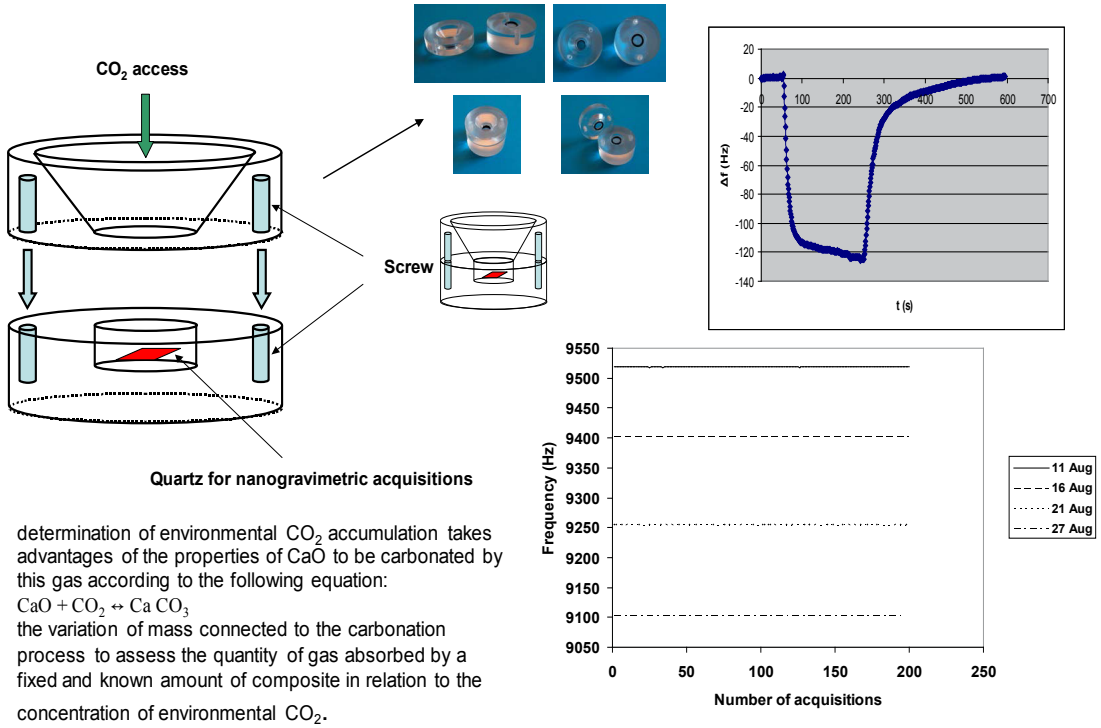

Figure 4: The long-term based on **Calcium Oxide** (left) versus short-termbased on Nanocomposites (right above) determination of environmental CO_2 accumulation. Variation of frequency in relation to the quantity of CO_2 absorbed by the composite. Experimental data showed a linear absorption, coherently with the human activity of the sampling period (right,below).

maximum flexibility when building an experimental set-up. The noise immunity was preserved and guaranteed by the oscillator design, which was based on a precision internal reference crystal, used as a timebase comparator for the working quartz crystal. Only the mixed, lower frequency signal was then transmitted to the base unit by dedicated twisted pair lines on the cable. The oscillator units were connected to the base unit by means of standard Ethernet class V cables.

The system was driven by an extremely user-friendly software running under MS-WindowsTM. It consisted of a lower level kernel, which implemented the necessary data transfer between the computer and the base unit, and of a higher level set of routines, which drove the user through an easy-to-perform data acquisition and analysis. In addition to the data acquisition service routine, a useful data display was implemented by which the user was easily able to retrieve the acquired signal values. Oscillating frequency versus time measurements can be performed following the data acquisition in real time on the computer screen, by means of strip chart plotting.

SWNTs and MWNTs embedded in POTO

Synthesis carried out by polymerizing the monomer in the presence of a dispersion of CNTs is very simple and can be effectively used for short-term monitoring of gas [6,7]. Polyaniline derivatives have been deeply studied among conducting polymers in the last decades for their good electrical properties, easy methods of synthesis and high environmental stability.

The chemistry of polyanilines is generally more complex with respect to other conducting polymers, due to their dependence on both the pH value and the oxidation states, described by three different forms known as leucoemeraldine base (fully reduced form), emeraldine base (EB) (50% oxidized form) and pernigraniline base (fully oxidized form). The most important is the EB form and its protonation by means of H+ ions generated from protic acids, issues the emeraldine salt form, responsible of the strong increment of the conducting properties [6,7]. This process is reversible and it is possible for the presence of imine group basic sites located along the conducting polymer backbone. Nanogravimetric analysis demonstrates that these materials are suitable for applications as sensors for carbon dioxide [34,35]. From the experimental results, the highest peak of oscillation is obtained in the presence of O-methylaniline. The standardized synthesis of nanocomposite materials was carried out by oxidative polymerization under controlled conditions, maintaining the temperature at 0-4°C by means of an ice bath for 24 h. The medium of reaction was 200 ml of 1 M HCl solution of the monomers. For the synthesis of the nanocomposites, we dispersed 100 mg CNTs in the medium of reaction by sonication. We performed the sonication, by means of SONIC 300 VT equipment, setting a 10% power for 1 min in order to only disperse CNTs without braking processes. We used a monomer/MWNTs weight ratio of 100/1 and a monomer/oxidant molar ratio of 4/1. In order to obtain the solvent processable undoped form, we filtered and subsequently treated the crude materials in the doped form (emeraldine salt form) with ammonium hydroxide for 2 h. We finally filtered the undoped materials (EB form) and performed a treatment with methanol and diethyl ether in order to eliminate the oligomers, followed by the evaporation of the residue solvents by vacuum. The final products in the EB forms were completely soluble in chloroform [36]. We fabricated the Langmuir monolayers in a LB trough (MDTcorp., Russia), 240 mm×100 mm in size and 300 mL in

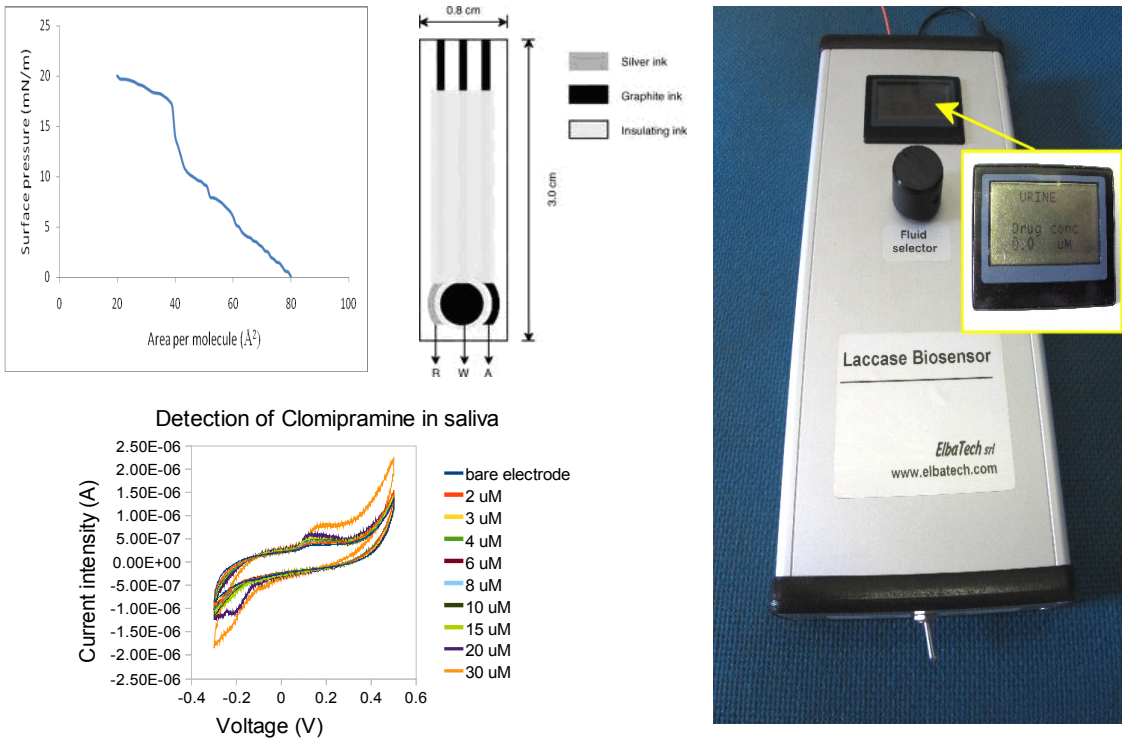

Figure 5: (above,left) Langmuir π-A isotherm of the recombinant laccase thin film (36,39)and screen-printed electrodes based on graphite / ruthenium ink and on silver one;(below,left) Cyclic Voltammetry of Laccase-based biosensor for detection of Clomipramine at increasing concentrations in saliva;(right) Prototype of Laccase Sensor ,compact and portable for all fluids indicated in Nicolini et al, Sensors 2012. The shown Cyclic Voltammetry is routinely obtained to water contaminants as phenoles with our device and by others (40,41).

volume, having a compression speed of 1.67 mm/s (100 cm^2/min). The spreading solutions of nanocomposites were prepared by dissolving 5 mg of materials in 20 ml of chloroform. The Langmuir-Schaefer (LS) films were deposited at air-water interface, maintaining the surface pressure at 25 mN/m. We used distilled water as sub-phase for the deposition in order to obtain films of the materials in the undoped form.

LB multi-layers of laccase

LB thin biofilm of recombinant laccase from *Rigidoporus lignosus* (formerly known as *Rigidoporus microporus*) and the corresponding sensor were obtained as reported [30], using highly concentrated sample of laccase enzyme.

The mixed chloroform solution in equimolar proportions had a concentration of 1 mg/ml. A volume of 50 µl of the mixture was spread on a Milli-Q water sub-phase and the monolayer was compressed with movable barriers at a rate of 70 mm/minute. The transfer pressure to obtain the LB film (Figure 5, above and left) was about 20 mN/m, at 22°C. The surface morphology and topology of the LB thin film of laccase was investigated *via* AFM. The roughness of the film was found to be 8.22 nm and the compressibility coefficient about 37.5 m/N, as determined from the LB π-A isotherm at the air-water interface. The enzyme was deposited onto the electrode *via* LS technique and a protocol of immobilization overnight was followed; after depositing the film, the electrodes were kept at 4°C up to a maximum of 16 hours. The employed electrodes were ruthenium and graphite ones, while the counter-electrode was of silver. The area of the electrode (Figure 5, above and center) was about 0.75 mm per 1 mm. The amperometric technique was used to polarize the electrochemical couple, and to obtain a current discharge related to the amount of the investigated drug, namely clomipramine, with an EG and G PARC model 263A potentiometer, equipped with dedicated in-house software [30]. This tricylic tertiary amine antidepressant is widely used for the therapy of depressive and obsessive compulsive disorders (OCD). Because of its clinical importance, many analytical methods have been developed to monitor its level: above all chromatographic techniques (like gas chromatography or GC, high performance liquid chromatography or HPLC), eventually coupled with tandem MS, but all these techniques are generally expensive, time-consuming and laborious. Moreover, the AGNP-TDM panel of experts has emphasized the importance of therapeutic drug monitoring. In our experiment, clomipramine was added at varying concentrations in the low micromolar range. The therapeutic dose is from 75 mg/day to 200 mg/day; the pharmacokinetics is extremely variable among the patients. Generally, the therapeutic concentration in the human blood of psychiatric patients is usually in the low micromolar range. The side-effects of the drug, especially in case of overdose, are seizures, hematological, cardiological and neurological adverse effects up to the coma (the so-called tricyclic antidepressant syndrome).

Results and Discussion

The growing concern for biosafety is here approached in three distinct different biosafety applications with sound nanotechnological methods, which have proven encouraging and promising, with profound implications in health and environment.

Vaccine design and biosafety

A miniature flow-cell (Figure 6, above and left) was designed for

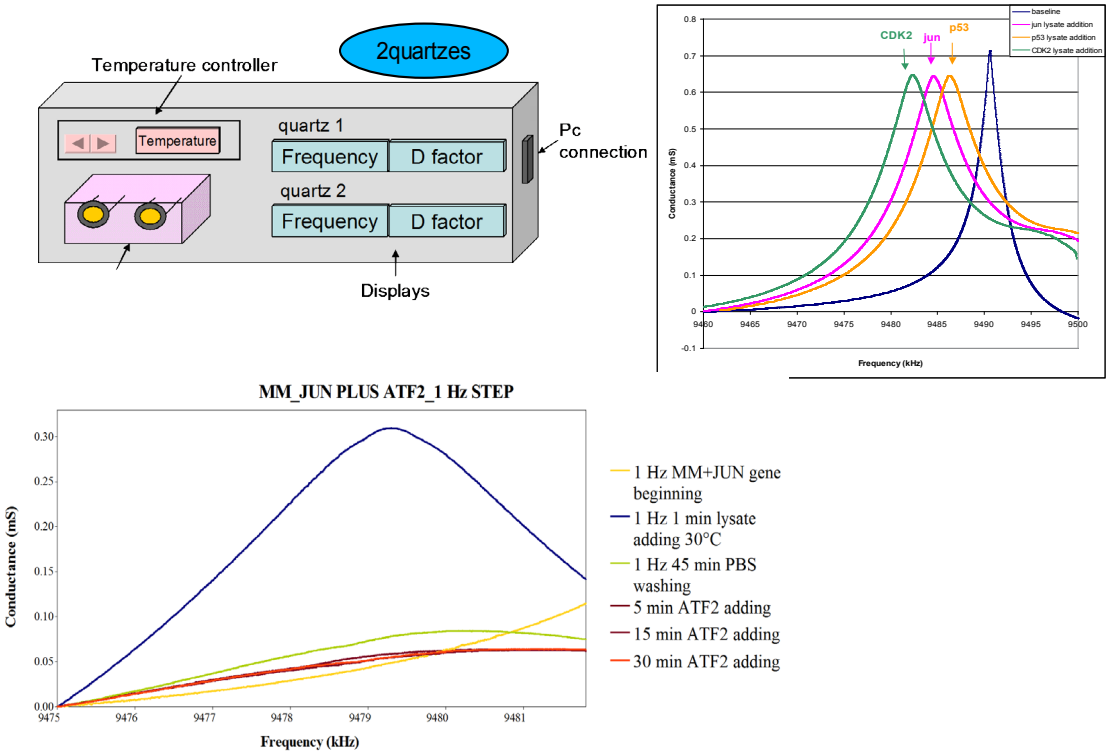

Figure 6: Flow-cell and static dual QMC_D (right) prototype built in house to follow each step of the protein expression versus time; (right,above) excellent discrimination of matching Conductance after lysate addition of Jun, CdK2 and p53 proves the identification of multiple genes in the same NAPPA spot (35), leding to its utilization for vaccine design presently in progress; (below) Conductance curves of JUN QCs (36) after the addition of ATF2 acquired with frequency acquisition steps of 1 Hz.

protein-protein interaction analysis *via* a conductometric QCM_D sensor [35]. The flow cell chamber volume was 100 µl and it was connected to a BioRad Econo Gradient Pump, able to pump solution in a flux range of 0.02-6 ml/min. The transducer consisted of 9.5 MHz, AT-cut quartz crystal of 14 mm blank diameter and 7.5 mm electrode diameter, and the electrode materials were 100 Å Cr and 1000 Å Au. Microarrays were produced on the quartzes previously described and connected to the nanogravimeter inside the incubator at 30°C for proteins synthesis, and then the temperature was decreased to 15°C to facilitate the proteins binding on the spot surface. After wash, the quartz was placed in the flux chamber for protein-protein interaction analysis [35], using genes of significant implications (both clinical and biological) and using 10×10 spots. In order to investigate the biosensor response to protein-protein interaction, we added a MDM2 solution to p53 quartz, once we had our proteins expressed. In Table 1 are reported for p53 quartz, before and after protein expression, the values of the frequency and the half width at half height (Γ) of the resulting QCM_D curve, along with the values of the variation in conductance YRE (mS). The ratio named normalized D factor, DN=2Γ/I, gives information on the shape of impedance curves and on sample viscosity, while the decrease in frequency f (Hertz) is related to the amount of p53 molecules being immobilized.

The resulting Michaelis-Menten constants of the p53-MDM2, as well as of other protein-protein interactions, appeared quite compatible with the literature [17].

The results obtained later using a human Lysate and 10×10 spots per quartz and with the most recent QCM_D configuration and set-up [35], suggest that the NAPPA based biosensor monitor with high selectivity the single protein being expressed even in a mixture of different genes (Figure 6, above and right), and from the analysis of the D-factor, allowed to acquire in real time information on the characteristics of each single protein being expressed with unique signature, and on the kinetics constant of the reactions. Then, in order to eliminate the background signal, we do routinely carry out the measurements starting from the crystal native frequency subtracted of 15 kHz and using a step of 1.0 Hz for collecting the whole impedance plots at the optimal resolution, and with a considerable number of data points. For this reason, we have used a dsPIC (Microchip Inc, USA), featuring at the same time good computational power and sufficient memory space. This represents a PC-driven prototype, in order to establish the proof of principle. The industrial prototype is being designed and realized under a different contract, in order to have a temperature-controlled ad hoc chamber, hardware and software optimized to increase speed, computational power and compactness to incorporate hardware, and to produce a friendly stand-alone device.

To establish some proofs of principle, we choose BRIP1, Jun and ATF2 [36]. JUN encodes the c-Jun protein, which is a well-known oncogene, being the putative transforming gene of avian sarcoma virus 17. It encodes indeed a protein with a strong similarity to the viral protein. It is encoded by the locus 1p32-p31 and its alteration leads to several malignancies. ATF2 gene (chromosome 2q32) encodes a 505 amino-acids long transcription factor, that belongs to the leucine zipper family of DNA-binding proteins, and is an important component of many signaling pathways whose alterations are a cause of malignant transformation (Figure 6, below).

Impedance curve	*f* (Hz)	*Γ (Hz)*	Y_{RE} *(mS)*	D_N *(Hz/mS)*
beginning	9542240	1205	0,698	3452
Lysate addition at 15C	9513830	8530	0.111	153694

Table 1: Peak frequency (*f*), half-width at half-height (*Γ*), maximum conductance increment (*YRE*), and normalized *D* factor for the impedance curves.

An interesting implication for potential clinical applications concerned the possibility to drastically reduce the time of protein expression [35,36], and capture under our experimental conditions (this is true, especially for BRIP1). By acquiring conductance curves each 5 minutes, we noticed that after the first 15 minutes after IVTT lysate addition at 30°C, the position and shape of the curves did not change anymore, likewise after few minutes at 15°C, for protein capture, position and shape of the curves did not change anymore. We deduced from these results that the protein expression took place in the first minutes and that also their capture needed only few minutes, and therefore, we performed experiments reducing the expression time from 90 minutes to 15 (at 30°C) and the capture time from 30 to 5 minutes (at 15°C). The results presented confirmed our hypothesis [35,36]. The conductance curves obtained showed that protein expression and capture and protein-protein interactions were successfully performed. Applying the calibration coefficients, we were able to estimate the amount of protein immobilized on the biosensor surface. To estimate the amount of molecules aspecifically captured on the QC surface after the NAPPA expression (human IVTT lysate molecules not specifically adsorbed on the QC surface), we employed a reference QC (MM QC), and we estimated an amount of 2 µg of molecules aspecifically adsorbed. For BRIP1 quartz, we obtained 2.28 µg, while for JUN quartz, the amount was 1.69 µg.

Future perspectives of this biosensor regard its clinical introduction for assessing cancer prognosis and make a personalized diagnosis, and/or to deliver an individualized treatment, and in the context of biosafety design, effective vaccines (Table 2). Only recently protein arrays were used to discover new antigenic determinants for vaccine development. NAPPA-based sensors could be used for screening the affinity between the identified proteins and the immunological synapse (CD4, TCR, MHC complex). Affinity kinetics can be evaluated also using classical techniques or *via* AFM and Surface Plasmon Resonance (SPR). In the right column of the Table 2 are shown the genes that interact with immunological human synapse (CD4+TCR+MHC).

Carbon dioxide monitoring for biosafety

We performed nanogravimetric acquisitions of frequency vs. time, in order to assess the variation of mass at regular intervals of time after placing the small plastic cell in a room. We also measured the mass of the deposited sample at time zero, in order to have the value of frequency related to the composite before reacting with environmental CO_2. We consequently carried out acquisitions of frequency vs. time in order to sample the indoor environmental CO_2. Basing on our previous experiments, we were able to calculate the variation of mass per week, and consequently, the average quantity of CO_2 in the environment. Experimental data, summarized in Table 1 for a clear vision, highlighted the variation of frequency in relation to the quantity of CO_2 absorbed by the composite issued a linear absorption, coherently with the constant human activity of the sampling period. Specifically, we found (Table 3) the variation of mass in relation to the quantity of CO_2 present in the environment (indoor), indicated the concentration of indoor CO_2 during the sampling period was 10 times less than the average concentration in the atmosphere, thus indicating good quality of the air in our laboratory. Since the very good results obtained from the usage of this long-term sampling device, we are presently programming new samplings both in indoor spaces affected by strong human activities and outdoor.

1. Microorganism	Antigen
Pseudomonas aeruginosa	PA0044, PA0807, PA0973 (OprL), PA1080, PA1148, PA1248, PA2300, PA3407, PA3724, PA3841 (ExoS), PA3931, PA4110, PA4922, PA5369, FlicA, OprI, OprH2, OprE, OprF, exotoxin A, flagellin
Vibrio cholera	Protein 1 (VC1085), 2 (VC2283), 3 (VC1893), 4 (VC2261), 5 (VC0339, PSD), 6 (VC1494), 7 (VC0556), 8 (VC0975)
Yersinia pestis	VA (V antigen), F1 antigen
Francisella tularensis	O-antigen
Bacillus anthracis	PA (protective antigen)

Table 2: Genes which interact with immunological human synapses.

Sampling period (days)	Variation of mass Dm (g)	Concentration of environmental CO_2 (ppm)
7	0.57 ± 0.007	48 ± 1
7	0.72 ± 0.007	55 ± 1
7	0.75 ± 0.007	55 ± 1

Table 3: Variation of mass in relation to the quantity of CO_2 present in the environment (indoor). The concentration of indoor CO_2 was found 10 times less than the average concentration in the atmosphere, thus indicating good quality of the air.

Fluid	Peak	Sensitivity
Blood	+75 mV; -150 mV	191.91 mA/M
Urine	+100 mV; -150 mV	138.93 mA/M
Liquor	+100 mV; -200 mV	31.7 mA/M
Breast-milk	+100 mV; -200 mV	9.8 mA/M
Saliva	+150 mV; -100 mV	11.14 mA/M
Sperm	+110 mV; -220 mV	19 mA/M

Table 4: Peak potentials and sensitivity of laccase-based biosensor measured at increasing concentrations in different human biological fluids.

Phenols detection for biosafety

Experiments carried out with cyclic voltammetry of our LB recombinant laccase [37] highlighted excellent reproducibility and linearity of the peaks of oxidation and reduction, related to the presence of the drug in several biological fluids Figure 5, below and Table 4).

These results allowed the design and the creation of a prototype of a biological sensor for antidepressants and to extend its utilization for biosafety in the phenols detection. The instrument consisted of a central unit, able to bias the working electrode on which the enzyme was deposited, and to detect the current generated as a result of the interaction of the enzyme with the analyte containing the substance of interest (Figure 5). By a multiple selector, the user can choose the fluid or substance to be analyzed, and through the proper calibration parameters on the display, the proper drug concentration will be provided. The instrument [30] was powered by two 9V batteries, in order to avoid noise from the main voltage. Using laccase, screen-printed electrodes technique and a portable device appear to be an emerging instrument suitable for investigation in biological, medical and environmental applications. The same enzyme [37] and the same proposed device [30] are presently being planned also in many different fields, such as in degradation of polyaromatic hydrocarbons, in textile industry, in food industry, in waste detoxification and finally for bioremediation and for biodegradation of industrial pullutants [38].

It must be stressed that laccase-based sensors can be used not only for medical purposes, but also for environmental and biosafety purposes, since they enable to detect contaminants, pollutants, such as phenols and pesticides [30,31].

Conclusion

In summary, our resulting prototypes appear to yield satisfactory proof of principles in the shown three specific applications to biosafety. Indeed, in order to measure CO_2, we have realized two new devices based on the use of a sensor technology (such as the nanogravimetric one), and an array of capture, highly specific for the gas of interest. CaO may be considered a valid solution to solve the problem of the long-term detection of carbon dioxide due to its ability to selectively absorb this gas through a chemical reaction of carbonation. The reaction of carbonation leads to a substantial variation of the molecular weight, and was, therefore, taken into account in the manufacture of nanogravimetric detection devices. The characteristics of the reaction allowed the construction of a dosimeter for the long-term analysis of the carbon dioxide, competitive with respect to the devices already available on the market based on infrared measurements (CO_2 reduces the incidence of infrared radiation on the sensor, then, depending on the concentration of CO_2), or on measurements of the variations of a voltage across a solid electrolyte depending on the concentration of CO_2.

For what concerns the NAPPA QCM_D conductance device, the results presented demonstrated a valid response for the protein-protein interaction analysis, exploiting the great advantage of this technique that allowed the real-time, label-free characterization of molecular binding kinetics to an immobilized receptor [36]. The most challenging prospective of the innovative biosensors emerging from this technology is the potential capability to develop a large number of sensors for molecules of biological and medical interest, simply changing the cDNA immobilized on the sensor, without changing the technology of detection. Among avenues being presently explored NAPPA-based vaccines identification appears to represent additional promising future perspectives for biosafety in the frame of the new Omics-based Public Health. Vaccinology has indeed emerged as a complex interdisciplinary science, especially because of the contributions of the new-Omics disciplines. Worthy of notice for future applications in human biosafety is finally the innovative kind of self-assembling protein microarray; the NAPPA expressed with the SNAP tag in E. coli coupled cell-free expression system, useful to develop a standardized efficient procedure to analyze in large scale the protein occurred on the array combining MS with bioinformatics [19]. By the coupling of our newly developed software SpADS [39] to K-Means Clustering algorithm with good results, both for known and unknown protein identification, up to 67% correct score, quite better than earlier MS

without SNAP [13]. The results obtained are encouraging, even with the quite low number of MS spectra so far acquired and without the subtraction of *ab initio* known MS spectra of recombinant components of the E. coli lysate (work in process).

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References

1. Biosafety and the Environment. An introduction to the Cartagena Protocol on Biosafety. The Secretariat of the Convention on Biological Diversity, Canada.
2. Nicolini C, Pechkova E (2010) Nanoproteomics for nanomedicine. Nanomedicine (Lond) 5: 677-682.
3. Nicolini C (2006) Nanogenomics for medicine. Nanomedicine (Lond) 1: 147-152.
4. Nicolini C, Singh M, Spera R, Felli L (2013) Analysis of gene expression on anodic porous alumina microarrays. Bioengineered 4: 332-337.
5. Terencio TB, Bavastrello V, Nicolini C (2012) Calcium oxide matrices and carbon dioxide sensors. Sensors (Basel) 12: 5896-5905.
6. Bavastrello V, Stura E, Carrara S, Erokhin V, Nicolini C (2004) Poly(2,5-dimethylaniline-MWNTs nanocomposite: a new material for conductometric acid vapors sensor. Sensors and Actuators B: Chemical 98: 247-253.
7. Bavastrello V, Bezerra Correia TT, Nicolini C (2011) Synthesis and characterization of polyaniline derivatives and related carbon nanotube nanocomposites-Study of optical properties and band gap calculation, Polymers 52: 46-54.
8. RacapÃ© M, Bragazzi N, Sivozhelezov V, Danger R, Pechkova E, et al. (2012) SMILE silencing and PMA activation gene networks in HeLa cells: Comparison with kidney transplantation gene networks. J Cell Biochem 113: 1820-1832.
9. Bragazzi NL, Nicolini C (2013) A leader genes approach-based tool for molecular genomics: From gene-ranking to gene-network systems biology and biotargets predictions. J Comput Sci Syst Biol 6: 165-176.
10. Bragazzi NL, Sivozhelezov V, Nicolini C (2011) Leader gene: A fast data-mining tool for molecular genomics. J Proteomics Bioinform 4: 83.
11. Nicolini C, Malvezzi AM, Tomaselli A, Sposito D, Tropiano G, et al. (2002) DNASER I: Layout and data analysis. IEEE Trans Nanobioscience 1: 67-72.
12. Nicolini C, Pechkova E (2010) An overview of nanotechnology-based functional proteomics for cancer and cell cycle progression. Anticancer Res 30: 2073-2080.
13. Spera R, Labaer J, Nicolini C (2011) MALDI-TOF characterization of NAPPA-generated proteins. J Mass Spectrom 46: 960-965.
14. Nicolini C, Correia TB, Stura E, Larosa C, Spera R, et al. (2013) Atomic force microscopy and anodic porous allumina of nucleic acid programmable protein arrays. Recent Pat Biotechnol 7: 112-121.
15. Ramachandran N, Hainsworth E, Demirkan G, LaBaer J (2006) On-chip protein synthesis for making microarrays. Methods Mol Biol 328: 1-14.
16. Ramachandran N, Raphael JV, Hainsworth E, Demirkan G, Fuentes MG, et al. (2008) Next-generation high-density self-assembling functional protein arrays. Nat Methods 5: 535-538.
17. Spera R, Bezerra Correia TT, Nicolini C (2013) NAPPA Based Nanogravimetric Biosensor: Preliminary Characterization. Sens Actuators B Chem 182: 682-688.
18. Nicolini C, Bragazzi N, Pechkova E (2012) Nanoproteomics enabling personalized nanomedicine. Adv Drug Deliv Rev 64: 1522-1531.
19. Nicolini C, Spera R, Festa F, Belmonte L, Chong S, et al. (2013) Mass Spectrometry and fluorescence analysis of SNAP-NAPPA arrays expressed using *E. coli* cell-free expression system.
20. Bae HS, Hou A (2013) 23S rRNA gene-based *Enterococci* community signatures in Lake Pontchartrain, Louisiana, USA, following urban runoff inputs after Hurricane Katrina. Microb Ecol 65: 289-301.
21. Nsuami MJ, Taylor SN, Smith BS, Martin DH (2009) Increases in gonorrhea among high school students following hurricane Katrina. Sex Transm Infect 85: 194-198.
22. Tohma K, Suzuki A, Otani K, Okamoto M, Nukiwa N, et al. (2012) Monitoring of influenza viruses in the aftermath of the Great East Japan earthquake. Jpn J Infect Dis 65: 542-544.
23. Kanamori H, Uchiyama B, Hirakata Y, Chiba T, Okuda M, et al. (2013) Lessons learned from a tuberculosis contact investigation associated with a disaster volunteer after the 2011 Great East Japan Earthquake. Am J Respir Crit Care Med 187: 1278-1279.
24. Berthet N, Leclercq I, Dublineau A, Shigematsu S, Burguière AM, et al. (2010) High-density resequencing DNA microarrays in public health emergencies. Nat Biotechnol 28: 25-27.
25. Xu Z, Hu W, Williams G, Clements AC, Kan H, et al. (2013) Air pollution, temperature and pediatric influenza in Brisbane, Australia. Environ Int 59: 384-388.
26. Semenza JC, Sudre B, Oni T, Suk JE, Giesecke J (2013) Linking environmental drivers to infectious diseases: the European environment and epidemiology network. PLoS Negl Trop Dis 7: e2323.
27. Appelgren P, Farnebo F, Dotevall L, Studahl M, Jönsson B, et al. (2008) Late-onset posttraumatic skin and soft-tissue infections caused by rapid-growing *mycobacteria* in tsunami survivors. Clin Infect Dis 47: e11-16.
28. Hashizume M, Chaves LF, Faruque AS, Yunus M, Streatfield K, et al. (2013) A differential effect of Indian ocean dipole and El Niño on cholera dynamics in Bangladesh. PLoS One 8: e60001.
29. Cutter SL, Emrich C (2005) Are natural hazards and disaster losses in the U.S. increasing? Eos Trans 86: 381-396.
30. Bragazzi NL, Pechkova E, Scudieri D, Terencio TB, Adami M, et al. (2012) Recombinant laccase: II. Medical biosensor. Crit Rev Eukaryot Gene Expr 22: 197-203.
31. CaÃ±as AI, Camarero S (2010) Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. Biotechnol Adv 28: 694-705.
32. Nicolini C, Adami M, Sartore M, Bragazzi NL, Bavastrello V, et al. (2012) Prototypes of newly conceived inorganic and biological sensors for health and environmental applications. Sensors (Basel) 12: 17112-17127.
33. Nicolini C, LaBaer J (2010) Functional proteomics and nanotechnology-based microarrays. Pan Stanford Series on Nanobiotechnology. Volume 2, Pan Stanford Publishing Private Limited, Singapore.
34. Bavastrello V, Bezzerra T, Nicolini C (2013) Conductive Polymers and Gas Sensors. Nanobiotechnology in energy, environment and electronics: Methods and applications. Pan Stanford Series on Nanobiotechnology Volume 4, London, UK.
35. Spera R, Festa F, Bragazzi NL, Pechkova E, Labaer J, et al. (2013) Conductometric monitoring of protein-protein interactions. J Proteome Res.
36. Bragazzi N, Spera R, Pechkova E, Nicolini C (2013) NAPPA-based nanobiosensors for the detection of proteins relevant to cancer. Chemosensors.
37. Nicolini C, Bruzzese D, Cambria MT, Bragazzi NL, Pechkova E (2013) Recombinant laccase: I. Enzyme cloning and characterization. J Cell Biochem 114: 599-605.
38. Asgher M, Bhatti HN, Ashraf M, Legge RL (2008) Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. Biodegradation 19: 771-783.
39. Belmonte L, Spera R, Nicolini C (2013) SpADS: An R script for mass spectrometry data preprocessing before data mining. J Comput Sci Syst Biol 6.