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BOOK OF ABSTRACTS

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INDICE

PROGRAMMA SCIENTIFICO

9:00 - 10:00	Registrazione
10:00 - 10:30	Apertura e saluti
	Prima sessione
10:30 - 11:10	DYE-DOPED SILICA NANOPARTICLES AS LUMINESCENT ORGANIZED SYSTEMS FOR NANOMEDICINE Luca Prodi, Dipartimento di Chimica "Giacomo Ciamician", Alma Mater Studiorum - Università di Bologna
11:10 - 11:50	ADVANCED DEVICES BASED ON NANOBIOMATERIALS Roberto Zamboni, Istituto per la Sintesi Organica e la Fotoreattività (ISOF), Consiglio Nazionale delle Ricerche
11:50 - 12:05	PROTEOMIC AND POST-TRANSLATIONAL PHOSPHORILATIONS STUDY OF WILD SILK COCOON *Cricula Trifenestrata* AND *Bombyx Mori* Francesca Ferraris, Dipartimento di Chimica, Università degli Studi di Roma "La Sapienza", Roma
12:05 - 12:20	DETECTION OF BINDING SITES ON THE SURFACE OF PROTEINS ADSORBING TRANSIENTLY ONTO NANOPARTICLES Michael Assfalg, Dipartimento di Biotecnologie, Laboratorio di NMR biomolecolare, Università di Verona, Verona
12:20 - 12:35	DEVELOPMENT OF NEW COMPOSITE MAGNETIC PHASES FOR PHOSPHOPEPTIDES ISOLATION IN SHOTGUN PHOSPHOPROTEOMICS Susy Piovesana, Dipartimento di Chimica, Università di Roma "La Sapienza", Roma
12:35 - 12:50	NEW ANALYTICAL SOLUTIONS FOR PHARMACEUTICAL NANOTECHNOLOGY Barbara Roda, Dipartimento di Chimica "Giacomo Ciamician", Università di Bologna, Bologna; byFlow srl, Bologna
12:50 - 13:50	***Lunch + sessione poster***
	Seconda sessione
13:50 - 14:05	A MODULAR CLAMP-LIKE MECHANISM TO REGULATE THE ACTIVITY OF NUCLEIC-ACID TARGET-RESPONSIVE NANOSWITCHES WITH EXTERNAL ACTIVATORS Andrea Idili, Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma Tor Vergata, Roma
14:05 - 14:20	POLYMETHYLMETHACRYLATE NANOPARTICLES FOR MOLECULAR BEACONS INTERNALIZATION IN CANCER CELLS Sara Tombelli, Istituto di Fisica Applicata "Nello Carrara", Consiglio Nazionale delle Ricerche, Sesto Fiorentino (FI)

14:20 - 14:35 NOVEL DETECTION STRATEGIES FOR PROTEIN TAU
Samuele Lisi, Dipartimento di Chimica “Ugo Schiff” ,Università di Firenze, Sesto F.no (FI); Département de Pharmacochimie Moléculaire, Université Grenoble Alpes, Grenoble (France)

14:35 - 14:50 UNEXPECTED BEHAVIOR OF FLUORESCENT PROBES DURING SPR EXPERIMENTS
Alberto Fringuello Mingo, Centro Ricerche Bracco, Bracco Imaging, Colleretto Giacosa (TO); Dipartimento di Fisica, Università degli Studi di Torino, Torino

14:50 - 15:05 INNOVATIVE APPROACHES IN IMMUNOCHROMATOGRAPHIC STRIP TEST TECHNIQUE
Fabio Di Nardo, Dipartimento di Chimica, Università di Torino, Torino

15:05 - 15:40 Coffee break + sessione poster

Terza sessione

15:40 - 15:55 TRANSPARENT CARBON NANOTUBE NETWORK FOR EFFICIENT ELECTROCHEMILUMINESCENCE DEVICE
Giovanni Valenti, Dipartimento di Chimica “Giacomo Ciamician”, Università di Bologna, Bologna

15:55 - 16:10 REDUCED GRAPHENE OXIDE AND CARBON NANOTUBES FOR THE DEVELOPMENT OF POLYPHENOLS AMPEROMETRIC BIOSENSORS
Ylea Vlamidis, Dipartimento di Chimica Industriale “Toso Montanari”, Università di Bologna, Bologna

16:10 - 16:25 A PAPER BASED-NANOMODIFIED ELECTROCHEMICAL BIOSENSOR FOR ETHANOL DETECTION IN BEERS
Fabiana Arduini, Dipartimento di Scienze e Tecnologie Chimiche, Università di “Tor Vergata”, Roma

16:25 - 16:40 AMPEROMETRIC GENOSENSOR ENHANCED BY DENDRIMER-LINKED PNA PROBES
Marco Giannetto, Dipartimento di Chimica, Università di Parma, Parma

16:40 - 16:55 ELECTROCHEMICAL DETECTION OF TNF-α BY USING A NON-IMMUNOGLOBULIN BIORECEPTOR
Bettazzi Francesca, Dipartimento di Chimica, Università degli Studi di Firenze, Firenze

16:55 - 17:10 SMARTPHONE-BASED BIOSENSOR FOR THERMOCHEMILUMINESCENT BIOASSAYS EMPLOYING SILICA FUNCTIONALIZED NANOPARTICLES DOPED WITH NEW ACRIDINE-1,2-DIOXETANE DERIVATIVES
Donato Calabria, Dipartimento di Chimica “Giacomo Ciamician”, Università di Bologna, Bologna

17:10 - 17:20 Conclusioni e saluti

POSTER

P01 A DNA-BASED NANOMACHINE FOR pH-REGULATED DRUG RELEASE

Alessandro Porchetta[a], Andrea Idili[a], Alexis Vallee-Belisle[b] and Francesco Ricci[a]

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P02 DEVELOPMENT OF A p53-SPECIFIC IMMUNOSENSOR BASED ON CARBON NANOTUBES-GOLD NANOPARTICLES COMPOSITE SUBSTRATE: A PERSPECTIVE FOR CANCER CLINIC TESTING

Maria Vittoria Bianchi[a], Marco Giannetto[b], Monica Mattarozzi[b], Maria Careri[b]

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P03 MICRO-FLOW IMMUNOSENSOR BASED ON THIN-FILM INTERDIGITATED GOLD ARRAY MICROELECTRODES FOR CANCER BIOMARKER DETECTION

Andrea Ravalli[a], Luca Lozzi[b] , Giovanna Marrazza[a]

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P04 IMPLEMENTATION OF A MULTIRESIDUAL ANALYTICAL APPROACH FOR THE CHARACTERIZATION OF FREE FATTY ACIDS AND CERAMIDES ON THE SKIN SURFACE LIPIDOME BY HPLC/(-)ESI-ToF-MS AND CHEMOMETRICS

Roberta Risoluti[a], Matteo Ludovici[b], Mauro Picardo[b], Maria Aurora Fabiano[a], Giuseppina Gullifa[a], Stefano Materazzi[a], Emanuela Camera[b]

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P05 PROPERTIES OF TEMPLATE-ENZYME CONJUGATES TO IMPRINTED SURFACES AND BULK MATERIALS

Claudio Baggiani[a], Cristina Giovannoli[a], Laura Anfossi[a], Fabio Di Nardo[a], Giulia Spano[a]

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P06 DETERMINATION OF OVER 60 DRUGS OF ABUSE IN HAIR BY PLE-dLLME EXTRACTION AND LC-HRMS ANALYSIS

Camilla Montesano[a], Gabriele Vannutelli[a], Flaminia Vincenti[a], A. Gregori[b], L. Ripani[b], D. Compagnone[c], Manuel Sergi[c], Roberta Curini[a]

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P07 SCREEN-PRINTED ELECTRODES TO DETECT CHLORINE DIOXIDE IN SWIMMING POOL WATER

Maria Rita Tomei[a], Daniela Neagu[b], Fabiana Arduini[a], Danila Moscone[a]
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P08 FUNCTIONALIZED TiO_2 AS AN ENZYME MIMICKING SIGNAL GENERATING PROBE FOR IMMUNOCHEMICAL TESTS
Laura Anfossi[a], Marco Sarro[a], Paola Calza[a], Marta Cerruti[b], Fabio Di Nardo[a], Cristina Giovannoli[a], Giulia Spano[a], Claudio Baggiani[a]
[a] *Dipartimento di Chimica, Università di Torino, Torino*
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P09 DEVELOPMENT OF AN IMPEDIMETRIC IMMUNOSENSOR FOR D-DIMER DETECTION IN BLOOD SERUM
Giorgio Scordo, Fabiana Arduini, Giuseppe Palleschi, Danila Moscone
Dipartimento di Scienze e Tecnologie Chimiche, Università degli Studi di Roma "Tor Vergata", Via della Ricerca Scientifica, 1 – 00133 Roma

P10 APPLICABILITY OF POLYDOPAMINE COATED MAGNETIC NANOPARTICLES FOR THE EXTRACTION OF NATURAL ESTROGENIC COMPOUNDS FROM WATER
Giorgia La Barbera, Anna Laura Capriotti, Chiara Cavaliere, Susy Piovesana, Roberto Samperi, Aldo Laganà
Dipartimento di Chimica, Università di Roma "La Sapienza", Roma

P11 MUSTARD AGENTS DETECTION USING A NANOMODIFIED ELECTROCHEMICAL BIOSENSOR
Noemi Colozza[a], Giulio Dionisi[a], Fabiana Arduini[a], Danila Moscone[a], Giuseppe Palleschi[a]
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P12 USE OF MULTIDIMENSIONAL LIQUID CHROMATOGRAPHY TO PURIFY AND IDENTIFY ENDOGENOUS BIOACTIVE PEPTIDES FROM DONKEY MILK
Riccardo Zenezini Chiozzi, Anna Laura Capriotti, Chiara Cavaliere, Roberto Samperi, Aldo Laganà
Dipartimento di Chimica, Sapienza Università di Roma, Piazzale Aldo Moro 5, 00185 Roma

P13 ANALYSIS OF DNA ADDUCTS WITH ACTIVATED PAHs BY MEANS OF MALDI-TOF/MS
Francesca Di Ottavio[a], Maria Chiara Simeoni[a],Martina Assanti[a], Camilla Montesano[b], Manuel Sergi[a], D. Compagnone[a]
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P14 A MINI APPARATUS FOR SURFACE PLASMON RESONANCE (SPR) AND LOCALIZED SURFACE PLASMON RESONANCE (LSPR) SENSORS ON D-SHAPED PLASTIC OPTICAL FIBER
Maria Pesavento[a], Simone Marchetti[a], Piersandro Pallavicini[a], Antonella Profumo[a], Luigi Zeni[b], Nunzio Cennamo[b]
[a] *Dipartimento di Chimica, Università di Pavia, Pavia*
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P15 SMARTPHONE-BASED BIOSENSOR INTEGRATING BIOLUMINESCENT "SENTINEL CELLS" FOR ENVIROMENTAL APPLICATIONS
Maria Maddalena Calabretta[a], Luca Cevenini[a], Elisa Michelinia[b], Giuseppe Tarantino[c], Antonia Lopreside[b], Aldo Roda[a,b]

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P16 A NEW SENSITIVE AND QUANTITATIVE CHEMILUMINESCENT ASSAY TO MONITOR INTRACELLULAR XANTHINE OXIDASE ACTIVITY FOR RAPID SCREENING OF INHIBITORS IN LIVING ENDOTHELIAL CELLS

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P17 FLOW FIELD-FLOW FRACTIONATION BASED APPROACH AS ANALYTICAL TOOL FOR NANOMATERIAL SELECTION AND APPLICATION IN HEALTHCARE

Valentina Marassi[a], Luisana Di Cristo[b], Barbara Roda[a], Andrea Zattoni[a], Sonia Casolari[a], Anna Luisa Costa[c], Adriele Prina-Mello[b], Pierluigi Reschiglian[a]
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P18 CHEMILUMINESCENCE ORIGAMI PAPER-BASED BIOSENSOR FOR GLUCOSE

Massimo Guardigli[a], Mara Mirasoli[a,b], Martina Zangheri[a], Donato Calabria[a], Cristiana Caliceti[a], Aldo Roda[a,b]
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P19 SMARTPHONE-BASED DEVICE FOR ULTRASENSITIVE QUANTIFICATION OF OCHRATOXIN-A IN WINE BY CHEMILUMINESCENT LATERAL FLOW IMMUNOASSAY

Martina Zangheri[a], Mara Mirasoli[a], Laura Anfossi[b], Fabio Di Nardo[b], Cristina Giovannoli[b], Massimo Guardigli[a], Claudio Baggiani[b], Aldo Roda[a]
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INVITED LECTURES

DYE-DOPED SILICA NANOPARTICLES AS LUMINESCENT ORGANIZED SYSTEMS FOR NANOMEDICINE
Prof. Luca Prodi
Dipartimento di Chimica "Giacomo Ciamician", Università di Bologna, Bologna

ADVANCED DEVICES BASED ON NANOBIOMATERIALS
Dott. Roberto Zamboni
Istituto per la Sintesi Organica e la Fotoreattività (ISOF), Consiglio Nazionale delle Ricerche

ABSTRACT COMUNICAZIONI ORALI

Proteomic and post-translational phosphorilations study of wild silk cocoon *Cricula Trifenestrata* and *Bombyx Mori*

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The development of functional materials that can interact with biological systems is nowadays of great interest. Such materials can be derived directly from nature or synthesized in the laboratory. However, despite the remarkable potential of man-made synthetics, their applications have been limited by challenges including biocompatibility, biodegradability and bioresorbability. The intrinsic advantages of natural materials lead to focus the research on silks, members of fibrous proteins family, with impressive mechanical strength, excellent biocompatibility, absence of immunogenicity, limited bacterial adhesion and controllable biodegradability.

Wild silks, not obtained from domesticated species, are primarily composed of proteins associated with certain macromolecules such as polysaccharides and lipids. The two primary proteins that comprise silk-cocoon silk are fibroin and sericin, consisting of 18 different amino acids: predominantly glycine, alanine and serine [1]. The amino acid sequences of silk proteins can vary from species to species, resulting in a wide range of mechanical properties [2].

This research is focused on wild silk cocoon of *Bombyx Mori* and *Cricula Trifenestrata* (no proteomic study has been done before on the latter) obtained from Indonesian source, and comprises a comparative proteomic analysis between the two cocoons and a final shotgun proteomic approach on the post translational modifications. In order to study these modifications, an upstream step of enrichment is required: for the specific enrichment of phosphopeptides, newly methods and stationary phases will be tested, i.e. coprecipitation methods [3] or spin columns with phosphate-specific-binding stationary phases [4].

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Detection of binding sites on the surface of proteins adsorbing transiently onto nanoparticles

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The successful application of nanoparticles (NPs) in biosciences necessitates an in-depth understanding of how they interface with biomolecules.[1] Indeed, it has been recognized that upon exposure to a biological medium, NPs adsorb a variety of biomolecules, forming a complex dynamic layer (protein corona).[2] NPs associating with proteins may interfere with protein-protein interactions and affect cellular communication pathways. Protein–NP interactions are also of interest for the development of hybrid devices. Thus, methodologies aimed at characterizing biomolecules bound to NPs represent an indispensable tool.

Solution NMR spectroscopy is a mature technique for the investigation of biomolecular structure, dynamics, and intermolecular associations, however its use in protein-NP interaction studies remains scarce and highly challenging, particularly due to unfavorable hydrodynamic properties of most nanoscale assemblies.[3] Here, we show that the transient adsorption of proteins onto NPs is accessible by NMR. We describe approaches based on either diamagnetic or paramagnetic protein NMR signal perturbations.

To contribute to an understanding of the interactions between a fundamental biomacromolecule, ubiquitin (Ub), and NPs, we investigated the adsorption of fullerenol on monomeric Ub and on a minimal polyubiquitin chain. Site-resolved chemical shift and intensity perturbations of Ub's NMR signals (Figure 1), together with (15)N spin relaxation rate changes were consistent with the reversible formation of soluble aggregates incorporating fullerenol clusters.[4] The specific interaction epitopes were identified, coincident with functional recognition sites in a monomeric and lysine48-linked dimeric Ub. Fullerenol appeared to target the open state of the dynamic structure of a dimeric Ub according to a conformational selection mechanism.

Biological media are heterogeneous mixtures of biomolecules and other components. Thus, investigation of specific protein-NP interactions is hampered by interferences from protein-protein binding. The incorporation of paramagnetic centers into NPs offers new opportunities to explore bio-nano interfaces. We propose NMR paramagnetic relaxation enhancement as a new tool to detect NP-binding surfaces on proteins with increased sensitivity, also extending the applicability to heterogeneous biomolecular mixtures. The adsorption of Ub onto gadolinium-doped fluoride-based NPs produced residue-specific NMR line-broadening effects mapping to a contiguous area on the surface of the protein. Importantly, an identical paramagnetic fingerprint was observed in the presence of a competing protein-protein association equilibrium, exemplifying possible interactions taking place in crowded biological media.[5]

Besides the mapping of binding surfaces, we also successfully applied NMR approaches to describe the dynamics of proteins adsorbed onto NPs.[6]

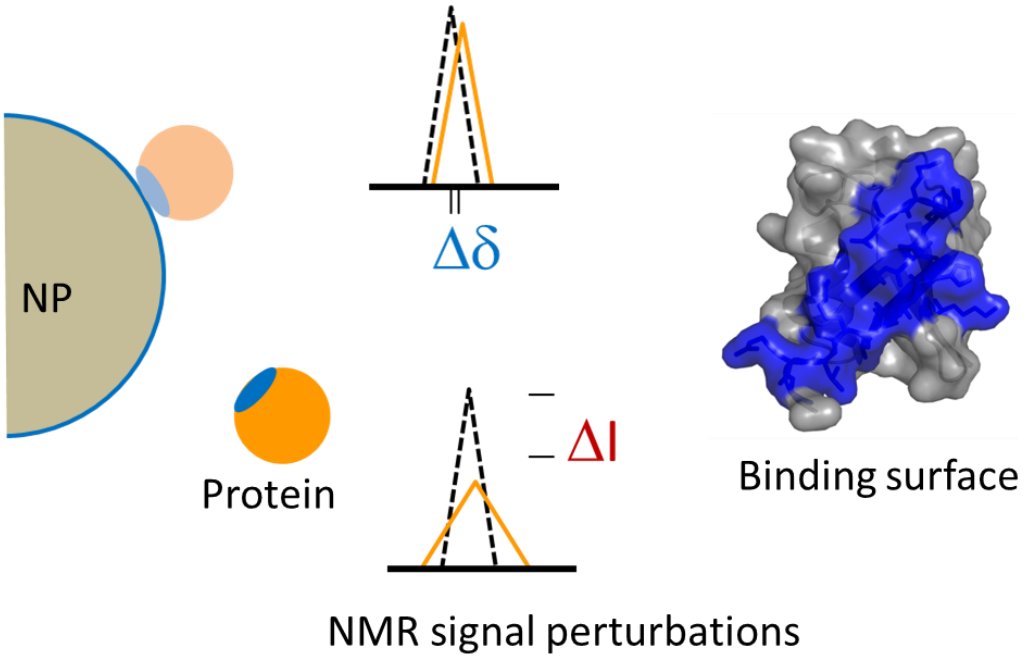

Figure 1: Mapping of binding sites on the surface of proteins interacting transiently with nanoparticles. Perturbations of NMR signals are observed for atoms forming the contact surfaces due to exchange averaging.

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Development of new composite magnetic phases for phosphopeptides isolation in shotgun phosphoproteomics

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Protein reversible phosphorylation is a central way to regulate cellular processes in cells but remains poorly characterized in classical proteomic screenings because phosphorylated proteins are present in very low amounts, which hinder their analysis even with the current high resolution mass spectrometry technologies. In this context, sample preparation is a fundamental step for the phosphoprotein mapping of complex samples analysis because the selective enrichment of modified peptides and proteins prior to HPLC-MS/MS analysis represents the best way to concentrate the target analytes and avoid suppression during ionization [1]. Several approaches were developed for such a purpose, with complementary enrichment capability [2].

Thus, the aim of our research was to develop new materials for the highly selective enrichment of phosphopeptides. The chosen approach was based on magnetic solid phase extraction, a technique easier to use than conventional packed miniaturized columns and applicable to small and large scale experiments. Two materials were developed: in one case magnetic Fe_3O_4 nanoparticles were covered with polydopamine, a biocompatible polymer easily produced by the spontaneous polymerization of dopamine under basic conditions. This polymer possesses catechol moieties, suitable for immobilization of cations, in this case Ti^{4+}. This stationary phase was tested for phosphopeptide enrichment and an optimized method was developed first on a standard protein digest of casein in bovine serum albumin, 1:100 ratio, then on the low abundant endogenous phosphorylated peptides in commercial cow milk, with good results compared to a standard commercial enrichment kit.

The second type of magnetic stationary phase developed exploited graphitized carbon black as support, due to its large surface area and special affinity for acidic compounds. The substrate was first magnetized and then covered with TiO_2 for selectivity to phosphopeptides. This new phase was first tested on the standard mix and then optimized on a more complex yeast whole protein extract. Different sample to stationary phase ratios were tested to maximize the selectivity of the protocol. The new system was compared to commercial TiO_2 spin column for assessing the enrichment efficiency on the yeast complex sample.

All the enrichment protocols, for both systems, were embedded within the framework of a typical shotgun proteomics workflow, where peptide identification was performed by nanoHPLC high resolution mass spectrometry and bioinformatics data analysis.

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New analytical solutions for pharmaceutical nanotechnology

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The rapid development of protein-based and nanostructured pharmaceuticals highlights the need for robust analytical methods to ensure their quality and stability. Between proteins, used in pharmaceutical applications an important and ever increasing role is represented by monoclonal antibodies and large proteins, often modified to enhance their activity or stability when used as drugs. Functionalized nanoparticles are used as active compounds for delivery in pharmaceutical field. The bioactivity and the stability of those proteins and particles are closely related to morphology and the maintenance of their complex structure, however, influenced from many external factors that can cause degradation and/or aggregation. The presence of aggregates in these drugs could reduce its bioactivity and bioavailability and induce immunogenicity. The choice of the proper analytical method for the analysis of aggregates is fundamental to understand their (size) dimensional range; their amount and if they are present in the sample as generated by an aggregation or are an artifact due to the method itself. Size exclusion chromatography is one of the most important techniques for the quality control of pharmaceutical particles, however its application is limited to relatively low molar masses aggregates. Among the techniques for the size-characterization of proteins, field-flow fractionation (FFF) represents a competitive choice since its soft mechanism due to the absence of a stationary phase and the higher dimensional range of applications form nanometer to micrometersized analytes. The microcolum variant of FFF, the hollow-fiber flow FFF (HF5), is on-line coupled with multi-angle light scattering (MALS) for the development of methods for the characterization of protein based drugs [1,2]. The HF5-MALS was shown able to size-separate therapeutic protein samples and their aggregates and to evaluate the nature of aggregates. The analytical performances, such as resolution, reproducibility and limit of detection, were also determined in the framework of quality control of particles-based drugs.

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A modular clamp-like mechanism to regulate the activity of nucleic-acid target-responsive nanoswitches with external activators

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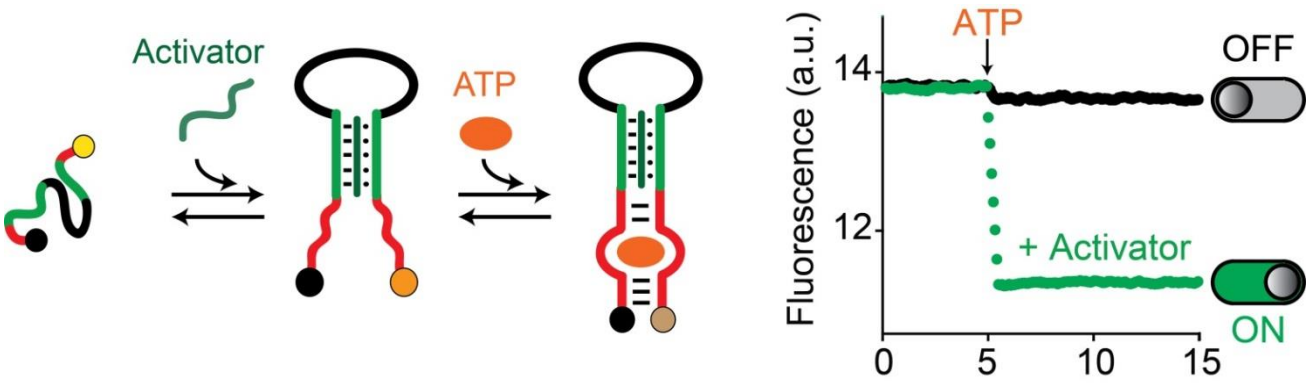
Here we demonstrate a general and modular approach to regulate the activity of target-responsive DNA-based nanoswitches. We do so by coupling together two DNA-based responsive elements: a triplex-forming clamp-like probe able to bind a specific DNA sequence and a split aptamer selected to bind a small molecule. In the presence of the specific target of one of the above responsive elements, the nanoswitch partially folds and its ability to bind the second target is restored. With this approach we can finely modulate the affinity of both DNA-recognition elements and aptamers using an external ligand. The modular nature of our strategy makes it easily generalizable to different DNA based recognition elements. As a demonstration of this we successfully designed five different DNA nanoswitches whose responsiveness can be regulated by different molecular effectors and targets. The convenience with which this mechanism is designed suggests that it may prove a useful tool by which sensors, genetic networks and other biotechnology devices employing nucleic-acid based receptors can be controlled with an external input.



Polymethylmethacrylate nanoparticles for molecular beacons internalization in cancer cells

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Effective drug delivery into cancer cells is a major objective for nanomedicine, which exploits the peculiar characteristics of cancer tissues, such as their enhanced permeability and retention (EPR) properties [1]. As delivery vehicles of anticancer drugs, nanoparticles (NPs) offer numerous advantages over conventional drug delivery approaches, such as the possibility of multiple functionalization for improving the imaging, diagnosis and targeted therapy [2]. Moreover, NPs can enhance active or passive targeting, thus increasing selectivity, reducing toxicity and prolonging the drug half-life in the human body with respect to the free drug [3]. The core-shell polymethylmethacrylate nanoparticles (PMMA-NPs) used in this study consist of a hydrophobic PMMA core covalently functionalized with fluorescein and an external hydrophilic shell decorated with primary amine groups and quaternary ammonium salts. PMMA-NPs have high biocompatibility, very low cytotoxicity, biological inertness and low costs of synthesis. Furthermore, the potential multiple functionalization for improving the selectivity and the ability to prolong the lifetime of the drug in the human body [4] represent relevant advantages with respect to classical transfection reagents such as Lipofectamine. Additionally, similar kinds of nanoparticles have been previously used as carriers of drugs, prodrugs and antisense oligoribonucleotides for inducing dystrophin restoration in body-wide muscles in the X chromosome-linked muscular dystrophy animal model [5]. Moreover, previous studies demonstrated that similar nanoparticles used for drug delivery purposes with different sizes, e.g. 40, 55, 100 and 180 nm, showed comparable ability to cross the cell membrane regardless to their size [6]. The idea developed in this work is to use biocompatible core-shell PMMA-NPs (Figure 1) as carrier of an oligodeoxynucleotide molecular beacon (MB) specific for survivin mRNA in A549 human lung adenocarcinoma epithelial cells. Survivin mRNA is considered a promising target for anticancer treatment. Indeed, survivin is a protein belonging to the Inhibitor of Apoptosis Protein family (IAP) that plays a key role in several cellular functions, such as the regulation of cell cycle, apoptosis and cell migration. Furthermore, its expression is very high in most cancer cells in which the protein levels correlate to poor prognosis and resistance to chemotherapeutic treatment, while it is rarely expressed in healthy tissues. Optical nanoprobes have been recently proposed as theranostic agents capable to conjugate the ability of sensing specific mRNA with the silencing activity, preventing the overexpression of proteins associated to cancer development [7]. An innovative strategy is represented by molecular beacons (MBs), oligonucleotide sequences generating a fluorescent signal when they hybridize with their target mRNA. MB is a stem-loop-folded oligonucleotide with fluorophore and quenching dye conjugated to the 3' and 5' ends, respectively. In the absence of the target, the fluorescence of the fluorophore is quenched by the closely located dye. Otherwise, the hybridization with the target opens the hairpin, generating a probe-analyte duplex with the fluorophore separated from the quencher and consequently able to

emit fluorescence once excited. In the present work, the survivin-MB was firstly characterized in solution in order to verify its functionality and then the PMMA-NPs ability to promote the MB internalization was verified in A549 cells by confocal microscopy. Confluent Human Dermal Fibroblasts from adult (HDFa) were used as healthy control. The results showed that PMMA-NPs promote endocytosis in A549 cells and the survivin-MB cellular up-take (Figure 2) and that the use of 10 µg/mL PMMA-NPs as carrier for survivin-MB for 1 h 30 min might be a promising strategy to reduce cancer cell proliferation avoiding detectable consequences on the healthy cells [8]. This research was supported by the national flagship project Nanomax-Encoder and by the European project Nanodem -NANOphotonic Device for Multiple therapeutic drug monitoring (FP7-ICT-2011 - contract 8318372).

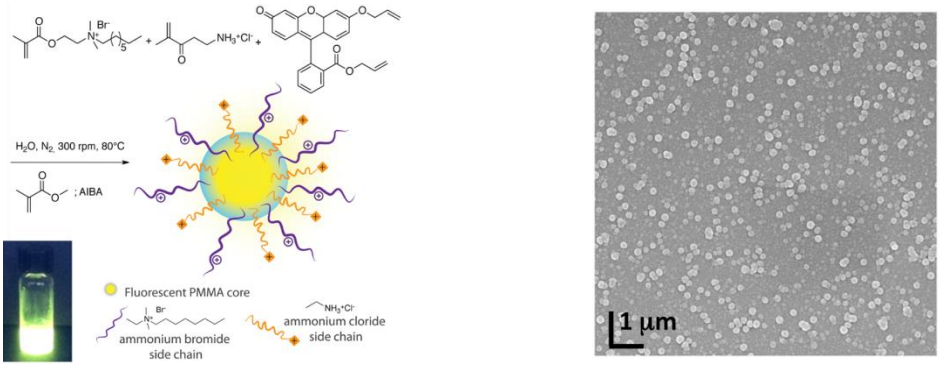

Figure 1: Left) Synthesis of core-shell PMMA-NPs; Right) SEM image of PMMA-NPs.

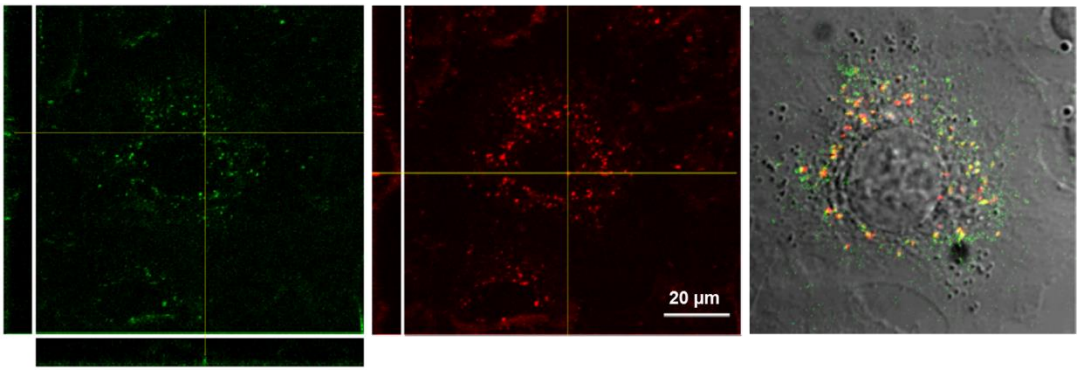

Figure 2: Confocal microscopy images of A549 living cells incubated with 100 nM survivin MB@10 µg/mL PMMA-NPs for 90 minutes in complete medium. Left) fluorescence image with excitation at 488 nm; Centre) fluorescence image with excitation at 638 nm; Right) merged image of the magnified region. Each panel shows an equatorial XY image and the relative XZ and YZ projections of the image stack.

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Novel detection strategies for protein tau

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Protein tau, together with amyloid beta peptide and phosphorylated tau, is one of the currently accepted biomarker for probable Alzheimer's disease diagnosis. Up to now quantification of tau protein in cerebrospinal fluid is usually realised by the conventional immunochemical methods such as ELISA, however such method is time consuming compared with bio-sensing techniques.
Here Surface Plasmon Resonance sensor is chosen as common platform to test the feasibility of two sensing approaches for detecting tau protein.
First approach deals with the development of an immunosensor (Figure1).
Commercial antibodies were selected and used as capturing receptor for tau, coupled to the sensor surface or as signal enhancers, by interacting with a different analyte epitope. Once the sensor was characterized in buffer and in simulated cerebrospinal fluid (aCSF) (showing a sensitivity in nM range), secondary antibody coupling with Carbon nanostructures was realized in order to further amplify the SPR signal. During the effect of sonication time, nanotubes-protein ratio, and coupling media was investigated. After optimization, thanks to the use of nano-architecture, signal was enhanced 150-times compared with direct assay.
The second strategy was based on the development of a new aptameric receptor for the protein. Aptamers, unlike antibodies, are selected *in vitro* and can be easily synthesized and modified. Selection is usually carried out by SELEX (Systematic Evolution of Ligands by Exponential enrichment), which is composed by separation step (here Capillary Electrophoresis), an amplification step by PCR and eventually a purification step. In this case aptamer selection was realized by Non-SELEX, a process which speeds up the identification of an aptamer population with increased affinity for the target.
In summary the approach based on immunosensing resulted successful in selective detection of tau even in artificial matrix. The selection for tau specific aptameric receptor resulted in few aptamers to be further characterised in terms of their affinity contrants (K_A) by SPR to be further used as alternative receptor to antibodies.

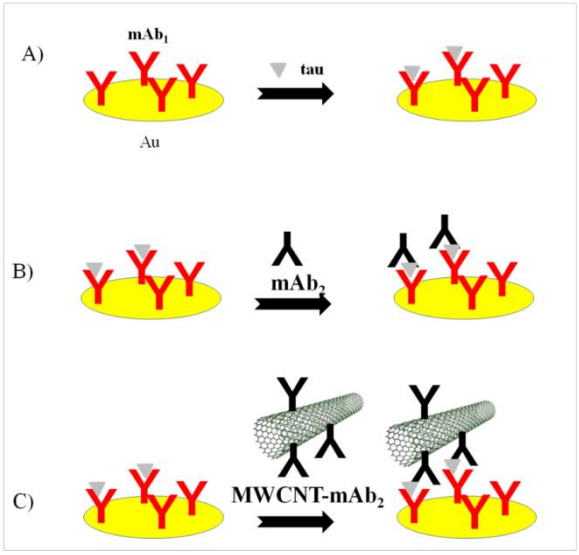

Figure 1: immunosensing strategies for protein tau. A) direct approach; B) conventional sandwich; C) Nanostructure as mass enhancers

Unexpected behavior of fluorescent probes during SPR experiments

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Surface Plasmon Resonance (SPR) is a highly sensitive technique aimed at investigating biological interactions between a surface possibly functionalized with a ligand of interest and a flowing analyte [1]. A typical SPR instrument consists of a p-polarized light beam, generated by a laser, that travels through a glass prisma and reaches a thin metal layer. The fraction of light reflected at the interface depends on the incidence angle and it is measured by a detector. A typical SPR curve (Figure 1A) is characterized by a marked dip, the fingerprint of the occurring resonance phenomenon, established when the wave vector of the evanescent field associated to the incident light matches the natural wave vector of the conduction electrons. The position of the resonance angle (θ_{res}) depends on the number and molecular mass of analyte molecules that interact with the (functionalized) metal layer. By plotting θ_{res} as a function of time when the analyte flows in contact with the metal layer one obtains a sensogram (Figure 1B). The resonance angle is shifted toward higher values when the ligand interacts with the surface (higher the shift, stronger the interaction).

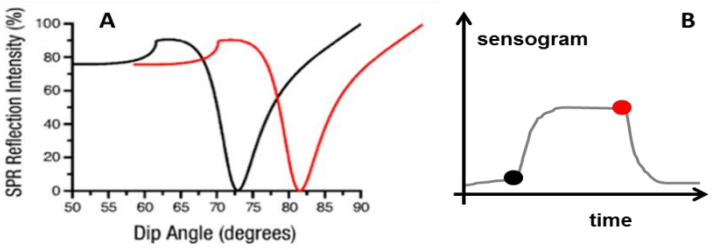


Figure 1 : Schematic representation of a SPR curve (A) and sensogram (B).

By recording sensograms at different concentrations of the flowing analyte, one can estimate the binding affinity between the ligand on the (functionalized) metal surface and the analyte itself. In the present work we adopted this method to assess the fatty acid free human serum albumin (HSA) binding properties of four different imaging probes, one for magnetic resonance and three for optical imaging applications (structures indicated in Table A and Figure 2).

	Binder	Reporter
A	Deoxycholic acid	Gd chelate
B	RGD peptide	Cy5.5
C	Deoxycholic acid	IRDye 800CW
D	Lithocholic acid	IRDye 800CW

Table A : Block composition of the four selected imaging probes.

The compounds (all synthesized internally) were dissolved in phosphate buffer saline (PBS) at different concentrations, from 0.5 to 100 □M. These solutions were then flowed into a MP-SPR Navi™ 210A (BioNavis, Finland) equipped with a gold sensor coated with branched carboxymethyldextran functionalized with HSA. SPR curves and relative sensograms were recorded under laser excitation at 670 nm and 785 nm, using the instrument software.

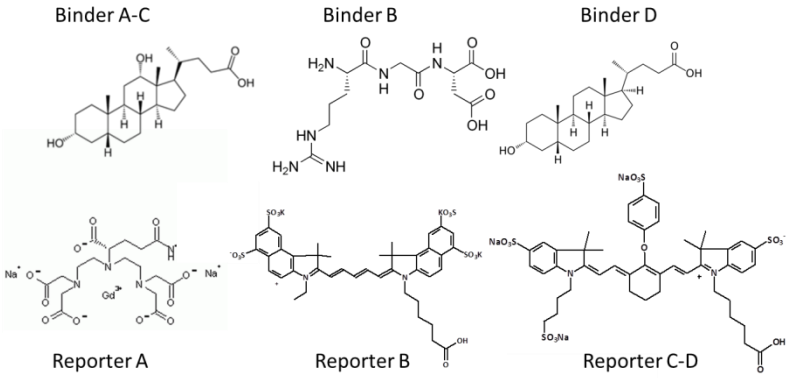


Figure 2 : Structure of the four selected imaging probes.

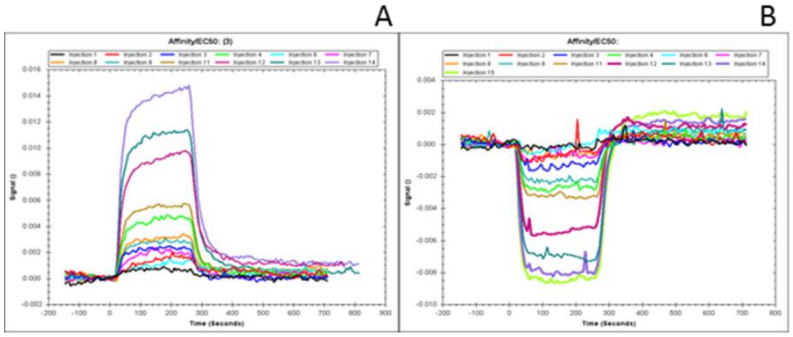
Representative examples of the recorded sensograms and a summary result table are reported in Figure 3. Surprisingly, in some cases, specifically when the probe absorption wavelength (λ_{probe}) was higher than the laser wavelength (λ_{laser}), negative sensograms were observed. Such experimental evidence corresponds to a reduction of θ_{res} when increasing the number of interacting analyte molecules, an effect not described in literature up to now according to the best of our knowledge. Moreover, a non linear response was obtained when $\lambda_{probe} \approx \lambda_{laser}$.

In order to comprehend the origin of the unexpected behavior and the reliability of the data extracted from negative sensograms a theoretical study was carried out. The classical description [2] of the refractive index η (the key SPR parameter) was thus applied to the SPR system, obtaining for the real component of the refractive index of the interacting layer:

$$\eta_S' = 1 + \frac{Nq_e^2\left(\omega_{probe}^2 - \omega_{laser}^2\right)}{2m\varepsilon_0\left[\gamma^2\omega_{laser}^2 + \left(\omega_{probe}^2 - \omega_{laser}^2\right)^2\right]}$$

, where N is the number of electrons, q_e the electron charge, m is the electron mass, ε_0 the dielectric constant of vacuum, $\omega_{laser(probe)}$ the angular frequency of laser (probe) and γ a damping coefficient. Since θ_{res} is directly proportional to η, when $\lambda_{laser} \gg \lambda_{probe}$ a decrement of the resonance angle is observed at higher N (and thus the number of interacting molecule). The linear dependence between θ_{res} and N is maintained in both regimes ($\lambda_{laser} \gg \lambda_{probe}$ and $\lambda_{laser} \ll \lambda_{probe}$) with the exception of the degenerative case $\lambda_{laser} = \lambda_{probe}$.

In conclusion, despite the sign reversal, obtained sensograms reliably represent the interaction between analyte and immobilized ligand in all those case where the wavelength of the laser and the investigated molecule do not overlap or are not nearly close.



C

Probe	λ_{probe} nm free(bound)	670 nm	785 nm
A	275	+	+
B	676(696)	-	+
C	775(786)	-	+/-
D	776(785)	-	+/-

Figure 3 : Representative examples of positive (A) and negative (B) sensograms obtained with probe B as flowing analyte. A summary of the results for the four agents, negative vs positive sensograms (C).

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Innovative approaches in Immunochromatographic Strip Test technique

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Nowadays, rapid immunoassay-based tests are widely applied in clinical, drug, food and environmental analysis. Among the different formats, the Immunochromatographic Strip Test (ICST) technique is increasing its relevance year by year. The importance of this technique can be noticed evaluating some economic data: in the 2015, the global market for ICSTs, used in routine, was estimated at about 5 billion dollars [1] and the perspective for the 2016 are even better.
From the development of the first commercial ICST in the 1988, the technique remained almost unchanged. ICST devices involve immunoassays in which the sample flows by capillary forces along an analytical membrane that contains immobilized bioreagents, dispensed in specific area called Test line and Control line, where the former gives information about the target analyte, while the latter ensures the correct functioning of the test.
Traditional ICSTs employ colloidal gold to generate visual signals and usually provide a binary yes/no answer. To enhance the ICST technique, new approaches need to be explored.
Recently, we reported the usage of Quantum Dots (QDs) as new labels for the development of a fluorescent ICST to detect fumonisins in maize [2]. Following the good results achieved, we started a study on an alternative use of these fluorescent semiconductor nanocrystals for the detection of small molecules.
Small molecules are characterised only by one epitope, therefore the competitive format must be applied for their detection. In the competitive format the response is inversely correlated to the analyte concentration, so, as the analyte concentration increases, the intensity of the Test-line decreases. For the end user, the inversely correlation might cause a misleading evaluation of the results since the presence of the analyte is expected to be correlated to the presence of a signal. In addition, the competitive format is intrinsically less sensitive than the non-competitive format, because it is more difficult to assess a signal decrease instead of a signal appearance.
For these reasons, our idea is to exploit the fluorescence quenching of QDs by gold nanoparticles (AuNPs) [3] to develop an ICST in competitive format in which the response is directly correlated to the analyte concentration.
In this kind of design, a conjugate between QDs and the analyte is immobilized on the membrane (Test line), while antibodies (Abs) anti-analyte labelled with AuNPs flow across the membrane. In the absence of analyte, the labelled Abs bind the Test line and the closeness of AuNPs to QDs cause their fluorescence quenching (signal absence under UV light). In the presence of analyte, less Abs bind the test line resulting in a lesser fluorescence quenching (signal increase under UV light).
More details about this approach will be showed in the communication.
Thanks to the simple preparation of AuNPs and their easy conjugation to antibodies, AuNPs-based ICSTs are still the most required from the market. The typical AuNPs used in ICST are mainly spherical, with a diameter of 30 nm; they exhibit a deep-red colour, which reflects the surface plasmon resonance (SPR) band in the visible region around 525 nm. Therefore, using these AuNPs, the generated visual signal is characterised by one or more red lines.

Sometimes the red colour of the lines can be a limitation: for weak signals is quite difficult to see a pale-red on the white background of the nitrocellulose; moreover for ICST designed for multianalyte analysis, the result interpretation can be tricky.
For these reasons, it would be very useful to have nanoparticles with the advantages of the typical spherical AuNPs, but characterised by a different colour.
It is well-known that size and shape of AuNPs have significant influences on the SPR band, therefore modulating these parameters it is possible to obtain nanoparticles with different colours.
We synthesised non-spherical AuNPs through a seeding growth approach. These AuNPs exhibit a SPR band at around 620 nm, which is consistent with the blue colour observed (fig. 1).

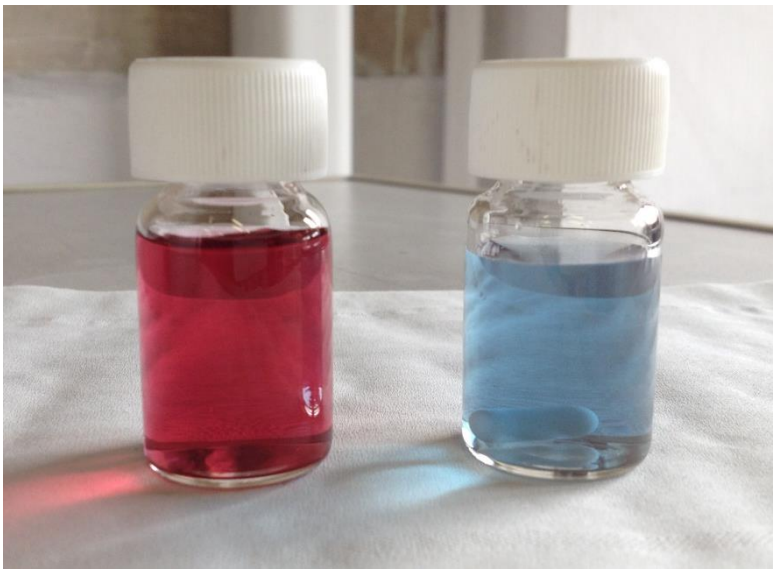

Figure 1: Resulting colours of AuNPs with SPR band at 525 (red) and 620 (blue) nm.

The use of these "blue AuNPs" in ICST is quite new [4] and seems to be very promising.
The preliminary results on the use of these "blue AuNPs" will be discussed in this communication.

Acknowledgments
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Transparent Carbon Nanotube Network for Efficient Electrochemiluminescence Device

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Electrochemically generated chemiluminescence (ECL), also called electrochemiluminescence, is a luminescence induced by an electrochemical stimulus.[1] As an analytical technique, it possesses several advantages over photoluminescence and chemiluminescence, in particular for (bio)sensor applications.[2] The electrochemically-induced way to generate luminescence signal allows to obtain sensors with low background signal and high sensitivity, good temporal and spatial resolution, robustness, versatility, and low cost. The peculiar analytical performances in terms of high detectability of conventional chemiluminescence (CL) are retained and, in addition, the electrochemical trigger of the reaction allows controlling the time and position of light emission from ECL probes. These properties make ECL systems particularly attractive also for microscopy imaging techniques in biological tissue sections or single cells, such as *in situ* hybridization (ISH) and immunohystochemistry (IHC). In this context, the nanostructured materials such as carbon nanotubes (CNTs) and graphene are particularly promising for sensing applications.[3]

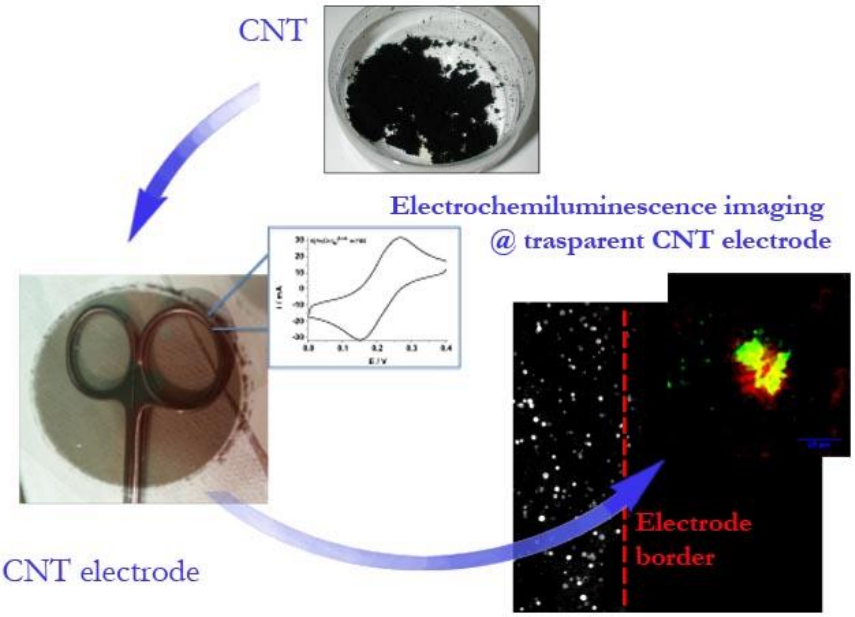

Figure 1: Schematic representation of the CNT-based electrode preparation from the pristine carbon material to the ECL imaging of single bead and single cell.

Here we present the application of optically transparent electrodes based on carbon nanotubes and graphene materials to ECL, [4] demonstrating the superiority of such materials *vis-à-vis* ITO electrodes. CNTs and graphene electrodes are excellent material for ECL application thanks to the very favorable overpotential of amine oxidation that represents the rate-determining step for the signal generation in both research systems and commercial instrumentation. The employ of

carbon nanotubes resulted in a ten times higher emission efficiency compared to commercial transparent ITO electrodes. We demonstrate as a proof of principle that our CNT device can be used for ECL imaging in which micro-beads were used to mimic a real biological sample, such as single cell visualization. Finally, we established a novel electrochemical detection technique based on the combination between CNT and ECL for single cells analysis with high throughput and low detection limit.

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Reduced Graphene Oxide and Carbon Nanotubes for the development of polyphenols amperometric biosensors

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Biosensors for sensitive, rapid and precise determination of phenolic compounds are attracting growing interest in environmental control and protection as well as in food industry.
Laccase (Lac) and tyrosinase (Tyr) are multicopper oxidase enzymes that catalyze the oxidation of phenol derivatives to the relevant quinones with the concomitant reduction of oxygen directly to water without the formation of reactive oxygen intermediates.
Here we present the development of amperometric biosensors based on Lac or Tyr physically adsorbed on glassy carbon electrodes modified with carbon-based nanomaterials.
Carbon-based nanomaterials are often employed in electrochemistry for their beneficial properties and in the last few years they have been frequently used in the development of biosensors to enhance the electron transfer between the electrode and the enzyme. Graphene represents an excellent material for sensing applications due to properties like fast heterogeneous electron transfer, large surface area, high mechanical strength, ease of functionalization, high conductivity and good biocompatibility. The electron transfer rates on graphene sheets obtained by electrochemical reduction of graphene oxide (GO) are similar to those observed for carbon nanotubes and higher than those of glassy carbon electrodes since the reduction of oxygen functional groups at edge and basal planes produces defect sites [1]. The electrochemical reduction of GO is a versatile method to obtain a graphene layer (rGO) on the electrode surface which still displays some controllable oxygen-containing functionalities, usable for covalent or physical immobilization of enzymes. The enzyme immobilization represents a rather critical issue because the methods used for this procedure significantly influence the biosensor properties, operability and long-term stability. Among the methods available in the case of tyrosinase and laccase, the most commonly employed is the physical entrapment, obtained by a cross-linking with bovine serum albumin (BSA) and glutaraldehyde (GA) [2].

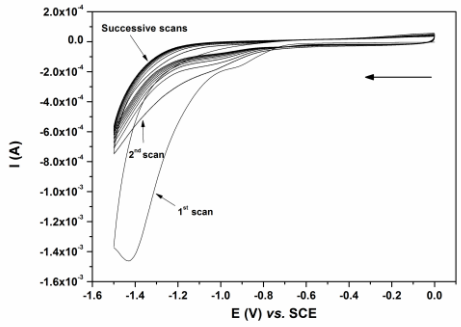

Figure 1: CV responses recorded during the electrochemical reduction of GO on a GCE in deoxygenated 0.1 M PBS, pH 4 at a scan rate of 0.05 V s^{-1}. The arrow indicates the direction of potential sweep.

In this work we investigated the performance of biosensors based on Tyr or Lac obtained by modifying glassy carbon electrodes (GCE) with different configurations of rGO and multiwalled carbon nanotubes (MWCNTs). After the deposition of the carbon based nanomaterials, the electrodes were submitted to an electrochemical procedure in order to reduce GO (Fig.1). The surface morphology and composition of the modified electrodes were monitored with scanning electron microscopy, energy-dispersive X-ray microanalysis and infrared reflectance spectroscopy.
The method for the immobilization of the enzymes was carefully studied and the best biosensors storage conditions were investigated in order to obtain long-life devices. The parameters studied for the optimization were the concentrations of the cross-linker GA and the extra protein BSA.
The current measured by the biosensors is mainly ascribable to the electrochemical reduction of quinones, at 0 V vs SCE, produced upon oxidation of monophenols or o-diphenols by Tyr or Lac, even if the so called DET (direct electron transfer) between the electrode surface and the active enzymatic site has been proven to occur. The operative conditions were found out using catechol as a reference compound which is known to show a good response towards laccase and tyrosinase, but other polyphenols were also investigated (Fig. 2). The effectiveness of the developed biosensors has been demonstrated by measuring the total concentration of polyphenols in samples of fruit juices expressed as epicatechin equivalents. The antioxidant capacity (AC) of those matrices had already been investigated by us by means of a recently proposed electrochemical sensor [3]. The proposed application of the biosensors aims to check if the AC can be essentially related to the presence of different classes of polyphenols.

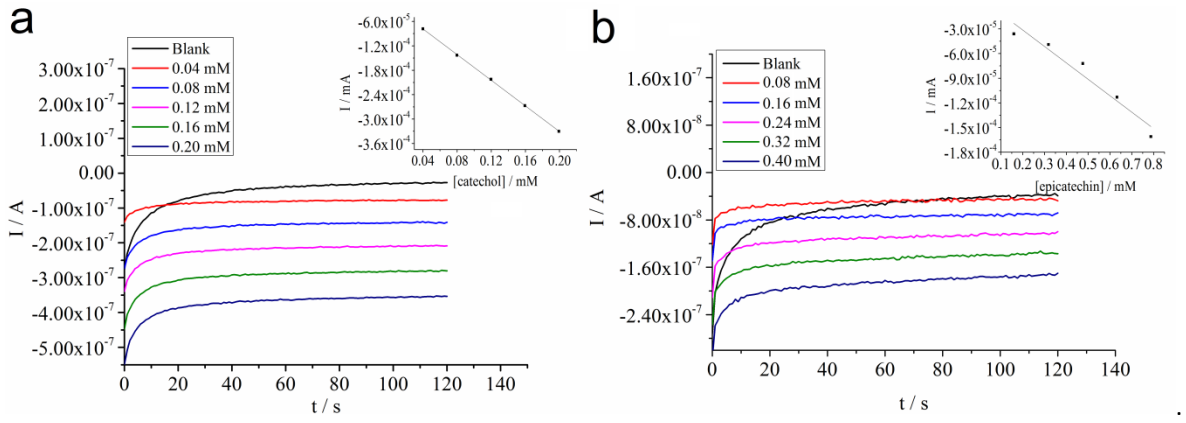

Figure 2 : chronoamperometric curves recorded in 0.1 M acetate buffer pH 4.5 and calibration plots (inset) obtained for increasing amounts of catechol (a) and epicatechin (b).

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A paper based-nanomodified electrochemical biosensor for ethanol detection in beers

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The needs for food process and quality control have entailed the requirements of reliable analytical tools. Besides the traditional methods such as chromatography, immunochromatographic, mass spectrometry, NMR, PCR, and light-dependent approaches (UV, IR, CD, fluorescence) [1], several research activities in analytical chemistry has recently focused on facile, cost-effective, miniaturized and sustainable approaches, particularly toward the analysis of the most consumed products, both food and beverages. Among several beverages, beer is the 3rd most drunk beverage in the world, the 1st just considering the alcoholic ones. Ethanol content is one of the most important ingredients that characterize a beer and, in terms of authenticity assessment, it is extremely important the development of analytical methods which allow rapid and affordable solutions.

Herein, we report the first example of paper-based screen-printed biosensor for the detection of ethanol in beer samples. Common office paper was adopted to fabricate the analytical device. The properties of this paper-based screen-printed electrode (SPE) were deeply investigated by means of physical, mechanical and chemical interrogations and compared with the well-established polyester-based SPEs. Paper evidenced similar properties when compared with polyester, highlighting suitability towards its utilization in sensor development, with the advantages of low cost and simple disposal by incineration. A nanocomposite formed by Carbon Black (CB) and Prussian Blue nanoparticles (PBNP), namely CB/PBNP, was utilized as electrocatalyst to detect the hydrogen peroxide generated by the enzymatic reaction between alcohol oxidase and ethanol. After optimizing the analytical parameters such as pH, enzyme concentration and working potential, the developed biosensor allowed a facile quantification of ethanol up to 10 mM with sensitivity of 16.2 μA/mM cm^2 and a detection limit equal to 0.52 mM. These satisfactory performances rendered the realized paper-based biosensor reliable over the analysis of ethanol contained in four different types of beers, including pilsner, weiss, lager and alcohol-free. The proposed manufacturing approach offers an affordable and sustainable tool both toward the food quality control and toward the realization of different electrochemical sensors and biosensors.

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Amperometric genosensor enhanced by dendrimer-linked PNA probes

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Genosensors are biosensors that base their recognition element on the interaction between nucleobases in nucleic acids, either natural, synthetic or mimics. Their purpose is to detect and quantify a nucleic acid in the sample, usually a specific strand of DNA, RNA, or microRNA. In a research program dealing with the development of innovative sensors as powerful analytical tools for assessing food safety[1,2], in this work we combined performance of DNA-mimic probes based on Peptide Nucleic Acids (PNA) with the enhancing properties of nanomolecular moieties such as Polyamidoaminic (PAMAM) dendrimers. Our previous studies were focused on the use of the same PNA probes on all-gold nanostructured Screen Printed Electrodes (SPEs). The findings of these studies[3] evidenced as best results those obtained suiting a non-competitive approach, based on the binding of target DNA by a PNA-Capture Probe (CP)-functionalized sensor, followed by the hybridation with a properly synthesized Ferrocene-tagged PNA-Reporter Probe (RP). In this prototype, the CPs were immobilized on the electrodic surface *via* Self Assembled Monolayers (SAM) from mercaptoundecanoic acid

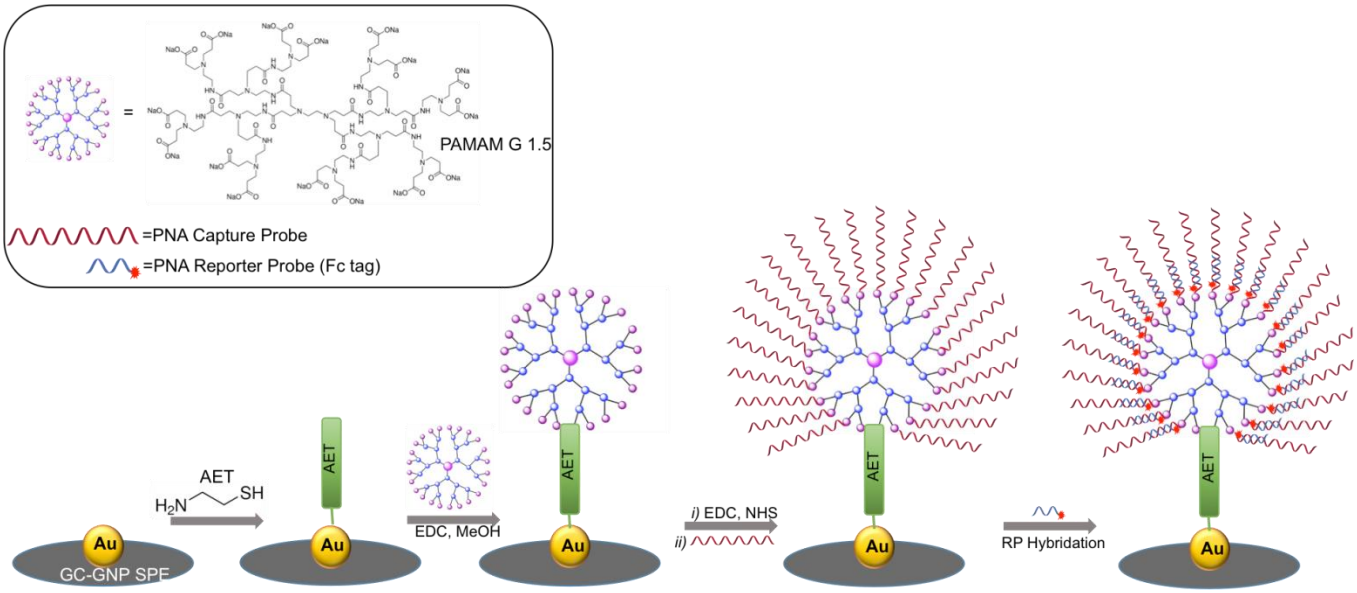

Figure 1: Development protocol of the GC-GNP PAMAM-enhanced PNA genosensor

Although this methodological approach allowed us to detect the DNA target (transgenic soy 15-mer sequence) at nanomolar scale, the non-competitive approach required the use of low concentrations of immobilized CP, thus reducing sensitivity of the genosensor. With the double aim of amplifying response of the device and simultaneously optimizing the conformational freedom of the bound CP strands (15-mer PNA) we combined the use of Glassy Carbon-Gold

Nanoparticles GC-GNP SPEs functionalized by covalent immobilization of a 1.5 generation PAMAM dendrimer, featuring a ethylenediamine *core* and 16 carboxyl-addressed terminal groups (Figure 1). Preliminary experiments showed highly promising results, with an amplification effect by two orders of magnitude in terms of current recorded upon the same concentration of the CP used to functionalize the electrodic surface. This strategy will be investigated by developing both competitive and non-competitive DNA-assay protocols aimed at specific recognition of transgenic material at trace levels, as possible contaminants of GMO-free foods.

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Electrochemical detection of TNF-α by using a non-immunoglobulin bioreceptor

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TNF-α is an inflammatory cytokine produced by the immune system. Serum TNF-α level is elevated in some pathological states such as septic shock, graft rejection, HIV infection, neurodegenerative diseases, rheumatoid arthritis and cancer. Detecting trace amount of TNF-α is, also, very important for the understanding of tumor biological processes. Detection of this key biomarker is commonly achieved by use of ELISA or cytofluorimetric based methods. In this study the traditional optical detection was replaced by differential pulse voltammetry (DPV) and an affinity molecule, produced by evolutionary approaches, has been tested as capture bioreceptor [1]. This molecule, namely a combinatorial non-immunoglobulin protein (Affibodys) interacts with TNF-α selectively and was here tested in a sandwich assay format. Moreover magnetic beads were used as support for bioreceptor immobilization and screen printed carbon electrodes were used as transducers. TNF-α calibration curve was performed, obtaining the detection limit of 38 pg/mL, the quantification range of 76–5000 pg/mL and RSD%=7. Preliminary results of serum samples analysis were also reported.

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Smartphone-based biosensor for thermochemiluminescent bioassays employing silica functionalized nanoparticles doped with new acridine-1,2-dioxietane derivatives

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We recently demonstrated that thermochemiluminescence (TCL), i.e. the photon emission originating from the thermolysis of a suitable molecule which leads to a singlet excited state product, is a powerful tool for biosensors development [1]. TCL has the unique feature of allowing reagent-less detection, as only a thermal shock is required to trigger light emission from the TCL label, thus simplifying the microfluidic network with respect to chemiluminescence (CL)-based miniaturized biosensors.
We previously synthesized a library of new TCL acridine-1,2-dioxetane derivatives, characterized by emission triggering temperatures down to 80−100°C and yielding highly efficient emitters (fluorescence quantum yields in the range 0.1-0.5) [2]. To obtain signal amplification, organically modified silica nanoparticles (ORMOSIL NPs) doped with TCL molecules were prepared and functionalized with biotin to be used as detection reagents for binding assays. A quantitative non-competitive immunoassay for streptavidin was developed, with analytical performance similar to that obtained with horseradish peroxidase-based CL detection (LOD 3 μg/mL).
As previously demonstrated for CL detection [3], herein we describe a smartphone-based compact 3D-printed device, developed for TCL bioassays, comprising a mini dark box and a Li battery-powered mini-heater.
A personalized App was developed for image processing and data transmission. The device was exploited to develop a competitive TCL immunoassay for the antiepileptic drug valproic acid, employing paper-based analytical format. The method presents a good precision and accuracy, with of LOD of 1 ng/mL.
We thus demonstrate that, for biosensors development, the smartphone is not auxiliary but rather forms the core of the analytical platform.

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ABSTRACT POSTER

A DNA-based Nanomachine For pH-regulated Drug Release

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Here we demonstrate a Nature-inspired approach to rationally design a DNA-based nanomachine that can load and release a molecular cargo at different pHs in a controlled fashion. We first demonstrated that it is possible to engineer allosterically regulated DNA-switches that can be activated or inhibited by different input such as oligonucleotides[4-5], heavy metals ions[3] and transcription factors[2].

By mimicking naturally-occurring mechanism, we propose a general strategy to design pH-regulated DNA-based receptors that can act as a DNA-based nanomachine to achieve controlled release of ligands. Nature, in fact, uses pH-regulated allostery to control the load and release profile of important species such as hemoglobin. This pH-induced allostery is often achieved through the exploitation of hydrogen bonds or other pH-dependent interactions in specific domains of the receptor that can either activate or inhibit its binding capacity. Inspired by naturally occurring pH-regulated receptors, we re-engineered two model DNA-based probes, a molecular beacon and a cocaine-binding aptamer, by introducing in their sequence a pH-dependent domain. We demonstrate that we can finely tune the affinity of these model receptors and, by doing so, finely control the load/release actvity of their specific target molecule by a simple pH change.

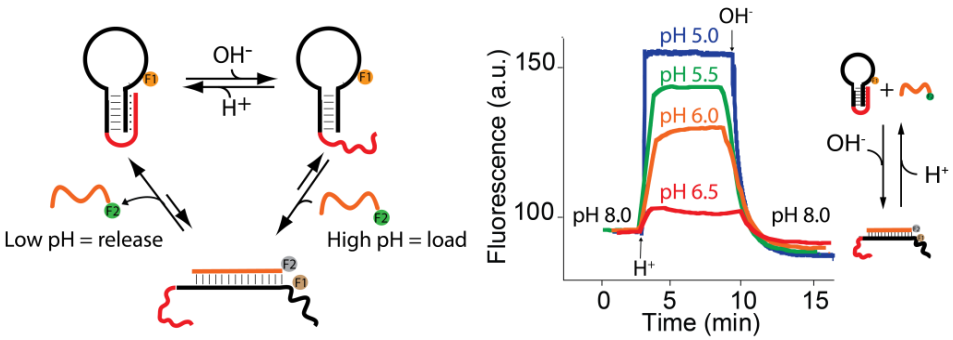

Figura 1: The pH-controlled DNA receptor we have engineered in this work can act as a DNA-based nanomachine that, through pH changes, can reversibly load and release its target in a controlled fashion. By gradually decreasing the pH of the solution from pH 8.0 to pH 6.5, 6.0, 5.5, and 5.0 we can observe a reversible and gradual loading and release of the target from the molecular beacon.

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Development of a p53-specific immunosensor based on carbon nanotubes-gold nanoparticles composite substrate: a perspective for cancer clinic testing

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P53 protein, a potent transcription factor that is activated in response to different stresses and environmental insults, is the most widely studied tumour suppressor. It acts as an important defence mechanism against cancer onset and progression, acting as a 'guardian of the genome'. It can be considered as a biomarker of crucial importance in diagnostic and for the optimization of therapeutic strategies [1]. To date, diagnostic techniques used for its determination require long run times and high costs. A valid alternative approach is represented by biosensors, which are small, portable devices, easy-to-use, and give rapid and quantitative results. The purpose of the present study is the development of a diagnostic system based on disposable nanostructured substrates for the determination of the p53 protein as clinical evidence of cancer [2].

The development of the immuno-device involves the identification of the most appropriate transduction method, the most suitable strategies for the immobilization of p53 on the surface of the biosensor and the best method of detection and quantification of the protein. For this purpose, we devised a method based on the use of electrochemical immunosensors with amperometric transduction, considered the most consolidated and effective technique. Disposable "Screen-Printed" Carbon Electrodes (SPCEs) developed on ceramic substrate were used. The most critical point in the realization of a biosensor lies in the immobilization of the biological sensing element on the electrode surface in order to keep its functionality and biological activity. For this reason, we chose electrodes with working nanocomposite electrode functionalized with carbon nanotubes and gold nanoparticles. (CNT-GNP) [3]. The presence of carbon nanotubes confers porosity, increasing the area available to bind the receptor to working electrode,, while the presence of nanogold involves the formation of covalent bonds by means of chemisorption of the proteins on their surface. Our attention focused on a competitive assay [4] after exploration of various experimental approaches (figure 1).

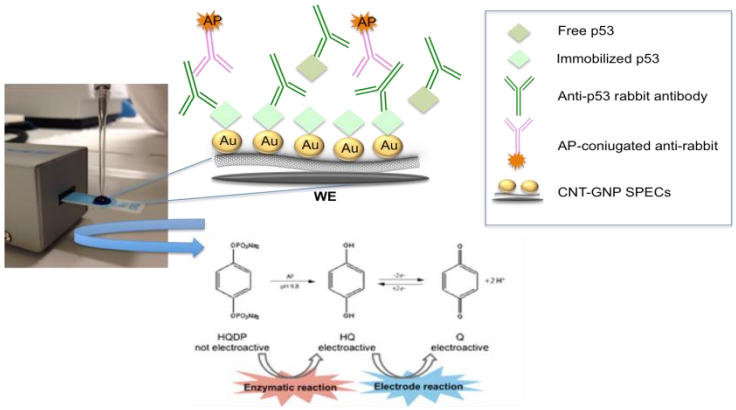

Figure 1: Schematic representation of the working principle and setup of the competitive immunosensor, implemented on disposable CNT/GNP SPCEs funtionalized with p53.

A two-factor three-level experimental design was used to determine the best conditions in terms of signal inhibition. For this purpose, the concentration used for the immobilization of p53 on CNT/GNP-SPCEs was explored over the 0.5-5 ug/mL range, whereas the Ab anti-p53 concentration was varied in the 2.5-10 µg/mL range. Two-way ANOVA results proved that both factors (p53 and Ab anti-p53) and their interaction have a significant effect ($p < 0.05$) on the immunosensor response. According to the ANOVA results, p53 concentration for the functionalization of CNT/GNP-SPCEs was fixed at 5 µg/mL and optimal concentration of Ab anti-p53 was 2.5 µg/mL. The absence of nonspecific responses, as well as the maintenance of the immunoreactivity of the p53 after its chemisorption on the nanotubes/nanogold substrate was verified by "blank" experiments carried out on p53-modified CNT/GNP-SPCEs blocked with BSA (albumin bovine serum) and incubated only with the reading secondary antibody (Rabbit Anti-Mouse IgG, conjugated with Alkaline Phosphatase). Figure 2 shows the inhibition rates recorded over the 10^{-4} –10^{3} µg/mL p53 concentration range, interpolated with a four-parameter logistic function; detection and quantification limits calculated according to Eurachem guidelines, were assessed at 0,1 and 0,4 µg/mL, respectively.

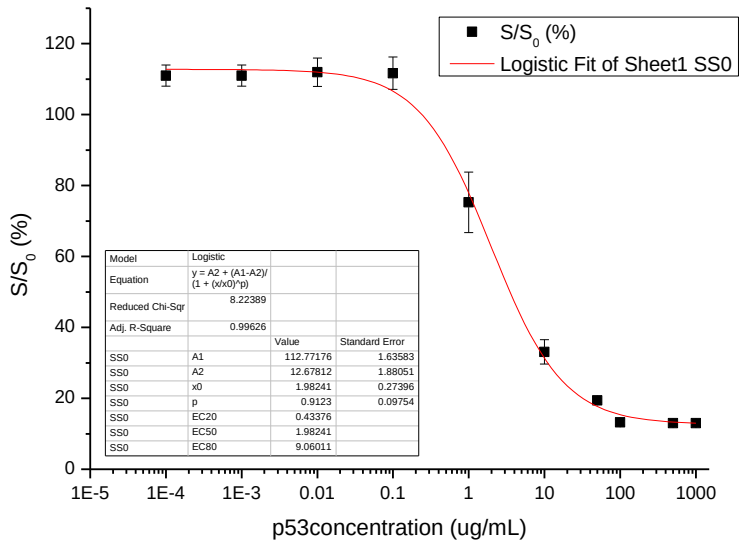

Figure 2: Inhibition curve recorded over the 10^{-4} – 10^{3} ug/mL p53 concentration range.
Inset table: fitted curve parameters.

Studies currently in progress are focused on validation of the device in clinical samples, such as urine, in order to apply the immunosensor as rapid and non-invasive screening tool for early diagnosis of bladder carcinoma.

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Micro-flow immunosensor based on thin-film interdigitated gold array microelectrodes for cancer biomarker detection

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In this work, we reported the development of a micro-flow label-free impedimetric biosensor based on the use of thin-film interdigitated gold array microelectrodes (IDA) for the detection of carbohydrate antigen 125 (CA125) [1]. IDA electrodes presents the advantages of the microelectrodes features increasing the sensitivity and the detection limits and, coupled with micro-fluidic system, allow the development of automatic device with easy data analysis, easy handling of chemicals (solution replacing and washing steps), the use of low volume of solutions which results in an increase of the precision and the accuracy of the measurements. Furthermore, due to the use of micro in-flow cell, both the mass transfer (from the mediator to the electrode surface) and the radial flow are increased which results in an improvement of the electrochemical response. IDA electrodes are also suitable for the use together with electrochemical impedance spectroscopy (EIS): this technique allows the estimation of bioreceptor-antigen affinity reaction without the use of a label which reduce the time and the costs of the analysis. In particular, the immunosensor is developed through the electropolymerization of anthranilic acid (AA) on the surface of IDA electrodes followed by the covalent attachment of anti-CA125 monoclonal antibody. CA125 protein affinity reaction was then evaluated by means of electrochemical impedance spectroscopy (EIS). The sensor was characterized by electrochemical techniques and scanning electron microscopy (SEM). Using the optimized experimental conditions, the developed immunosensor showed a good analytical performance for CA125 detection from 0 to 100 U/mL with estimated limit of detection (LOD = 3Sblank/Slope) of 7 U/mL.

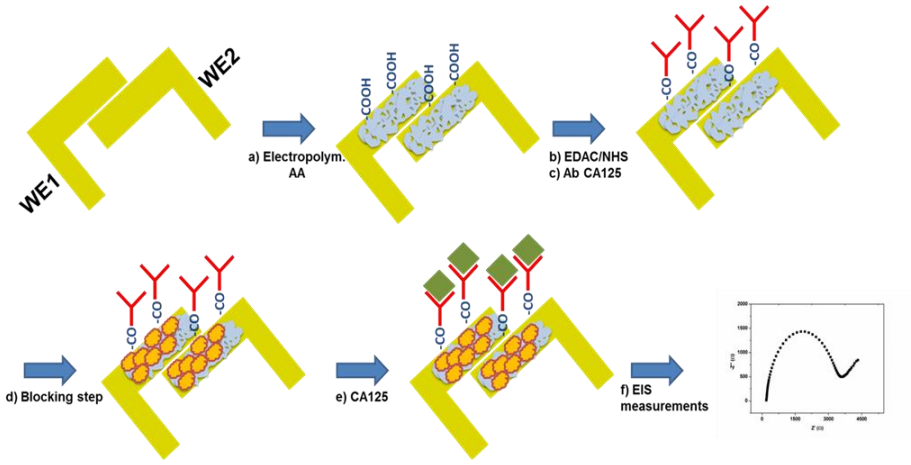

Figura 1: Schematic representation of interdigitated gold array microelectrodes-based micro-flow immunosensor for CA125 determination.

Implementation of a multiresidual analytical approach for the characterization of free fatty acids and ceramides on the skin surface lipidome by HPLC/(-)ESI-ToF-MS and chemometrics

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Skin is a lipid-rich tissue and the largest organ delimiting our body from the external environments. The involvement of skin lipids in the maintenance of the body homeostasis is well documented [1]. Sebum, an amorphous lipid matrix secreted by the sebaceous gland (SG), and epidermal lipids in the stratum corneum (SC), represent the main skin surface lipid compartments. SC has an exceptional lipid composition with ceramides [2], free fatty acids (FFA) [3] and cholesterol [4] as main lipid classes. The SC extends to the entire body surface, whereas SG presents elevated density at the face, scalp, and trunk. In this work, a multiresidual method for the determination of free fatty acids and ceramides was optimized in order to evaluate the influence of the SG on the epidermal lipid arrangement. In 10 healthy adults (8F, 2M) SC has been sampled from forearm, chest, and forehead following sebum removal. Total lipid extracts were profiled by HPLC/(-)ESI-ToF-MS. Detected species were extracted with the molecular features extraction algorithm (Agilent Technologies, USA) and a chemometric approach based on Principal Component Analysis (PCA) was used for processing results of aligned/normalized data. Numerous lipid species emerged as relevant in the discrimination of SC sampled from areas at different SG density. The majority of discriminating species belonged to ceramides, CH derivatives, and FFA. The results indicated that the SG plays a role in the epidermal lipid composition.

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PROPERTIES OF TEMPLATE-ENZYME CONJUGATES TO IMPRINTED SURFACES AND BULK MATERIALS

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Molecularly imprinted materials are often described as "plastibodies", a sort of artificial antibodies which share with natural antibodies the same binding behaviour. It is therefore not surprising that many investigators have thought of using polymers as artificial receptors in immunoassay-like analytical applications, by replacing natural antibodies with artificial receptors in the so-called "molecularly imprinted sorbent assay".[1] Despite the feasibility of such assays has been shown, and their potential efficacy has been demonstrated in many studies, with respect to other fields of application typical of molecular imprinting technology, molecularly imprinted sorbent assay is still in a developmental stage of proof-of-principle, and a certain number of relevant issues remain to be solved.

One of the most relevant issues concerns the generalized use of enzyme-labelled templates as traces in ELISA-like assays. In fact, unlike the binding sites of antibodies that are easily accessible and exhibit antigen induced fit, imprinted materials show narrow porosity and rigidity of the polymer structure. This implies problems of steric hindrance at the entrance of the ligand in the binding sites. As a consequence, binding kinetics can be slow and unfavourable to the development of an assay. More drawbacks are present when the ligand is covalently conjugated to an enzyme tracer. In fact, imprinted materials work well in organic or mixed aqueous/organic solutions but enzymes are sensible to inactivation in such media. Moreover, enzymes are biomacromolecules characterized by slow diffusion in nanometer-sized pores typical of imprinted materials, making impractically slow the assay kinetics. Last but not least, imprinted materials have moderately hydrophobic surfaces, prone to irreversibly adsorb biomacromolecules like enzymes, thus increasing the analytical signal due to non-specific interactions.

To study the binding behaviour of template-enzyme conjugates towards imprinted materials we prepared two types of cortisol-imprinted polymers: (i) imprinted layers grafted onto glass surfaces, and (ii) sub-micron imprinted particles adsorbed onto microtitration plates. The adsorption of unconjugated or cortisol-conjugated horseradish peroxidase was studied, evaluating the effect of the different experimental conditions on the binding of the enzyme, with the ultimate goals to maximize the tracer selective binding and minimize the non-selective binding by the imprinted surfaces.

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Determination of over 60 drugs of abuse in hair by PLE-dLLME extraction and LC-HRMS analysis

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The use of natural substances that can change the perceptives, cognitive, relational and emotional life of a person is common to different ages and cultures. These substances are known as "psychoactive or psychotropic substances", however, over the years, the way they are abused has changed. Nowadays, the abuse of illegal drugs has increased and a lot of new psychoactive substances (NPS) are widespread on the illegal market. More than 500 NPS have been notified in EU since 2005 [1]. Due to their rapid spread and the strong variety in chemical composition, it is not an easy task to keep up with the emergence; in fact, NPS are also a heterogeneous group (e.g. phenethylamines, tryptamines, cathinones and synthetic cannabinoids). The identification of these new drugs is a never-ending challenge for forensic toxicologists.

Blood and urine are the conventional specimens to determine drug exposure; however, in the last twenty years hair testing has gained increasing attention and recognition as a complement to blood and urine analysis. Hair is a unique material for the retrospective detection of drug consumption, due to its large detection window, and it is easy to collect, store and transport [2]. Consequently, hair analysis of illicit drugs and medicines is currently employed to address a wide range of challenges, such as drug abuse history, workplace testing, post-mortem toxicology, therapeutic drug monitoring or drug facilitated assault (DFA) investigations [3].

The extraction of psychoactive substances from the inner of the hair structure is a critical point of the analytical process, and different strategies have been proposed for this purpose. Hair incubation is often performed by digestion of hair matrix with NaOH. Alternative method consists in the incubation of hair with methanol or ethanol for several (4 to 18) hours. Recently, our research group has proposed pressurized liquid extraction (PLE) demonstrating that this technique provides excellent yields in a short time [4,5].

In this work a multiclass method for the determination of several traditional drugs of abuse and NPS in hair has been developed. The analytes include all the most diffused drugs such as cocaine, amphetamines and opiates as well as new drugs belonging to the classes of synthetic cannabinoids, cathinones and phenetylamines. The main metabolites are also included in the method in order to differentiate between passive exposure and effective assumption. The extraction of drugs is based on PLE extraction while the clean-up is carried on by dispersive-liquid liquid microextraction (dLLME). This miniaturized technique uses a very low amount of extraction solvent resulting in considerable enrichment factors for the tested compounds [6]. Several parameters can strongly affect the recoveries of the dLLME procedure and were been extensively studied as showed in figure 1.

The detection was performed by liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS). The use of HRMS as detection technique allows to easily add new analytes in the method with minimal validation steps. In addition, it is possible to re-interrogate retrospectively the already acquired HRMS data for the search of unknown compounds, based on

the determination of the accurate molecular mass; this feature is particularly valuable for NPS analysis in view of their dynamic expansion. The method is being validated according to SWGTOX and SoHT guidelines.

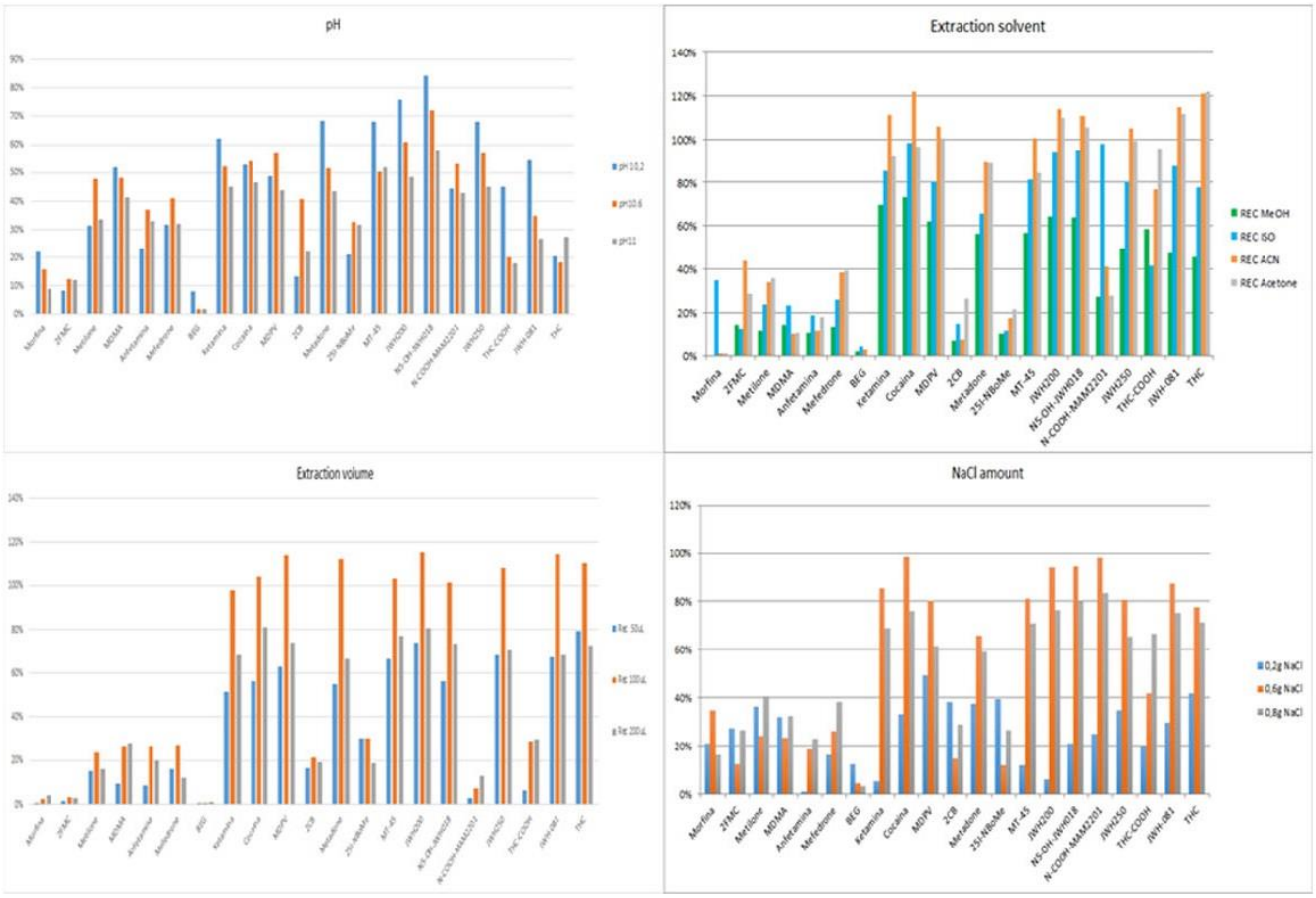

Figure 1: dLLME recoveries for model compounds in different experimental conditions

The developed method allows rapid analysis of a large number of substances. The limits of detection obtained, in the low pg/mg, may allow the detection of single dose drug exposure for some analytes, with possible application in DFA cases. On the other hand the linearity range is wide enough to allow the investigation of chronic exposure to drugs

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Screen-printed electrodes to detect chlorine dioxide in swimming pool water

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Chlorine dioxide is a chemical compound used as a bleaching agent in paper mills and textile industries as well as in the water disinfection.
Chlorine dioxide is also employed in combination with other chlorine compounds, such as sodium hypochlorite in swimming pool water. The use of mixtures of hypochlorite and chlorine dioxide allows a synergistic action, but, in excess of chlorine, there is the formation of chloramines which are irritant compounds for the eyes and mucous membranes. The advantages of chlorine dioxide use rely on the better organoleptic properties of the treated waters compared with hypochlorite and the reduction of organic by-products, such as trihalomethanes (THM) [1].
The contact with a chlorine excess, coming from the decomposition of chlorine dioxide, can cause irritation, blurred vision, cough, sore throat, severe headaches, pulmonary edema and bronchial spasms. For this reason, the allowable concentration is 0.1 mg/l, but it is usually used with other chlorine derivatives and so the amount of free chlorine is measured. The WHO establishes that acceptable levels of free chlorine can be set at the local level, but in public and semi-public pools these should not exceed 3 mg/l and in public/semi-public hot tubs these should not exceed 5 mg/l.
Herein, we report a novel, sensitive, and cost-effective electrochemical sensor for the *in-situ* determination of chlorine dioxide in swimming pool water.
The sensor was fabricated using screen-printed electrodes modified with carbon black (CB), a cost effective and raw nanomaterial with excellent electrocatalytic properties as demonstrated by our research group and corroborated also by other groups, like Compton's group and Pumera's group [2-5].
The working electrode was modified by drop casting method with 2 µl of a carbon black dispersion 1 mg/ml prepared in a mixture of N N-Dimethylformamide/H_2O 1:1 (v/v).
Under optimised conditions (potential of + 0.1 V vs Ag/AgCl and Britton-Robinson buffer 0.02 M + KCl 0.02 M, pH =2 as working solution), the sensor showed a linear response up to 10 ppm (0.989), sensitivity and LOD equal to 13.4 nA/ppm and 0.03 ppm, respectively. The matrix effect as well as the accuracy were tested in swimming pool water, obtaining a satisfactory recovery value (78 ± 8) %.

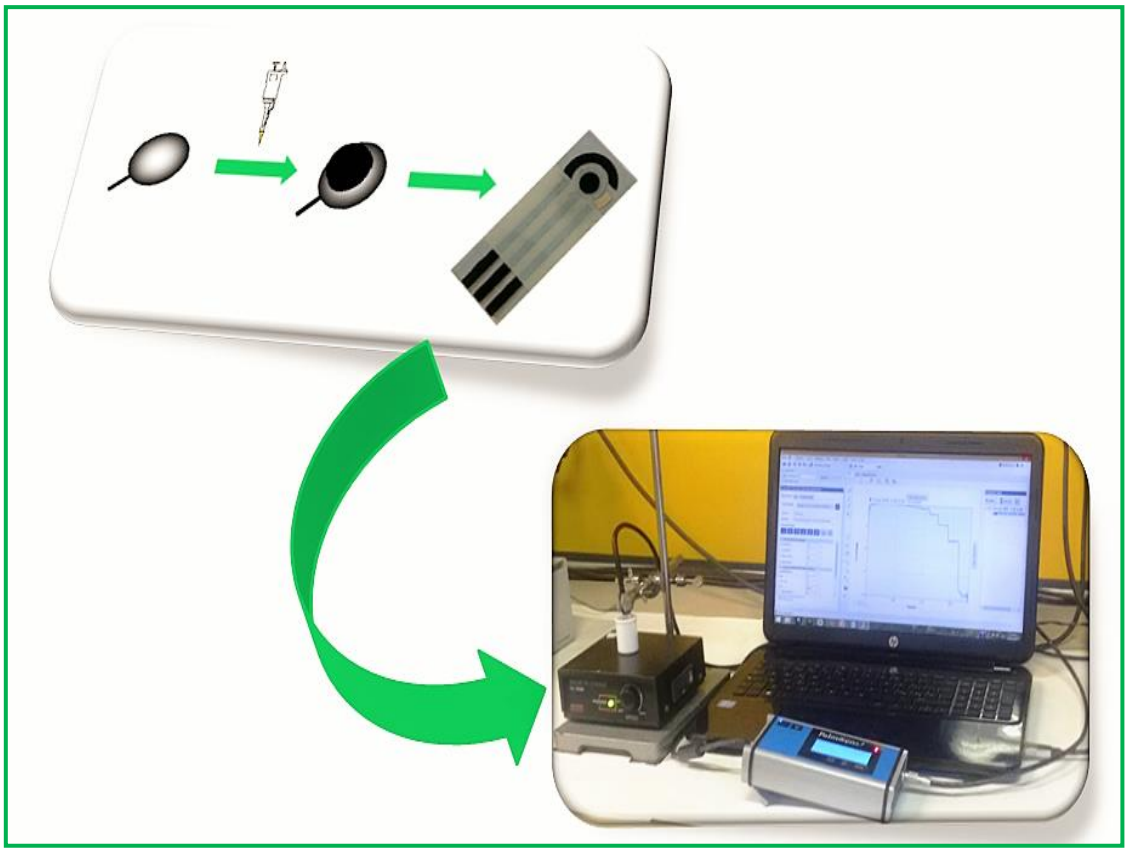

Figure 1: Developed electrochemical system for the chlorine dioxide detection

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Functionalized TiO_2 as an enzyme mimicking signal generating probe for immunochemical tests

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Immunochemical methods of analysis are widely used in many applications, including clinical, veterinary, environmental, forensic investigation, and food safety assessment, due to their sensitivity and selectivity, combined with rapidity, cost-effectiveness, and simplicity. In most cases, immunoanalytical techniques require a probe that generates a signal upon immunocomplex formation. The first immunoassay probes were radioisotopes; however, they have been almost completely replaced with other probes, such as fluorophores, nanoparticles, and primarily, enzymes. Enzymes owe their popularity as probes for immunoassays to the huge amplification of the signal obtained through their catalytic activity, and to the versatility of the detection afforded by the variety of available substrates. Still, enzymes are limited by their stability as a function of several parameters, such as temperature, pH, ionic strength, and the presence of inhibitors [2]. Thus, to widen the applicability of immunoassays and, more in general, to increase their ruggedness, more robust probes need to be developed.

In this context, we designed robust inorganic probes based on TiO_2 nanoparticles (NP) and graphene-TiO_2 NP hybrid materials as versatile labels for developing immunological tests. Similarly to enzymatic probes traditionally used in immunoassay, the TiO_2-based materials could be exploited as signal generating probes, as they could be covalently linked to antigens or antibodies through the superficial modification of the TiO_2 NP or by exploiting graphene nanoplatelets modified with carboxylic groups (GNP). Both materials allowed revealing the rate of antigen-antibody complex formation by exploiting the photoactivity of the TiO_2 which promoted the oxidation of a suitable chromogenic substrate with absorption in the Vis region. We studied the catalytic activity of the functionalized TiO_2 NP and of the GNP-TiO_2 hybrid material conjugated to a model antigen (bovine serum albumin, BSA) under UV irradiation. Oxidation of the substrate showed a clear dependence on H_2O_2 concentration that confirmed the peroxidase-like activity of the inorganic probes [2]. In particular, the conversion rate obtained by the TiO_2 NP catalyst was sufficient to achieve signals comparable to those usually observable with enzymatic probes, by employing the same substrate and a simple colorimetric detection. At the same time, we observed that the TiO_2 NP probe did not prevent the formation of complexes between the antigen conjugated to the probe and its specific antibodies anchored to the wells of a standard microtitre plate.

Instead, contrarily to natural probes, the modified TiO_2 preserved almost identical activity in acidic, neutral and alkaline conditions and could be stored for 14 days at room temperature without any detrimental effect on its catalytic ability. Also, the TiO_2-based probes resulted unaffected by thermal treatments (i.e.: 50°C for 1 hour).

To prove definitely the applicability of TiO_2 NP as probes for developing effectively working immunoassays, we developed a direct competitive assay for measuring the urinary content of human serum albumin for diabetes diagnosis. The optimized TiO_2-based assay showed high sensitivity (detection limit 1.4 mg l^{-1}), wide dynamic range (6-1270 mg l^{-1}) and acceptable precision

(within- and between-assay coefficient of variation <20%) and accuracy (75-95% compared to a reference turbidimetric assay). The assay is fully compatible with materials and equipment of standard ELISA (except for the need of UV irradiation), and the overall time required to complete the analysis is comparable to usual immunoassays based on enzymatic probes, while the inorganic probe is more robust towards chemical and physical conditions and shows better long term stability compared to enzymes.

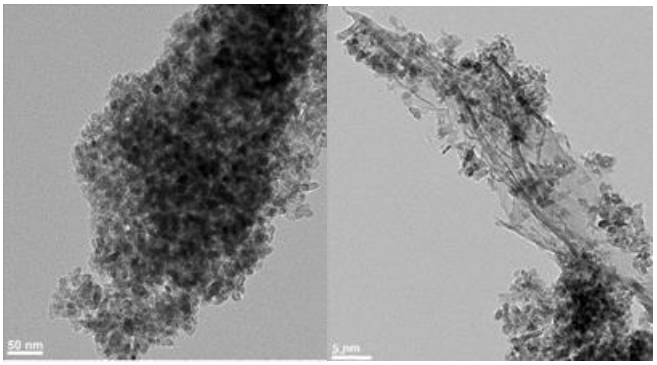

Figure 1: TEM micrographs for TiO_2 NP (left) and GNP-TiO_2 (right)

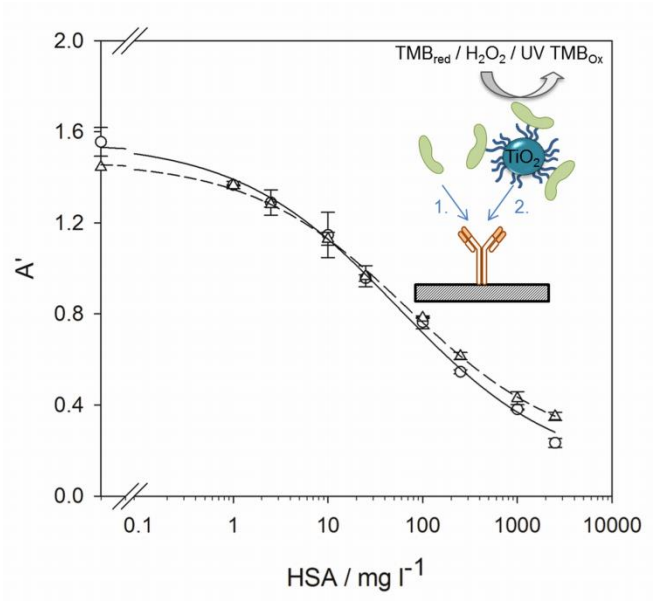

Figure 2: Typical inhibition curves for the direct competitive immunoassay for HSA, in which HSA calibrators were prepared in PB (open circles, ○) and artificial urine (open triangles, △). The assay involved two steps: first, calibrators or samples were allowed to react into immunoreactive wells for 30 min, then unbound components were removed and the HSA-TiO_2 tracer added. It was allowed to react for further 30 min, followed by color development. The absorbance of oxidized TMB was recorded and plotted against HSA concentrations, after subtracting the signal due to the photo-oxidation of TMB (A').

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Development of an impedimetric immunosensor for D-Dimer detection in blood serum

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Cardiovascular diseases like acute coronary thrombosis (ACT) or acute myocardial infarction (AMI) are considered as a major threat to global health and today the development of portable, low cost, rapid biosensing devices for their detection are considered as necessary.
During cardiovascular diseases, many biomarkers such as Myoglobin, Troponin (cTnI/T) or D-dimer etc., significantly increases their levels in blood.
For this reason, we developed a rapid and sensitive method for detection and quantification of D-dimer in serum; this biomarker is a protein of 180 kDa and its physiological level is lower than 500 µg/L. A higher D-dimer level indicates the presence of abnormally increased fibrin degradation products that may results in clot formation in blood.
We developed an impedimetric immunosensor utilizing screen-printed gold electrodes (AuSPEs) for the detection of D-dimer. and the electrochemical impedance spectroscopy (EIS) as detection technique. The detection is based on increase of electron transfer resistance (Rct) in presence of the target. In details, this label free impedimetric immunosensor was fabricated immobilising a self assembled monolayer of 11-mercaptoundecanoic acid onto treated gold electrode, followed by blockage step with 6-mercaptohexanol and reaction with 1-Ethyl-3-(3-methylaminopropyl) carbodiimmide (EDC), N-hydroxysuccinimide (NHS) to immobilise the antibody for D-dimer onto the working electrode surface as schematised in Figure 1.
In this study, every modification step of the electrode surface was optimized, such as the most suitable immobilization time (2 hour) for thiols, the choice of different blocking solutions (dry milk 0.5% w/v, the best antibody amount to be immobilized (50 µg/ml), and, finally, several antigen concentrations were analyzed.
Preliminary results show that this immunosensor is able to detect physiological levels of D-dimer after an incubation time of 10 minutes.

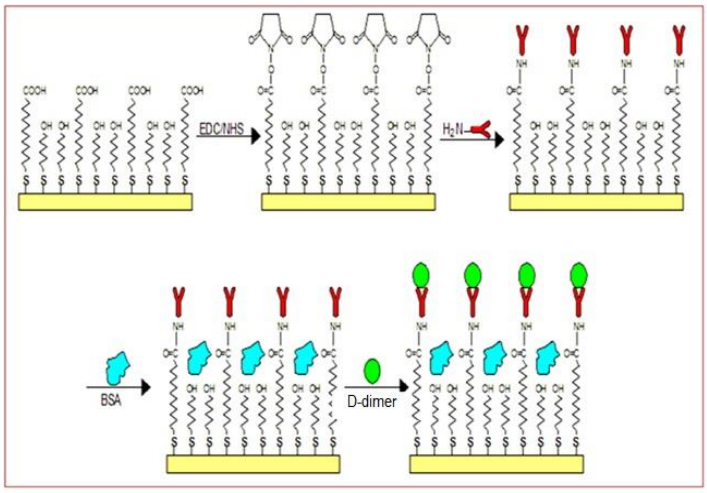

Figure 1: Scheme of the developed immunosensor.

Applicability of polydopamine coated magnetic nanoparticles for the extraction of natural estrogenic compounds from water

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The widespread presence in the environment of endocrine disrupting compounds (EDCs) and the risk associated with their ability to interfere with the hormonal systems and/or block the action of natural hormones of organisms [1] has led to an increasing attention towards these compounds, in particular those with estrogenic activity [2].
Natural estrogen hormones are 17β-estradiol (β-E2), 17α-estradiol (α-E2), estrone (E1), and estriol (E3). However, there are other naturally occurring compounds possessing a certain degree of estrogenic activity, such as phytoestrogens and mycoestrogens.
It has been reported that estrogens, phytoestrogens and mycoestrogens may entry into the surface waters from different sources, such as effluents of municipal wastewater treatment plants, industrial plants and animal farms, and runoff from agricultural areas. Indeed, several studies suggested that also phytoestrogens and mycoestrogens, though exerting much lower estrogenicity than that of natural hormones, could contribute to the estrogenic activity detected in surface water samples [3]. Therefore, analytical strategies able to extract a large number of compounds possessing estrogenic activity from water could be useful to estimate the total exposure to these EDCs.
A multiresidue analytical method for the determination of seventeen natural estrogenic compounds, including four steroid estrogens, six mycoestrogens and seven phytoestrogens, in river water samples has been developed. (Fe_3O_4)-based magnetic nanoparticles coated by polydopamine (Fe_3O_4@pDA) were used for dispersive solid phase extraction, and the final extract was analyzed by ultra-high performance liquid chromatography coupled with tandem mass spectrometry with negative electrospray ionization (ESI).
The Fe_3O_4@pDA magnetic nanoparticles were prepared by a co-precipation procedure. After that, dopamine was added to magnetic nanoparticles and allowed to polymerize *in situ*, on their surface, adjusting the pH to 8.5 and leaving the reactions overnight under magnetic stirring at room temperature. The prepared Fe_3O_4@pDA nanoparticles were finally characterized by scanning electron microscopy, infrared spectroscopy and elemental analysis.
The sample preparation method was optimized in terms of extraction recovery, matrix effect, selectivity, trueness, precision, method limits of detection and quantification (MLOQs). For all the seventeen analytes, recoveries were >70% and matrix effects were below 30% when 25 mL of river water sample were treated with 90 mg of Fe_3O_4@pDA nanoparticles. Selectivity was tested by spiking river water samples with other fifty compounds (mycotoxins, antibacterials, conjugated hormones, UV filters, alkylphenols, etc.); only aflatoxins and some benzophenones showed recoveries >60%. Moreover, the in situ polymerization of pDA on MNP core has been shown to be facile and reproducible from batch to batch.
We tested the applicability of dispersive magnetic SPE on milk samples. However, results showed that the performance of this system is unsatisfying in a complex matrix. In particular, matrix effect

(signal suppression observed in ESI) dramatically increased changing the matrix from river water to milk.

In this work, we have proved the ability of pDA to selectively capture estrogens and structurally similar compounds, i.e., mycoestrogens and phytoestrogens, from river water samples. Even if the importance of phytoestrogens as environmental contaminants has been reported, however, generally these substances are not included in multi-residue methods for estrogenic compounds. Therefore, in this work, we wanted to fill this gap using magnetic SPE for the simultaneous determination of these three groups of estrogens in river water. Moreover, the obtained MLODs and MLOQs are lower than those reported in the literature for estrogens and mycoestrogens using the same pDA-coated MNPs.

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Mustard Agents detection using a nanomodified electrochemical biosensor

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Blister or mustard agents (MAs) are one of the most common Chemical Weapon Agents, and are defined as blistering compounds owing to the similarity of the lesions caused by these substances to burns and blisters. MAs compounds are able to generate severe toxic effects on living organisms and to persistently contaminate soils and water. They embrace sulphur mustards, such as Yperite (bis(2-chloroethyl)sulphide), and nitrogen mustards, such as HN1 (bis(2-chloroethyl)ethylamine), HN2 or Mustine (bis(2-chloroethyl)methylamine), and organic arsenical Lewisite (dichloro(2-chlorovinyl)-arsine). During the war between Iran and Iraq in 1979–88, Iraq used large quantities of chemical agents, leading to about 5000 Iranian soldiers killed, 10–20 % by mustard agents, and 40000 to 50000 injured. Moreover, the dumping of chemical weapons in the waters off the Danish and Swedish coasts, dating back to the Second World War, are still nowadays causing incidents, which mainly involve fishermen accidentally exposed to mustard agents brought to the surface by fishing nets.

Classical analytical techniques such as Gas Chromatography (GC) and Liquid Chromatography (LC), also combined with Mass Spectrometry (MS), are employed to detect and quantify these compounds. Nevertheless, they require complex and expensive instrumentation, a laboratory-setup and qualified personnel, which make them unsuitable for in-field analysis. Biosensors represent an important alternative characterised by sensitivity, selectivity, and the possibility to realise portable, easy-to-use, and cost-effective analytical devices. Furthermore, they offer the advantage of giving a fast response, which is critical for an alarm system in case of chemical attack. Recently, we have demonstrated the capability of nitrogen mustard simulant bis(2-chloroethyl)amine and the sulfur mustard simulants 2-chloroethyl ethyl sulfide and 2-chloroethyl phenyl sulfide to inhibit the choline oxidase enzyme.[1] Herein, we present the preliminary results obtained in the development of an amperometric biosensor based on choline oxidase enzyme immobilised onto the working electrode surface of screen-printed electrode (SPE). To monitor the enzymatic activity, choline oxidase was used as substrate and the enzymatic by-product hydrogen peroxide was detected at low applied potential (-50 mV vs Ag/AgCl) using SPE modified by drop casting with a dispersion of Carbon Black and Prussian Blue nanoparticles hybrid nanocomposite , which confers to the sensor good analytical performances in terms of limit of detection and sensitivity.[2] The choline oxidase was immobilised by means of glutaraldehyde, Nafion, and Bovine Serum Albumine. The sensibility towards the choline as well as nitrogen and sulphur mostard simulants was assessed.

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Use of multidimensional liquid chromatography to purify and identify endogenous bioactive peptides from donkey milk

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Donkey milk is valuable product for the food industry, and nowadays it is considered a "pharmafood" for its nutraceutical, nutritional and functional properties [1]. Donkey milk strongly resembles human milk because it contains similar amounts of lactose and minerals and similar fatty acid and protein profile; for these reasons, it is considered the most suitable mammalian milk for infant consumption, especially in case of cow milk allergy.
In the last years, interesting biological activities, such as anti-inflammatory, antiallergic, antimicrobial, antioxidant, immunological properties related to the prevention of atherosclerosis, antiviral activity and the antiproliferative effect on A549 human lung cancer cells were ascribed to donkey milk [2]. In this work, a combination of consecutive chromatographic separations, including reversed phase liquid chromatography (RP-LC) and hydrophilic interaction chromatography (HILIC), was used to purify endogenous peptides in donkey milk. The most active fractions, with the highest antioxidant (AO) and angiotensin-converting enzyme (ACE)-inhibitory properties obtained from second chromatographic dimension, were further analyzed by RP-nanoHPLC with direct injection into a Orbitrap mass spectrometer for peptide sequencing. In silico analysis using Peptide Ranker was then employed to ascribe a bioactivity rank to each peptide. Finally, on the basis on the composition and the probability as calculated by Peptide Ranker algorithm, five peptides were selected and synthetized, and their AO and ACE-inhibitory activities were determined by in vitro bioassays. Finally, for further confirmation, the synthetized peptides were compared to the natural occurring ones checking their retention times and fragmentation patterns of the five selected peptides in donkey milk alone and donkey milk with spike-in peptides.

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Analysis of DNA adducts with activated PAHs by means of MALDI-TOF/MS

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Polycyclic aromatic hydrocarbons (PAHs) are a group of structurally related chemicals produced during a variety of combustion processes [1] and characterized by unusual chemical stability. These ubiquitous environmental pollutants constitute an important class of carcinogenic compounds [2–5] because they can bioaccumulate and then their electrophilic derivatives (i.e. dihydrodiol epoxide metabolites) have been proved to interact with DNA by covalent bonding with the bases adenosine (Ado) and guanosine (Guo). Covalent modification of DNA by a chemical has been demonstrated to be the initial step in chemical carcinogenesis. The presence of adducts can thus be an useful biomarker both in cancer risk assessment [6] and monitoring of environmental and occupational exposure to chemical hazards [7].
Recently, different studies have been conducted also on the toxicity of PAHs exposed to UV and visible light: the photodegradation products have been demonstrated to be mutagenic and to induce DNA damage [8]. This has been hypothesized to occur indirectly, via reactive oxygen species (ROS), or directly, via formation of adducts between some of the photooxidized products of PAHs and DNA.
Detection of DNA adducts is, however, a rather complex matter, because of the low frequency of DNA adduction that occurs in vivo; in fact, techniques that can detect down to 10 pg adducts on 1 mg DNA are required. Mass spectrometric (MS) detection can be an useful tool for the identification of these adducts [9]: most of these studies have been carried out using benzo[a]pyrene (B[a]P) and its derivatives, as model compounds; in fact, B[a]P is known to be the most widespread and probably the most carcinogenic compound among the PAH. In particular, LC–MS–MS has been proved to address sensitivity and specificity issues in the analysis of DNA adducts [10].
In this work, a procedure for detection of activated PAH–oligonucleotide adducts using MALDI-TOF/MS is reported. In the first part of the work, a procedure for detection of the adducts was developed using B[a]P-dihydrodiol epoxide (B[a]PDE) as model compound. B[a]PDE is the major metabolite of B[a]P and its ability to bind DNA is well known. The structures of adducts of B[a]PDE with guanosine and adenosine have been identified and characterized, as well as the adducts with oligonucleotides (GGGG and CCCC).
The developed MALDI-TOF/MS procedure enables rapid, sensitive, and selective analysis of B[a]PDE–nucleosides making possible their detection directly in the reaction mixture without isolation of the adducts and with minimum clean-up. Moreover, it can be potentially applied to any compound forming adducts with nucleosides. In order to prove this concept the second part of the work was devoted to identification and characterization of photodegradation products of B[a]P and to detection of the formation of stable adducts with oligonucleotides under the experimental conditions.
For this purpose different MALDI matrices were tested in order to achieve best sensitivity and specificity due to effective ionization and less interfering peaks: 2',4',6'-Trihydroxyacetophenone monohydrate (THAP) was chosen for its ability to promote oligonucleotide ionization. It was dissolved in a water/acetonitrile solution (75/25 or 50/50) added with formic acid 0.1%.
Photooxidation was carried out using a low-pressure (LP) Hg vapour UV lamp placing 100-µL aliquot of 500 ppm B[a]P in acetonitrile, irradiated for 24 h (1 max 365 nm) at room temperature.

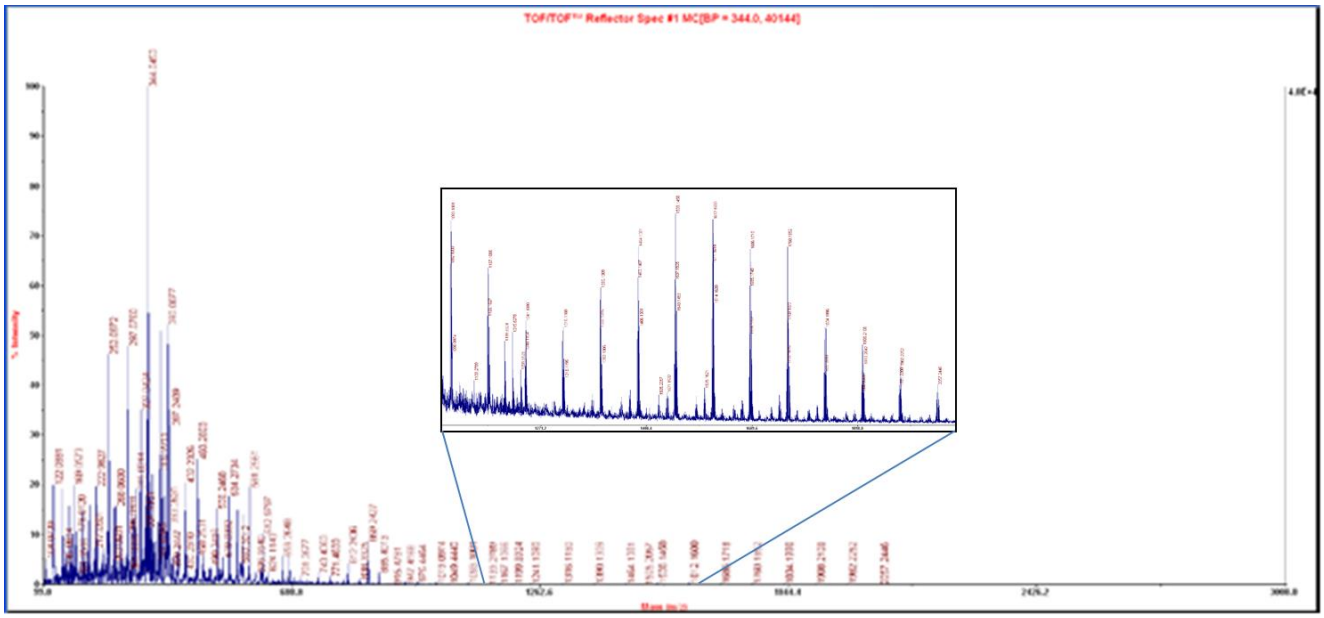

Figure 1: *MALDI-TOF spectrum of a mixture containing GGGG and activated BaP with some adducts highlighted in the zoomed box.*

The use of this powerful technique enables adduct detection without previous isolation of the analytes in complex mixtures, as was apparent from the minimal clean-up carried out on the synthesized adducts. Therefore MALDI-TOF/MS seems to have significant potential for detection and characterization of DNA adducts formed with compounds different from PAHs.
The results reported here represent a further evidence of the ability of some photoactivated compounds to bind DNA, pointing out their genotoxicity.

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Acknowledgements: Project IZS ME 07/13 RC - Valutazione del rischio chimico tossicologico degli integratori alimentari connesso alla presenza di idrocarburi policiclici aromatici IPA.

A mini apparatus for surface plasmon resonance (SPR) and localized surface plasmon resonance (LSPR) sensors on D-shaped plastic optical fiber

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Surface plasmon resonance (SPR) is a sensitive techniques for determining small refractive index (RI) changes at the interface between a metal and a dielectric medium. This can be an effective and label free way of signal transduction for the combination of a receptor with a substrate, when an RI variation is induced by the combination. Actually the SPR based methods are widely employed in the sensor technology, in association with bio and chemo receptors.
The localized surface plasmon resonance methods (LSPR) are based on the plasmon resonance at nano metal surfaces. Metal nanoparticles (NPs) are often implemented to enhance the SPR response, which has been previously ascribed to different effects [1].
The Kretschman configuration is most often employed for the transduction of the signal, obtaining either wavelength or angular resonance spectra. A large number of instruments based on this configuration are commercially available, but generally they are bulky and expensive, so that they are hardly suitable for the development of sensors of practical utility.
Recently, the implementation of optical fibers in SPR platforms made it possible a noticeable reduction of the dimension and the cost of this kind of optical label-free transduction, in particular when plastic optical fibers are employed [2, 3]. The very small and low cost experimental set-up illustrated in Fig.1 has been demonstrated to be effective for sensor technology. The aim of the present work is to compare the SPR and LSPR transduction in the case of the particular experimental set-up presented in Fig 1.
It employs an halogen lamp (Model no. HL-2000-LL, manufactured by Ocean Optics) with a wavelength emission range from 360 nm to 1700 nm, while the spectrum analyzer detection range was from 200 nm to 850 nm. A small Ocean Optics "USB2000 + UV-VIS" spectrometer was used.
Particularly interesting for sensor application is the shape of the POF, which presents a D profile with a flat surface, and is obtained in a simple and reproducible way, starting from a usual multimodal POF (960 μm diameter). The flat surface makes the deposition of the receptor relatively easy both in the case of natural receptors as proteins [2] and of polymeric layers as molecularly imprinted polymers (MIP) [3]. Fig 1B and 1C illustrate schematically the SPR and LSPR platform concept. In the first case the surface plasmons are excited at the large surface of a gold layer interposed between the optical fiber and the dielectric, while in the second case at the nano surface of gold nano stars, shown in Fig 1, embedded in the MIP layer over the optical fiber.

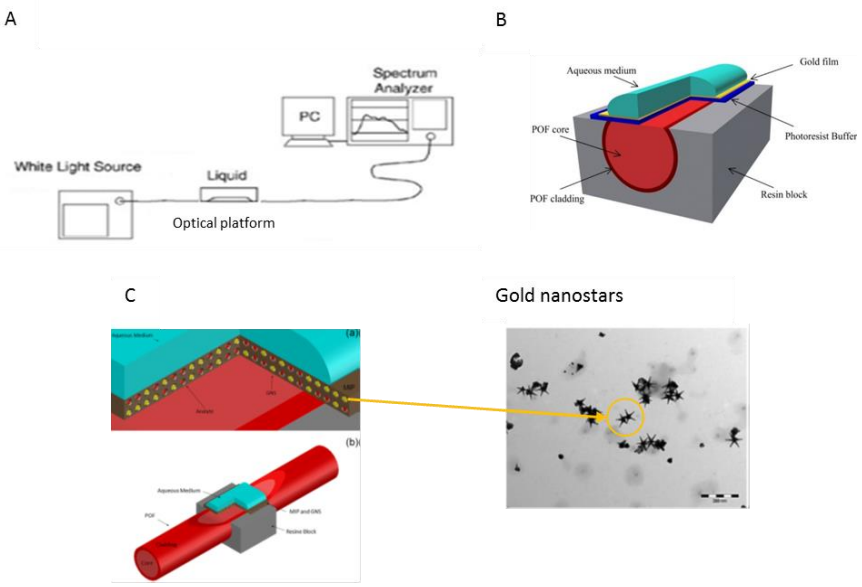

Figure 1: SPR and LSPR sensors based on D-shaped optical fiber. A) experimental set-up. B) schematic view of the SPR platform with thin gold layer at the interface POF/dielectric. C) schematic view of the LSPR platform with gold nanostars dispersed the dielectric (MIP).

SPR and LSPR platforms show very different surface plasmon resonance, and dependence from RI in the range from 1.33 (pure water) to 1.40.

This has been investigated by measuring the resonance wavelength shift in function of the RI of a liquid placed as a drop over the bare sensor chip, i.e. without the receptor layer. In the case of LSPR sensor the gold nanostructures were placed directly over the core of the optical fiber, which is a structure not exactly similar to that of the nanoparticles embedded in the MIP. However could give indications about the possibility of using this particular kind of metallic nanostructures. The dips in the transmittance wavelength spectrum from 450 to 800 nm, obtained in water as the dielectric medium, are at about 620 nm for the SPR sensor and at 718 nm for the LSPR sensor.

They are both shifted to higher wavelengths at increasing RI of the liquid in contact, with a linear dependence for small RI ranges variations.

To investigate the behavior of the two proposed sensors in the presence of MIP as a specific receptor, as shown in Fig 1, we compared the results obtained in the case of two sensors for trinitrotoluene (TNT), with the same specific MIP as the receptor, and based respectively on SPR and LSPR. The resonance wavelengths are at 757 nm for the SPR sensor and at 495 nm for the LSPR sensor. These resonance wavelengths are both shifted to higher values at increasing concentrations of TNT, indicating that the adsorption of the analyte on the MIP induces an increase of its RI. Notice the large shift of the resonance wavelength of the SPR sensor, which clearly support the hypothesis that the RI of the MIP is noticeably higher than that of water.

The sensitivity of the two platforms, i.e. that with a thin gold layer (50 nm thick) and that with gold nanoparticles embedded in MIP, toward TNT in aqueous solution is respectively 2.7 10^4 nm/M and 8.5 10^4 nm/M, with a 3-times enhancement in the case of LSPR based sensor.

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SMARTPHONE-BASED BIOSENSOR INTEGRATING BIOLUMINESCENT "SENTINEL CELLS" FOR ENVIROMENTAL APPLICATIONS

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Global security threats have become a major concern and their early detection represents a major challenge to current monitoring technologies. The routine monitoring of water, food and the environment for chemical and biological threat agents is often hampered by the fact that available techniques usually require clean samples and sophisticated equipment based on high performance liquid chromatography-tandem mass spectrometry and are thus unsuitable for real-time, cost-effective and on-field routine monitoring. We report a compact stand-alone toxicity sensor incorporating bioluminescent cells into a smartphone-based device. We fabricated 3D printed cartridges to integrate an array of bioluminescent cells into ready-to-use cartridges and smartphone adaptor. We used human embryonic kidney cells (Hek293T) constitutively expressing a green-emitting luciferase as "sentinel cells" and an Android app was developed to provide a user-friendly built-it data analysis. To confirm the suitability of this approach the toxicity test showed performance comparable to that obtained using portable cooled CCD camera. The analytical performance of the smartphone-biosensor was evaluated with model and real samples [1]. We also explored the feasibility of using as reporter genes both Nanoluc and its destabilized version NlucP; dose-response curves for Tumor Necrosis Factor α, used as pro-inflammatory analyte, showed the same limit of detection (0.4±0.1 ng/ml) and an EC50 of 1.3±0.4 ng/ml and 1.7±0.2 ng/ml for Nluc and NlucP, respectively. Conscious that huge efforts will be required to extend the lifespan of the integrated cells without affecting the analytical performance of the system, we believe that it could find significant application as rapid alerting tool, suitable for detecting the presence of harmful pollutants in civil and military water supplies, for terrorism surveillance, and for detection of health threats in drinking water in developing countries.

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A new sensitive and quantitative chemiluminescent assay to monitor intracellular xanthine oxidase activity for rapid screening of inhibitors in living endothelial cells

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Xanthine oxidase (XO), an important enzyme expressed at high levels in the vasculature in endothelial cells, catalyses the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid. Excessive production of uric acid results in hyperuricemia linked to gout and cardiovascular diseases [1]. Testing inhibition of XO is important for detection of potentially effective drugs or natural product that could be used to treat diseases caused by an excessive uric acid production. Several methods to detect selectively XO activity are reported in literature but the majority of them use cell-free systems [2] or lysed/scraped cells [3][4]. In the present study, for the first time we present an *in vitro* chemiluminescent (CL) bioassay to determine XO activity in living endothelial cells and the IC50 value of oxypurinol, the active metabolite of the standard inhibitor drug allopurinol. As proof of principle, intracellular XO activity was measured in less than 30 minutes with a luminol/catalyst-based CL assay able to measure XO [(6±1)*10-7 mU/ml cell] with detection limit of 0.4 μU/mL and quantification limit of 1.3 μU/mL. Oxypurinol addition (ranging from 5.0 to 0.0 μM) caused a linear decrease in luminol-based CL intensity, with an IC50 of 1.0 ± 0.5 μM.

Next, to demonstrate the applicability of the CL cell-based assay for intracellular XO inhibition on real samples, endothelial cells were treated with extracts of two fungi, *Ganoderma Lucidum* and *Cordyceps Sinensis*, to which are attributed several therapeutic properties (from anti-cancer activity to cardiovascular diseases prevention) [5][6]. In literature, the data relating to the chemical and pharmacological behaviour of these two fungi are few and in any case, have been carried out with old and outdated analytical methods. Once verified that the two families of fungi are not able to interfere with the CL detection reaction, their effects on XO activity were evaluated in cells (fungi concentrations ranging from 0.2 to 0.0 mg/ml), determining a decrease in CL signal intensity, with an IC50 of 28 ± 4 μg/ml and of 14 ± 3 μg/ml for *Ganoderma Lucidum* and *Cordyceps Sinesis*, respectively. The detection system developed is low-cost, rapid, reproducible and could be used on small quantities of cells (3.500 cells).

Since the bioavailability of the compounds, especially the ability to cross cell plasma membranes, is an important issue to take into consideration, this method, utilising whole cells, is more representative and predictive to study XO inhibitors *in vitro*.

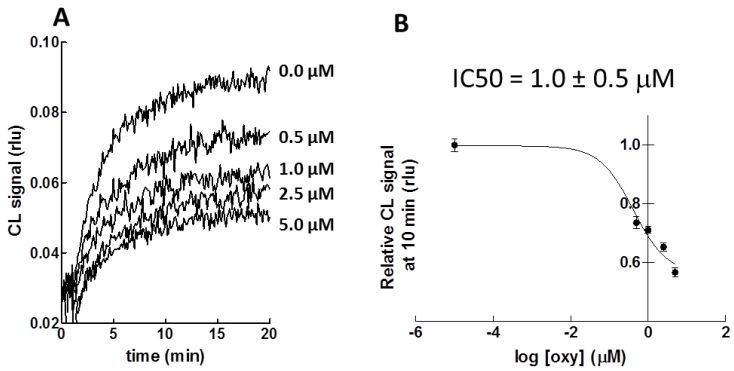

Figura 1. A. CL kinetic profiles of the intracellular light emission (HUVECs, $5x10^3$ cells/well) in presence of different concentrations of oxypurinol (range 5.0 – 0.5 µM). **B.** Concentration-Response plot of intracellular XO inhibition obtained by analyzing CL signals after 10 minutes of incubation in presence of different concentrations of oxypurinol (range 5.0 – 0.5 µM).

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Flow field-flow fractionation based approach as analytical tool for nanomaterial selection and application in healthcare

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In a world where silver nanoparticles (AgNPs) are more and more employed in various aspects of life, either as part of textiles [1] , or as drug carriers [2] , additives [3] or medical devices [4] , it is important to determine their properties and to understand to what extent –and in which way- such materials in the nanoscale form interact with living organisms and the environment. The access to suitable analytical tools for characterisation is of primary importance. The assessment cannot be limited to an accurate description of the newly composed material, but needs to be equally reliable when considering the modified state of AgNPs, which undergo changes in composition, size, shape and core-shell properties when in the exposure medium (biological testing or environment).[5] These modifications in fact influence the overall toxicity of engineered nanoparticles and can cause adverse effects upon exposure.[6]

Currently employed characterization techniques like transmission electron microscopy (TEM) and dynamic light scattering (DLS) present various limitations: batch analyses in DLS cannot provide information about the shape of the particles but only their hydrodynamic radius, while TEM analyses requires manipulation (drying) of the sample and is not representative of a suspension. The introduction of an in-flow separation technique as a characterisation step can both provide reliable data regarding samples in suspension and provide collectable fractions to be individually characterised/tested.

The aim of this work is to analytically distinguish among the colloidal properties of differently coated silver nanoparticles (AgNPs) when dispersed in aqueous media. In this study we will present a dataset based on their physicochemical properties, in order to provide the selection criteria for ranking the best performing material for healthcare applications.

By exploiting the coupling of a soft separation technique (hollow fiber flow field flow fractionation, HF5) with various detectors online and offline, we determined size, shape, surface charge and ionic release of the particles at the native state. This is a non-destructive method, allowing for the collection of filtered nanoparticles that can be therefore tested individually to investigate particle-specific activity. Outcomes can be the introduction of HF5-based characterization as part of protocols to investigate the component-specific hazard of a nanoparticles; the Flow Field-Flow Fractionation characterisation approach is also proposed as a tool to establish the most relevant parameters to consider when developing new metal-based materials in order to accelerate candidate screening.

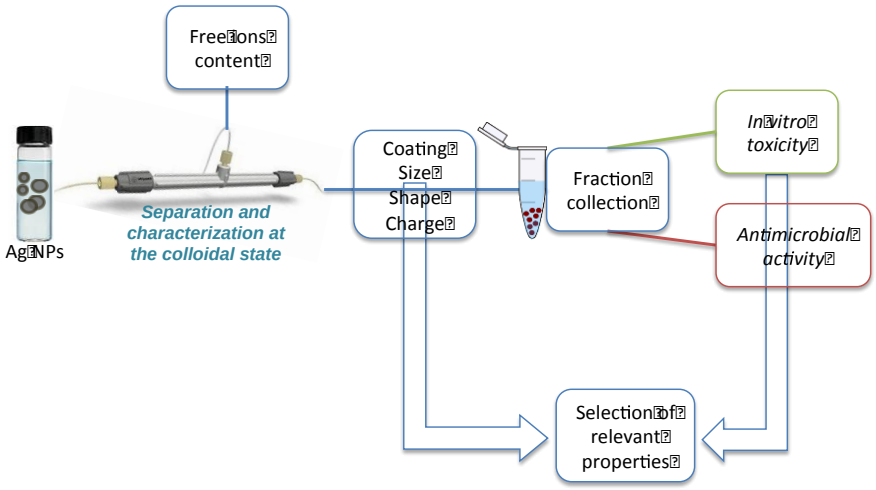

Figure 1: Schematic representation of the HF5-based analytical approach used to investigate AgNPs properties and activity

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Chemiluminescence origami paper-based biosensor for glucose

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The development of Point-of-Care (POC) analytical devices for diagnostic applications is an ever growing field of research. In this context, paper-based microfluidics is emerging as a very promising approach for the development of disposable, easy-to-use, rapid and cost-effective devices, while the exploitation of the origami concept allows convenient translation of multistep analytical procedures.

We have developed a portable paper-based analytical device for the measurement of glucose that combined the glucose oxidase-catalyzed enzyme reaction for the production of hydrogen peroxide from glucose and the luminol-hexacyanoferrate(III) chemiluminescent system for its detection. The use of paper as analytical support allowed to obtain a simple and economical device, which already contained all the reagents necessary to perform the analysis. Moreover, the origami approach (i.e., folding the device in a suitable configuration during the analytical procedure) provided additional flow control in order to avoid any premature contact between the reactants. A commercial 3D printer was used to produce additional components (a device holder and a miniaturized dark box) for performing the chemiluminescence measurement by a portable CCD camera.

The device proved suitable for the measurement of glucose with a limit of detection of about 10 µM and a linear response up to 250 µM, and the total analysis time was 15 minutes. It was therefore adequate for measurement of glucose blood levels, even after a significant sample dilution to eliminate any possible matrix effect (physiological glucose blood levels range between 4.0 and 5.5 mM).

In perspective, the field of application of this device could be much broader since it can be easily modified to measure by oxidase-catalyzed reactions other clinically relevant analytes (e.g., uric acid and lactic acid). Moreover, use of alternative light detectors, such as silicon diodes or CMOS cameras integrated into smartphones and tablets, is under investigation.

Smartphone-based device for ultrasensitive quantification of ochratoxin-A in wine by chemiluminescent lateral flow immunoassay

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Ochratoxin A (OTA) is a mycotoxin produced by several species of Aspergillus and Penicillium fungi that is detected worldwide in various food and feed sources. Since OTA represents a potential hazard for human health, the European Community (EC) has established a maximum level for OTA in various feed and foods, in particular 2 μg L^{-1} in grape juices, wine and must. Several instrumental analytical methods are currently available for detecting these toxins in foodstuff, but they require complex sample preparation and dedicated laboratory equipment. Biosensors are very promising analytical tools for rapid on-site detection of analytes in complex matrices. We recently described a biosensor for multiplex detection of type-B fumonisins and B1 aflatoxin in maize samples based on a chemiluminescence Lateral Flow ImmunoAssay (CL-LFIA) coupled with a portable ultrasensitive CCD-based "contact" imaging device [1]. The use of CL detection allowed accurate and objective analytes quantification, down to picomoles, rather than qualitative or semi-quantitative information usually obtained employing conventional LFIAs based on colloidal gold labelling.

Here, we report on the development of a smartphone-based simple, rapid and accurate biosensor based on CL-LFIA method for quantitative detection of OTA in wine. The biosensor is based on a direct competitive immunoassay using peroxidase (HRP)-OTA conjugate, which is detected by adding the CL substrate luminol/enhancer/hydrogen peroxide and by using a smartphone camera for the image acquisition and data handling [2].

A self-standing microfluidic cartridge was developed, which houses the LFIA membrane and all the reagents necessary for the analysis and that can be easily coupled with the smartphone camera to perform CL measurements in an integrated device. The developed system is suitable for the quantitative analysis of OTA in wine samples with limits of detection that comply with EC legislation.

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