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Impact of miniaturized concepts for the rapid and generic detection of bacterial contamination

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Introduction :
Blood safety is a major concern for the health surveillance. Preventive measures implemented to reduce the viral risk allowed unprecedented levels of blood safety to be reached. The risk of transmission of the main viruses (HIV, HTLV, HCV and HBV) is currently very low in the developed countries. The bacterial risk then became the most frequent infectious risk in transfusion despite of the current preventive strategies. A better control of the bacterial risk will enhance the safety of the cellular products. The technological approaches developed up to now, which are mainly based on the detection of bacteria after culture or on their metabolism, are long and present many limits. Alternative strategies were proposed. Pathogen reduction technologies are in strong development and are evaluated in several countries. These techniques are performed during the process of product preparation. They cannot be applied to products derived from cellular engineering. The rapid control of a labile blood product or of a product derived from cellular engineering, carried out just before its delivery could represent another safety strategy. This control requires a simple, sensitive, and fast system for bacterial detection. Second generation biosensors coupling micro- and nano-technologies allow to meet these requirements and consequently to further develop miniaturized tests at a lower cost for the diagnosis.

Aim : To develop microsystems integrating ultrasensitive electrochemical biosensors to test the presence of bacteria in a cellular product or any other biological fluid. These systems are expected to be very sensitive, easy to use by a non specialised personnel and of low cost at the end of development.

Methods / Results: The first development is based on immunosensors: addressable nanoparticles under magnetic field and coupled with anti-LTA and anti-LPS antibodies, directed against Gram (+) and Gram (-) respectively, are used for the generic capture. Preliminary conductivity measurements performed on *E. coli* cultures showed specific, label-free and real-time detection from 1 to 10^5 CFU / ml. The immunosensors will then be integrated in a microfluidic "lab on chip" system. The second development is to design a peptide biosensor: human natural defensins HNP1-3 patented in the laboratory will be used for the recognition of bacteria. Antimicrobial peptides offer new strategic opportunities already identified in highly innovative areas related to the diagnosis.

Conclusions : The development of miniaturized technologies is expected to improve the detection of bacterial contamination in a biological product.

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Development of flexible process to manufacture lab on Chip systems on polymer substrates to predict the first earlier detection of the transplanted organs rejection

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Abstract:

Heart failure (HF) is the most increasing cause of death in Western Countries. For that reason, together with the difficulty of having a sufficient number of donor organs, it is recognized that the device-based therapeutic approaches will assume an increasingly important role in treating the growing number of patients with advanced heart failure, not only as bridge to transplant, but also as destination therapy, by considering also the ageing population. In fact, HF is primarily a disease of the elderly. Approximately 6% to 10% of people older than 65 years have HF, and approximately 80% of patients hospitalized with HF are more than 65 years old. During the last 10 years, the annual number of hospitalizations has increased from approximately 550,000 to nearly 900,000 for HF as a primary diagnosis and from 1.7 to 2.6 million for HF as a primary or secondary diagnosis. Nearly 300,000 patients die of HF as a primary or contributory cause each year, and the number of deaths has increased steadily despite advances in treatment.

Clinical inflammation is both a cellular and biochemical event (1-3). Both pro-inflammatory cytokines such as Tumour necrosis factor-alpha, Interleukin-1, and Interleukin-6 and anti-inflammatory cytokines such as interleukin 10, and interleukin 1 receptor antagonist are participants in the basic inflammatory process and mediators of cellular infiltration (4-5). These biomarkers are at high level in the serum of patients experiencing a cytokine storm.

Increasing need for a fast, real-time and reliable medical diagnosis has led to growing interest in new point-of-care biological sensors capable of the sensitive and specific detection of biomolecules [6]. Lab on Chip field constitutes a peculiar research area of SensorART project. From the evaluation of early detection of inflammation and/or sepsis markers as well as water retention sensor for heart failure monitoring in patients with VADs.
This work surveys a few of the emerging Lab on a chip technologies based on the combination of the “Soft” fabrication techniques and Self-assembled monolayers (SAMs) at Laboratoire de Sciences Analytiques (LSA) for improved, inexpensive health care. The developed lab-on-a-chip system is devoted to the detection of Tumour Necrosis Factor-alpha (TNF alpha) as a biomarkers in the serum of patients experiencing an inflammation due to the rejection of the transplanted organ. The Lab on a Chip is based on a flexible polymer substrate (Polyimide or PI) (Figure1) and it consists on the integration of a platinum counter electrode, a silver pseudo reference microelectrode and an array of gold microelectrode. A specific

monoclonal antibodies are immobilized on a gold substrate by several methods [7] in order to detect the corresponding cytokines involving in the inflammation process (TNF alpha) by Electrochemical impedance spectroscopy (EIS). The limit detection of cytokine in vitro is 0,1 Pg/ml (See Fig.2). Finally, the developed Lab on chip will be used to detect a specific cytokine (IL-1, TNF alpha and IL-10) and predict the first earlier detection of the transplanted organs rejection.

Figures :

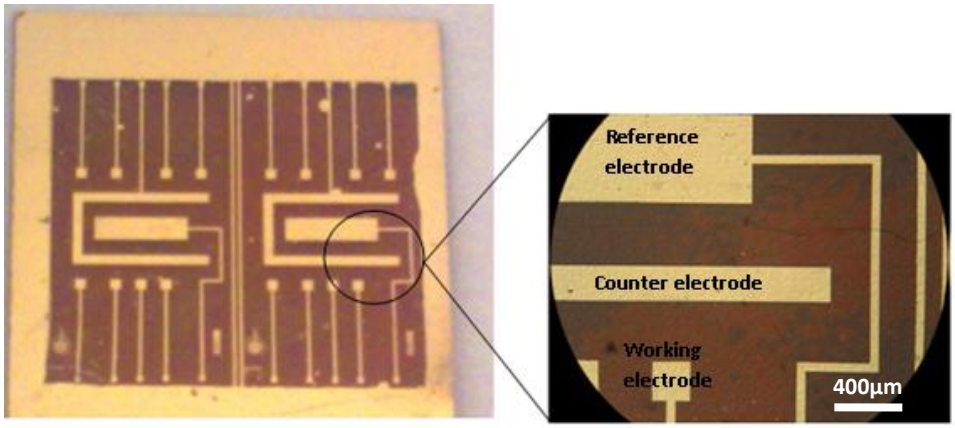

Fig. 1: Gold microelectrodes on polyimide substrate

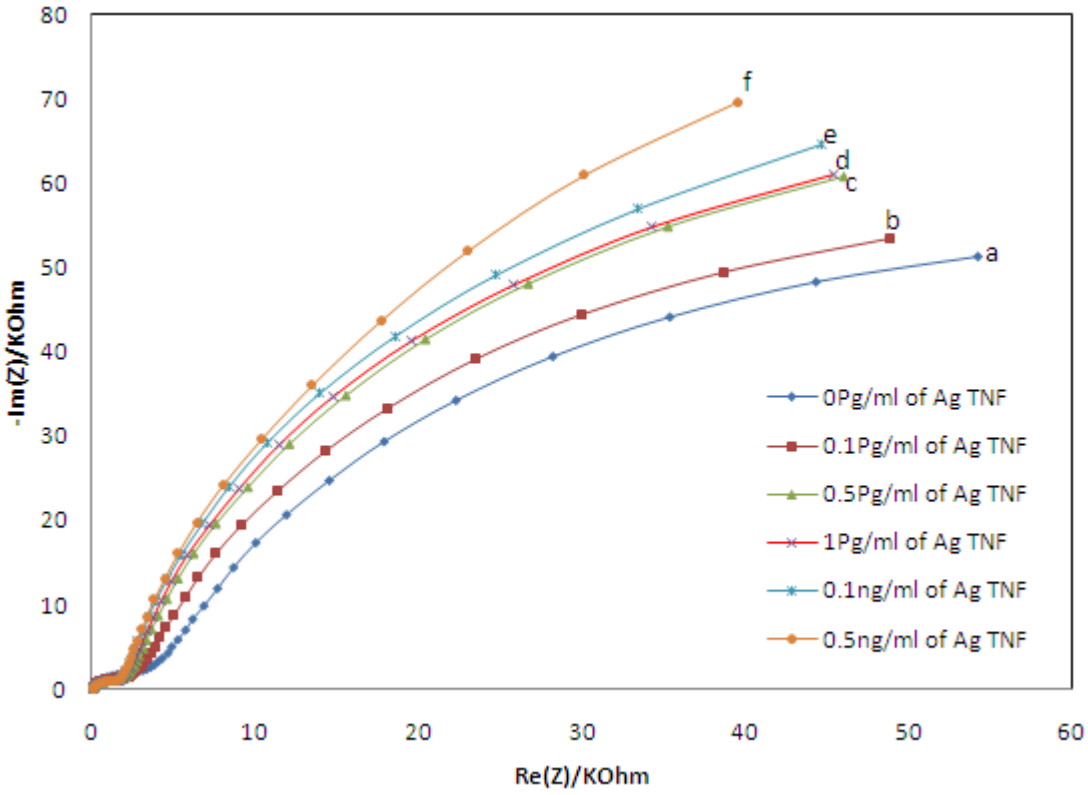

Fig. 2: Nyquist plot impedance (Z_r vs Z_i: at 5mM of $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ in PBS pH7,4 solution) at various TNF aplha antigen concentrations on its specific antybody : (a) 0Pg-ml; (b) 0.1Pg/ml;(c) 0.5Pg/ml; (d) 1Pg/ml; (e) 0.1ng/ml et (f) 0.5ng/ml

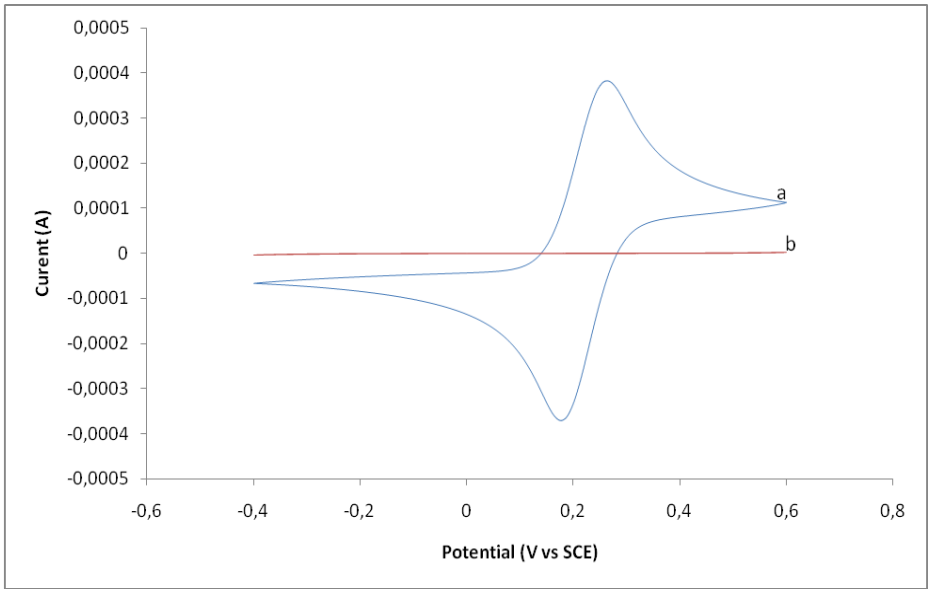

Fig. 3 : Cyclic voltammetry for :(a)bare gold electrodes and(b) mixed SAM electrodes in 5mM $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ in PBS pH 7.4 Electrodes were scanned at a rate of 100 mV/s

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New technology for the read-out of arrays of micromechanical sensors for biomedical applications

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In the last years, microcantilevers have been increasingly used as mechanical transducers of molecular recognition events[1] [2] [3] [4]. The transduction signal of nanomechanical sensors is a nano-scale motion. This nanometric deformation arises from the intermolecular forces that are generated by molecular recognition interactions on the sensitized surface of a microcantilever. Main techniques for the readout of the nanomechanical response include the optical lever method, interferometry-based methods integrated optical waveguides and the use of piezoresistive cantilevers. The optical lever method is the most used due to the extreme accuracy and easy implementation for measuring cantilevers.

In this contribution we present new instrument, SCALA, based on the scanning of a laser beam across a surface (up to centimeters). Deflection of the reflected beam during the scanning is collected on a position sensitive detector (PSD). This allows the instruments to perform the read-out of arrays of nanomechanical systems without limitation in the geometry of the sample, high sensitivity and a spatial resolution of few micrometers. Simultaneously our technology provides a complete map of the resonant properties. The instrument is able to operate in vacuum, air and liquids.

Measurement of nanoscale deformations on surfaces of cm^2 are performed automatically, with minimal need of user intervention for optical alignment. To exploit the capability of the instrument for high thoughput biological and chemical sensing we have designed and fabricated a two-dimensional array of 128 cantilevers (Fig 1(a)). We measured the nanometer-scale bending of the 128 cantilevers (Fig. 1(b)), previously coated with a thin gold layer, induced by the adsorption and self-assembly on the gold surface of several self-assembled monolayers[5].
In addition, we applied the technique for analyzing the vibration mode shape of a variety of nanomechanical systems that range from microcantilevers (Fig. 1(c)) to coupled nanomechanical resonators[6]. The technique is simple, allows imaging in air, vacuum and liquids, and it is unique in providing synchronized information of the static and dynamic out-of-plane displacement of nanomechanical systems. Additionally, it can create 3-D images of any arbitrary object (Fig. 2).

Finally we use the environmental chamber of SCALA for label-free detection of nucleic acid and proteins based on surface stress variations induced by tuning the environmental conditions[7].

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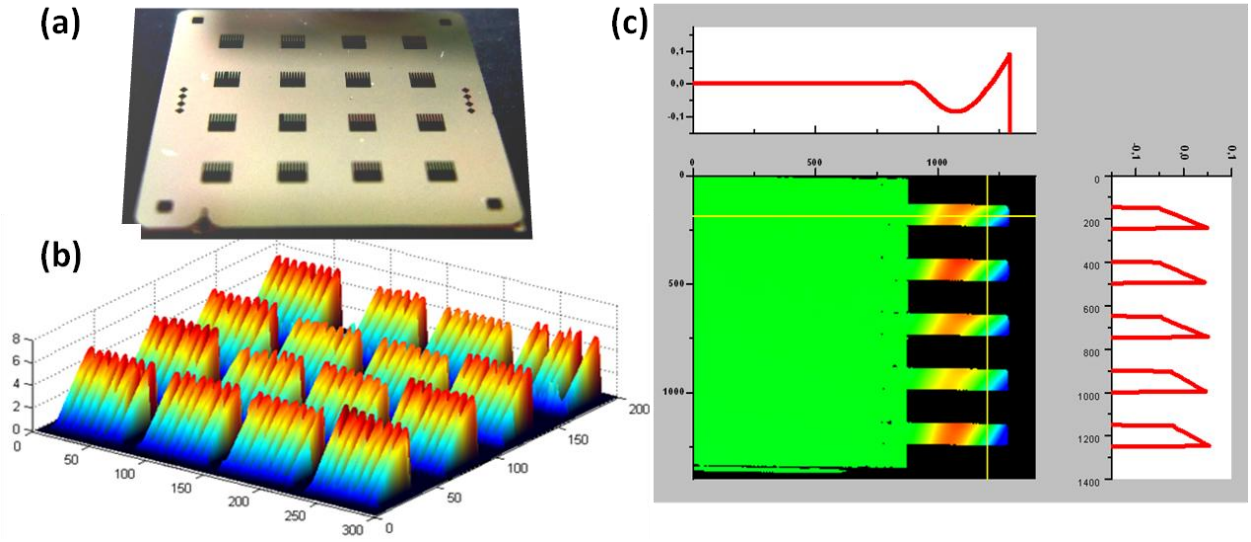

Fig. 1 *(a) Optical image of an 2D array of 128 microcantilevers arranged in 4x4 wells Each cantilever has 400 microns in length and 50 microns in width.(b) SCALA height image of the whole array.(c) SCALA vibration image of an array of 5 microcantilevers of the second vibration mode at 60 kHz.*

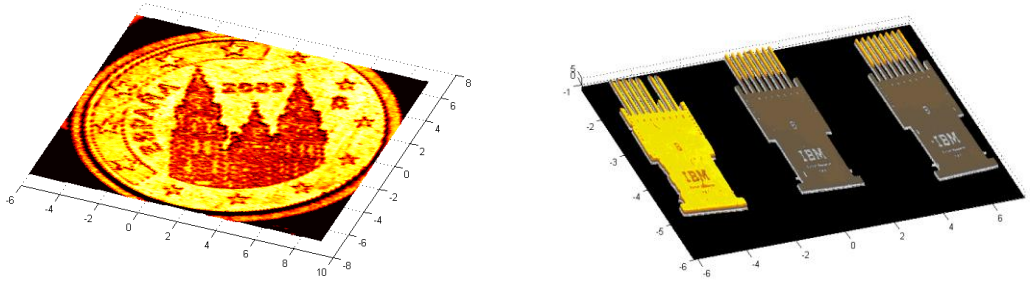

Fig. 2 *SCALA scanning of a one cent coin and three IBM cantilever arrays.*

SIZE DOES NOT MATTER … BUT SOMETIMES IT DOES: SMART NANOHYDROGELS

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Since the early twentieth century science advanced greatly to the point of being able to modify the structure of a large number of molecules, polymers among them, which nowadays are everyday materials and have great industrial and technological importance. Thanks to these advances, current polymer research is centered not from the perspective of inert materials, but in developing materials for applications that are beyond its typical use.

In the last decade, nanoscience has taken a growing interest because of the wide range of potential applications that can bring in areas such as biomedicine, agriculture, the cosmetics industry, polymer science, etc. [1]. Undoubtedly, the complexity of nanotechnology involves the use of new techniques for structuring and chemical functionalization exploiting the most of the properties of nanoparticles to enable its use on a selective or specific manner [2]. In this regard, the science of polymers makes it possible to obtain macromolecules with new chemical structures and physical-chemical properties with extraordinary because the polymers are complex materials that have a wide variety of properties that may change before, during and after their synthesis [3].

The so-called stimulus-response smart nanogels have emerged as a promising new class of materials with pharmaceutical applications. In these systems, small changes in some environmental variable such as temperature, pH, ionic strength, leads to a reversible phase transition in the structure of the gel. The poly(N-isopropylacrylamide) hydrogel presents a well-defined LCST in water near 32°C, above this temperature the gel structure collapses resulting in a sharp deswelling, which is reversible if the system temperature returns to below 32ºC. Furthermore, when these materials have ionizing functional groups are sensitive to changes in pH. The pH affects these systems similarly to temperature, so that a given change in pH of the medium makes the nanogel to swell, leading to an increased pore size of the polymer network, this facilitate molecules migration toward outside of the nanogel. This process is known as "release" and is the principle governing the current drug dispensing systems. Within the drug dosing systems we can find two basic types, which are the starting point for designing new mechanisms of transport and drug delivery, these are called "controlled release" and "targeted release".

The hydrogels are polymers that have similar characteristics to those of a living tissue. This feature has allowed them to be the focus of the biomedicine [4]. The combination of nanoscience, biomedicine and polymer science has become the main topic of research in recent years because it is a new discipline that exploits the potential of nanoparticles for use in biomedical applications [5]. Undoubtedly, the complexity of the nanoscience implies the use of new chemical structuration and functionalization techniques that exploit

the properties of nanoparticles that let express them in selective manner specific qualities. In this regard, the polymer science is the nearest to allow selective chemical structure due to the polymers are complex materials that have a wide variety of properties that can be changed before, during and after their issuance.

Here is reported the chemical functionalization of smart nanogels by incorporating pH-sensitive functional groups and folic acid as a tumor targeting ligand into the same initial polymer network. One of the most promising strategies in anticancer therapies is the targeted delivery through malignancy-associated cellular markers. Recently, Katime and coworkers [6] have designed new synthetic devices with enhanced stimuli-responsive sensitivity and targeting ligands that can be a promising field for the development of cancer-specific delivery systems. The new devices would lead to a reduction in the minimum effective dose of the drug required for each target [7]. The over-expression of folate receptors on many cancers identifies them as a potential target for a variety of ligand receptor-based cancer therapeutics. In fact, folate receptors are qualified as a tumor-specific target. Furthermore, folic acid molecules have numerous advantages in comparison with other ligands, such as their small size, availability, simple chemical conjugation, and no inmumogenicity. Due to these unique characteristics folic acid is actually presented as an ideal ligand for targeted delivery into tumors. Katime have developed new synthetic molecules based on microemulsions systems that can offer an interesting and potentially quite powerful alternative carrier system for drug delivery because of their high solubilization capacity, transparency, thermodynamic stability, ease of preparation, and high diffusion and absorption rates when compared to solvent without the surfactant system [8]. The microemulsión polymerization process is one of the most versatile methods to obtain smart nanocarriers ranged between 10-100 nm with a very homogeneous particle size distribution (≈ 1.1).

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A novel human TGF-β1 fusion protein and type I collagen serve as a scaffold for adult mesenchymal stem cells in bone regeneration

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The regulation of stem cell functions including cell adhesion and migration, mitosis and differentiation into specific tissue cells is now considered a crucial strategy to improve the regenerative potential of scaffolding matrices used for tissue engineering [1,2]. The stem cell niche, which is a pool of microenvironmental cues that control the fate of stem cells, is highly specified depending on the tissue types in terms of physical, chemical and mechanical characteristics [3]. Therefore, providing a physiological situation that mimics the native tissue environment may switch on the action of stem cells to enter into an appropriate stage and undergo tissue differentiation [4,5].

Mesenchymal stem cells (MSCs) are known to have multipotency to develop into a series of cell lineages, including osteoblast, adipocyte, chondrocyte and neural cells, in response to appropriate chemical and physical cues [6,7]. When compared with embryonic stem cells, MSCs are accessible without the possible concerns regarding ethical issues and can be readily obtained from adults, allowing their clinical application [8,9]. Recent studies have demonstrated the potential usefulness of MSCs for the treatment of defective and diseased tissues, including bone, cartilage and nerves [9]. Above all, the importance of microenvironmental control has been highlighted to enable complete use of MSCs in tissue engineering and regenerative therapy [10,11].

Transforming growth factor-betas (TGF-βs) are a superfamily of molecules closely related in structure and function. It is now well established that members of the TGF-β family play a prominent role in the development, growth and maintenance of the vertebrate skeleton [12,13]. The effect of TGF-β1 on the proliferation and osteoblastic differentiation of MSCs in vitro –causing an increase in total cell number, alkaline phosphatase (ALP) activity, and osteocalcin (OC) production- is well documented [14,15].

Collagen gel has been a model for culturing cells in three-dimensional environments, which is a condition much more similar to native tissue extracellular matrix than two-dimensional culture dishes [16,17]. Specifically, the collagen fibrous network and surrounding medium fluid constitute a soft and flexible gel matrix that allows cells to freely reach out and migrate in three dimensions [1,18]. Many groups have used collagen gel matrix to investigate the behavior and differentiation of MSCs into specific cell lineages, such as chondrocytes, osteoblasts and endothelial cells [2,17].

To define the responses of mesenchymal osteoprogenitor stem cells to TGF-β1, we cultured Fischer 344 rat bone marrow (BM) cells in a collagen gel medium containing 0.5% fetal bovine serum for prolonged periods of time. Under these conditions, survival of BM MSCs was dependent on the addition of TGF-β1. Recombinant human TGF-β1-F2 (rhTGF-β1-F2), a fusion protein engineered to contain an auxiliary collagen binding domain (von Willebrand's factor-derived), demonstrated the ability to support survival colony formation and growth of the surviving cells, whereas commercial TGF-β1 did not. Initially, cells were selected from a whole BM cell population and captured inside a collagen network, on

the basis of their survival response to added exogenous growth factors. After the 10-day selection period, the surviving cells in the rhTGF-β1-F2 test groups proliferated rapidly in response to serum factors (10% FBS), and maximal DNA synthesis levels were observed (Fig. 1). Upon the addition of osteoinductive factors, osteogenic differentiation *in vitro* was evaluated by the induction of ALP expression (Fig. 2), and the formation of mineralized matrix (data not shown).

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Figures

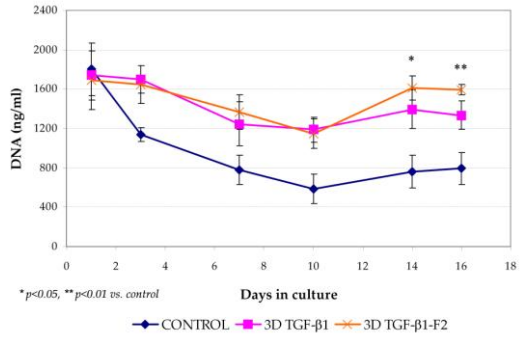

Figure 1. Quantification of DNA content as indication of cell replication.

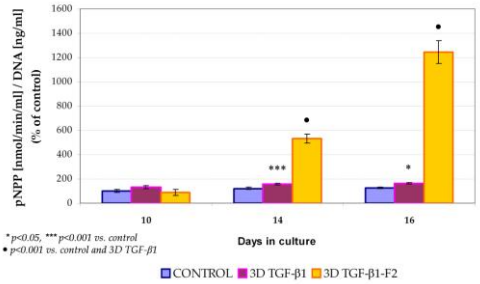

Figure 2. Effects of culture conditions on ALP activity.

Antimicrobial activity of different silver-containing nanostructured materials

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Silver compounds have been used as antibacterial agents for centuries, from the use of coins to preserve water in ancient Greece and Rome, to the XIX century use of silver nitrate to treat a variety of ailments, from typhoid to post-partum infections. [1] Today a variety of applications are contemplated, including Ag-coated medical devices, dressings for chronic wounds and burns, cosmetics, food preservation and water treatment. [1], [2]. The silver mechanism of action is far from understood: the biochemical explanations proposed include (i) inactivation of proteins, enzymes and DNA by Ag attachment to groups containing sulfur or phosphorus, (ii) cell membrane and mitochondria damage, (iii) inhibition of respiratory processes and (iv) generation of reactive oxygen molecules and free radicals.

In this work, we describe the bactericidal ability of different forms of silver on highly adherent slime producing *Staphylococcus aureus* strain 9213 [3]. We show that silver-exchanged zeolites (ZSM5) at low Ag loadings are more effective against *S.aureus* than other materials with higher amount of silver such as silver (I) oxide and nanoparticulated (< 100 nm) silver.

NH_4-ZSM-5 microparticles with a Si/Al ratio of 20 were purchased from Zeolyst Int.; other commercial Ag-exchanged zeolites with different silver loadings (i.e.,15-20 wt. % and 35 wt. %) and geometries (pellets and granular, respectively) were purchased from Sigma-Aldrich as well as silver (I) oxide and nanoparticulated (< 100 nm) silver. The ammonium form of the zeolite was calcined to obtain its protonic form and ion-exchanged by using 1 wt. % $AgNO_3$ solution (atomic absorption standard solution 1 wt. % (HNO_3), Sigma Aldrich) under stirring, for up to 24 hours. In this way we obtained Ag loadings around 0.2 wt. %. [4].

For the antibacterial assays, 2 mL of an overnight stationary growth phase of TSB bacterial culture were added under sterile conditions to glass tubes, each of which contained the 60 mg of Ag-ZSM5, H-ZSM5 (as control), silver (I) oxide, commercial pellets, commercial granular particles and nanoparticulated silver. Samples were subsequently incubated at 37 ºC for 4, 6 or 24 hours in the dark. After incubation tubes were placed in a 50 Hz ultrasonic bath for 15 minutes (tubes at 4 and 6 hours) or 30 minutes (tubes at 24 hours). Seven 1:10 dilutions of the contents of each ultrasonicated tube were made and then three 25 μL drops of each suspension were spread on Triptone Soy Agar (TSA) plates. Bacterial colonies were counted after incubation overnight at 37º C. Silver content on the released media was determined using atomic absorption spectrophotometry (AAS) in a Varian Spectra A110. Media were centrifuged using exclusion centrifugal filters (5 nm cut–off) to obtain supernatants free of any lose nanoparticles. [4].

The bactericidal action when using the same amount of material added to the stationary grown bacteria (10^9 CFU/mL) was found maxima for the Ag-exchanged zeolite compared to the other commercial silver-exchanged zeolites (pellets and granular) (Figure 1).

Silver exchanged zeolites showed nanosized clusters on their surfaces indicating that in those materials silver is present as cation in the zeolite network as well as metallic silver as clusters or nanoparticles on their surfaces. The higher the bioavailability of cationic silver, the higher the bactericidal action of the silver-carrier material. Silver (I) oxide showed the highest bactericidal action of all the materials tested. Ionic silver content, particle geometry, silver solubility, particle size and crystallinity are some of the variables controlling the bactericidal effect of silver.

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Figures

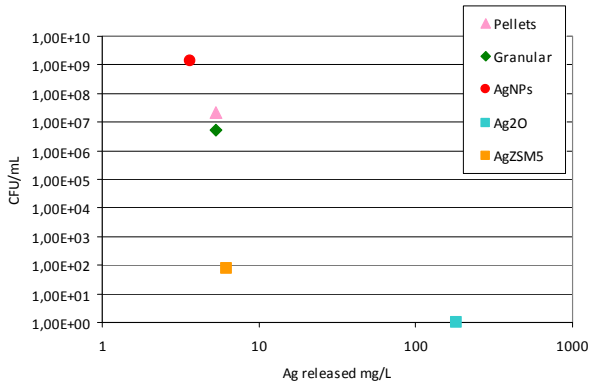

Figure 1. *Bactericidal action of the different silver-containing materials.*

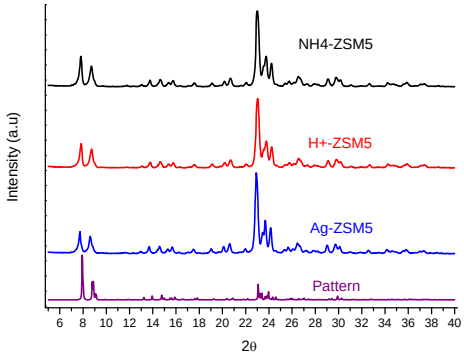

Figure 2. *XRD ZSM5 before and after calcination and ion-exchange.*

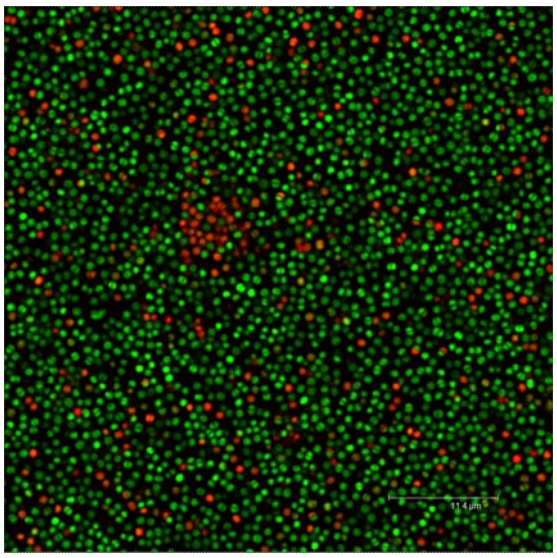

Figure 3. *S.aureus by CLMS.*

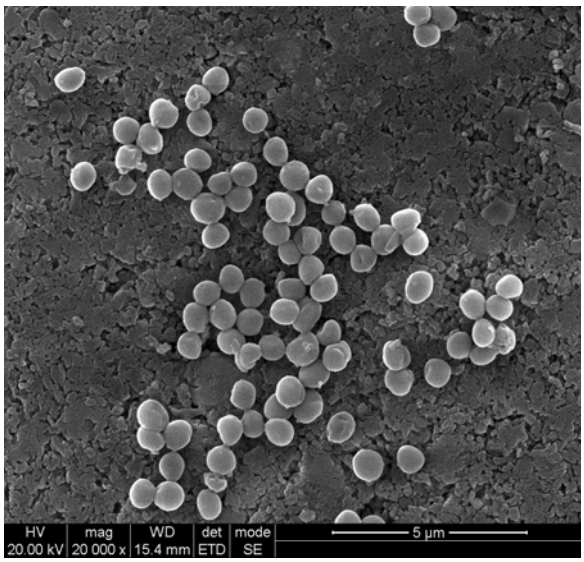

Figure 4. *S.aures by SEM.*

Tip Based Printing of Functionalized Hydrogels Microscale Patterns

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Introduction: Hydrogels are cross linked hydrophilic polymers that swell in water and can be synthesized from a variety of natural and synthetic polymers. Hydrogel scaffolds can be tuned to closely mimic the chemical and mechanical properties of extra cellular matrix and therefore could play a crucial role in tissue engineering research. However, in bulk hydrogels, it is difficult to control the 3D architecture and may cause cell necrosis due to diffusion limitation. A solution to this problem is the construction of microscale hydrogel patterns. Currently, microscale hydrogel patterns are generally constructed using techniques like photolithography and micromolding. But each of these methods has significant drawbacks.
In this report we present a novel method for the construction of chemically functional hydrogel microscale patterns. We use a tip based lithography method to directly deposit the hydrogel precursors at defined location and polymerize them to form hydrogels. This method allows for rapid fabrication of high resolution patterns.

Methods: A 1:2 (wt/wt) solution of Poly(ethylene glycol) dimethacrylate (PEG-DMA) with a molecular weight of 0.55 kDa and 1 kDa was used for printing hydrogels. A photoinitiator (2,2-diethoxyacetophenone) was added into the PEG-DMA ink solution to aid in polymerization and formation of hydrogels. A NLP 2000™ desktop nanolithography platform (NanoInk Inc., Skokie, IL USA) equipped with a 12 pen array (M-expV1 type) was used to construct the patterns (Fig 1) on chemically functionalized glass substrates. The hydrogel precursor patterns were polymerized by exposing them to 10 mW UV irradiation for 20 mins. To construct the functionalized hydrogel patterns, a 1:2 (wt/wt) solution of PEG-DMA (1 kDa) and 4-arm PEG-thiol (2 kDa) was used. For functional study, the hydrogel pattern was incubated with thiol-reactive rhodamine red C2 maleimide (Invitrogen) for 2 hrs and characterized by fluorescence microscopy.

Results: We have demonstrated that microscale hydrogel patterns can be easily generated using tip based lithography process. Uniform and size controllable hydrogel patterning could be obtained by temperature and humidity control during the deposition process. At 37ºC and 20% RH, relatively larger droplets (~5 µm) were printed while at 25ºC and 20% RH, smaller droplets (~2 µm) were deposited (Fig 2). AFM imaging of the polymerized hydrogel patterns confirmed the size and homogeneity of the array.
We have also successfully demonstrated the precise deposition of thiol functionalized PEG hydrogel on glass substrates. In addition, we have shown specific immobilization of the thiol-reactive rhodamine red C2 maleimide molecules on the surface of our microscale hydrogel patterns (Fig 3). Red-fluorescent was observed exclusively at the patterned area. By simply adjusting the ratio between the two PEG components, we should be able to fine-tune the number of free thiol functional groups in the hydrogel, and hence the amount of conjugated biomolecules. These hydrogels with different composition can be also loaded simultaneously onto the writing tips by an inkwell to create concentration gradient patterns in a single array.

Summary: Functionalized hydrogels have been proven to be very useful in biomedical applications. The methodology we report herein is an effective way to produce well-defined size arrays of hydrogel with selective immobilized biomolecules printed on glass substrates which are ideal systems for addressing different biological relevant issues.

Figure 1. *The desktop nanolithography platform NLP 2000™ was used for patterning hydrogels.*

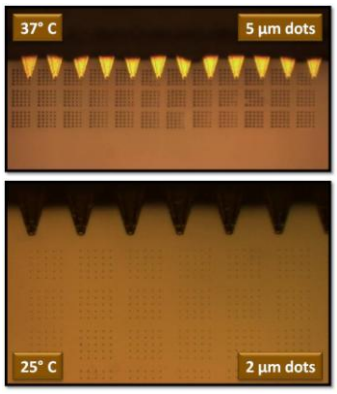

Figure 2*. Hydrogel spot size as a function of temperature. Environmental (temperature and humidity) conditions play a crucial role in the spot sizes that can be obtained by tip based lithography. At a constant relative humidity of 20%, larger spots can be printed at 37 ºC (top) compared to 25 ºC (bottom).*

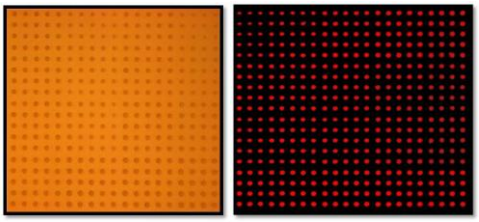

Figure 3*. Functionalized Hydrogel. (Left) Bright field image of functional hydrogel patterns that were constructed with excessive thiol groups. These patterns were incubated with thiol-reactive rhodamine red C2 maleimide molecules and interrogated by fluorescence microscopy (Right).*

NEPHH - nanomaterials related environmental pollution and health hazards throughout their life cycle

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Besides the positive multipurpose nano-reinforcement in polymeric materials and expanded devices applications, limited know-how presently exists on the environmental and health risks of certain manufactured nanomaterials. Initial research has indicated that engineered nanoparticles can have a negative impact on human health and environmental pollution. More importantly, and fundamental to the success of nanotechnology, is the perceived safety of the technology by the public. As activity shifts from research to the development of applications, there exists an urgent need to understanding and managing the associated risks, but in particular to personnel working with these materials.

NEPHH Project aims to identify and rate important forms of nanotechnology-related environmental pollution and health hazards that could result from activities involved in silicon-based polymer nanocomposites throughout their life cycle, and also to suggest means that might reduce or eliminate these impacts. NEPHH will consider the safety, environmental and human health implications of nanotechnology-based materials and products. This project accounts that nanoparticles, for most applications, are surface modified and generally embedded in the final product and therefore do not come into direct contact with consumers or the environment. Consequently, NEPHH will be going beyond the primary nanomaterials and will specially focus into the secondary and tertiary polymer nanoproducts with silicon nanoparticles compounds as base of their composition.

Present communication will include a general overview of the Project, including its main structure and work packages interrelation. Main outcomes of the Project to date will also be described including materials selected and procedures for samples production.

Design of multifunctional nanocarriers for biomedical applications

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The design of nanocarriers able to reach selectively the disease site at therapeutic concentrations and at the same time easy to be monitored at real time is a promising way to improve efficacy and decrease adverse effects of drugs. [1-3]. Polymeric nanoparticles are good candidates as starting materials for the preparation of multifunctional nanocarriers. They can be obtained in O/W nano-emulsions by incorporating preformed non-water-soluble polymers in the oily dispersed phase followed by solvent evaporation [4]. Although this method has been known for some time, most studies are based on the use of toxic organic solvents or high temperature procedures. Moreover, commonly, polymeric nano-emulsions are prepared by high-energy methods. Therefore, there is a need of mild procedures combining the use of low-energy emulsification methods and low toxicity components suitable for biomedical applications [5].

The aims of this work were the preparation of biocompatible O/W polymeric nano-emulsions by low-energy methods at 25ºC, their use for nanoparticle preparation by the solvent evaporation method and their functionalization. Nano-emulsions have been formed in water / nonionic surfactant / polymeric organic solution systems by the phase inversion composition method (PIC) at relatively high oil/surfactant (O/S) ratios and characterized by means of several techniques (cross correlation spectrometry, light backscattering, cryoTEM, etc). The nano-emulsion average droplet size (around 200nm) (Figure 1) was found to depend on the O/S ratio as well as the polymer concentration in the organic solution. Some nano-emulsions were chosen for nanoparticle preparation by the solvent evaporation method The nanoparticle average size (typically below 50 nm) was shown to be related to that of the precursor nano-emulsion as determined by tramsmission electron microscopy (TEM) image analysis and their shape was rounded (Figure 2) as evidenced by transmission (TEM) and scanning (SEM) electron microscopy [6]. Further, coumarin-6 was incorporated in the nanoparticles for imaging purposes and folic acid was coupled to the polymer for tumour targeting.

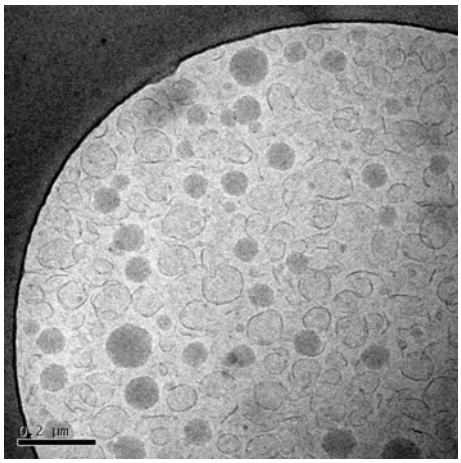

Figure 1: *Cryo-TEM image of the polymeric O/W nano-emulsion with an O/S ratio of 70/30 and 90 wt% of water content.*

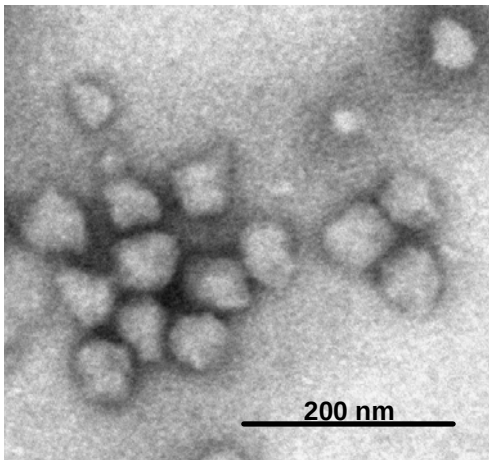

Figure 2: *TEM micrograph of polymeric nanoparticles as observed after negative staining with phosphotungstic acid.*

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Dual drug release of triamterene and aminophylline from Poly(N-isopropylacrylamide) hydrogels studied by UV-Vis, ESEM and DLS

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An alternative to conventional dosage of antineoplastic agents is controlled and localized release from a polymer. A large variety of drugs could be released in a localized manner, alone or combined, from hydrogels. Recently, combination therapy have shown to be an effective way to treat diseases and regenerate tissues. In order to optimize these effects, different drugs have to be employed in the optimal dose and the adequate periods of action on damaged tissue.

We used temperature-sensitive Poly(N-isopropylacrylamide) hydrogels as drug delivery systems, so changes in body temperature induced by pathogens can act like external stimulus to activate controlled release of the drug incorporated in the hydrogel.

In the combined release studies, we chose two model drugs: aminiphilline and triamterene. Triamterene (2,4,7-triamino-6-fenilpteridina) is an antidiuretic used with success in combination with diuretics in the treatment of hypertension and edema. Aminophylline is a bronchodilator used successfully in the treatment of asthma in combination with other drugs.

We found no interaction between drug and polymer (linear dependence between the amount of drug loaded and the PNIPAM concentration). The ammount of drug released was measured by UV-Vis spectroscopy following the evolution of the absorption peaks of aminophylline (271 nm) and triamterene (365 nm). The maximum release time is greater for triamterene (days) than for aminophylline (hours). By changing the shape of the hydrogel (from a disk of 1 cm to a cylinder of 10 cm of thickness), and with increasing molecular weight or solubility of the drug, we observed that the diffusion coefficient decreases. On the contrary, with increasing hydrophobicity of the drug diffusion coefficient increases.

The evolution of pore size distribution of hydrogels during loading and release was obtained by dynamic light scattering (DLS) and the algorithm NNLS. When loading and releasing the drug, pore size of the hydrogel decreases and increases again without reaching the initial pore size of hydrogel, respectively. We observed that the greater the concentration of drug loaded in the hydrogel greater the reduction in pore size.

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Figures

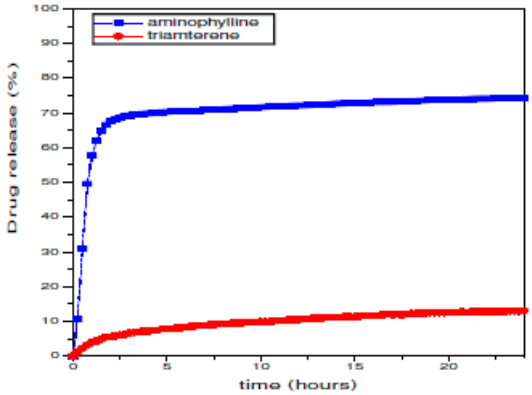

Figure 1. Release percent of triamterene and aminophylline from PNIPAM hydrogels in distilled water at 37 ºC.

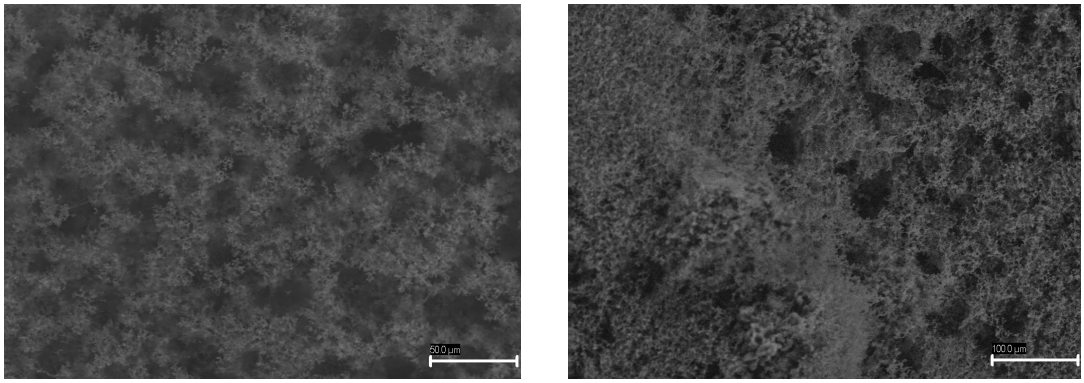

Figure 2. ESEM micrograph showing the change in pore size with the release of the drugs from the hydrogels.

Optical hyperthermia conducted by Silica/gold nanoshells to induce transgenic expression

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Introduction and Objective

Nanoshells (NS) consist of a spherical dielectric nanoparticulated core surrounded by a thin metal shell [1]. By varying the relative thickness of the nanoparticle (NP) core and its metallic shell, it is possible to generate NPs that can either absorb or scatter light at a desired wavelength. We generated NS with a dense silica core of 160-170 nm in diameter and a gold shell of 12-15 nm thick optimized to absorb near-infrared (NIR) light, particularly in a spectral range known as the "water window" (700-1200 nm). This window represents a region of the electromagnetic spectrum characterized for a minimal absorption by water and biological chromophores [2]. Laser irradiation of NS at their peak extinction coefficient results in the conversion of light energy into heat, which raises temperature in the NPs surroundings. In this study we explored the capability of NS to induce transgene expression in human cells activated by optical hyperthermia.

Materials and methods

The preparation of NS followed the general procedure outlined by Oldenburg *et al.* [3]. The dense silica cores were prepared using a modified Stöber process. The surface of the silica NPs was functionalized with amine groups via silane coupling. Separately, a suspension of Au seeds (from 1-2 nm to 3-4 nm) was prepared following the method reported by Duff *et al.* [4]. The gold seeds were anchored on the amino groups of the functionalized silica cores. The gold shell was then completed by adding additional gold solution and potassium carbonate. NS were characterized by transmission electron microscopy (TEM). Laser irradiation was carried out with an IR Laser system consisting in a 808 nm-wavelength laser diode coupled to an optic fiber of 400 µm and a power controller, placed in a thermostatized chamber. Absorption measurements of the NP-based dispersions were obtained using a UV-Vis-NIR spectrometer. A HeLa-pDsRed-Monomer clonal cell line harboring the encoding sequence of the fluorescent fusion protein DsRed-Monomer under the control of the extremely heat-sensitive human hsp70 promoter was generated. Hela-pDsRed-Monomer cells respond to subtle increases of culture temperature by increasing the expression of the transgene. Cell viability was assessed in cultures treated for 24 h in the presence or absence of NS, using the alamarBlue assay. Cell internalization of NS was visualized using the reflection mode of the spectral confocal microscope (CLSM). For *in vitro* photothermal studies, HeLa-pDsRed-Monomer cells were exposed to NS and irradiated from the top surface with the 808 nm infrared diode laser. After irradiation, the fluorescence signal derived from the DsRed-Monomer protein was visualized using CLSM.

Results and conclusions

TEM micrograph in Figure 1A showed the NS structure. NS presented a clear dense silica-core coated with a high density layer corresponding to the gold shell. It is worth noting the homogeneity in the size distribution of synthesized NS. These NPs showed a strong absorption of the light radiation in the 500-900 nm range of the NIR region (Figure 1B). After addition of NS to the culture medium, the material was exposed to a 808 nm-wavelength laser system integrated in a thermostated chamber to establish

the environmental temperature at physiological conditions. The heat dissipated from NS significantly increased, in a dose-dependent manner, the temperature of the culture medium where they were immersed (Figure 1C). Prior to the development of the optical hyperthermia protocols, cell viability and NS internalization were tested in cells exposed to the material for 24 h. NS treatment did not impaired metabolic activities of the cells, indicating a good biocompatibility of the material. Cells were able to internalize NS, which appeared located as clusters inside the cells. Application of NIR light at 808 nm on cultures treated with NS generated an increase of intracellular temperature that efficiently activated the expression of fluorescent DsRed-Monomer protein controlled by the thermo-sensitive promoter (Figure 2A). In the absence of NIR laser treatment, cells were not able to activate the expression of the fusion protein (Figure 2B). In summary, we demonstrated the suitability of NS as photothermal agents to induce transgene expression after sub-lethal heat treatment conducted by NIR radiation. This finding points to the great potential of NS in multimodal therapeutic protocols based in the combination of optical hyperthermia and gene therapy.

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Figures

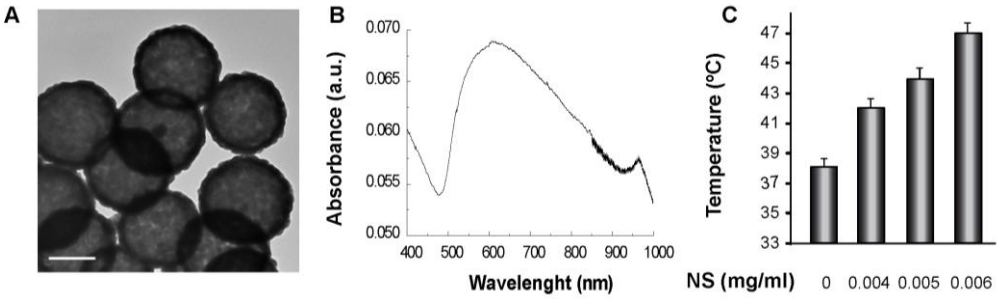

Figure 1. (A) TEM image of NS. Bar = 50 nm. (B) Absorbance spectra of NS. The peak absorbance wavelength corresponds to the NS surface plasmon resonance. (C) Temperature measurements of culture media containing increasing concentrations of NS, after 10 min of exposure to NIR laser.

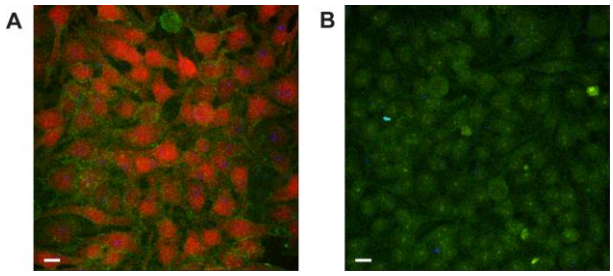

Figure 2. Transgene expression after sub-lethal optical hyperthermia. Hela-pDsRed-Monomer cells were exposed to NS for 24 h, washed to remove non-internalized NS and exposed (A) or not (B) to NIR laser at 808 nm for 30 min. The fluorescent signal derived from the expression of DsRed-Monomer protein was observed using CLSM after 24 h. Confocal maximum projections show DsRed-Monomer (red) and actin (green) stained cells. NS (blue) were detected by reflection. Bars = 10 µm.

A fully synthetic carbohydrate vaccine based on gold nanoparticles

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Since carbohydrates are usually poorly immunogenic, strategies have been developed to improve their immune response. Current advances in the identification and synthesis of carbohydrate epitopes have opened new ways to rationalize vaccine design. Several strategies for the production of synthetic carbohydrate-based vaccines have been developed that have overcome the hurdles encountered when using complex bacterial capsular polysaccharides.[1] The selection of a carrier in the preparation of a conjugate vaccine is another parameter in the design of carbohydrate vaccines.

This study explores the potential application of hybrid gold glyconanoparticles (GNPs) as vaccine candidates against *Streptococcus pneumoniae*, a major cause of invasive respiratory tract infections in both children and the elderly. The conjugation of biomolecules to metal nanoclusters has opened new opportunities in the design and synthesis of multifunctional and multimodal assembled systems for biomedical applications. Gold nanoparticles have been extensively explored because of their relative inertness, low toxicity, and easy manipulation, and because the chemistry of their surface is easy to control.[2] Gold surface can be simultaneously tailored with different ligands in a controlled way *via* thiol chemistry affording multivalent and multifunctional nanoparticles.[3],[4] In search for new conjugate vaccines not based on protein carriers, we here present gold nanoclusters as a versatile platform to construct a potential carbohydrate-based vaccine against *S. pneumoniae* type 14.

The branched tetrasaccharide Gal(β1-4)Glc(β1-6)[Gal(β1-4)]βGlcNAc, corresponding to the repeating unit of the pneumococcal type 14 capsular polysaccharide (Pn14PS), is able to induce anti-Pn14PS specific antibodies when conjugated to CRM_{197} and thus it was selected as candidate for the development of a synthetic conjugate vaccine against *S. pneumoniae* type 14.[5],[6]

Antigenic tetrasaccharide Gal(β1-4)Glc(β1-6)[Gal(β1-4)]βGlcNAc, ovalbumin peptide-fragment $OVA_{323-339}$ which is well known as immunodominant T-cell epitope, and glucose were suitably functionalised with different thiol-ended linkers and employed as ligands for the construction of hybrid GNPs (scheme 1).

These biofunctional GNPs, having mean gold core diameters of ~2 nm, were used to immunise intracutaneously BALB/c mice. Depending on the density of the different ligands, GNPs coated with the tetrasaccharide conjugate induced significant levels of specific IgG antibodies that recognize both the native polysaccharide of Pn14 and the very same branched tetrasaccharide fragment of Pn14PS as determined by ELISA. Glucose was used as inner and inert component to assist water dispersibility and biocompatibility and to allow the tetrasaccharide moiety, armed with a long amphiphilic linker, protruding above the organic shell of GNPs.

Details on the synthesis of ligands, the one-step preparation of GNPs using different ratios of the ligands, and the techniques used for characterization (TEM, NMR, IR, UV-Vis) will be presented together with the experiments for evaluating the type-specific antibodies, opsonophagocitosis and cytokine levels after spleen cell stimulation. Although further optimization of vaccine efficacy is necessary, this study presents the first example of a fully synthetic carbohydrate vaccine based on nanoparticles that is able to induce specific IgG antibodies that react with native capsular polysaccharide.These results confirm that a suitable presentation of antigenic carbohydrates is essential to induce a specific immune response and should encourage the use of gold GNPs as new systems in the development of a synthetic carbohydrate-based pneumococcal vaccine.

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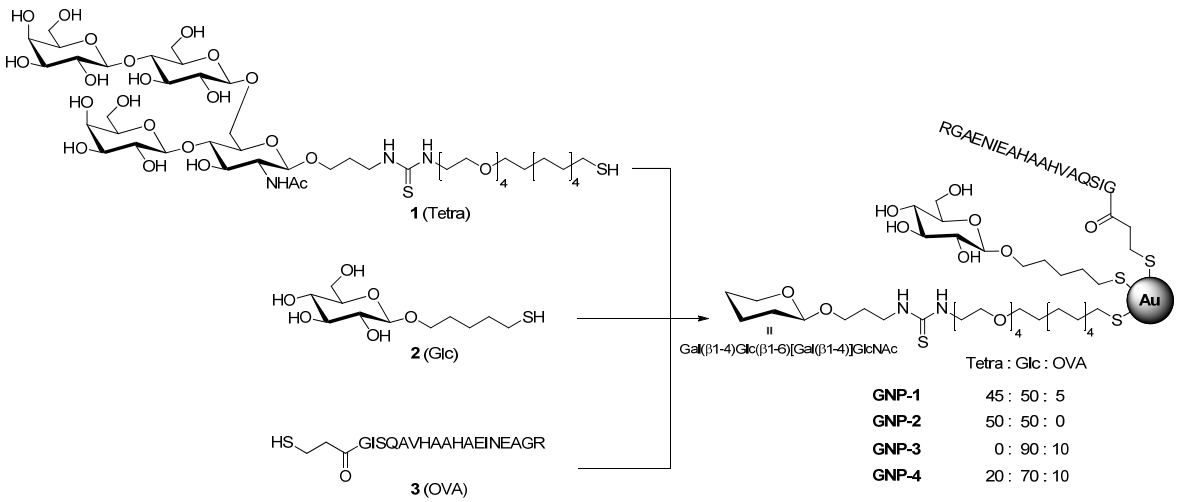

*Scheme 1: One-step synthesis of hybrid gold nanoparticles (GNPs) incorporating different molar ratios of branched tetrasaccharide (Tetra; Gal-Glc-(Gal-)GlcNAc) (**1**), D-glucose (Glc) (**2**), and $OVA_{323-339}$-peptide (OVA) (**3**) conjugates. Reagents and conditions: $HAuCl_4$, $NaBH_4$, H_2O/MeOH, 2 h, 25 ºC. For clarity, all conjugates are depicted as thiols. The dimension of the gold nanoclusters is ca. 2 nm and is not in scale with the size of conjugates.*

Magnetoliposomes open up new horizons as MRI contrast agents

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Over the past few decades, the interest in magnetizable nanocolloids has steadily increased as a result of the growing number of applications which can be designed both in the field of biotechnology and biomedicine. To improve the colloidal stability the particles have to be wrapped in a cushion of adsorbing molecules such as dextrans which are often used for the production of magnetic resonance imaging (MRI) agents. However, the dextran coating is vulnerable to aging and may induce alterations in the behavior and morphology of cells in culture. As a promising alternative we introduced in the late 1980's so-called magnetoliposomes (MLs) [1]. These constructs consist of nanometer-sized magnetite (Fe_3O_4) cores covered with a bilayer of phospholipid molecules. The inner leaflet molecules are very strongly chemisorbed with their polar headgroup on the Fe_3O_4 surface, whereas those residing in the outer layer are more loosely physisorbed. Since phospholipids are components of all biological membranes, MLs can be considered as highly biocompatible, which is essential, for instance, for *in vivo* cell labeling [2].

In the present work we further exploit the high flexibility of the lipid bilayer, enabling easy and well controllable **particle functionalization**. In this respect it is shown that ***cationic lipids*** built in the outer leaflet of the ML envelope, ultimately, result in much higher cellular uptake [3,4]. For instance, using 3.33 mol% 1,2-distearoyl-3-methylammonium propane [DSTAP] containing MLs, 3T3 fibroblasts can be efficiently labeled with up to 47.66 pg Fe/cell, one of the highest values reported in literature, without inducing any toxic effects [5]. Also, upon incorporating 1 mol% of galactose-modified phospholipids into the ML coat, the particles are ***specifically targeted*** and taken up by HepG2 (hepatocellular carcinoma) cells and primary hepathocytes, which typically express the complimentary asialoglycoprotein receptor [6]. In vivo experiments have also shown better retention in the liver of functionalized MLs compared to non-functionalized ones.

Besides our attempts to improve (specific) particle uptake, the **intracellular fate** of the MLs is further investigated and compared with the behavior of other well known MRI contrast agents. As, in general, internalized particulates end up in the 'acidic' endosomes/lysosomes, we first studied nanoparticle susceptibility to **pH-induced degradation** and the consequences thereof for MR contrast generation. Kinetic experiments were performed in a test tube setup at pH 7.0, 5.5 and 4.5. Both by Fe^{3+} determinations and analysis of T2* maps of MRI phantoms it is found that **MLs withstand much better the harsh acidic conditions** as compared to Endorem (dextran-coated), Resovist (carboxydextran-coated) and VSOP (citrate-coated) particles [7].

Besides the above-mentioned pH-induced effect, the lipid bilayer is also partially destroyed by the action of lysosomal phospholipase A2. The kinetics of this enzymatic degradation are monitored in an *in vitro* lysosomal model system. A gradual decrease in pH caused by liberated fatty acids (Fig. A) as well as a concomitant lowering of the phospholipid/iron ratio of the MLs is observed (Fig. B). At equilibrium the remaining lipid amount on the Fe_3O_4 core equals about one third of the value found for an intact ML. This observation strongly points to the fact that the inner lipid layer is very resistant to further hydrolysis due to an unfavorable orientation and, thus, avidly protects the iron oxide core. Monolayer-coated particles, indeed, display a hydrophobic surface and – due to the hydrophobic effect

- will **cluster** in the aqueous environment, resulting in a 6-fold increase in hydrodynamic diameter (as measured by dynamic light scattering – Fig. C), an enhanced magnetic attraction in the presence of an external magnetic field (Fig. D), and a 2-fold reduction of T2* relaxation time [7]. This aggregation event observed with MLs also occurs intracellulary as shown with C17.2 neural progenitor cells. By contrast, with Endorem, at a similar initial intracellular iron oxide level, no aggregation occurs [7].

In conclusion, MLs show up as highly versatile, biocompatible nanocolloids which are extremely prone for **long-term MRI follow-up studies**.

Acknowledgements

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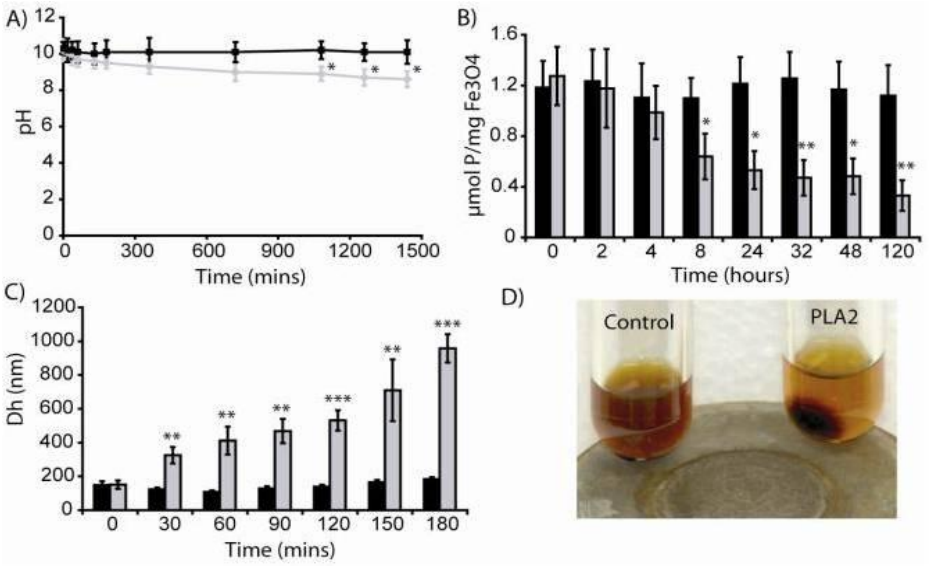
Figures



Effects of PLase A_2-action on MLs, shown by A) the decrease in pH, B) the ratio of phosphate over magnetite, C) increase in hydrodynamic diameter and D) visual confirmation of an incresae in aggregation of PLA_2-treated MLs. A-C) grey indicates PLA_2-treated samples; black represents control samples.

Magnetoresistive biosensors for quantitative lateral-flow bioassays using magnetic nano-markers

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Lateral-flow assays are commonly used for the detection of biological activity in a solution. In the standard format, a solution flows through a membrane which has been locally imprinted with proteins targeting the bioanalyte under study. Upon immunorecognition at the test area of the membrane, the biological agent is immobilized and its presence can be readily identified by the photoluminescence of the colored particles that the analyte has been labeled with. Quantitative measurements of the concentration can only be achieved via chromatographers (immune-chromatic test). However, in the past decade the magnetic thin film technology has been successfully implemented into the bioanalytical science [1]. In this paper we propose an efficient and unexpensive method substituting the colored particles by magnetic nano-beads and the photodiode detection by magnetoresistive sensors [2]. The sensing mechanism is thus the same as in the reading heads for the magnetic bits of commercial hard drives: giant magnetoresistence (GMR) or tunnel magnetoresistence (TMR). We pattern a chip containing arrays of such sensing units with micrometric footprint. A strip-shaped sample membrane is scanned by the chip, resolving the magnetic signature of the sample with micrometric resolution. In terms of sensibility, this method is at the same level as former techniques based on the coil inductance [3,4], but in addition, the large spatial resolution brings about the possibility of multianalyte single shot assays. Furthermore, reference magnetic motifs can get immobilized/sensed in the same membrane and environment as the test region, extending the concept to a field-deployable device.

We will describe the design and performance optimization of the biosensensing station utilized to carry out immune-magnetic tests. It features micrometric xyz positioning and controlled pressure (ΔP<4 psi) of the sample membrane over the sensor surface. The detection limit has been pushed beyond the visual limit, with a figure of merit of 1 μemu sensibility (equivalent to 2 nano-grams of maghemite), which surpasses the SQUID sensibility in this sort of sample system. Test measurements on both artificial sample membranes and real biological assays will be shown, and compared with results from alternative characterization tools.

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Magnetic particles functional characterization at the Bioinstrumentation Laboratory of the Centre for Biomedical Technology

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Nanoscience and nanotechnology are in the spotlight nowadays because researchers from these fields are now able to explain and control the phenomena observed at the nano and microscales. They are trying to create new medical applications like treatment and diagnosis using magnetic micro and nanoparticles by breaking down the frontiers between biology, chemistry and physics.
Researchers need to overcome several problems with these substances, such as toxicity issues or the understanding of the human body complexity, if magnetic micro and nanoparticles (MNPs) are wanted to be used in medical applications. Besides, there's a huge gap between in vivo and in vitro experimentation with MNPs that researchers are trying to remove. Following this idea, the Bioinstrumentation Laboratory is working on setting up a characterization platform of MNPs where researchers or fabricants send their samples and receive several data, such as the magnetic and mechanical response, relaxation time when are used as contrast agents in MRI, and movement behavior of the particles.
The magnetic and mechanical response is measured using the Alternating Gradient Field Magnetometer (AGFM) *MicroMag Model 2900 AGM System*®. Due to its high sensitivity, low noise floor and its capability to accommodate a large range of samples of very different properties, such as solid samples, ultra thin films, powders, liquids and even slurries, the AGFM can be used for characterizing the magnetic and mechanical properties of MNPs in biological samples. Some examples of actual research lines with the AGFM are the discrimination of the behavior of MNPs acquired by cells or situated on the extracellular matrix and the use of the AGFM as biosensor. The final goal is to use the AGFM combined with other techniques for the detection and identification of engineered MNPs as contaminant in ex-vivo samples (see Figure1) [1-4].
The *SMARTracer* Relaxometer, in conjunction with a 2T Electromagnet, allows the acquisition of the T1 and T2 relaxation rates of water and biological solutions containing MNPs when are employed as Contrast Agents in MRI. The relation between relaxation rates and the morphological and physical properties of the particles can be obtained by adapting the information contained in the Nuclear Magnetic Relaxation Dispersion (NMRD) profiles (dependence of the relaxivity on magnetic field strength, Figure 2(a)) into a Theoretical Model. The main goal of this research line is to obtain the concentration of MNPs on the tissue directly from a MRI using the information of the NMRD profiles and the data from the Theoretical Model (see Figure 2 (a,b)) [5-7].
To achieve dynamic characterization, which means in this case movement of magnetic particles, it was made a set up that involves: magnets, auxiliary geometries for focalization, viscometer, analysis and simulation software as well as random laboratory material. With all these things together plus theoretical phenomena, it is possible the understanding of this kind of magnetic particles characteristics. The principal research lines are oriented to cellular filters fabrication, focusing of magnetic particles using magnetostatic fields, mathematical model optimization of magnetic phenomena, and human and animal models for in vitro experimentation (phantoms). The final goal is the use of these techniques to separate cells and its implementation for treatment and diagnosis (see Figure 3) [8-12].

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Figures

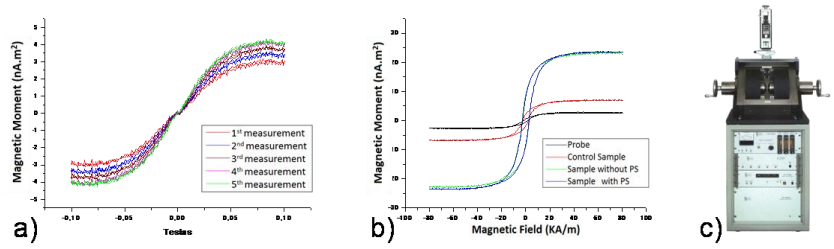

Figure 1. Magnetic and mechanical response measured using an AGFM. The hysteresis loops can been seen, a) behavioral differences between solid and liquid samples with MNPs, and b) MNPs detection inside cells; a picture of the AGFM is shown on c).

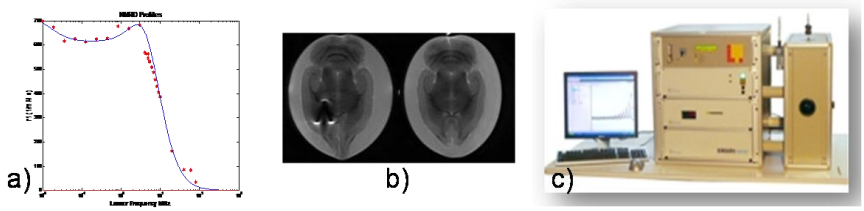

Figure 2. MNPs Characterization as Contrast Agent in MRI: a) NMRD Profiles of a sample of MNPs. b) Differences between images with (left) and without (right) Contrast Agents. c) Fast Field Cycling Relaxometer.

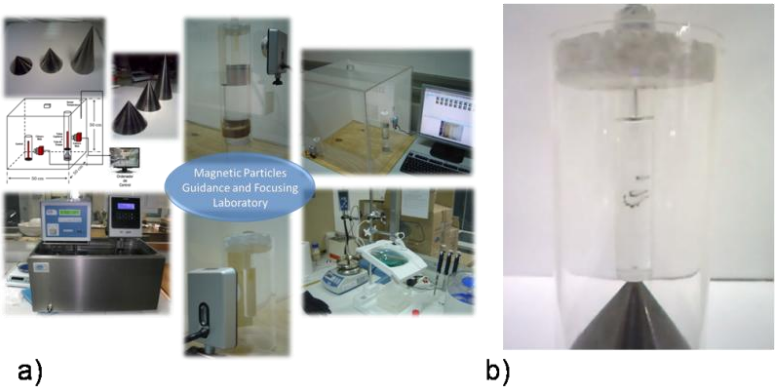

Figure 3. Dynamic characterization of magnetic particles. On a) different equipment used for characterization, and on b) an image of magnetic particles dynamic characterization.

Self-polarization phenomenon and control of dispersion of synthetic antiferromagnetic nanoparticules for biological applications

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Using a top-down approach, synthetic antiferromagnetic micro/nanoparticles [1] usable for biological applications were prepared. They are made of magnetic multilayers consisting of ferromagnetic layers antiferromagnetically coupled between them through a thin ruthenium spacer. These particles exhibit "superparamagnetic-like" properties. Their magnetic susceptibility can be accurately controlled by the thickness of the constituting layers. We focused on particles of composition $(NiFe/Ru0.7nm/)_n/NiFe$ with a total NiFe thickness kept constant at 120nm and varying the number of repeats n between 1 and 7. The size of particles that we investigated was 1μm*1μm*120nm. These particles have much higher moment and susceptibility than conventional magnetic oxide based nanoparticles prepared by chemical routes. This allows manipulating them with lower magnetic fields. The preparation technique is illustrated in Figure 1 with various examples of realization. The technique benefits from all the knowhow in microelectronic technology in particular nano-imprint lithography. Particles as small as 30nm could be prepared. The interest of this approach is that both the shape and composition of the particles can be optimized depending on the foreseen biotechnological applications. Besides, these particles are not spherical. They exhibit some shape anisotropy or intrinsic magnetic anisotropy which opens new possibilities to manipulate them with magnetic fields rather than gradients of magnetic field. This may allow controlling their motion at much longer distance than with conventional approach. Indeed gradients of magnetic field decrease very rapidly as a function of distance from field source whereas field can be produced over much longer distance.

When dispersed in solution, striking differences in the interactions between these synthetic antiferromagnetic particles are observed depending on their susceptibility [2]. Above a susceptibility threshold, a phenomenon of self-polarization is observed in zero applied field resulting in a gradual agglomeration of the particles. In contrast, below this susceptibility threshold, the particles get redispersed in zero field. For practical use, this second situation is of course preferable. It is therefore possible to optimize the magnetic susceptibility to keep it much larger than in conventional magnetic nanoparticles but still avoiding their magnetic agglomeration. This control of agglomeration.dispersion is illustrated in Fig.2. This phenomenon was interpreted by a self-consistent model taking into account dipolar interactions between particles and their magnetic susceptibility [2]. The model is similar to the mean field theory of ferromagnetism wherein the mean field is due to the dipolar field created by all neighboring particles on a given one.

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Figures

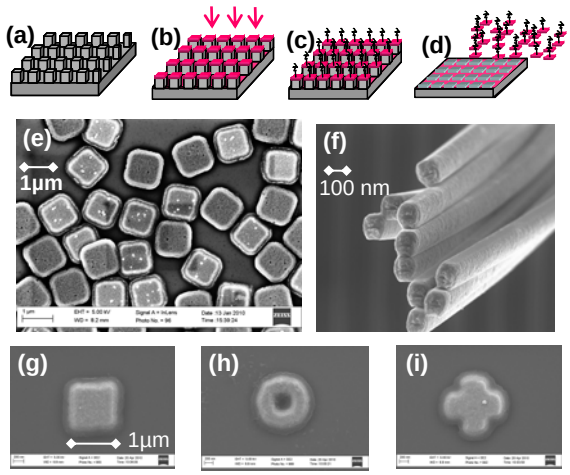

FIG.1. "Top-down" approach: schematic steps of the nanoparticles fabrication. (a) Prestructuration of wafer (nanoimprint or lithography) (b) Deposition of magnetic stack (c) Functionalization of surfaces (d) Release in solution by lift-off. SEM Photos: (e) SAF "superparamagnetic-like" particles, NiFe/Ru/NiFe, 1µmx1µmx120nm (f) Multilayered magneto-resistive nanowires 100nm in diameter, 100 m long, prepared by this top-down approach. (g) Square (h) Ring (i) Cross.

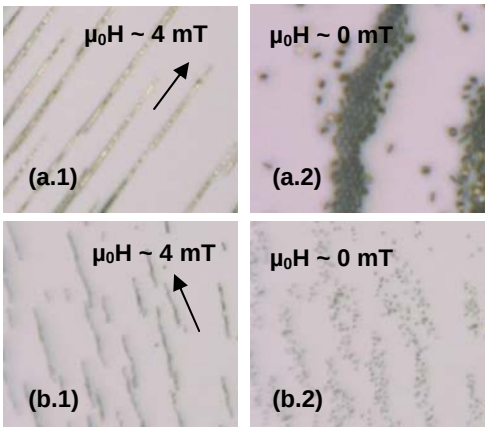

FIG.2. Optical microscopy images of SAF particles in acetone, after lift-off. (1µmx1µmx120nm) of (NiFe/Ru/)$_n$NiFe (a) n=1, $\mu_0 H_{SAT}$ ~4 mT. (b) n=7, $\mu_0 H_{SAT}$ ~32 mT. (a.1) Chains in μ_0H~4 mT (a.2) Self-polarization in zero external H~0 (in earth field) (b.1) Chains in μ_0H~4 mT (b.2) Dispersion in external H~0 (in earth field).

Gold and silver nanomaterials based biosensors : a comparative study

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Noble metal nanoparticles (NPs) are intensively studied due to their particular optical properties, mainly high optical absorption and diffusion yields, leading to interesting applications in biochemical sensing, molecular tracking and imaging, drug delivery and photothermal therapies [1]. These unique optical properties arise from a physical process named surface plasmon resonance (SPR) which is a resonant coupling of incident light to the collective motion of electrons along the nanoparticles surface [2].

Optical SPR biosensors are able to measure complex formation in real time. Indeed, the SPR absorption spectrum band of the NPs is sensitive to the shape, size, inter-particle distance and composition of the NP as well as the dielectric properties of the surrounding medium [2]. Due to the sensitivity of SPR to the local dielectric environment, plasmonic NPs can thus act as transducers that convert small changes in the local refractive index or in the inter-particle distance into spectral shifts and broadenings of the absorption spectral bands [3].

Among metals, silver and gold NPs have received considerable interest for many reasons. For instance, they are stable in ambient atmosphere and exhibit good biocompatibility even if particular surface treatments are sometimes required. The Ag and Au NPs are also relatively easy to fabricate with different sizes and shapes allowing the tuning of the SPR optical absorption band from the near ultraviolet (400 nm) to the near infrared (1000 nm) wavelengths.

In this study, our aim is to characterize two biosensors based on silver and gold spherical NPs in order to detect which one seems the best. Both NPs have a diameter close to 15 nm. We use the well-known biocytin-avidin complex as a model system because the bonding of avidin with biocytin is extremely strong with a dissociation constant three order of magnitude higher than the typical constants of antigen-antibody interactions. More precisely, we compare the intensities, the band shapes and the spectral locations of the SPR adsorption bands before and after the biomolecular recognition of avidin by biocytin molecules adsorbed on the Ag and Au NPs. The kinetic of the interaction is also discussed.

Before surface treatment with biocytin, the NPs SPR bands are located around 390 and 520 nm for Ag and Au NPs, respectively. The SPR band intensity is higher for silver than for gold.

Biocytin adsorption does not significantly modify the SPR spectral features. NPs do not therefore form aggregates and the local refraction index has not significantly changed.

After avidin addition, a SPR red-shift and a broadening of the SPR bands are observed with both NPs as shown on Figure 1. These parameters evolved with time and reach their final values after around 45 min for each system. The aforementioned spectral changes arise from the biomolecular recognition process between biotin and avidin which leads to the NPs aggregation. The recognition process also induces a variation of the local refractive index around these NPs which contributes to the red-shift. The maximum SPR shifts are equal to 25 nm and 12 nm for silver NPs and gold NPs, respectively. Our results highlight the smaller dielectric sensitivity of gold NPs compared to the silver NPs one for a same particles' size and for an equivalent concentration of avidin. The detection limit, described as the lowest concentration for clear identification of wavelength shift due to biomolecular recognition, is equal to 4 nM for both silver and gold NPs. With this protein concentration, 3 nm is the typical wavelength shift.

The specificity of the biocytin - avidin biosensors is verified by replacing avidin by Bovine Serum Albumina (BSA). When BSA is added, we observe a SPR band shift which is smaller than the detection limit of 3 nm attesting the biosensor selectivity.

Our work demonstrates the superiority of Ag over Au NPs for the elaboration of biosensors based on SPR. However, it is well-known that Ag NPs are less biocompatible than gold. This problem can be circumvented by an appropriate coating of the NPs surface prior ligand adsorption.

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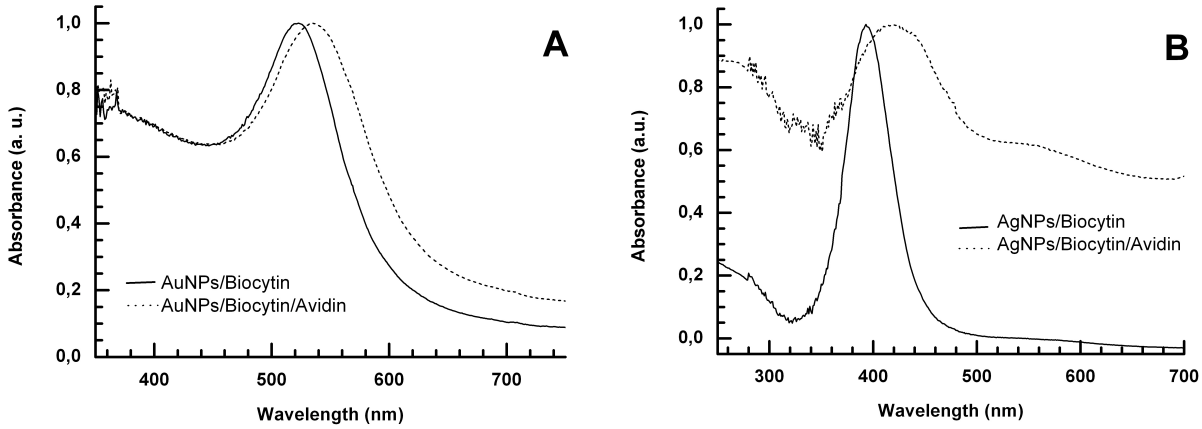
Figure 1



UV-VIS absorption spectra of biocytin - AuNPs (spectra A) and biocytin – AgNPs (spectra B) before (continuous line) and after (dashed line) avidin interaction. The spectra are normalized by the adsorbance maxima.

Self assembled nanogels

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Amphiphilic molecules, such as surfactants or lipids, spontaneously self-assemble in water, forming self-aggregates, such as micelles, bilayer membranes, tubes and vesicles. Amphiphilicity of biopolymers is one of the important factors for their self-organization in water [1]. By self-assembling, the hydrophobic segments are segregated from the aqueous exterior, to form an inner core surrounded by hydrophilic chains. This kind of structure is suitable for trapping hydrophobic substances, such as fluorescent probes, proteins, and hydrophobic pharmaceuticals. Size, density and colloidal stability of nanoparticles can be controlled, by changing the degree of substitution of hydrophobes and its hydrophobicity. The association mechanism is mainly governed by the alkyl chain concentration and length and is little influenced by the molecular weight of the polymer backbone.
The amphiphilic molecule dextrin-VA-SC_{16} ($dexC_{16}$) was produced and studied in this work. $DexC_{16}$ has a hydrophilic dextrin backbone with grafted acrylate groups (VA), substituted with hydrophobic 1-hexadecanethiol (C_{16}). The dextrin degree of substitution with the hydrophobic chains (DS_{C16}, number of alkyl chains per 100 dextrin glucopyranoside residues) may be controlled. Materials with different DS_{C16} were prepared and characterized using ^{1}H NMR. $DexC_{16}$ self assembles in water through association of the hydrophobic alkyl chains, originating hydrogel nanoparticles. The properties of the hydrogel nanoparticle were studied by dynamic light scattering (DLS), fluorescence spectroscopy and atomic force microscopy (AFM). Nanostructures spontaneously form when the concentration of the polymer is higher than a concentration called critical micelle concentration (cmc). The self-assembly of hydrophobized dextrin in water ($dexC_{16}$) was investigated in this work. The structural change upon the dilution of the $dexC_{16}$ self-aggregates, in water, was investigated by fluorometry, in the presence of pyrene as the fluorescent probe. Other relevant properties of the nanoparticles, such as the size, stability, and shape were also evaluated in this work. Furthermore, the interaction of the nanoparticles with cells (fibroblasts and macrophages, studies on the biodistribution, intracellular trafiking, the encapsulation of therapeutic proteins such as IL10 and anticancer drugs such as curcumine were analysed [2,3,4,5,6]

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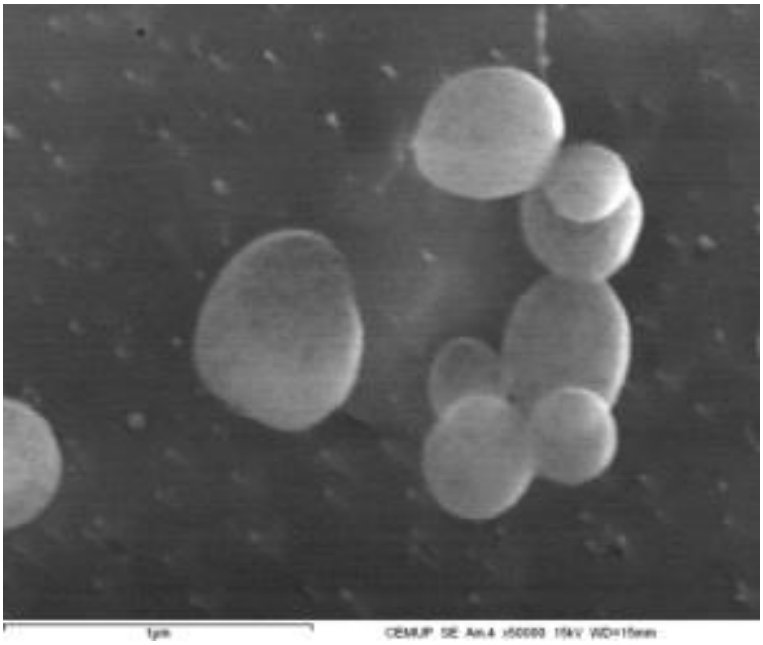

Cryo SEM images of dextrin nanogel

Detection of cancer marker ebna-1 by aptamer based biosensors

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The Epstein-Barr virus (EBV) is a human herpes virus that infects the majority of World´s adult population. Following primary infection, EBV immortalized a portion of hosts B-lymphocytes and establishes a latent infection that persists in the patient for life. EVB is the causative agent of infectious mononucleosis and has been associated with several malignancies including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and lymphoproliferative disorders in immunodeficient individuals. All EVB associated disorders shows a distinct viral gene expression patter, but in all of them the nuclear antigen 1 (EBNA-1) is constitutively expressed [1].

EBNA-1 is a DNA binding protein located in the nucleus that is necessary for the maintenance and replication of the viral genome. In EVB associated tumors and latently infected B-lymphocytes, viral genome persist as a multicopy episome. For viral genome maintenance and replication, EBNA-1 forms homodimers that recognize specific sequences in the EVB genome. In addition to its role in viral episome maintenance, it was suggested that EBNA-1 can contribute to the oncogenic process by up-regulating the apoptosis suppressor protein in EVB-associated B-lymphomas [2]. Furthermore, it was reported the direct binding of EBNA-1 to cellular promoters and the correlation between EBNA-1 bound promoters and changes in gene expression [3].

In the present work, we develop an aptamer based system to detect EBNA-1 using Quartz Crystal Microbalance (QCM). The EBNA-1 binding aptamer was obtained by annealing 30-mer consensus oligonucleotides, and 16 T bases and a thiol group were added at the 5´end to facilitate its immobilization on the gold electrode. QCM experiments were performed using a Q-Sense E4 unit (Q-Sense AB, Sweden) and the shifts in frequency (Δf) was monitored online.

Scheme 1A show the label free detection of EBNA-1. In order to get a calibration curve, different standard solutions of EBNA-1, ranging from 20 nM to 0.5 nM, were applied to the biosensors. As shown in Fig. 1A, there is a linear relationship between the shift in frequency and the concentration of EBNA-1 in the range of 0.5 and 10 nM, with a correlation coefficient of 0.991 and a detection limit of 0.5 nM.

To detect EBNA-1 concentrations below 0.5 nM, we used a signal amplification system based on the alkaline phosphatase (AP) catalyzed oxidative hydrolysis of 5-bromo-4-chloro-3-indolyl phosphate [4], showed in scheme 1B. The oxidative hydrolysis of 5-bromo-4-chloro-3-indolyl phosphate yields the accumulation of the insoluble indigo derivative on the surface of the electrode that provides an amplification route for the detection of EBNA-1. The precipitate generates an increase of mass on the QCM sensor, resulting in a decrease in the resonance frequency of the electrode.

As shows the in Fig.1B, the frequency shift shows a linear relationship with the logarithm of the EBNA-1 concentration over a range of 5-100 pM, with a correlation coefficient of 0.994 and a detection limit of 5 pM. This means that using the signal amplification system the sensitivity was improved 100 times in comparison with that of the label free detection of EBNA-1 (0.5 nM).

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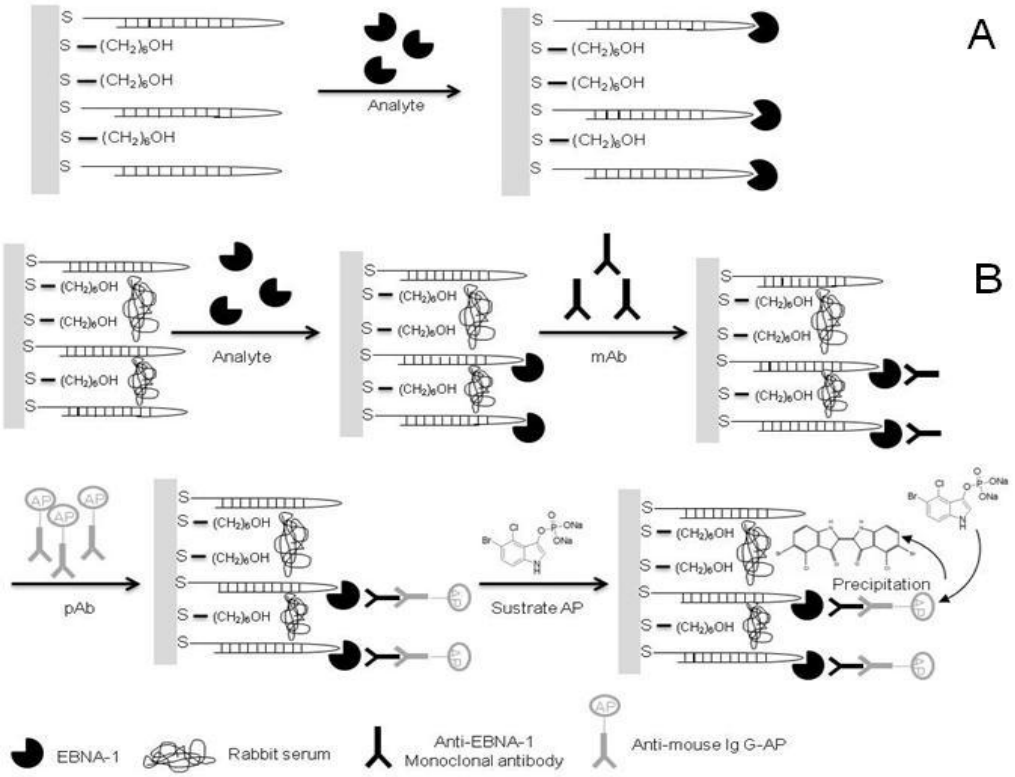

***Scheme 1**. Schematic diagram of A) the label free detection of EBNA-1 and B) detection of EBNA-1 by the 5-bromo-4-chloro-3-indolyl phosphate based amplification system.*

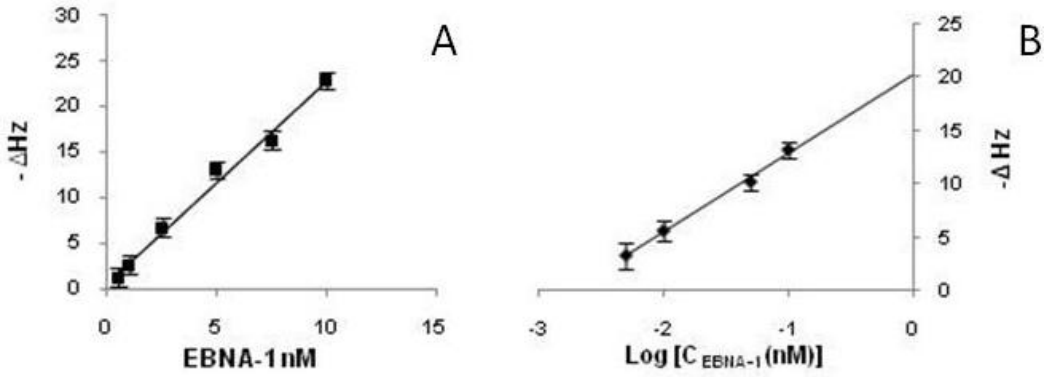

***Figure 1**. Calibration curve A) linear relationship between EBNA-1 concentration and frequency shift in the range of 0.5-10 nM. Calibration curve B) linear relationship between the amplified frequency shift and the logarithm of EBNA-1 concentrations. The error bars represent the standard deviation of three measurements.*

New antiangiogenic polymer drugs. From synthesis to biological activity.

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Introduction: Inhibition of angiogenesis is one of the main strategies carried out in the treatment of solid tumors. Heparin-dependent growth factors (GF), such as fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF) are directly involved in this disease being over expressed in the tumoral tissue. The aim of this work is the inhibion of the pro-angiogenic activity of these GF by the action of new heparin-like copolymers synthesized in our laboratory based on a methacrylic derivative of 5-amino-2-naphthalene sulfonic acid (MANSA) and 2-acrylamide-2-methylpropane sulfonic acid (AMPS). The molecular structure-bioactivity relationship of four new families of copolymeric systems is discussed. The physico-chemical characterization of the macromolecules is reported. Also the *in vitro* and *in vivo* antiangiogenic effect of these polymers is demonstrated.

Materials and Methods: Four copolymeric systems were obtained by free-radical copolymerization. AMPS [1] and MANSA [2] were copolymerized with a hydrophilic or a hydrophobic monomer (N-vinylpyrrolidone, VP, or butylacrylate, BA, respectively) in order to prepare macromolecules with different hydrophilic/hydrophobic balance.

Reactivity ratios were determined by *in situ* ^{1}H-NMR in order to estimate the monomer distribution along the polymeric chains. The copolymers conformation was studied using light scattering and zeta potential measurements.

The biological behavior of the new materials was studied *in vitro* using 2D cultures of endothelial cells to determine the inhibitory activity of the polymers on cell proliferation induced by pro-angiogenic factors and 3D cultures on fibrin matrix to determined the inhibition of different steps in the angiogenic process [3].

Finally we studied the *in vivo* activity using 6-week-old female nude mouse. Two silicon tubes filled with Matrigel that contained either PBS (negative control), bFGF (positive control) or bFGF plus the polymers were inserted into a skin pocket in the back of each anesthetized nude mice. Eleven days later, the mice were injected intravenously with FITC–dextran and 10 minutes later, the tubes were removed from the skin pockets and the amount of fluorescence trapped in the implants was measured to evaluate the volume of blood circulating through the newly formed vessels.

Results and Discussion: The reactivity ratios of the AMPS systems (VP-AMPS: r_{VP}=0.12; r_{AMPS}=0.28; BA-AMPS: r_{BA}=3.60; r_{AMPS}=0.28) indicated that VP-AMPS system is azeotropic presenting a moderate

alternating structure whereas BA-AMPS preferably added the most reactive monomer BA to the growing chain.

The reactivity ratios of the different systems (VP-MANSA: r_{VP}=13.40; r_{MANSA}=0.12; BA-MANSA: r_{BA}=14.78; r_{MANSA}=0.26) indicated that both propagating species preferably added the most reactive monomer (VP or BA). Consequently, there was a tendency toward consecutive homopolymerization of the two monomers. At the beginning of the reaction VP or BA-rich copolymers were formed; when these monomers were almost consumed MANSA-rich copolymers were subsequently formed.

This monomer distribution along the copolymeric chains lets the formation of micelles in the case of BA-MANSA and BA-AMPS copolymers with a BA (hydrophobic) core and a MANSA (hydrophilic) corona. The particular self-assembling of these macromolecules, locating the sulfonic groups on the surface of the micelles, made them more accessible to interact with the proangiogenic growth factors.

The anti-angiogenic activity was measured by 2D and 3D *in vitro* assays. Higher activity of the BA-MANSA copolymers was observed, being the micelles more active than linear polymers with similar amount of MANSA in their structure.

Finally the *in vivo* assays also showed a better inhibition in the case of BA-MANSA systems reaching almost a total inhibition.

Conclusions: Anti-angiogenic activity of new copolymers bearing sulfonic groups in their structure (heparin-like synthetic macromolecules) was tested by 2D and 3D *in vitro* and *in vivo* assays, showing good inhibition of different steps of the angiogenesis process. This bioactivity was related to the particular distribution of the monomers along the copolymeric chains and their organization in supramolecular structures.

Acknowledgements: This study was supported by grant SAF2009-13240-C02-01 (Spanish Ministry of Science and Innovation) and CIBER-BBN.

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SPS sintered YTZP-MWCNT nanocomposites with an outstanding crack and ageing resistance

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Yttria Stabilized Zirconia (YTZP) ceramics are widely used in biomedical applications such as orthopedic and dental implants. The biocompatible YTZP shows high crack resistance for a ceramic due to a phase transformation reinforcement mechanism [1-2]. However, the major drawback of YTZP is its lack of stability: zirconia is prone to ageing especially (but not only) under humid atmosphere. Ageing is referred to a slow surface transformation of the zirconia from its high temperature structure (tetragonal structure), obtained by the stabilization of the ceramic with yttria, into the stable monoclinic phase in the presence of water or water vapor. This transformation induces surface roughening, microcracking and, for the most severe cases, failure and loss of functionality. The most dramatic case of ageing was reported at the beginning of 2002 for zirconia hip joints heads, when several hundreds of implants failed within a short period [3]. There have been several attempts in the recent literature to increase the ageing resistance of YTZP. However, increasing the ageing resistance of YTZP led so far to a decrease of toughness and crack resistance. This is due to the fact that to avoid the ageing it is necessary to reduce the transformability of the zirconia, reaching a more stable tetragonal phase. But this will also imply less transformability under stress, which results in lower mechanical strength. On the one hand, the reduction of the zirconia grain size to a submicrometric or nanometric level, limits the phase transformation and, therefore, ageing is delayed [4-5]. But, on the other hand, the fracture toughness is reduced, because the transformation toughening mechanism is lost.

In this work the addition of a small volume fraction of multiwall carbon nanotubes (MWCNT) in a polycrystalline, nano-structured YTZP, sintered by Spark plasma Sintering (SPS) leads to a material exhibiting a balance between ageing and crack resistance never reached before.

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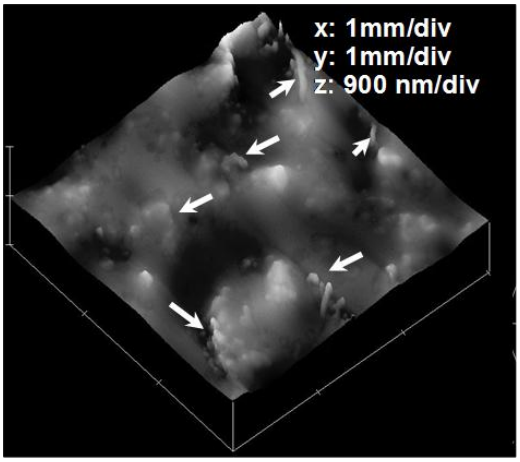
Figures



AFM image of an YTZP-MWCNT sample sintered by SPS showing the carbon nanotube pull-out on the fracture surface

Novel paradigms for biological sensing based on nanomechanical systems

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The development of ultrasensitive protein spectrometers and ultrasensitive biological sensors will speed up the identification of disease biomarkers and their rapid detection [1,2]. Nanomechanical resonators have emerged as promising candidates for ultrasensitive mass sensors [3,4]. The continuous advancements in top-down micro- and nanofabrication techniques has made possible increasingly smaller nanomechanical resonators with detection limits in the subattogram range. Moreover, resonant nanowires and nanotubes fabricated by bottom-up methods can weigh masses below a zeptogram (1.66 10-21 g). However, the implementation of these devices is hindered by several obstacles such as the need of operation in high vacuum, low specificity and low reproducibility and still little understanding of the effect of biomolecular adsorption on the mechanical properties of nanoresonators.
In this talk, I will present our recent developments oriented to apply nanomechanical systems for biological detection. In particular, I will present two novel paradigms for sensing that opens the door to develop ultrasensitive biological sensors. The first approach is the use of coupled nanomechanical resonators fabricated by standard silicon technology [5,6] (Fig. 1). When the resonators are identical, the vibration of the eigenmodes is delocalized over the array. In a similar way to the Anderson's localization, the addition of the mass on one of the resonators leads to the spatial localization of the eigenmodes. Since vibration localization is insensitive to uniform adsorption, coupled nanomechanical resonators allows decoupling of unspecific and specific molecular adsorption in differentially sensitized resonators. The main advantage of measuring eigenmode localization, is the posibility of tune it sensitivity through the modification of the coupling constant. By decreasing the coupling they become more sensitive, being able even to beat current frequency shift measurements with isolated cantilevers. On this way, we can improve the resonator sensitivity without fall in miniaturation, that could be feasible in some applications. In adittion, eigenmode localization is no longer depending on the extreme miniaturization.
The second approach uses resonant nanowires/nanotubes (Fig. 2) and it is based on the fact that if a molecule alights on a perfectly axisymmetric resonant nanobeam, the frequency degeneration of the stochastic two-dimensional orbits is abruptly broken, and the vibration can be described as the superposition of two orthogonal vibrations with different frequency. The tracking of both frequencies and the orentation of the vibrational planes, enables the determination of the adsorbate's mass and stiffness, as well as the azimuthal direction from which the adsorbate arrives [7]. We experimentally demonstrate such sensing paradigm with resonant silicon nanowires, which serves to add kPa resolution in Young's modulus determination to their currently established zeptogram mass sensitivity. The proposed method provides a unique asset for ultrasensitive mass and stiffness spectrometry of biomolecules by using nanowire-like resonant structures.

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Figures

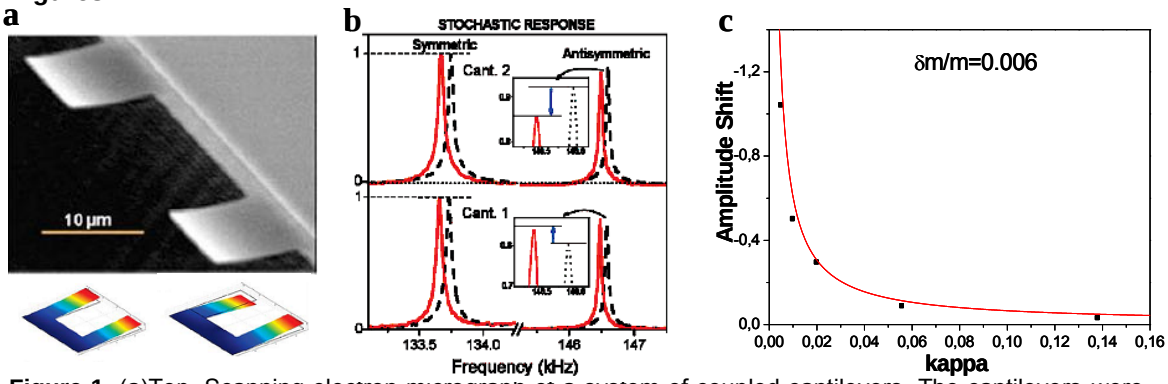

Figure 1. (a)Top. Scanning electron micrograph of a system of coupled cantilevers. The cantilevers were fabricated in low stress silicon nitride. The length, width, and thickness of the cantilevers were 25, 10, and 0.1 µm, respectively. The gap between the cantilevers is 20 µm. The structural coupling between the cantilevers arises from the overhang connecting the cantilevers at the base, Lo, which is about 8 µm long. Botton. FEM simulation of symmetric and antisymmetric mode of vibration of this coupled array modes (b) Stochastic response driven by ambient thermal excitation, of a two couple resonators before (black, broken line) and after (red, solid line) the deposition of 170 fg of mass on cantilever 2. All the spectra have been normalized such that the height of the symmetric peak equals 1.The insets show a zoom of the relative change in the amplitude of the antisymmetric mode. Ratio between the relative changes of the antisymmetric/symmetric amplitude ratio and resonance frequency shift versus the coupling constant. The symbols are experimental data and the dashed red line is a theoretical prediction based on coupled harmonic oscillator theory and the fluctuation-dissipation. c) Relative changes of the antisymmetric/symmetric amplitude ratio produced by the deposition of 100 fg on cantilever 2, versus the coupling constant. The coupling constant is decreased by increasing the pitch between cantilevers. Pitch range from 2- 100 µm. The symbols are experimental data and the dashed red line is a theoretical prediction based on coupled harmonic oscillator theory and the fluctuation-dissipation.

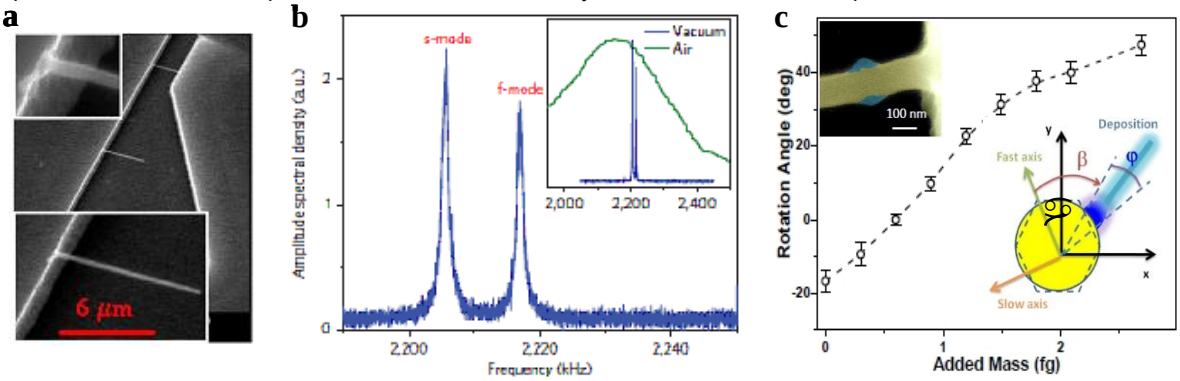

Figure 2.(*a) Scanning electron micrograph (SEM) of a typical nanowire used in this work. Nanowires anchored normal to the trench wall were selected, with lengths and diameters of 5–10 µm and 100–300 nm, respectively. (b) A fast Fourier transform of the signal from the photodetector is dominated by the displacement thermal fluctuation of the nanowires. Only a single resonant peak can be seen in air (green line in inset), but two resonant peaks can be clearly seen in vacuum (blue lines). Depending on the nanowire dimensions, the resonance frequencies range from 2 to 6 MHz. s-mode and f-mode refers to the spliting of the resonance frequency into slower and faster vibration modes vibrating at orthogonal directions. (c) Evolution of the angle between the fast vibration axis and the optical axis (α) versus mass added to the clamped end of the nanowire.after successive depositions of ~0.6 fg. Deposition was performed at an angle of ~45° to the optical axis (inset). Each deposition rotates the fast vibration plane through 7–12° towards the deposition direction, as indicated by the green arrows.*

Biosensors Based on DNA Aptamers and Antibodies for Medical Diagnostics

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DNA aptamers are single stranded oligonucleotides (<100 bases) with high affinity to proteins or other ligands, comparable to those of antibodies. The aptamers are selected in vitro by the SELEX method [1]. Once the sequence is selected, aptamers can be multiplied with high precision and purity. In solution, the selected sequence maintains an unique 3D configuration that contains specific binding site to the ligand. Aptamers can be easily modified by biotin, SH or amino- groups, leading to a variety of immobilization strategies on solid supports. Thus, aptamers can serve as simple and highly sensitive artificial receptors. Currently there is increased interest in development of aptamer based biosensors for detection proteins and other molecules using various method of detection, such us optical, acoustical and electrochemical [2,3]. These biosensors could be used in medical diagnostics as a fast and low cost method of detection the dissease indicators, such are increased concentration of thrombin or prions. The sensitivity of detection depends not only on the selectivity of binding site, but also on supporting part of the aptamer that serve for immobilisation onto a solid support. Aptamers are rather flexible and using simple molecular engineering it is possible to increase their sensitivity. This has been demonstrated simultaneously by Hasegawa et al. [4] that used thymine linker for connection of two DNA aptamers sensitive to fibrinogen and heparin-binding exosites of thrombin and by us for thrombin aptamer dimers (so called aptabodies) that were prepared by hybridization of aptamer supporting parts [5,6]. Using multiwalled carbon nanotubes (MWCNT) as an immobilization matrix we developed high sensitive biosensor for detection human thrombin [5] and cellular prions (PrP^C) [7] in biological liquids.

In this work we extended our study to comparative analysis of efficiency of DNA aptamers and antibodies for detection human cellular prions using electrochemical quartz crystal microbalance (EQCM), quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) methods. We also analyzed possibility of amplification the detection using gold nanoparticles conjugated to aptamers or antibodies. The DNA aptamer specific for PrP^C was designed according to Takemura et al. [8] and purchased from Thermo Fisher Scientific (Germany). This aptamer was extended by dT_{15} spacer (5`-CGG TGG GGC AAT TTC TCC TAC TGT $dT_{15}$-3`) and has been modified at 3` end either by SH group or biotin. Gold nanoparticles (diameter 10 nm, Sigma Aldrich, USA) were used for modification of aptamers or antibodies. Monoclonal antibodies BAR 233 and PRI 308 which recognizes the PrP^C sequence within amino acids 141-152 and 106-126, respectively, were from Spibio (Montigny France), AG4 and AH6 antibodies recommended for detection of N-terminal amino acid residues 31-51 and C-terminal amino acid residues 90-230, respectively of PrP^C were from TSE Resource (UK). The receptors

(aptamers or antibodies) were immobilized either on MWCNT, polyamidoamine dendrimers of four generation (G4) conjugated with protein A, or on a surface of conducting co-polymer.

The highest sensitivity of detection of PrP^C indicated by limit of detection (LOD) has been obtained by EQCM method and using aptamers or BAR 223 antibodies immobilized on MWCNT (LOD 50 and 20 pM, respectively). Less sensitivity was obtained when antibodies were immobilised on a surface of QCM transducer modified by G4 dendrimers conjugated with protein A. In this case the detection considerably depends on the type of antibody used as it is demonstrated on (Fig. 1) where the plot of the frequency changes as a function of PrP^C is shown. Best LOD has been obtained for PRI 308 (0.8 nM), however amplification of the QCM detection by means of DNA aptamers (Fig. 2) or antibodies AH6 conjugated

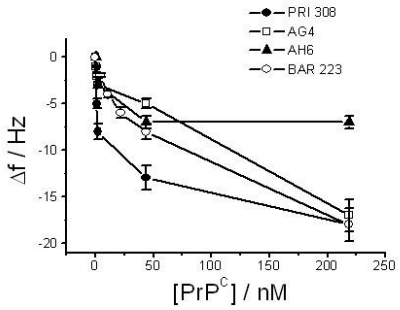

Fig. 1. The plot of the changes of the resonant frequency as a function of PrP^C concentration for QCM biosensors based on various antibodies against PrP^C.

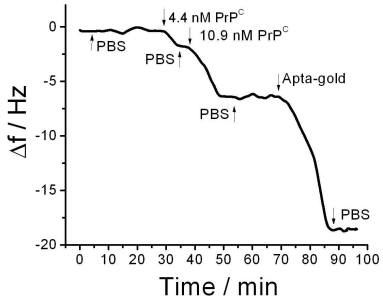

Fig. 2. The kinetics of the frequency changes following addition of PrP^C and amplification of the sensor response by the injection DNA aptamers modified by gold nanoparticles (Apta-gold). PBS is phosphate buffer used for removing non specifically adsorbed molecules.

With gold nanoparticles improved LOD substantially (110 pM). The sensitivity of detection PrP^C by QCM using antibodies and SPR using DNA aptamers was comparable. However, the advantage of aptamers-based SPR detection, consisted in possibility of sensor regeneration by means of washing the surface with 0.1 M NaOH.

Acknowledgements
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Strategies for molecular imaging with inorganic nanoparticles

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Targeted nanoparticles have great potential for application as radionuclide molecular imaging agents but are subject to several limitations, including complex radiolabelling procedures, slow pharmacokinetics, low uptake in target tissue, and potential toxicity. We propose a targeted nanoparticle system comprising biocompatible materials with intrinsic affinity for readily-prepared radiotracers such as [^{18}F]-fluoride and [^{99m}Tc]-technetium bisphosphonate derivatives. Such a system would offer simple labelling, and signal amplification (each particle can deliver many radionuclides). To overcome slow pharmacokinetics we propose to exploit pretargeting whereby the radionuclide-nanoparticle bond can form *in vivo*. Methods: We screened many inorganic nanoparticulate materials for binding to [^{18}F]-fluoride and [^{99m}Tc]-bisphosphonates, and synthesised bifunctional linkers comprising a bisphosphonate group for binding to nanoparticles and a maleimide group for conjugation to biomolecules. Results: Of the materials tested, hydroxyapatite showed the most efficient binding to both [^{18}F]-fluoride and [^{99m}Tc]-bisphophonates. The radiolabel remained associated with nanoparticle in serum, and *in vivo* in mice until taken up in the reticuloendothelial system. Conjugation of the maleimide derivative to thiol groups of proteins and peptides led to efficient binding of the biomolecules to hydroxyapatite particles. Conclusion: hydroxyapatite, bisphosphonate bioconjugates and bone-affine radiopharmaceuticals can be assembled into a targeted nanoparticulate biocompatible system for radionuclide moelcular imaging.

Use of a new family of biomarker : fluorescent noble metal (Au, Ag) nanoclusters for protein-labelling

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Fluorescent labelling techniques have been used extensively in both biological research (in vivo imaging & targeting) and clinical diagnosis because of its high sensitivity, simplicity, and diversity. Biological studies are often limited by existing fluorophores which suffer from inherent deficiencies. For example, organic fluorophores, which are most commonly used in fluorescence spectroscopy, are easily photobleached. Large fluorescent tags can also perturb labelled biomolecules, causing artificial movement within cells and altered protein-protein interactions. Another fluorophore, quantum dots, shows great promise in biolabelling due to their unique optical properties which cause them to emit light of different colours depending on their size. Unfortunately, quantum dots are commonly synthesized using harsh conditions and toxic precursors, are difficult to surface passivate, have large physical size comparable to proteins after biocompatible surface modification, and tend to photoblink.

Noble metal nanoclusters (NCs) have gained a tremendous interest in the last five years due to their photophysical properties and their applications in nanomedicine. Several groups have studied the origin of the fluorescence of NCs and their structure–property inside different cavities such as polymers[1], dendrimers[2] and proteins[3, 4]. Few-atom nanoclusters differ from gold or silver nanoparticles in that they can be highly fluorescent, do not support a surface plasmon, and do not have the metallic and bulk-like properties of nanoparticles/nanocomposites. This fluorescence is likely due to the transition of molecule-like electronic levels when subnanometre sizes are smaller than the Fermi wavelength (i.e. < 1 nm) and the Jellium $E_{fermi}/N_{1/3}$ energy scaling law was used as a model to describe the size dependent electronic structure and relative electronic transitions of the small clusters[5, 6].

Gold and silver clusters were labelled to three common proteins : bovine serum albumin (BSA) and human transferrin (Tf) by wet chemistry, and glutathione by the etching process. Structural investigations indicate a covalent binding between the metal cluster to the proteins via the sulfur groups. Photophysical studies suggest a relative high quantum yield with a fluorescence emission tuneable in the visible range (Figures 1&2). Cytotoxycity and cell studies performed on gold labelled BSA and Tf exhibit biocompatibility and the cell uptake in lung tumor cell lines, A549 (Figure 3). This survey highlights the potential of a new type of non-toxic fluorescent proteins for imaging and targeting.

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Figures

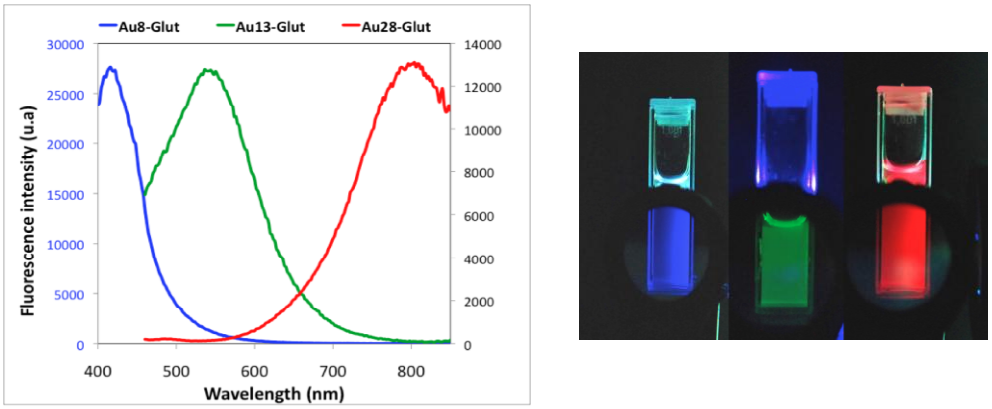

Figure 1. Different size gold nanoclusters (Au NCs) labelled to Glutathione prepared by the etching process.

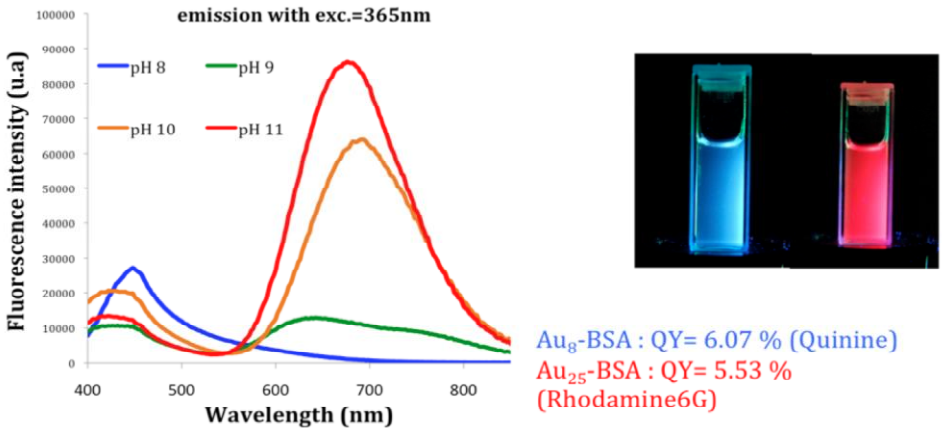

Figure 2. pH dependent synthesis of blue and red fluorescent gold nanocluster labelled BSA.

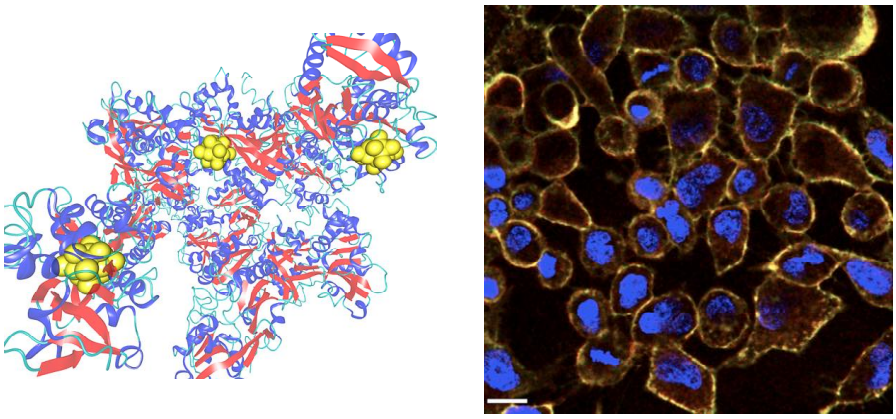

Figure 3.Uptake of gold labelled iron loaded human transferrin (with a red fluorescence emission) in lung tumor cells line, A549. Bar 21μm.

Physical DNA sequencing: codon thermoelectric signature

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The proliferation of large-scale DNA-sequencing projects for applications in clinical medicine, health care and criminal research has driven the quest for alternative approaches to the commonly used Sanger sequencing method in order to reduce time, error rate and cost. Since Sanger's method relies on chemistry to read the bases guanine (G), adenine (A), cytosine (C), and thymine (T) in DNA, this quest has spurred new perspectives in nanotechnology looking for sequencing methods entirely based on physical differences between the bases for non invasive detection of nucleotides along the DNA strands [1]. Measuring transverse (i.e., perpendicular to the helix axis) tunnel currents through single-stranded DNA as it translocates through a nanopore has been proposed as a suitable physical method enabling single-base resolution [2,3], and it has experimentally been shown that the four bases provide a distinguishable transverse electronic signature when measured with a scanning tunnel microscope which directly detects the molecular levels of single DNA bases [4]. On the other hand, improvements of current nanotechnology allow us to confidently measure thermoelectricity at the molecular level as well. Thus, the thermoelectric properties of molecular junctions containing different benzene related moieties chemically bond to gold nanocontacts has been investigated in a series of experiments with a suitably modified scanning tunneling microscope [5,6]. Positive values of the Seebeck coefficient (indicating that the Fermi level is closer to the highest occupied molecular orbital (HOMO) level) were obtained for all considered molecules when contacted through thiol groups, indicating that the charge carriers are holes in this case. On the contrary, a negative value is obtained for a benzene molecule contacted to gold electrodes with cyanide-groups. Thus, end-groups are key to controlling the very nature of charge carriers and by properly varying end groups and molecular junction constituents, one can engineer metal-molecule heterostructures with targeted thermoelectric properties.

Motivated by these experimental results in this talk we will review previous works proposing the possibility of sequencing short DNA fragments by employing thermoelectric measurements [7-11]. The working hypothesis inspiring our proposal is the following. The basic unit of information in the genetic code is the so-called codon. A codon is an ordered sequence of three consecutive nucleotides that specifies a particular amino acid in a protein or initiation (stops) sites where translation into protein synthesis begins (ends). From the viewpoint of condensed matter physics each codon is characterized by its electronic structure, stemming from orbital overlapping among neighboring nucleobases. Therefore, the resulting electronic structure provides a characteristic spectral portrait, also determining the codon transport properties. Then, one may regard charge carrier propagating through the oligonucleotide as a physical probe sensing its electronic structure as a whole. In this way, rather than a one-by-one nucleotide reading, typical of chemistry based techniques, we will be able to directly sensing triplet nucleobases associations (including codons in codifying regions) at once. In order to substantiate this approach, we shall analyze the thermoelectric spectral curves of codons trapped between appropriate contacts at different temperatures, determining their characteristic thermoelectric signature. Due to the extreme sensitivity of thermopower to finer details in the codon-electrode electronic structure the thermoelectric response of trimer nucleobases forming a molecular junction exhibits several narrow resonant features where the Seebeck coefficient attains very large values (200-2000 μV/K at room

temperature). The position of these peaks sensitively depends on the characteristic electronic structure of the considered trimer, hence providing a very accurate method to properly identify different codons of biological interest [12].

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Nanomaterials based electrochemical biosensors for diagnostics applications

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Biosensors represent an interesting alternative for an efficient, fast, low-cost and user-friendly clinical analysis in general and cancer diagnostic particularly. Between different biosensing alternatives the nanotechnology oriented biosensors or nanobiosensors represent a very attractive tool for clinical applications. The need for nucleic acid and protein based diagnostic tests has increased enormously in the last few years and the design of novel nanostructures with special optical and electrochemical properties is bringing significant advantages in several fields being diagnostic one of the most important.

Electrochemical biosensors based on the use of nanoparticles (NPs) as electroactive labels may offer several advantages in terms of cost-efficiency in comparison to traditional methods of bioanalysis such as ELISA or PCR. Gold nanoparticles (AuNPs) stand out from the variety of nanoparticles used as labels in biosensing due to their simple synthesis, narrow size distribution, optical and electrochemical properties and easy bioconjugation alternatives. The advantageous properties of AuNP-based immuno and DNA electrochemical assays have given rise to an increased number of publications and other reports in the last years.

The aim of this work is to present the different strategies developed in our group for the direct (redox properties) [1] and indirect (electrocatalytic properties) [2-4] electrochemical detection of AuNPs tags in immunosensing assays, avoiding their previous dissolution in a separate and highly acidic media. Furthermore different platforms (i.e. magneto - screen-printed electrodes, nanoporous surfaces [5-7] etc.) that improve the performance of the performed assays have been used so as to improve the biosensing performances. Of special interest is the use of nanoporous materials not only as sensing systems but also as filters, minimizing matrix effects in human blood analysis.

The optimized immunosensors have been applied for the detection of protein biomarkers in real samples. The obtained results show that the developed technologies can be valid alternatives to the traditional methods. These works and their final validation in real samples analysis are still in progress at our laboratories and will be object of future presentations.

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Gold nanorod for LSPR based biosensors

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Noble metal nanoparticles have attracted great attention due to their unique optical properties, in particular the localized surface plasmon resonance (LSPR). Because of the LSPR phenomenon, the nanoparticle extinction spectrum is affected by the particle size and shape as well as the constituting material and the surrounding dielectric media. Nanorods are an extreme case of anisometric nanoparticles that typically display two plasmon resonance peaks: transversal, fixed at around 530 nm, and longitudinal that changes with the GNR aspect ratio and exhibit a high sensitivity to refractive index changes [1]. Several analytical and bioanalysis applications, in which the nanorod optical properties are exploited, have been presented in the last years. Within them, various LSPR based biosensor formats have been proposed. The identification of an analyte could be accomplished by recording the shift of the LSPR peak. A specific bioreceptor is immobilised on the nanoparticle surface and the analyte/receptor biomolecular interaction modifies the dielectric properties of the surrounding medium, with consequent changes in the resonance peak.

In view of this, a study for the development of an immunosensor for anabolic androgenic steroids (AAS) based on the use of gold nanorods has been performed. In recent years the assumption of dietetic supplements and drugs is drastically increased because of current social and cultural habits. Most of these substances are included in the list of prohibited compounds of the World Anti-Doping Agency and within them the AAS. A World Anti-Doping Code has been drawn up in order to coordinate effective anti-doping programs [2]. In this context, the demand of new rapid, efficient and throughput detection systems is always present.

In this work, first, a surface chemistry for conjugating antibodies specific for AAS to gold nanorods has been optimized and the obtained bioconjugates have been characterized. Moreover, the analytical performances of the bioconjugates are under evaluation in a capture competitive sensor format, in which the competitors are directly immobilised onto the sensor surface and the antigen-antibody recognition took place when the specific antibodies labeled with gold nanorods were introduced (Figure 1). Preliminary results demonstrated that the detection signal is due to the appearance of a plasmon peak only where specific antigen/antibody interaction occurs and that it is related to the different amounts of nanorods interacting with the surface.

In future, the identification of several prohibited substances can be accomplished by using bioconjugate nanoparticles differing in shape and size (label-encoded microarray) or by the location in the microarray (site-encoded microarray).

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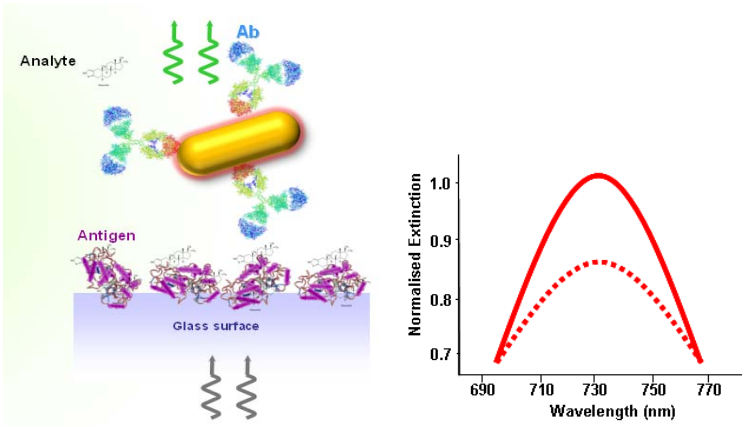

Figure 1

Gold nanorods addition over functionalized surface produces a change in the intensity of the plasmon peak.

Cellular Uptake and Cytotoxicity of metal oxide Nanoparticles

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Metal oxide and metal NPs are widely used in various industrial processes as catalysts, UV protectors and so on. Interactions of the colloidal materials with biological systems and environment could lead to unintended adverse effects, which are recently recognized and attracted broad attentions [1-3]. Our goal is to study the cell uptake, intracellular distribution and the influence of the metal oxide nanoparticles on cell viability and functions. In this work, we shall introduce the interactions between TiO_2, ZnO, and Fe_3O_4 particles and NCI H460 epithelial cells, and Raw264.7 macrophage cells.

The size and surface charge properties of these nanoparticles in cell culture medium with and without serum as well as their morphology were studied by DLS, zeta potential measurements and TEM. All the particles were agglomerated to over 100nm aggregates with similar surface zeta potentials in the cell culture medium, especially in the medium containing serum. The solubility of those nanoparticles in the cell culture medium was also tested by AAS (atomic absorption spectrometry). The ZnO particles could be dissolved largely with a high Zn ions concentration (~1 μg/ml) in 4 hours. The Fe_3O_4 particles showed lower solubility (~0.01 μg/ml), while the TiO_2 particles showed very poor solubility (<0.00001 μg/ml). The difference of solubility could play an important role in the toxicity generated by the particles, e.g. the ZnO particles caused significant decrease of viability of the epithelial cells and the macrophages, while the other two types of the particles showed lower toxicity at the tested concentrations and culture time. We also found that the cytotoxicity was generated through weakening of mitochondria membrane potential (MMP) and inducing cell apoptosis. The cellular loadings of the particles were quantified by AAS, showing that the uptaken amount of the Fe_3O_4 and TiO_2 particles increased with the co-incubation time at the first 12h and reached an equilibrium state afterwards until 48h. The cellular loading of the ZnO particles increased at the initial 12h, and then the cells were dead and the final metal content in the cells was much higher than that of the other particles. Distribution of the three kinds of particles in both types of cells as a function of co-incubation time was followed by TEM. We found that all the particles entered into the NCI-H460 and raw264.7 cells and located inside the vesicles in the cytoplasm, but did not cross into the nucleus during 48 h.

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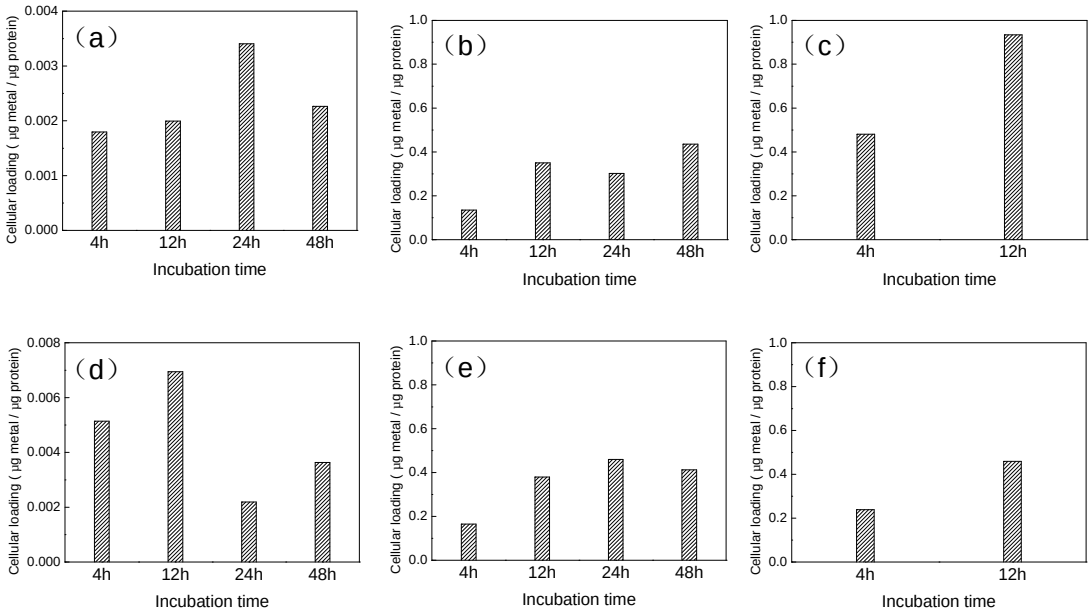
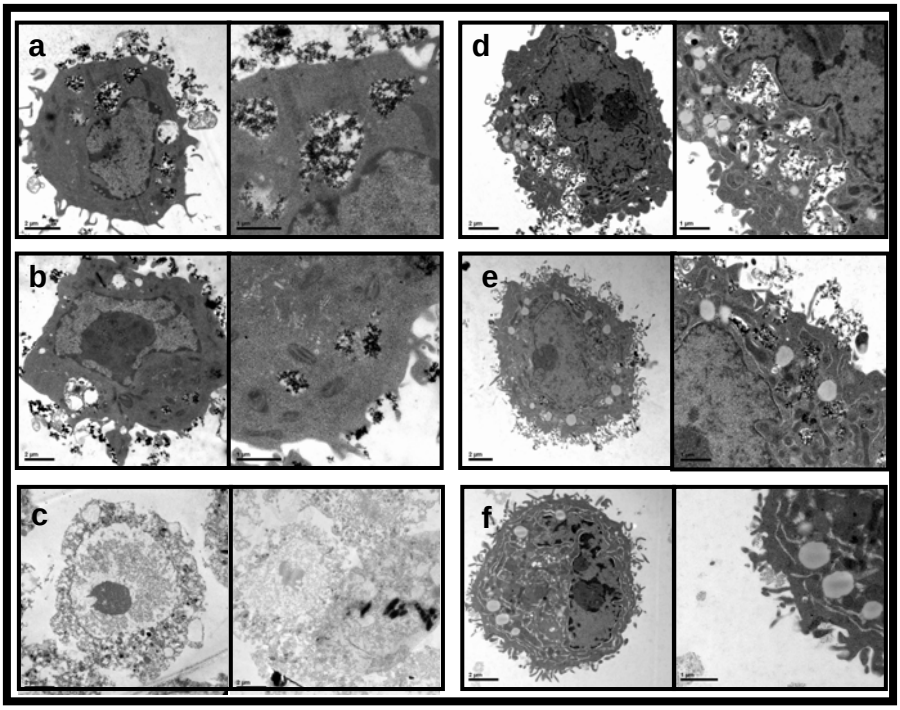


Fiugre 1 The cellular loading of metal oxide particles as a function of co-incubation time. (a-c) Raw 246.7 cells and (d-f) H 460 cells co-incubated with (a, d) TiO_2, (b, e) Fe_3O_4, and (c, f) ZnO particles.



Fiugre 2 The TEM images of the cells internalized metal oxide nanoparticles (right: corresponding magnified TEM images): (a-c) Raw 246.7 cells, (d-f) H460 cells; (a, d) Fe_3O_4, (b, e) TiO_2, and (c, f) ZnO.

Paramagnetic Gd-based gold glyconanoparticles as probes for MRI

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Magnetic Resonance Imaging (MRI) is primarily used to obtain anatomical images, but it also gives information on the physico-chemical state of tissues, flow diffusion and motion. No use of harmful high-energy radiation, clinic resolution close to 1 mm^2, and exceptional soft tissue contrast are some of the characteristics which make MRI an ideal technique for medicinal diagnostics. Nowadays, most of the MRI contrast agents in clinical use are paramagnetic complexes, usually gadolinium [Gd(III)] chelates. [1] These contrast agents enhance the signal in T_1-weighted images (signal-brightening effect). Due to the toxicity of Gd(III), the conventional paramagnetic contrast agents are in the form of ionic complexes with chelating ligands, which are thermodynamically stable and kinetically inert and less toxic.
Recently, gold nanoparticles have been used in the preparation of multimodal and multifunctional paramagnetic contrast agents. [2] Our laboratory has a great expertise in preparing gold nanoclusters and semiconductor nanocrystals functionalized with carbohydrate antigens (glyconanoparticles, GNPs).[3] These gold GNPs have been shown to be excellent platforms for basic studies of carbohydrate interactions and potential tools for biotechnological and biomedical applications. GNPs are water soluble, biocompatible and non-toxic to cellular lines or mice,[4] thus being good candidates for *in vivo* use. The methodology developed in our laboratories allows us to introduce multifunctionality in a controlled way.[5]
We have recently reported the preparation of hybrid GNPs having on the same gold nanoplatform sugar conjugates and Gd(III) chelates as new paramagnetic probes for imaging of glioma in mice by MRI. [6] The insertion of both Gd-complex derivatives and suitable glycoconjugates onto the same gold nanocluster enhanced the relaxation properties of the Gd-chelate depending on the sugar stereochemistry and the relative position of the sugar with respect to the Gd(III) ion. These paramagnetic GNPs were prepared in one-pot fashion using different ratios of thiol-ending sugar conjugates and 10% of tetraazacyclododecane triacetic acid (DO3A) ligands (DO3AC_5S or DO3AC_{11}S) in the presence a gold salt ($HAuCl_4$) and a reductive agent ($NaBH_4$). [6] The amount of Gd present in the GNP synthesized by this method was not under complete control. In order to solve this problem and obtain GNPs with a control amount of Gd, a new protocol was developed. The new GNPs were synthesized in two steps: (i) preparation of GNPs 100%-coated with thiol-ended glycoconjugates β-glucose (GlcC_2SH, GlcC_3SH, GlcC_5SH, GlcC_7SH, GlcC_9SH), β-galactose (GalC_5SH), α-mannose (ManC_5SH), β-lactose (LacC_5SH), β-maltose (MaltoseC_5SH), and β-cellobiose (CellobioseC_5SH)) using the *in situ* procedure [7]; (ii) functionalization of the so-obtained GNPs by thiol-for-thiol ligand place exchange (LPE) reactions [8] using thiol-armed DO3A:Gd complexes. (Scheme 1) Transmission electron microscopy (TEM), UV-Vis, IR, ^{1}H NMR and elemental analysis were used for their characterization. The amount of Gd(III) present in the GNPs has been measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES).
The longitudinal and transversal relaxation times (T_1 and T_2) of our GNPs were measured to confirm their potentiality as MRI contrast agents. They showed very good relaxivities values (r_1 and r_2), even better than commercial available contrast agents. These GNPs are being tested as paramagnetic contrast agents for tumour detection in mice.

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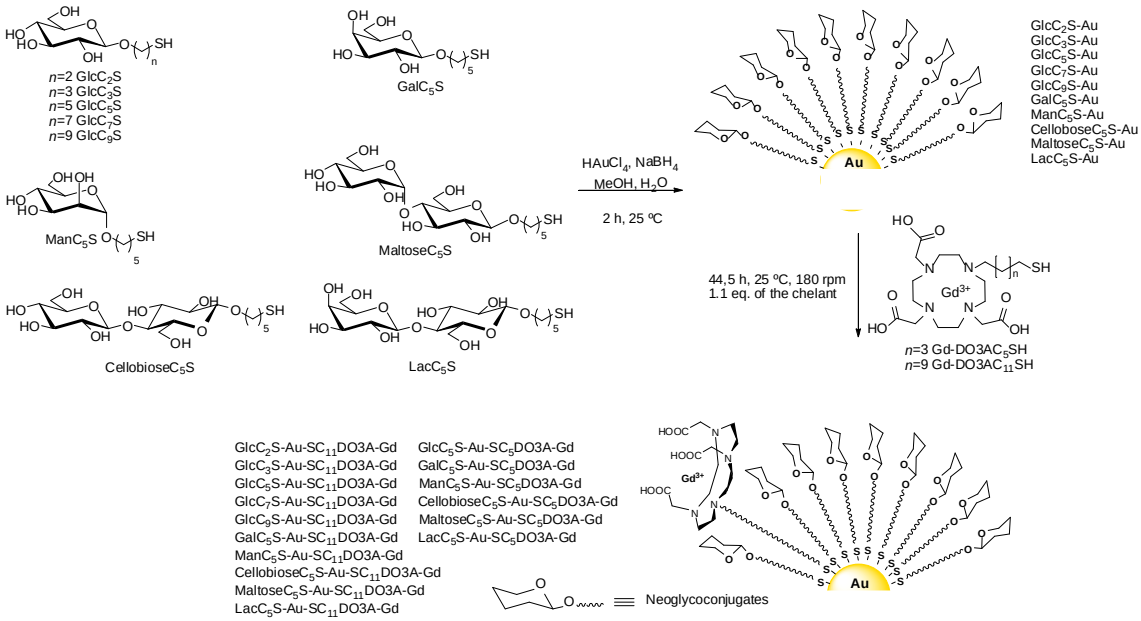

Scheme 1: Synthesis of paramagnetic Gd-based gold glyconanoparticles (GNPs) by Ligand Place Exchange (LPE) reactions with Gd-chelates.

Protein Detection with Scanning Light Analyzer (SCALA)

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In the last years, microcantilevers have been increasingly used as mechanical transducers of molecular recognition events. The transduction signal of nanomechanical sensors is a nano-scale motion. This nanometric deformation arises from the intermolecular forces produced from molecular recognition interactions on the sensitized surface of a microcantilever. Main techniques for the readout of the nanomechanical response include the optical lever method, interferometry-based methods, integrated optical waveguides and the use of piezoresistive cantilevers. The optical lever method is the most used due to the extreme accuracy and easy implementation for measuring cantilevers.

In this contribution we present an application of the SCAnning Light Analyzer instrument, SCALA,[1],[2] to the detection of proteins. This instrument is based on the scanning of a laser beam across a surface (up to centimeters). The reflected beam during the scanning is collected on a position sensitive detector (PSD). This allows the instruments to perform the read-out of arrays of nanomechanical systems without limitation in the geometry of the sample, with high sensitivity and a spatial resolution of few micrometers. The read-out can be made both in one-point deflection measurement or the whole cantilever profile deflection measurement.

We have used this technology to measure the response of cantilever arrays covered with a layer of goat anti-rabbit IgG to hydration forces. We have followed similar protocol described in [3] to form a layer of proteins on the surface of the cantilever sensor. After that, we have measured the cantilever arrays deflection as we increase the relative humidity in a controlled environmental chamber. In order to perform detection, we have incubated the cantilever arrays in Rabbit IgG solution (100 ugr/mL) for different times. The response of the sensor with anti-rabbit IgG layer shows a peak with increasing relative humidity. The position of this peak is around 10-15 %. This type of response is similar to other published in previous works with different biomolecules immobilised on the surface of the cantilever[4]. This could be explained as the response of a monolayer of standing up proteins. When the sensor is incubated in rabbit IgG solution this peak response is decreased. For an incubation time of 500 minutes the peak response fully disappears and the sensor response is characterized by a monotonous decrease with increasing relative humidity. This response can be explained as the monolayer of anti-IgG being fully bonded to rabbit-IgG so that water molecules can't diffuse into holes as described in [4].

This results show how detection of proteins can be made through measurements of hydration forces on cantilever arrays. As the technology of SCALA is capable of reading-out tens of cantilevers per second, it could be possible to develop a cantilever array chip for medical diagnostics purposes providing test results in few hours.

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Figure 1

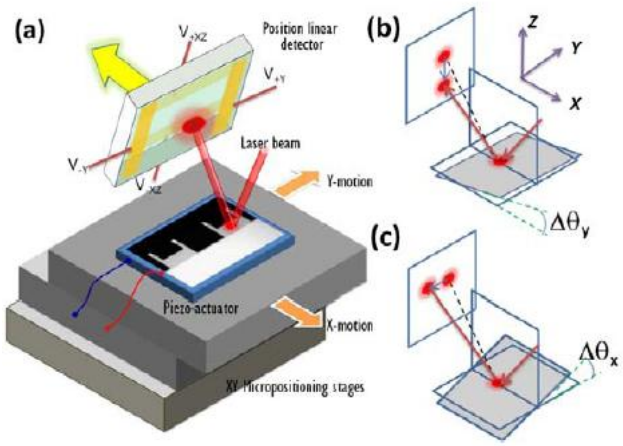

Figure1. Schematic of the SCAnning Laser Analyzer system. (a) Set up, (b) Detection of vertical deflection (c) Detection of lateral deflection.

Figure 2

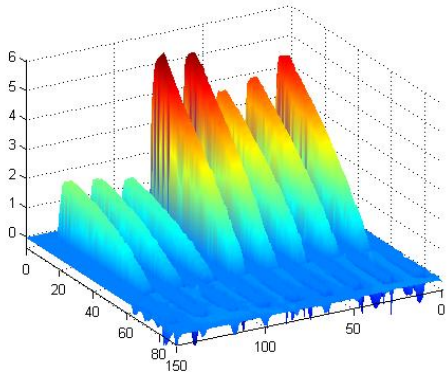

Figure2. Profile detection of an array of 8 cantilevers with SCALA.

Figure 3

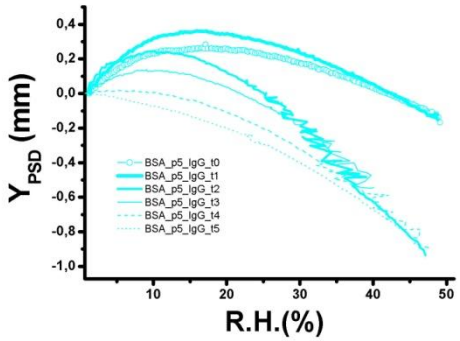

Figure3.Deflection measurement of the free-end of one cantilever showing how the peak response decreases with incubation time.

Membrane scaffolds exhibiting biochemical and physical signals for tissue engineering

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The use of thin bioactive membrane scaffolds in tissue engineering and regenerative medicine could have many applications *in vivo*, directly replacing or stimulating tissues, and *in vitro*, facilitating well-controlled studies of cell-cell communication or nutrient permeability. It is well known that both biochemical (specific epitopes) [1,2] and physical (topography, porosity, stiffness) [3,4] signals can be used to control and guide cell behavior.[5] In this work we report on the use of a top-down/bottom-up approach to develop thin self-supporting bioactive membranes exhibiting both biochemical and physical signals specifically designed to elicit a specific biological response.

Elastin-Like Polymers (ELP) containing the cell adhesive epitope arginine-glycine-aspartic acid-serine (RGDS) were synthesized using standard recombinant protein production techniques and cross-linked with 1,6-hexamethylene-diisocyanate (HMDI). The ELP membranes were fabricated by a drop-casting/evaporation technique using a spin-coater to precisely control membrane thickness. Membranes were fabricated with a variety of nano and micro topographical patterns on either one or both sides, uniform and well-defined through-holes, and exhibiting multi-layers. Membrane swelling and stiffness were characterized by atomic force microscopy (AFM), nanoindentation tests, and scanning electron microscopy (SEM). The membrane biocompatibility and bioactivity were assessed by *in vitro* culture using rat mesenchymal stem cells (rMSCs) and quantifying cell adhesion, morphology, and proliferation.

Membranes were reproducibly fabricated with thicknesses varying between 500 nm – 100 µm depending on the fabrication conditions, exhibited sufficient structural integrity to be handled and sutured, and served as *in vitro* cell culture substrates. Membranes were also fabricated comprising topographical features with heights ranging between 500 nm and up to 10 µm. Optical, inmuno-fluorescence, and scanning electron microscopy demonstrated that rMSCs adhered on the ELP membranes exhibiting a spread morphology and well-defined actin cytoskeleton. However, cell adhesion tests did not reveal a significant increase in cell number after 4 hrs of incubation between membranes comprising the RGDS epitope and those that did not exhibited the epitope ($p>0.05$). Nonetheless, cells were observed to spread faster on the RGDS membranes compared to those not exhibiting RGDS. Furthermore, cell morphology was qualitatively affected by the different topographical features present on the membrane's surface.

We have developed a variety of fabrication techniques based on micro and nanotechnologies to create thin self-sustained membranes of controlled thickness that comprise bioactive epitopes and a variety of topographical and structural components that could be fine-tuned to stimulate specific biological processes. These structures could potentially serve as thin bioactive, biomimetic, multifunctional, and biodegradable scaffolds for a variety of applications in tissue engineering and regenerative medicine.

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Figures

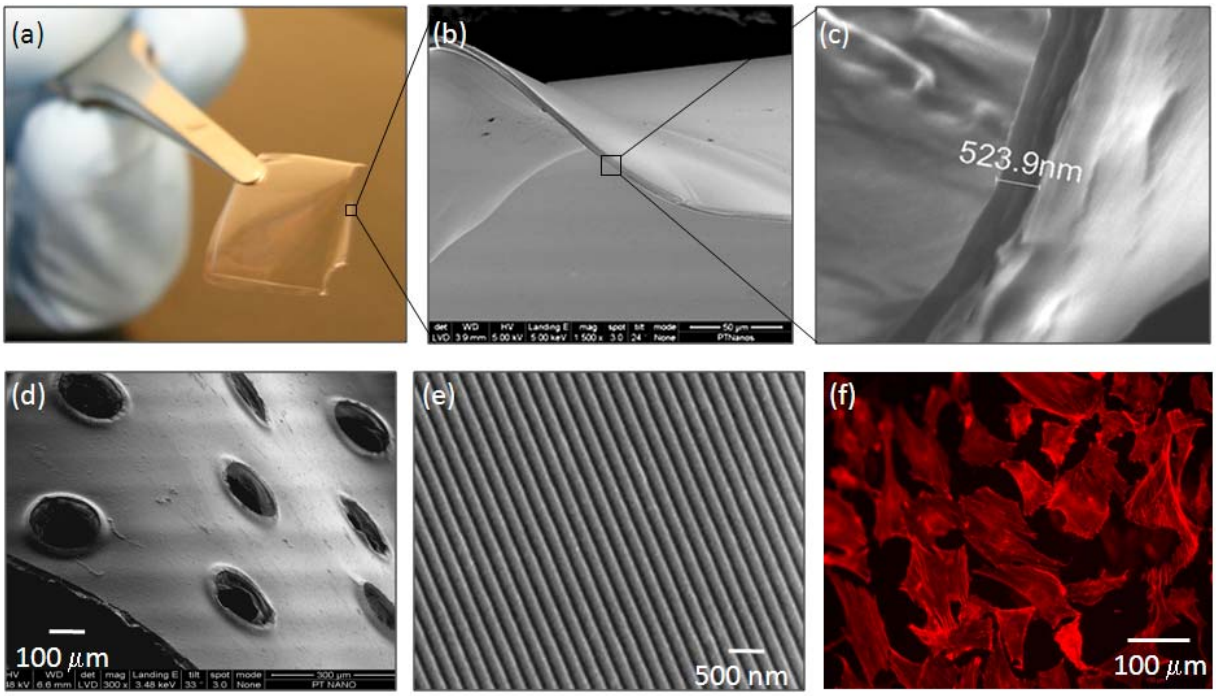

Figure 1: (a-c) Self-sustained membrane scaffolds exhibiting a controlled thickness. The membranes are made from Elastin-like Polymers (ELP) comprising the RGDS epitope to promote cell adhesion and a variety of topographical and structural components including (d) pores and (e) micro/nanotopographies that can be designed to provide biochemical and physical stimuli to (f) mesenchymal stem cells.

Phase Separated Cu@Fe_3O_4 Heteroparticles from Organometallic Reactants - Potent Agents to Track and Kill Caki-1 Cancer Cells

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Cu@Fe_3O_4 heteroparticles with distinct morphologies were synthesized from organometallic reactants. The formation of these nanoparticles is unexpected, because (i) Cu is not a noble metal and (ii) stable binary copper oxides and several ternary Cu-Fe-O compounds such as $CuFe_2O_4$ or $CuFeO_2$ are known. They display magnetic and optical properties that are useful for simultaneous magnetic and optical detection. After functionalization, the Cu@Fe_3O_4 heterodimers become highly water soluble allowing their use in biomedical applications. The high toxicity of Cu@Fe_3O_4 heteroparticles may lead to a new design of anticancer nanomedicines based on Cu cytotoxicity. A special advantage of these heterodimers lies in the fact that nanodomains of different composition can be addressed separately and specifically and can be used as inorganic drug-delivery agents and simultaneously as strong MRI contrast agents. The probes were characterized by transmission electron microscopy (TEM), high-resolution transmission electron microscopy (HRTEM), superconducting quantum interference device (SQUID), powder X-ray diffraction (XRD), and confocal laser scanning microscopy (CLSM).

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Figures

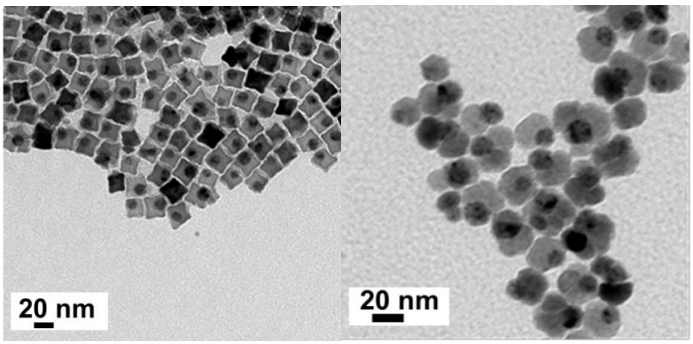

Figure 1. *Transmission electron microscopy (TEM) images of asymmetric (cubes) and symmetric (cloverleafs) Cu@Fe_3O_4 heteroparticles*

Very small lipid nanoparticles for clinical use

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Nanoparticles have attracted growing scientific interest during the last few years as an alternative material for biological and medical applications. Especially, nanotechnology could overcome some major drawbacks in the fields of diagnosis (novel and sensitive contrast agent) and/ or therapy (non viral carriers for active molecules delivery). In this context, lipid-based carriers present some advantages for industrial transfer compared to inorganic nanoparticles since they are biodegradable and not toxic for organism.

Recently, we have developed lipid nanoparticles (LNP), called Lipidots™, for the encapsulation of hydrophobic compounds such as dyes or small drugs. Manufacturing process is simple, fast, highly reproducible and clean (solvent free). The nanoparticle core is composed of a complex lipid mixture stabilised by a monolayer of phospholipids. A Pegylated coating provides them not only an excellent colloidal stability in buffer (at least 6 months at room temperature) but also stealth properties to reticulo-endothelial system after their injection *in vivo* as demonstrated by *in vivo* fluorescence imaging in mice. Ligands like saccharides, peptides or antibodies could be grafted to PEG chains in order to preferentially reach the targeted tissue. As an example, cRGD grafting around these nanoparticles facilitates their cellular internalisation *in vitro* and may promote their tissue content in tumor as observed in tumor bearing nude mice thank to the well known EPR effect, rendering them very promising for development of new strategies in cancer therapy.

The nanotoxicological assessment of these novel very small lipid nanoparticles (hydrodynamic diameter around 30 nm) is on going but we have already observed that (1) they remain stable for few hours (>15h-24h) in plasma, (2) they are very well tolerated *in vitro* (IC50~500 µg/mL in 3T3 fibroblasts, even more in Hep G2 cell line), (3) their uptake by macrophages or dendritic cells are very slow likely due to their pegylated coating and at last (4) they do not activate these dendritic cells. Moreover, they do not induce an inflammatory reaction after their systemic injection in rat as revealed by circulating cytokines dosage or complement activation studies.

Further experiments are needed in order to better understand LNP behaviour in biological fluids like the characterisation of the potential protein corona or whenever they reach biological barriers such as blood-brain, placenta, skin or intestinal barriers.

Enzymatic Growth of Quantum Dots for Activity Assays

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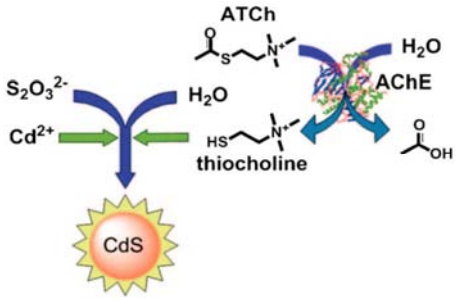
Nanoparticles (NPs) of different nature, for instance, semiconductor and metal NPs are very broadly utilized as labels to read out biorecognition events by optical, electrochemical and other physical methods [1]. In most cases, pre-synthesized metal NPs were linked to recognition elements proteins, polysaccharides, antibodies, RNA and DNA aptamers, DNA oligonucleotides which have affinity for respective target analyte molecules such as proteins, DNA fragments, small organic and inorganic molecules, cations and anions and so on [2,3]. The key advantage of semiconductor NPs is their intrinsic capacity to become photoexcited to produce electron/hole couples, which can recombine to yield fluorescent emission of light. The size, nature and environment of semiconductor NPs define the wavelength and intensity of emitted light [2]. The emission of light by these NPs is explained by quantum effects, therefore they are referred to in the literature as quantum dots (QDs).

We developed three new analytical assays for detection of enzymatic activities in which generation of fluorescent CdS NPs was induced by products of bio-catalytic reactions. The first assay for enzymatic activity of acetylcholine esterase (an enzyme that participates in the termination of the synaptic transmission by breaking down acetylcholine at cholinergic synapses) is shown in Scheme 1.



This enzyme breaks the artificial substrate acetylthiocholine (ATCh) to acetate and thiocholine. The latter catalyzes decomposition of $S_2O_3^{2-}$ according to equation: $S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + H_2S$. The resulting hydrogen sulfide interacts with Cd^{2+} to yield CdS NPs: $Cd^{2+} + H_2S \rightarrow CdS + 2H^+$. We found out that the formation of CdS NPs in this system obeys auto-catalytic mechanism.

Scheme 1. *Enzymatic generation of CdS QDs for the detection of AChE activity.*

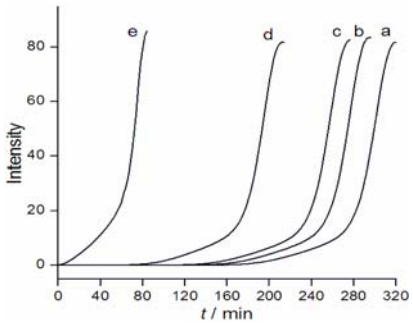
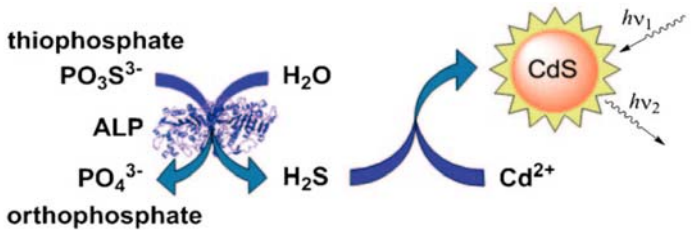


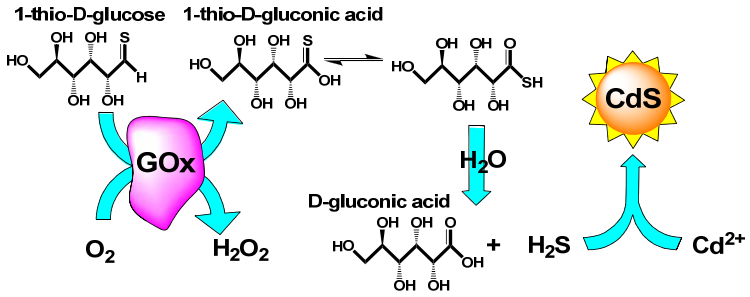
Figure 1. *Evolution of the fluorescence intensity of the CdS QDs formed with different concentrations of AChE: a) 0 mU mL^{-1}; b) 25 mU mL^{-1}; c) 50 mU mL^{-1}; d) 100 mU mL^{-1} and e) 250 mU mL^{-1}.*

The second assay was designed to detect enzymatic activity of alkaline phosphatase (ALP) see Scheme 2 and Figure 2A. This enzyme finds wide application in bioanalysis as a label in enzyme linked immunosorbent assays. ALP hydrolyzes thiophosphate to orthophosphate and H_2S. The latter reacts immediately with cadmium cations to give CdS QDs. When these CdS QDs are excited at 360 nm a strong fluorescence signal was observed.



Scheme 2. Enzymatic generation of CdS QDs for the detection of ALP activity.

The third analytical assays was developed to detect catalytic activity of glucose oxidase (GOx). We used 1-thio-D-glucose as a substrate for GOx. This enzyme oxidizes 1-thio-D-glucose to D-gluconic acid and H_2S according to Scheme 3 and Figure 2B.



Scheme 3. *Enzymatic generation of CdS QDs for the detection of glucose oxidase activity.*

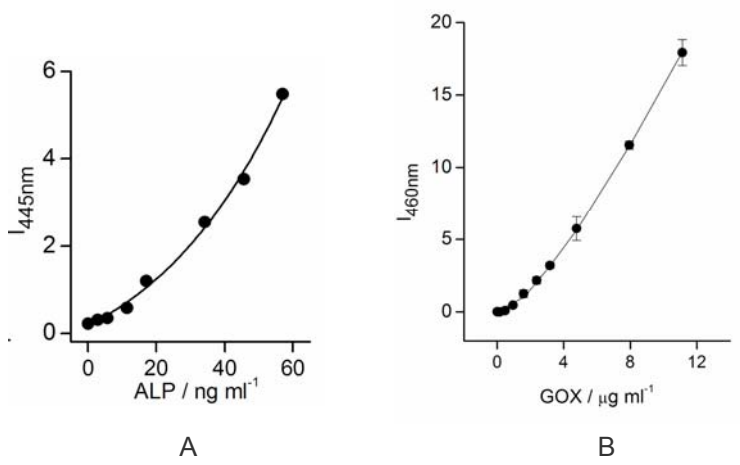


Figure 2. *(A) Calibration curve for ALP; (B) Calibration curve for GOx.*

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Immobilization of enzymes on the biogenic magnetite for biological applications

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Superparamagnetic nanoparticles of iron oxides with a suitable surface modification have proven useful in various biological applications including immobilization of biocomponents, magnetic resonance imaging, anticancer therapy using hyperthermia, magnetically guided site-specific drug delivery, cell labeling, immunoassays, bioseparation, purification of nucleic acids and so on. In order to become an appropriate biomaterial, magnetic nanoparticles need to be stabilized to avoid agglomeration[1]. Such a stabilization may reside in coating with polymeric substances (chitosan and its derivates, PEG, PVA, dextran, alginate etc.) resulting in biocompatibility, biodegradability and nontoxicity. These coating materials contain active groups which can bind bioparticles or biomolecules such as whole cells systems, proteins (including enzymes), hormones or drugs. Magnetotactic bacteria are interesting microorganisms that are able to produce by biomineralization membrane-enveloped crystals of magnetite called magnetosomes. Magnetic nanoparticles isolated from *Magnetospirillum gryphiswaldense* can be used as an excellent solid support for carrying immobilized enzymes[2]. After immobilization, the enzymes display improved properties like e.g. increased resistance to temperature, denaturants or organic solvents. Their stability is enhanced, which results in the possibility of repeated use. Whereas immobilized oxidoreductases are advantageous for constructing biosensors, immobilized proteases are attractive from the point of view of proteomics.

In this work, the biogenic magnetite nanoparticles from *M. gryphiswaldense* were used as a carrier for the immobilization of oxidoreductases (amine oxidase, peroxidase, sulfite oxidase) and proteases (trypsin, prolyl endoprotease). First, the material was covered by chitosan which provides appropriate hydroxy and amino groups for enzyme immobilization. Then the enzymes were attached to the surface using glutaraldehyde as a coupling agent. The immobilized amine oxidase and peroxidase were used for the preparation of a modified carbon paste electrode. This modified bioelectrode was then characterized by cyclic voltammetry and chronoamperometry, which indicated the possible applicability for the determination of amines in biological samples. A platinum electrode was modified by the immobilized sulfite oxidase and utilized for the determination of sulfite in food and beverages. Magnetic carriers containing the immobilized trypsin and prolyl endoprotease were applied for a rapid protein digestion. After immobilization, trypsin was more thermostable than its soluble form and this system was used repeatedly. Both free and immobilized enzymes were characterized by their kinetic parameters. The morphological properties and size of the biogenic magnetite before and after linking enzymes were measured by transmission electron microscopy.

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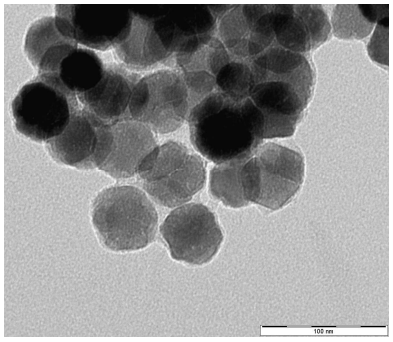
Figures



Magnetite nanoparticles isolated from magnetotactic bacteria and covered by chitosan

Biomolecules immobilization as a models of nanobiosensors

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Immobilization of biomolecules or organic molecules on a biocompatible surface such as gold or silicon is a process that has been received much attention, specially in the search for nanobiosensors[1]. In this context, the main objective of our research project consists in to give biological or chemical properties to a surface in order to make them capable of actuate as a sensor or a tag in a biosystem.

The immovilization approach is based in the formation of a self assembled monolayer (SAM) and the posterior linkage to the functional (bio)molecule through a covalent bond. Different monolayers have been studied in order to find the optimal conditions for the SAM formation on both silicon and gold surfaces. Different nanotechniques were used to characterize the functionalized surface, such as contact angle, AFM and fluorescence confocal microscope. The choice of the biomolecule is based on the ultimate use of the device. Thus, wheat germ agglutinin (WGA), has been chosen because it is a protein that provides an specific cellular recognition due to its capacity to recognize some specific sugars of the cell membrane[2]. We are also involved in the immobilization of fluorescein derivatives, in order to build sensors of reactive oxygen species [3]. Biofuncionalization of WGA and fluorescein have been performed on silicon micronanosubstrates in order to probe their activity in living cells and also WGA has been immobilized in AFM tips in order to measure adhesion forces.

Also, we have been exploring bi-functionalization solution processes to be able to immobilize two diferent (bio)molecules on a substrate formed by two different materials, like gold and silicone. These experiments will permit to combine in one micronanotool two different activities such as cellular recognition and sensing. .

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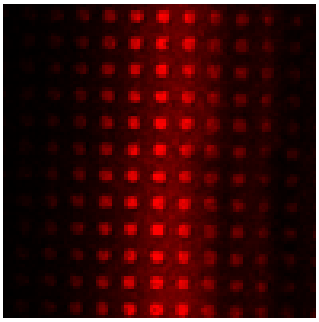
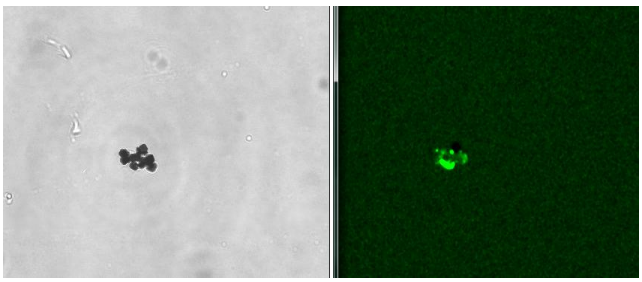
Acknowledgements. This work has been supported by the Ministerio de Educación y Ciencia (MICINN-Spain) through project TEC2008-06883-C03-02 and the Generalitat de Catalunya (2009SGR158). O.P. thanks the MEC for a predoctoral grant.

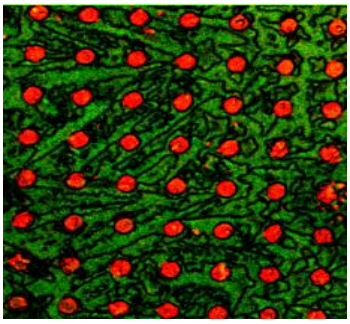
Figures



WGA immobilization



Optical and fluorescence image of fluorescein immobilization in a micronanosubstrate



Bifunctionalization confocal image

Doubly PEG-modified Folate targeted chitosan nanoparticles

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Introduction

Nowadays, an important point in cancer therapy is specific targeting of tumor cells to achieve higher drug levels in tumor tissue and to overcome the side effects. Polymer nanoparticles have been widely investigated as a carrier for drug delivery in cancer therapy. In this regard, it is well known that polymer nanoparticles targeting with folic acid are an interesting way to obtain intracellular site-specific delivery of cancer therapy [1]. For this purpose biocompatible polymer and crosslinking agents are good candidates in the obtaining of covalently crosslinked nanoparticles. Chitosan has been reported to possess potentials as a drug carrier because of its high positive charge density and relatively low cytotoxicity, biocompatibility, low toxicity, biodegradability, low immunogenicity, and antibacterial properties [2]. A limiting factor in the application of chitosan is its poor solubility because it is insoluble in neutral or basic pH range. PEGylation have resulted a good procedure to get biocompatible crosslinked networks of chitosan as well as to improve chitosan water solubility [3].

On the other hand, folate-receptor mediated endocytosis has been exploited for tumor-specific targeting of nanocarriers. It has been observed that the linkage with a suitable spacer molecule, such as PEG, improves the accessibility of the targeted ligand and enhances the cellular association of the nanocarrier [4].

Taking into account the previous knowledge, the aim of this work is to synthesize chitosan nanoparticles functionalized with folic acid for site-specific targeting and PEG, for a triple purpose: water-dispersability, biocompatible crosslinking and long spacer between chitosan network and folic acid as potential drug nanocarriers for cancer therapy.

Synthesis and characterization of FA-PEG-Chi nanoparticles

FA-PEG-Chi nanoparticles also crosslinked with PEG were prepared by w/o microemulsion. Three different microemulsions were separately prepared mixing their corresponding aqueous solutions with cyclohexane, n-hexanol and Triton X-100: modified folic acid, activated PEG and chitosan microemulsion. The microemulsions were formed in various functionalization steps. Firstly, the previously activated (NHS and EDC) folic acid was reacted with monoprotected 2,2′-(Ethylenedioxy)bis(ethylamine) to obtain a NH_2-Folate aqueous solution for the obtaining of modified folic acid microemulsion. Secondly, PEGBCOOH was activated (EDC and NHS) to provide an amine reactive PEG-succinimide diester aqueous solution for the activated PEG microemulsion. Thirdly, chitosan microemulsion was prepared and finally the three microemulsion were mixed in a last unique reaction microemulsion that was keeping under stirring at room temperature during 24 hours.

The success of reaction was determined by ^{1}H NMR. The decreased of the peak located at 3.2 ppm assigned to the proton of H-2 of glucosamine moieties indicated that the addition of PEG to chitosan was successfully. The success of the amidation reaction was confirmed by the resonance at 4.0 ppm corresponding to the new H-2 protons with amide moieties. Chemical shifts from folic acid 6.64, 7. 66 and 8.7 ppm are visible in the 1H NMR spectrum, which corresponded to the aromatic protons of folic acid, suggesting the successful conjugation of FA to the Chi-PEG.

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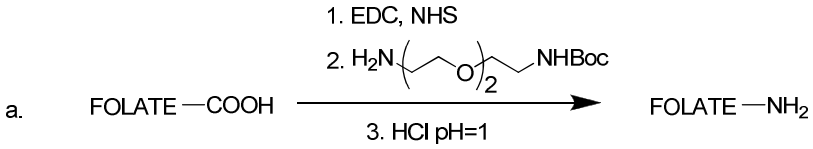

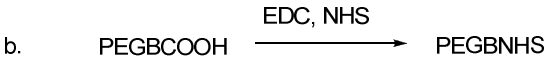

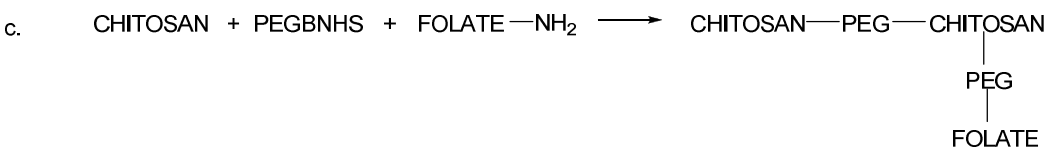

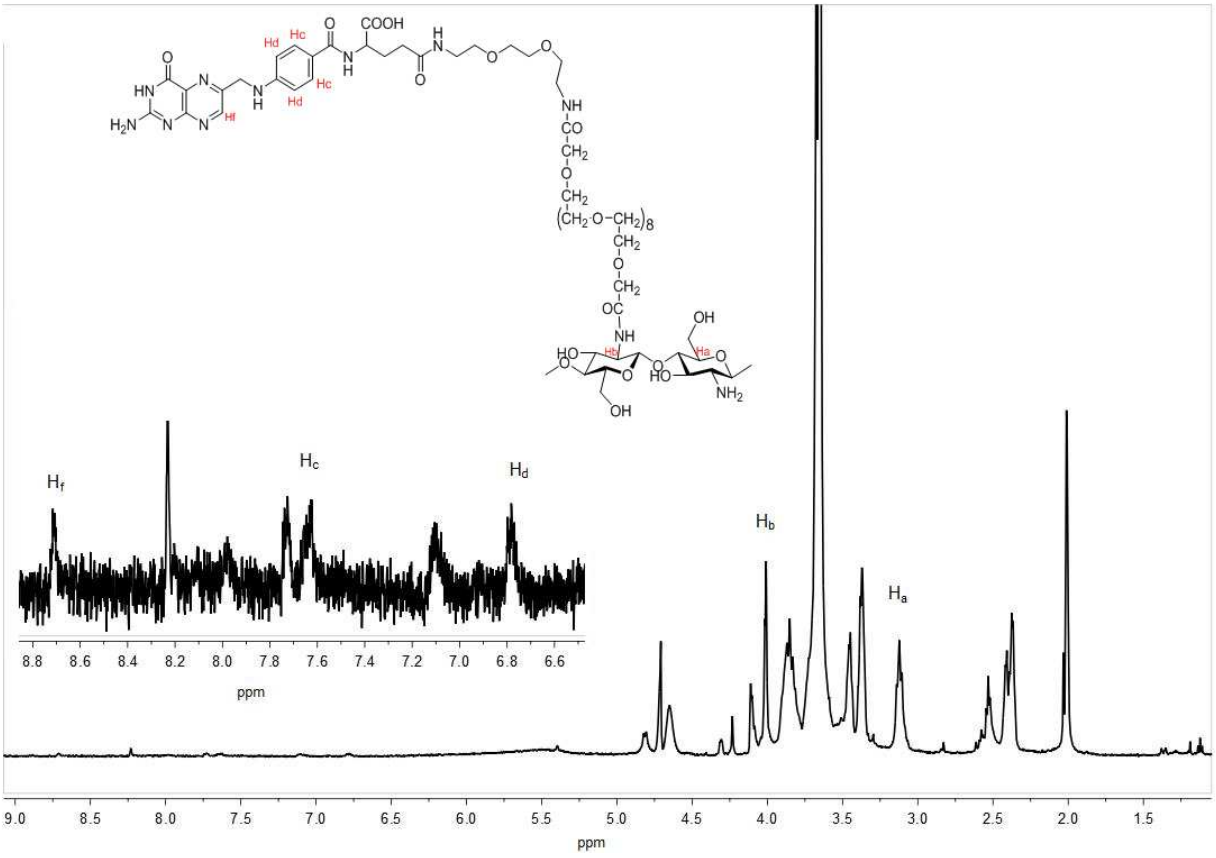

Figure Caption

Scheme 1. a) Folate modification b) PEGBCOOH Activation and c) Funtionalization sequence of FA-PEG-chitosan nanoparticles.

Figure 1. ^{1}H NMR spectra of obtained FA-PEG-chitosan nanoparticles.

Synthesis, characterization and activation of metal oxide nanoparticles

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Metal and metal oxide nanoparticles (NPs) are commonly used in various industrial processes and are employed in commercial products such as sun-creams and paints. Therefore, the assessment of pharmacokinetic (PK) properties of NPs and potential toxicological effects due to long term exposure has recently become a challenge for the scientific community. Positron Emission Tomography (PET) is a powerful tool for the (non-invasive) pharmacokinetic characterization of new chemical entities, although a positron emitter has to be introduced in the chemical structure prior to image acquisition.

The objectives in this project are:

1- To develop a new strategy for the introduction of a positron emitter in the core of metal oxide NPs.
2- To characterize NPs before and after irradiation to evaluate the effects of activation in the physico-chemical and radiological properties.

Aluminium oxide NPs incorporating oxygen-18 were synthesized by reacting an aluminium salt ($Al_2(SO_4)_3 \cdot 16H_2O$[1] or anhydrous $AlCl_3$) with a base (Urea/reflux or NH_3(g)/Room temperature) in enriched water ($[^{18}O]H_2O$). The resulting NPs were bombarded with high energy (18 MeV) protons in an IBA 18/9 cyclotron to produce (*in situ*) ^{18}F (nuclear reaction $^{18}O(p, n)^{18}F$). After irradiation, the decay curves derived from positron annihilation were determined in a PET-CT camera (eXplore Vista-CT, GE Healthcare) and the number and relative amounts of positron emitters in the samples were calculated by adjustment of multi-exponential equations. NPs were also characterized by TEM, DLS, Raman Spectroscopy[2], and XRD before and after bombardment to assess the effect of irradiation on physico-chemical properties.

In general terms, no significant differences were observed between samples before and after irradiation, regarding particle size and chemical composition. However, variations in morphism were observed when $Al_2(SO_4)_3 \cdot 16H_2O$ was used as aluminium salt and Urea was used as a base. Regarding the radiochemical characterization, ^{18}F and ^{13}N (produced from the undesired nuclear reaction $^{16}O(p,\alpha)^{13}N$) were found in all samples, the most favourable case being when anhydrous $AlCl_3$ and NH_3 were used as starting aluminium salt and base, respectively (86% ^{13}N / 14% ^{18}F). Further experiments to improve the production of ^{18}F are being currently carried out.

Metal oxide NPs containing ^{18}O could be synthesized by reaction of aluminium salts with a base in $[^{18}O]H_2O$. Activation of such NPs with high energy protons led to the formation of ^{18}F and ^{13}N. The irradiation process did not introduce significant changes in particle size and crystal structure in most cases.

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Biomedical Application of Multifunctional Synthetic Cholesteric Liquid Crystal Polymers.

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Chiral Cholesteric Liquid Crystal Polymers (ChLCP) PTOBEE $[C_{26}H_{20}O_8]_n$ and PTOBDME $[C_{34}H_{36}O_8]_n$ have been synthetized in our laboratory.

These ChLCPs behave both as thermotropic and lyotropic, confering interesting macromolecular properties to these compounds indicative of potential application on the biological and engineering field.

Besides they proved to be biocompatible against macrophages and fibroblasts cellular lines.

The amphiphilic shape of their monomers makes them polymerize along helical chains, being able to entrap smaller molecules inside [2, 3], such as Lycopene.

They are also able to interact with biomacromolecules such as lipids both neutral and cationic and nucleic acids. Their structures in the complexes, identified by synchrotron radiation source [4, 5, 6], have been applied successfully as non-viral vectors in gene therapy [7].

New functionalized ChLCP have been synthesized attached to commercial DNA of increasing complexity [Poly-A]; [Poly-C], [Poly-G], [PolydT], calf thymus DNA and a plasmid.

In order to determine their interaction mechanism the complexes have been dispersed in aqueous media with three different proportions ChLCP:DNA respectively: (1:2), (1:1), and (2:1).

The structure of the complexes has been studied by SAXS at the BM16 beamline at ESRF, at room temperature. A monochromatized beam at λ= 0,9795 Å was used. A 2D detector camera was placed at 6 m from the sample. The spectra were converted into 1D and with Fit2D, and normalized.

Information about size [Rg (Guinier)] and shape could be estimated based on *Ln I(q)* versus *Ln q* slope[8].

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Figures

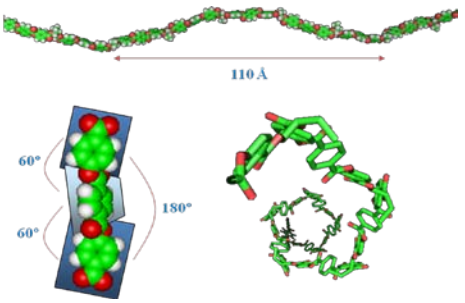

Figure1: Molecular model of PTOBEE

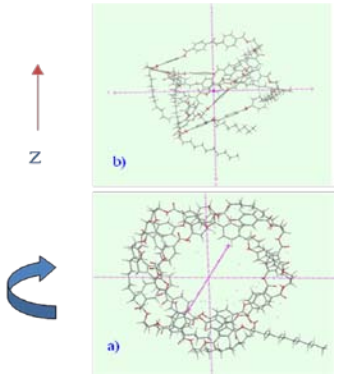

Figure 2: Molecular model of PTOBDME

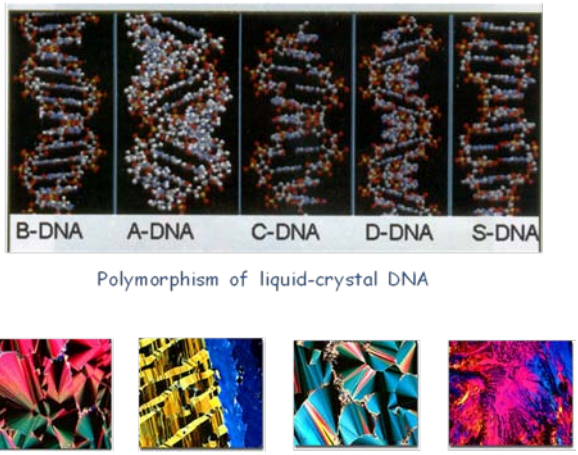

Figure 3: Cholesteric morphology of DNA

Exploring new molecular hydrogelators for therapeutical applications

Serena Carelli,[1,2] Bibiana Izquierdo,[1,2] Oriol Penon,[1,2] David Amabilino,[3] Lluïsa Pérez-García[1,2]

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Hydrogels are very promising materials for a number of therapeutical applications,[1] amongst them, controlled drug release. The release properties are controlled by the constitution and chemical properties of the gelator, and depend on the ultimate use of the new nanomaterial.

In this context, we have been interested in finding different molecular entities that could be used for the preparation of nanomaterials for the local release of both photosensitizers for photodynamic therapy[2] and anionic drugs for cancer therapy.[3]

In the poster we will report the preparation of new porphyrin based and imidazolium based gelators, designed to self-organize in water and other therapeutically compatible solvents. The experimental conditions for the gelification process (see Figure) will be established, and characterization of the new materials will be carried out by optical, scanning electron, transmission electron and atomic force microscopies. Experiments of incorporation of selected drugs and the study of their controlled release are also aimed at.

Gels formed in acetonitrile using imidazolium based gelators.

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Functionalized magnetic nanoparticles of iron oxides for biomedical applications

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Nanoparticles of iron oxides have been extensively studied in the last decade due to their unique magnetic, electric and optical properties. Mainly two of iron oxide polymorphs, maghemite (γ-Fe_2O_3) and magnetite (Fe_3O_4), with the desirable superparamagnetic behaviour connected with large surface area, non-toxicity and suitable surface biocompatible functionalization, are nowadays widely used in many biomedical applications such as MRI (Magnetic Resonance Imaging), drug delivery, hyperthermia or cell labelling. In biotechnologies, strong magnetic properties of magnetic nanoparticles play a key role for immobilization, separation and purification of various biosubstances such as enzymes, proteins, antibodies and nucleotides by using an external magnetic field[1].

Here we describe the synthesis of new MRI negative per-oral contrast agent consisting of superparamagnetic maghemite nanoparticles prepared from iron(II) acetate homogenously adsorbed on the surface of bentonite sheets (smectite mineral). Such prepared nontoxic biocompatible nanocomposite showing a high effective negative contrast was clinically tested on patients with various small bowel diseases. From the statistic analyses the results of the clinical tests demonstrate a desirable applicability and high diagnostic value in imaging the abdomen mainly in MRCP (Magnetic Resonance Cholangiopancreaticography). Nowadays MREg (Magnetic Resonance Enterography) investigations of patients with inflammatory bowel diseases are being evaluated. It seems it should be a promising tool especially for investigation of coeliac disease, where mesenterial edema, mural edema in jejunal loops and extraluminal free fluid are visible[2].

The second example of applicability of magnetic iron oxides nanoparticles shows the labeling of cells, which are used in cell therapy. For advanced cell-based therapy it is necessary to monitor the spatio-temporal distribution of transplanted cells *in-vivo*. Labeling the cells with superparamagnetic nanoparticles as MRI contrast agents appears to be a promising tool for the cell monitoring[3]. In our work the efficacy of labeling of mesenchymal stem cells with magnetic nanoparticles differing in size, surface shell and charge is studied (in-vitro viability tests and characterization by microscope techniques). At the same time contrast effect of magnetically labeled cells is measured by MRI phantom experiments. The results will be used to monitor stem cells in-vivo for patients after myocardial infarction or to follow-up the stem cells therapy for patients with diabetic diseases (such as diabetic foot).

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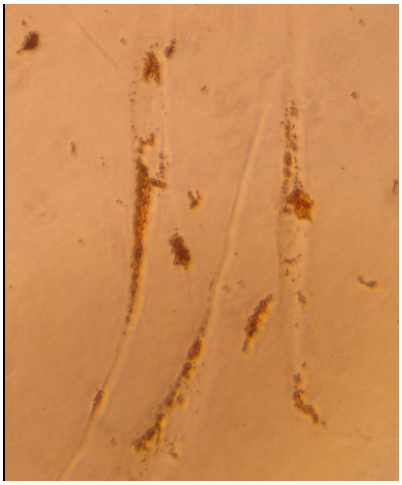

Image of mesenchymal stem cells after incubation of magnetic nanoparticles, Notice: nanoparticles incorporated into the cells did not affect cellular proliferation and viability.

DNA-templated silver nanowires on silicon surface

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New challenges of solid-state electronics oblige to search the methods for nanosize devices creation. One way of solving this problem is to use self-organizing processes at the molecular level. Biological molecules are an attractive material for this. In particular, DNA molecule possesses some unique properties: great rigidity of a molecular chain, high charge density and 2 nm diameter of helix. These properties open the comprehensive facilities for manufacturing of new devices on the base of DNA for nanoelectronics. High resistance of DNA complicates its application in electronic schemes. Modification of DNA by metal allows to increase the conductivity considerably. Thus, it is possible to produce ultrathin nanowires from various metals, or nanoclusters on a surface of a DNA molecule. It is called as DNA-templated self-assembly. DNA nanowires can be usefull, for example, for manufacturing of field transistors and other elements of nanoelectronic devices, and as high-sensitivity biosensors.
The unique method of creation of DNA-sample silver nanowires was carried out in our work. It is based on chemical reduction of binding with DNA silver ions. The special process of DNA fixation on n-type silicon surface makes macromolecules are gathered into fibrils. The realization of metallization process provides the forming of extended nanowires with the length of several micrometers, consisting of Ag^0 clusters with diameter about 30 nanometers. Novelty of a described method consists of two things. Fist, the using of silicon reducing properties is a key moment of this method. This helps to minimize the number of chemical reagents. Second, the best result of metallization was obtained not for a single DNA molecule but for fibrils of oriented DNA chains generated on silicon surface. So, all that techniques allow to simplify considerably procedure and to achieve more efficiency. The explanation of the possible mechanism of metallization is offered, and, for its proof, the comparative analysis of metallization of DNA molecules fixed on mica, glass and p-type silicon surfaces is provided. Thus we have shown that the concentration of free electrons plays the most important role in the process of metallization. Images of DNA molecules and fabricated nanowires have been obtained using the atomic force microscope (AFM) and the scanning ion helium microscope Zeiss ORION *.
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Reference:
Metallization of DNA on silicon surface, Puchkova A. O., Sokolov P., Kasyanenko N. A., Petrov U. V.// Journal of Nanoparticle Research (in press)

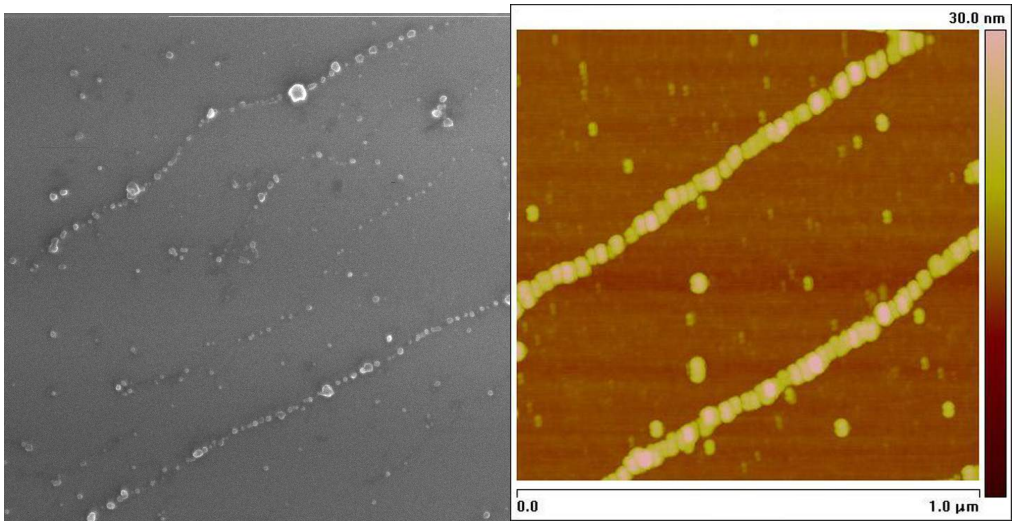

Fig.1. DNA-templated silver nanoeires on n-type silicon surface. Left: scanning helium microscope image (scan size 1.5 µm); right: AFM image.
(*) Interdisciplinary Resource Center for Nanotechnology of St-Petersburg State University.

Fluorescence studies of new potential antitumoral di(hetero)arylethers derivatives of a thieno[3,2-*b*]pyridine encapsulated in nanoliposomes

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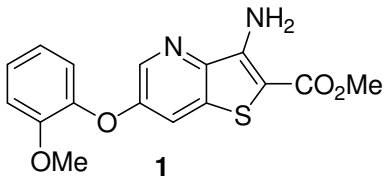
Nanoliposomes are new technological developments for the encapsulation and delivery of bioactive agents. Because of their biocompatibility and biodegradability, along with their size, nanoliposomes have potential applications in a vast range of fields, including nanotherapy. Nanoliposomes are able to enhance the performance of bioactive agents by improving their bioavailability, *in vitro* and *in vivo* stability, as well as preventing their unwanted interactions with other molecules [1].

Nanoliposomes may contain, in addition to phospholipids, other molecules such as cholesterol (Ch) which is an important component of most natural membranes. The incorporation of Ch can increase stability by modulating the fluidity of the lipid bilayer preventing crystallization of the phospholipid acyl chains and providing steric hindrance to their movement. Further advances in liposome research found that polyethylene glycol (PEG), which is inert in the body, allows longer circulatory life of the drug delivery system [2].

In this work, new potential antitumoral di(hetero)arylethers derivatives of a thieno[3,2-*b*]pyridine, **1** and **2**, synthesized by us, have been encapsulated in different nanoliposome formulations, composed of egg-yolk phosphatidylcholine (Egg-PC), dipalmitoyl phosphatidylcholine (DPPC), dioleoyl phosphatidylcholine (DOPC), dimyristoyl phosphatidylglycerol (DMPG), distearoyl phosphatidylcholine (DSPC), with or without Ch and distearoyl phosphatidylethanolamine-(polyethylene glycol)2000 (DSPE-PEG).



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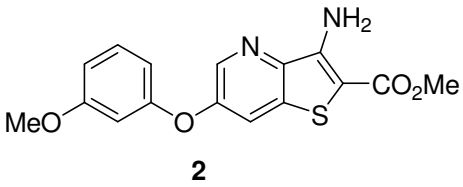

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Compounds **1** and **2** were evaluated for the *in vitro* cell growth inhibition on three human tumor cell lines, breast adenocarcinoma (MCF-7), non-small cell lung cancer (NCI-H460) and a melanoma cell line (A375-C5), after a continuous exposure of 48 h, exhibiting very low GI_{50} values (μM) in the three tumor cell lines (Table 1).

Table 1. Values of compounds **1** and **2** concentration needed for 50% of cell growth inhibition (GI_{50}).

	GI_{50} (μM)		
	MCF-7	**NCI-H460**	**A375-C5**
1	1.4 ± 0.1	1.4 ± 0.2	1.1 ± 0.1
2	2.5 ± 0.1	2.5 ± 0.3	2.3 ± 0.1

Doxorubicin was used as control: GI_{50} MCF-7 = 43.3 ± 2.6 nM; NCI-H460 = 130.2 ± 10.1 nM; A375-C5 = 35.6 ± 1.6 nM.

Nanoliposomes were prepared by injection of an ethanolic solution of the different lipid mixtures in aqueous media under vigorous stirring, above the melting transition temperature of the lipids, followed by six extrusion cycles through 100 nm polycarbonate membranes. Dynamic light scattering (DLS) measurements indicated that the nanoliposomes with the incorporated compound are generally monodisperse and with diameters between 70 nm and 120 nm.

Compounds **1** and **2** exhibit fluorescence in non-polar media, while no emission is observed in protic solvents (like ethanol, methanol and water). The two compounds show significant emission when incorporated in nanoliposomes (Figure 1) and fluorescence anisotropy values are generally high. This behavior indicates that both compounds can be transported in the hydrophobic region of the lipid bilayer. These results may be important for future drug delivery applications of the new potential antitumoral di(hetero)aryletherthienopyridines, using nanoliposomes as drug carriers.

Acknowledgements

This work was funded by FCT-Portugal through CFUM, CQ/UM, Project PTDC/QUI/81238/2006 cofinanced by FCT and program FEDER/COMPETE (FCOMP-01-0124-FEDER-007467).

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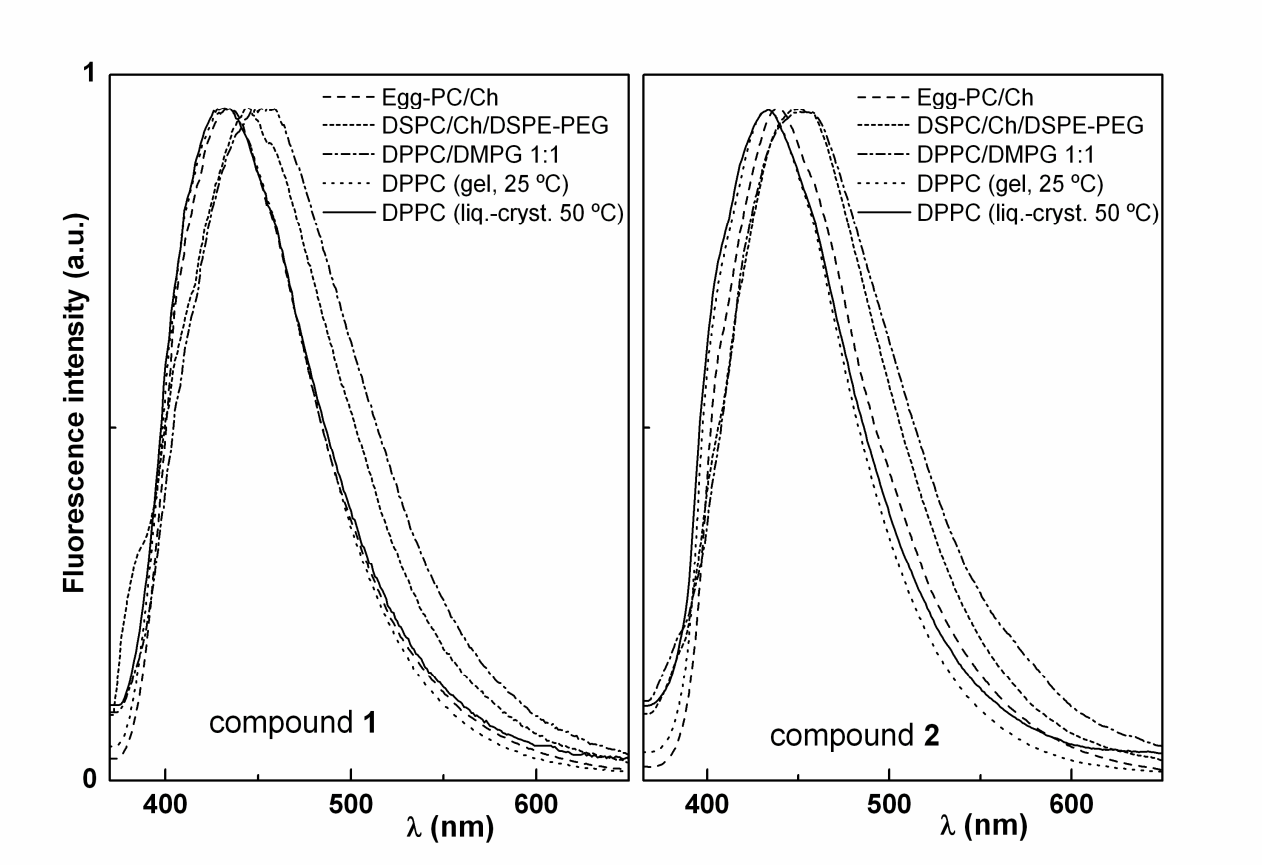

*Figure 1. Normalized fluorescence spectra of compounds **1** and **2** in several nanoliposome formulations.*

Phosphatidyl serine containing liposomes on titania: phase behaviour, bilayer formation, and lipid asymmetry.

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Interactions between surfaces of inorganic materials and biological systems are important in numerous technological contexts (implant integration, biosensor development). They also present basic challenges. For example, the role of surface ion equilibrium in the biological response to the material is not well understood, although a casual link between the two has been proposed.[1] Here, we investigate the behavior of phosphatidyl serine (PS)-containing liposomes on TiO_2. Our interest in the PS is due to its crucial role in blood coagulation: platelets (small anuclear cell fragments circulating in the blood responsible for maintaining haemostasis) expose PS upon activation, converting an inert outer membrane surface into a reactive, pro-coagulant one.[2] Blood coagulation, on the other hand, is one of the first responses of the body to the introduction of a foreign implant material. As a model biomaterial surface we focus on titania, because of its wide range of applications in implants.[3] Unraveling its interactions with biological systems, in particular various blood components,[1,4,5] is crucial for understanding implant integration and rejection processes.
It is known that Ca ions bind to both TiO_2[6] and to PS.[7] Therefore, we studied the behavior of PS-containing liposomes on TiO_2 as a function of PS content and Ca concentration (Figure 1).[8, 9] We determine a "phase diagram", where a percolation-type transition between adsorbed liposomes and supported bilayers is observed, describe the driving force for this transition, and identify the role of surface heterogeneities in this process. Finally, we quantify the distribution of PS in the resulting supported bilayers by neutron reflectometry (Figure 2).

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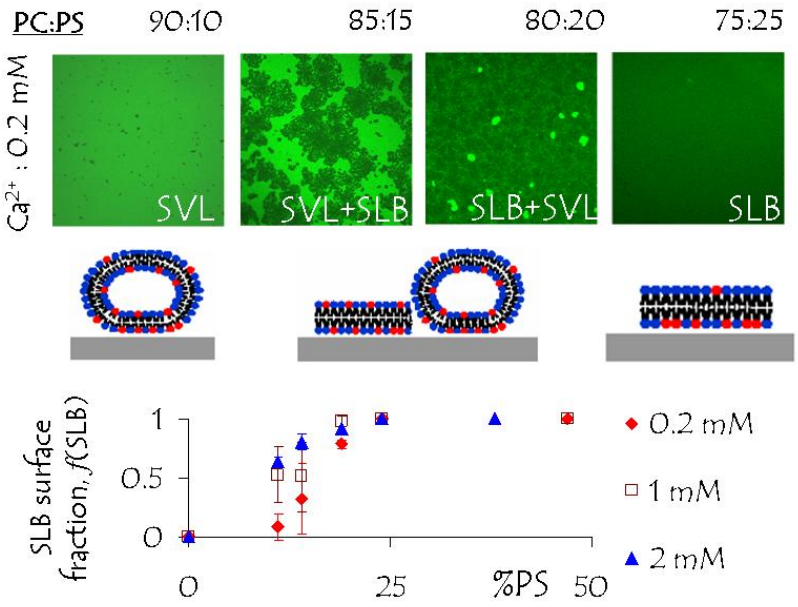

Figure 1: Studying "phase behavior" of PS-containing liposomes on TiO_2. Top row: Phosphatidyl serine containing liposomes of various compositions were incubated with TiO_2-coated glass slides at a Ca^{2+} concentration of 0.2 mM. Three types of structures were observed, as indicated with the labels and diagrams below the images. SVL, or supported vesicular layer, consists of liposomes that adsorb to the surface and remain intact. At higher PS contents, the adsorbing liposomes open and form a continuous SLB, or supported lipid bilayer. Intermediate PS contents lead to intermediate structures, where both non-ruptured liposomes and continuous bilayers are present on the surface. Identification of the structures was based on the fluorescence images, as well as atomic force microscopy and quartz crystal microbalance data (now shown). Fluorescence images are 149 x 149 μm^2 **Bottom row:** The fraction of the surface occupied by the supported lipid bilayers was quantified from the fluorescence images such as the ones shown above, and plotted as a function of PS content in the liposomes for three different Ca concentrations.

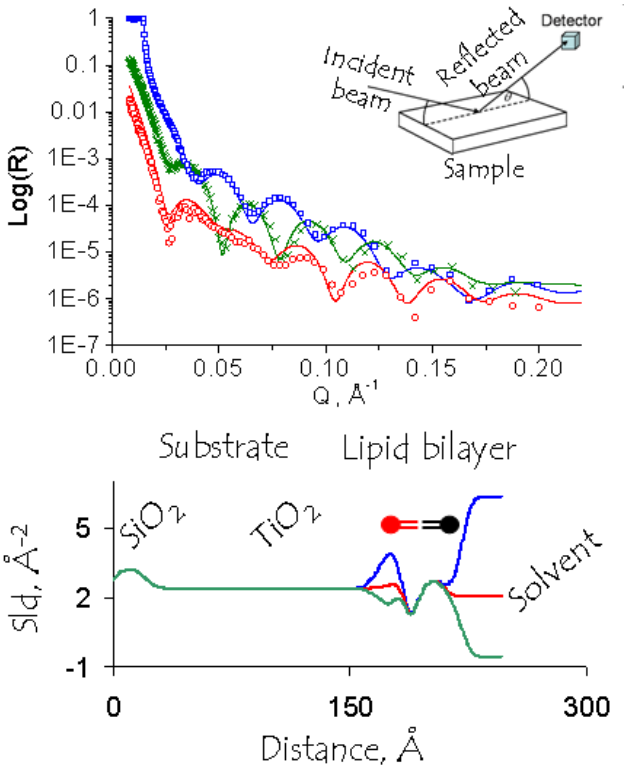

Figure 2: Quantifying bilayer composition by neutron reflectometry. Top: Experimental reflectometry profiles acquired from a supported lipid bilayer prepared on TiO_2 from liposomes containing deuterated POPC and hydrogenated DOPS in a ratio 65 : 35. Experimental geometry is shown in the inset. The measurement probes the distribution of material along the direction normal to the surface. The results are plotted as the ratio of reflected beam intensity to that of the incident beam as a function of inverse spacing. This inverse spacing can be thought of as the size of the "window" probing the interface. The smaller the window (the larger the inverse spacing), the more fine details can be resolved. Lines are calculated reflectometry profiles based on the model shown below. **Bottom:** The substrate consists of an SiO_2 layer and a TiO_2 layer. The bilayer is modeled as four layers: two leaflets, one facing the surface (red) and one facing the solvent (black), with each leaflet divided into the headgroup region of the lipids (circles) and the chains region of the lipids (lines). Scattering length density (sld), plotted on the Y-axis, is analogous to refractive index in optics and is a function of layer composition. It is used to calculate the composition of each layer in terms of %dPOPC and %DOPS. In this case, the surface-facing layer is found to contain ~ 70% DOPS, while the solution facing layer – only ~ 10%; the bilayer is highly asymmetric. Three colors in both plots correspond to measurements performed in solvents that have different sld's. If a measurement is done in one solvent only, it may be impossible to find a unique model.[10, 11]

Bis-imidazolium amphiphile-based gold nanoparticles for drug delivery

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In recent years we have assisted to the rapid evolution of nanotechnology and its applications in many fields, of which medicine is an example. One of the areas of interest is the development of nanocarriers for drug delivery. Gold is among the most common used materials to prepare nanoparticles, because of its physical and chemical characteristics [1]. Gold nanoparticles can be synthesized by the Brust-Schiffrin method, which allows obtaining nanoparticles with narrow size range: It is based in a two-phase system (water /toluene), where a phase-transfer agent is needed to transfer gold ($AuCl_4^-$) from water to toluene, where it is reduced by $NaBH_4$ and stabilized by a thiol, which has a natural affinity to interact with gold. The size of the nanoparticles can be controlled by the thiol:$AuCl_4^-$ proportion [2].

In our group we synthesized an amphiphilic imidazolium-based molecule, capable of interacting with anions [3] and gold [4]. This molecule was successfully used to prepare gold nanoparticles with a size ca. 8 nm (see Figure 1), a novel procedure where the bis-imidazolium amphiphile plays three roles: transfer agent promoting the synthesis, stabilizer of the gold nanoparticles, and due to its interaction with anions, complexation of negatively charged drugs and therefore role as a delivery vehicle.

We present here the results of the synthesis of gold nanoparticles with this novel amphiphilic molecule, as well as the release profile of a model drug that was associated to these nanoparticles. Given the fact that their purpose is to serve as vehicles for delivery of drugs in the human body, the determination of the toxicity is of great importance. It was studied the cytotoxicity and the genotoxicity of the nanoparticles, and also their internalization into human cells (see Figure 2).

The potential application of the synthesized nanostructures to other types of therapy relies on the development of a method to obtain water soluble nanoparticles, a goal which is also being pursued in our group.

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Figures

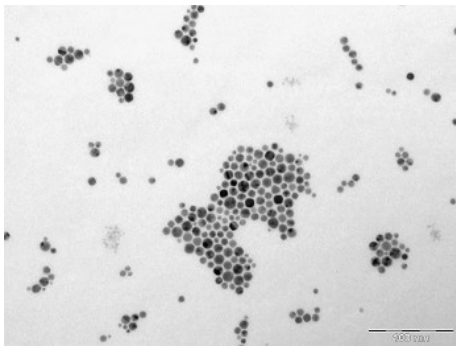

Figure 1: TEM micrograph of gold nanoparticles stabilized with amphiphilic molecule

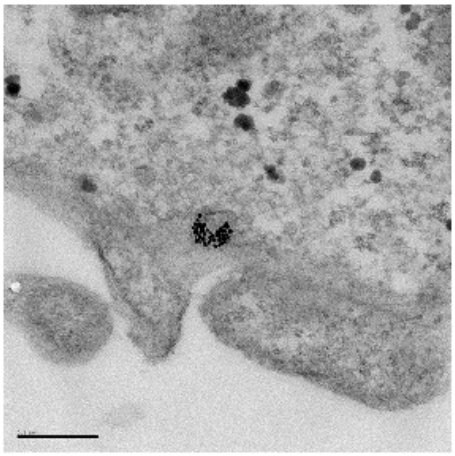

Figure 2: TEM migrograph of gold nanoparticles inside Caco-2 cells after 30 minutes

A localized surface plasmon sensor for early cancer detection (SPEDOC)

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Surface plasmon resonances (SPR) have become a standard tool for performing concentration analysis of biological samples, determining affinity constants of biomolecules or measuring chemical reaction kinetics. Commercial SPR systems are widely available and can be found in many modern bio-chemistry labs. Recent experiments suggest that using nanostructures exhibiting localized surface plasmon resonances (LSPR) could be the basis for biosensors of comparable or even better performace [1].

SPEDOC (Surface Plasmon Early Detection of Circulating Heat Shock Proteins) is a research initiative supported by the European Comission's 7th Framework programme that aims at combining the latest advances of nano-optics, optical manipulation and microfluidics with recent discoveries about Heat shock Proteins (HSP) to develop the precursor of future individualized cancer diagnosis and treatment follow-up devices.

COSINGO is a company that unifies great expertise in optical engineering, in applied research with optical technologies and long experience building optical sensors. For SPEDOC, COSINGO teams up with four research institutions from France, Switzerland and Spain; ICFO, UB, INSERM and EPFL.

The platform developed during this project will exploit the surface plasmon resonances supported by gold nanostructures integrated in a microfluidic environment to track HSP70 proteins in the peripheral blood. This innovative sensor should permit providing treatment to cancer patients at an earlier stage and at lower doses with the consequent decrease of secondary effects.

We will present the project objectives and show first results achieved with the first generation prototype of an optical detection platform (ODP) developed by COSINGO. This ODP is designed for the detection of proteins in solution and uses a PDMS microfluidic chip of the type shown in figure 1. The setup will be presented and results of the first experiments measuring the plasmon resonance shift of nanodimers (illustrated in figure 2) when exposed to solutions of different refractive indices will be presented.

The measurements are performed by data acquisition software using the centroid tracking method [2]. This allows for live monitoring of binding dynamics and greatly improves the sensitivity of the associated sensor.

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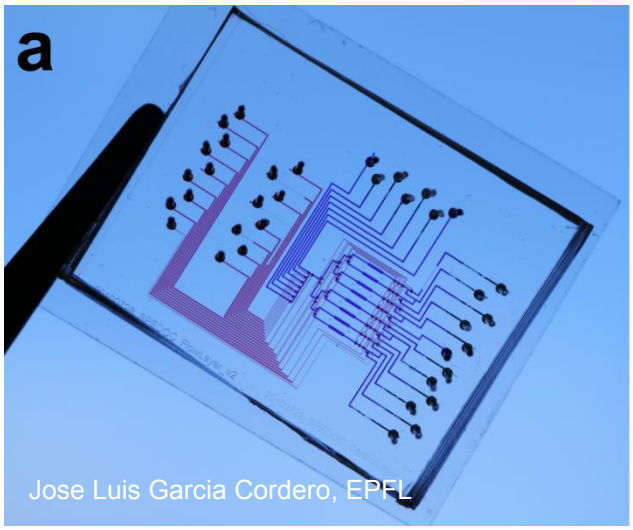

Figure 1: SPEDOC first generation microfluidic chip

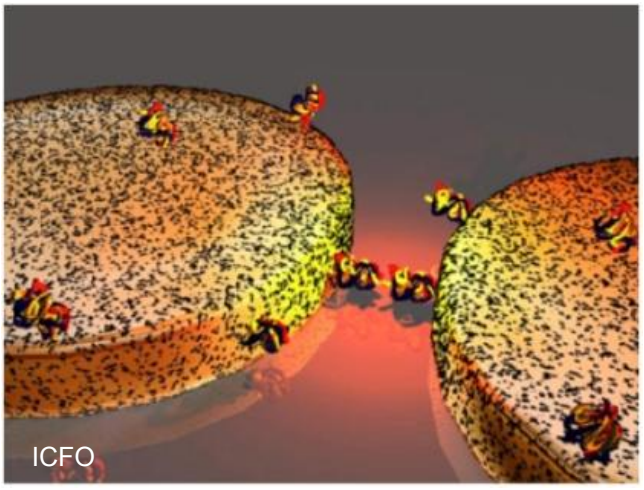

Figure 2: Illustration of functionalized nanodimers binding HSP70

Cell uptake and cytotoxicity studies of Metal Oxide Nanoparticles

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Metal oxide and metal NPs are widely used in various industrial processes and common products. Some examples of these are TiO_2 and ZnO as catalysts and UV protectors, CuO in anti-fouling paints, Al_2O_3 as a surface protector, CeO_2 in polishing, indium-tin oxides forming anti-electrostatic coatings and various rare earth oxides in electronics manufacturing.

Metal and metal oxide NPs may be toxic for two reasons:

1) They may possess increased catalytic activity due to nanoscale structure or chemical modification of their surface. These catalytic properties may interfere with numerous intracellular biochemical processes.

2) The decomposition of NPs and subsequent ion leakage may result in a continuous formation of free radicals and metal ions, and, in this way, may heavily interfere with the intracellular free metal ion homeostasis, which is essential for cell metabolism and requires that metal ions are kept at extremely low levels in the cytoplasm. [1]

A key issue regarding the study of the toxicological effects of metal oxides NPs is their localization and biological fate at cellular level and the quantification of their uptake by cells. Confocal Scanning Laser Microscopy (CLSM) and Flow Cytometry are normally applied for NPs uptake studies but require the labelling of cell interior and as well as the NPs, which may change their properties and their toxicological end points.

We will show that intracellular localization of NPs is possible by means of Spontaneous Confocal Raman Spectroscopy. Both NP and cells have characteristic Raman spectra [2] that can be employed for their detection avoiding labelling. [3] In parallel, NP localization has also been studied by TEM to provide a reference for the Raman Studies (Figure 1).

Besides localization, Raman can be used to asses changes in the cellular machinery after exposition to the NPs like DNA fragmentation or protein conformation that can be related to the toxicity of the NPs. As control cell viability studies were conducted with MTT.

In addition, Metal oxide NPs have been characterized by Transmission Electron Microscopy (TEM), Dinamic Light Scattering (DLS), UV-Visible Spectrophotometry, Confocal Raman Microscopy, Energy-dispersive X-ray Spectroscopy (EDX) and X-ray photoelectron Spectroscopic (XPS). The size/aggregation and charge of NPs were characterized in cell culture by means of DLS.

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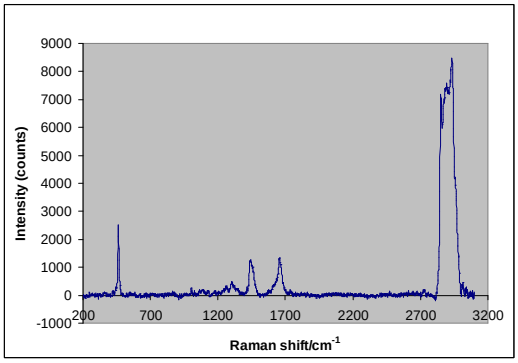

Figure 1. Spectrum recorded from a cell (HepG2 line) wich was incubated together with CeO_2 NPs during 48h. Here it is possible to see NP characteristic peak (at 460) and the rest of peaks coming from different compounds of the cell.

DNA concentration, elution, and real-time PCR amplification inside a Labcard platform

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We present a hand held system consisting of a portable platform and disposable Labcard capable of performing a nucleic acid concentration, amplification and detection (Figure 1). The Labcard comprises two inlets, two outlets, five microvalves, and two chambers (a concentration chamber and an amplification chamber with stored reagents) (Figure 2). It is made of a Cyclo Olefin Copolymer (COC) substrate sealed by a polypropylene film coated with a pressure sensitive adhesive. The assay is carried out in a sequential manner and automatically controlled by a laptop. First, magnetic bead-based concentration, washing and elution are carried out in the first chamber. Then, the eluted DNA is transferred to the second chamber where the stored reagents are rehydrated. Finally, a quantitative Polymerase Chain Reaction (qPCR) takes place in the second chamber. The fluorescence created during the qPCR is recorded in real time by the platform. The use of the magnetic bead protocol allows the Labcard to process a wide range of sample volumes (from 10 µl to 10 ml). The reagents are stored inside the labcard allowing long-term storage, reduction of reagent volumes, simplification of the Labcard design, and finally easier automatisation. The cross contamination is avoided by making the Labcard disposable. The low cost labcard is guaranteed by a demonstrated mass production process and by limiting the integration of components within the Labcard. Hence, the portable platform keeps all the costly components outside the labcard: an external fluorescence detector, a peristaltic micropump, two heaters and their temperature sensors, two magnets and 5 pin actuated micromotors. This platform is connected to a laptop PC by an Universal Serial Bus (USB) connector where the final result is displayed in the form of a typical realtime curve (Figure 3).

Miniaturisation of biological assays in Lab on a Chips (LOCs) has very well known advantages[1,2]. However, it still needs to select the target molecules out of a large complex sample and placed them precisely on top of the biosensor area[3]. In the particular case of sample preparation and PCR, Kim et *al.*[4] fabricated a Polymethylmethacrylate (PMMA) microchip where PCR reagents were stored under a paraffin layer in the same chamber where the amplification took place. Gulliksen *et al*[5]*.* presented a COC LOC where RNA purification and a nucleic acid sequence-based amplification (NASBA) was carried out by injecting a crude *E. coli* cultured lysate. El-Ali *et al.* performed Cell dielectrophoresis and PCR in a SU8 chip with a PDMS cover[6]. Agirregabiria *el al.* developed a DNA sample preparation and PCR on a SU8-one-chamber chip for gram positive bacteria such as Salmonella *spp.* in human faeces.[7] This chip design was further used to detect Campylobacter *spp.* in chicken faeces by PCR[8] and identify influenza viruses in nasopharyngeal human sample by Retro Transcriptase qPCR[9]. More recently, Lee *et al.* developed a similar LOC together with its reader for gram negative bacteria[10].

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This paper shows a portable system and nucleic acid LOC in the form of a Labcard. Unlike previous works: (i) The sealing process is carried out at a low temperature by a pressure sensitive tape avoiding any damage of the previously stored PCR reagents; (ii) The use of integrated valves in the Labcard; and (iii) The complex sample preparation, transport of the eluted DNA and qPCR are carried out in an automatic manner without the user intervention.

Figures

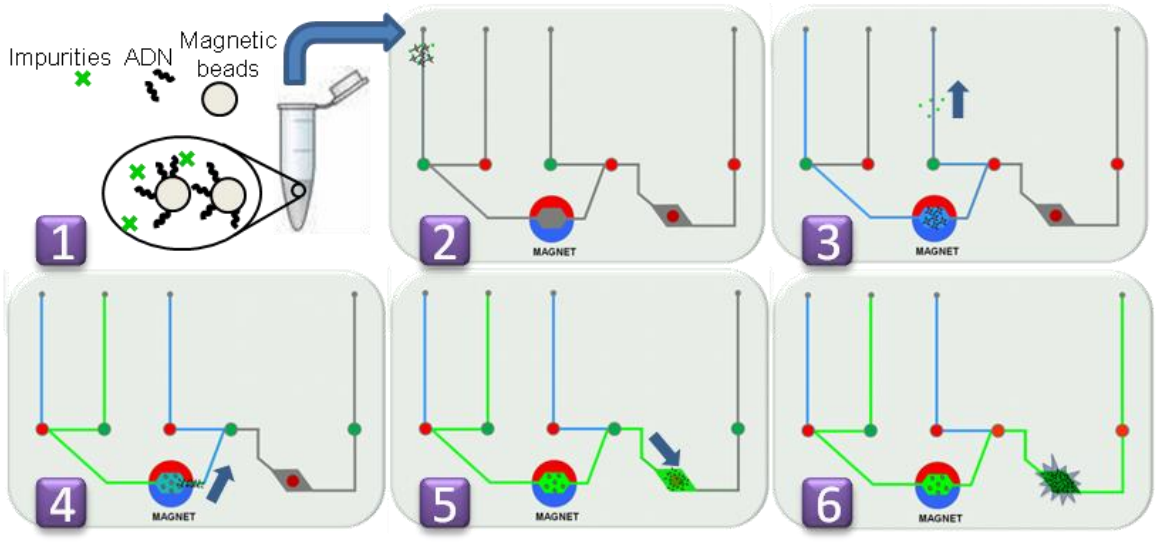

Fig. 1. 1) 5µL of DNA, 10µL of magnetic beads, 50µL of isopropyl alcohol are added to 995µL of nuclease-free water; 2) The DNA concentration is done by magnetic separation using two magnets placed on both sides of the first reaction chamber; 3) As the sample containing the magnetic beads and DNA passes through the chamber, the beads are retained in the magnetic field generated by the two magnets. The impurities leave the labcards and only the beads and bound DNA remain inside the chamber; 4) The DNA is eluted using elution buffer injected and carefully transferred to the PCR chamber; 5) The DNA and PCR reagents are mixed rehydrating the stored reagents; 6) Once the amplification chamber is completely filled with eluted DNA, the appropriate valves are closed and the thermocycling begins with the fluorescence detector activated.

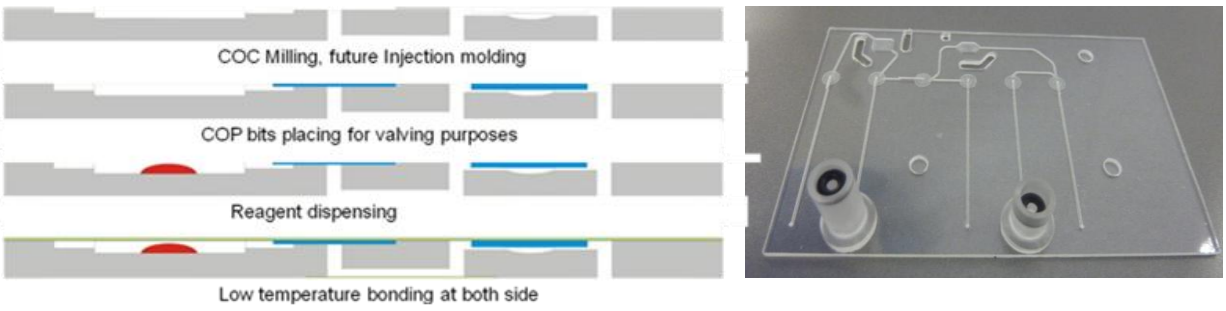

Fig. 2: Left) Schematic representation of the fabrication steps. **Right)** Picture of the verification labcard.

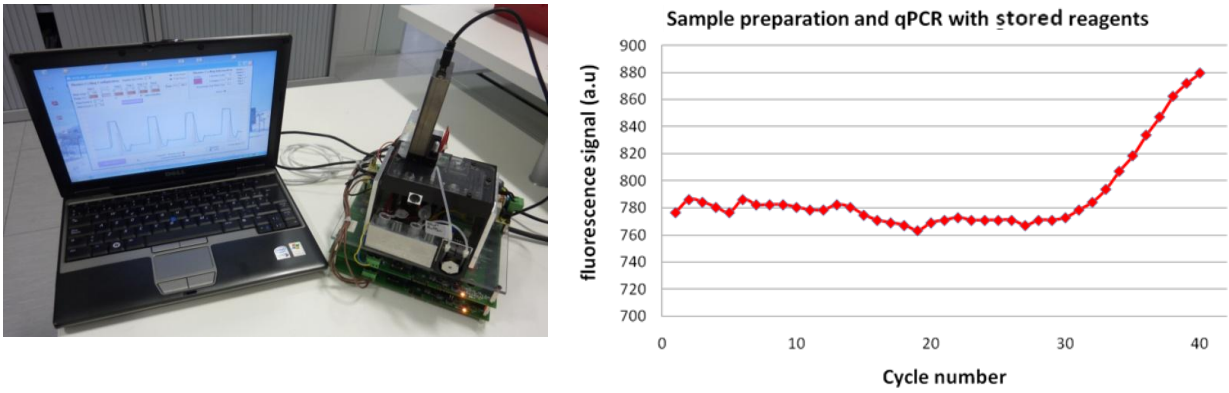

Fig. 3. Left): Picture of the developed labcard platform. **Right)** Graph showing the final result once the nucleic acid concentration, amplification and detection took place.

NIR laser-triggered drug release from mesoporous-silica core/gold-shell nanoparticles

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In 2008 the U.S. demand for drug delivery systems was predicted to increase by more than 10 % a year, reaching $132 billion by 2012 [1]. Those desired drug delivery systems can be classified based on the method of administration either topical, enteral or parenteral as well as classified by the delivery mechanism active or passive. Passive drug delivery is controlled by the molecular diffusion of a previously attached, adsorbed or enclosed drug from its carrier towards the surrounding media depending exclusively on a concentration gradient and usually taking advantage of the specific human physiology or of the natural response of the immune system in order to accumulate the drug at a targeted site. Active drug delivery allows for the controlled triggered release of a therapeutic molecule (drug, gene or protein) by using a chemical characteristic of the carrier or of the drug itself (i.e., pH responsive [2,3], chemically responsive [4], enzymatically responsive [5], red-ox responsive release [6], etc.) responding to a an environmental stimulus, or by using a physical response of the carrier upon external stimulation (i.e., magnetically aided drug release [7], optically [8] or sonic [9]) or even by means of combinations of several triggering mechanisms (i.e., pH and temperature responsive hydrogels [10]).

Both active and passive delivery systems are ideally designed to avoid non-specific drug distribution throughout the body, to regulate drug-release kinetics, to minimize side-effects and to improve the therapeutic efficacy compared to systemic applications of the corresponding drug.

Of all those physically-triggered release systems, optically triggered systems in the near infrared (NIR) region (around 650–900 nm) of the electromagnetic spectrum have the main advantage of not being invasive. Also, NIR light is preferable as trigger in biomedical applications because it has maximal penetration in tissues or whole blood due to their minimal absorbance at those wavelengths [11]. Hemoglobin and water, the major absorbers of visible and infrared light, respectively, have their lowest absorption coefficient in the NIR region. NIR light has been shown to travel at least 10 cm through breast tissue, and 4 cm of skull/brain tissue or deep muscle using microwatt laser sources (FDA class 1) and with higher power levels (FDA class 3) light has been shown to penetrate through 7 cm of muscle and neonatal skull/brain [12].

In this work, we introduce a new system for the external control of drug release from mesoporous silica nanoparticles coated with a porous gold shell. The release of the guest molecule, adsorbed on the pores of the mesoporous silica, is triggered by applying a NIR laser.

The nanoparticles were prepared modifying a previous method described by Halas and co-workers [13] for the preparation of silica/gold core/shell nanoparticles in which the core is non-porous. In the work described here nanoshells were obtained from mesoporous silica nanoparticles [14] modified with amine groups where gold nanoparticles of 3-4 nm were used as seeds [15]. Following a re-growing process mesoporous silica/gold nanoshells were obtained with core and shell both porous. These nanoparticles were characterized by TEM, EDX, UV-VIS-NIR spectrophotometry, DLS, SAXS and

nitrogen adsorption. Ibuprofen sodium salt was used as a model drug and the capacity of heating and the controlled release from this system was demonstrated (Figure 1).

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Figures

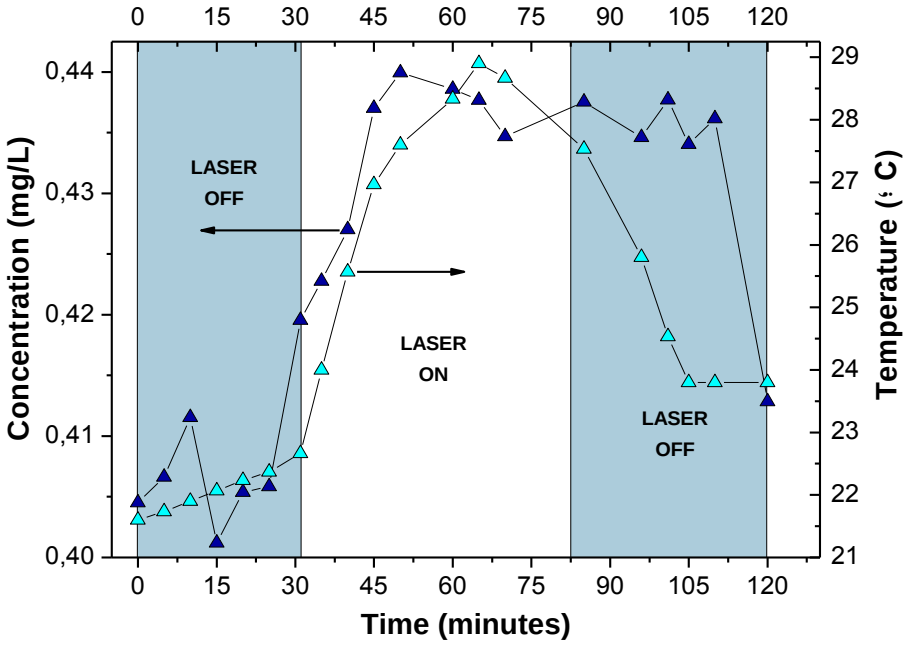

Figure 1. *Release of ibuprofen sodium salt from porous SiO_2@Au nanoshells externally controlled by NIR-laser radiation.*

The Potential of Combining Silica-Based Mesoporous Materials with Osteoprogenitor Cells for Bone Repair

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The development of new biomaterials is acquiring increasing importance in Traumatology and Orthopaedics. Biomaterials can facilitate bone repair by serving as delivery systems for drug administration, or by providing a substrate where cells can be seeded to fabricate an engineered tissue. The present work explores the possible application of silica-based mesoporous materials to bone tissue engineering by evaluating the interaction of these materials with osteoprogenitor and mesenchymal stem cells.

Biocompatibility and osteoconductivity of the particulate and consolidate materials were evaluated in vitro by culturing rodent and human osteoprogenitor cells in the presence of SBA-15, HA-SBA-15 and MCM-41 materials. Osteoblasts and adult MSC were cultured with these materials under standard 2D and micromass (3D) culture conditions. Cell viability and proliferation were assessed by vital staining and cell quantitation assays. Organ explant culture was also performed in order to further evaluate the potential osteoconductivity of SBA-15, HA-SBA-15 and MCM-41.

2D cultures of mouse osteoblasts, as well as mouse and human MSC, grew normally in the presence of up to 100 ug/mL particulate SBA-15, HA-SBA-15 or MCM-41. Mouse calvarium explants also behaved normally in the presence of these materials. Spontaneous attachment of cells to the materials was observed in all cases suggesting osteoconductive characteristics. Micromass culture showed that mouse and human MSC and osteoblasts could grow in three dimensions when cells use these materials as scaffolds. Culture on consolidate porous materials also confirmed this data.

In conclusion, silica-based mesoporous materials are biocompatible and osteoconductive, since they sustain MSC and osteoblast cell adhesion and proliferation. They then provide a suitable scaffold for three-dimensional culture. These data support further experimentation for bringing them into tissue engineering arena.

Layer by Layer RNA encapsulation for genetic therapy nanodevices construction

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RNA based therapies, like siRNA based gene expression silencing, offer a series of advantages over the challenges of traditional heterologous DNA based therapies for the treatment of genetic disorders, including the endogenous control of the expression of the target gene and the relative shorter therapeutical agents [1].

One of the main issues of RNA based therapies is to find a carrier system that allows controlled delivery of the nucleic acid, improving transfection efficacy in the target cells and protecting therapeutical agents from nuclease degradation.

The Layer by Layer (LBL) technique, based on the alternative assembly of oppositely charged polyelectrolyte molecules, is a very versatile tool for the fabrication of thin polymer film with controlled features at the nanoscale [2]. We have applied the LBL technique to encapsulate purified total RNA between polyelectrolyte layers as a negative charged biopolyelectrolyte in Chitosane/Alginate/RNA and PAA/PEI/RNA systems on planar surfaces and colloidal PLGA particles.

RNA assembly in the multilayers was monitored trough Quartz Crystal Microbalance with Dissipation (QCM–D) and ζ-Potential measurements. RNA delivery after multilayer degradation was measured trough a fluorescence assay with intercalating fluorescent tags.

Layer by Layer encapsulation offers several advantages in the development of therapeutical carriers, because allows sustained delivery of one or different encapsulated agents. Besides that, LBL multilayer are suitable for further surface modification [3] which can provide recognition functions to the carrier improving selective uptake in target cells.

Figure

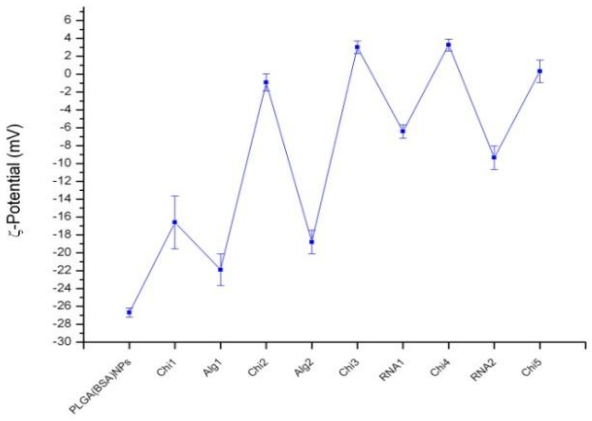

Figure 1. Changes in ζ-Potential in Layer by Layer assembling of Chitosan, Alginate and RNA polyeletrotolytes on PLGA nanoparticles.

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Environmental contamination by colloidal nanoparticles via vapors and water

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During the last decade, biomedical research has been identified as one of the fields that can greatly benefit from the advancement in nanotechnologies. In particular, the use of various kinds of nanoparticles (for example: metallic, metal oxides, semiconductors, silica, etc.) in biotechnology merges successfully the fields of material science and biology [1-3]. Although the potential of nanoscale objects in biology and medicine is really tremendous, a lot of questions remain about the safety of nanomaterials and the risk/benefit ratio of their usage. Thus, a whole field called nanotoxicology has emerged. It refers to the study of the potential negative impacts of the interactions between nanomaterials and biological objects. In almost absolute majority of cases, nanoparticles (NPs) dispersed in liquid solutions are manipulated and studied from cytotoxicity point of view. However, in several number of pulmonary toxicity studies, for example, various NP aerosols were used. Indeed, specific aero-dynamical characteristics of nanoparticles allow them, in particular, to be efficiently dispersed in gas phase.

We'll show that even if the NPs were initially dispersed in liquids they can be nevertheless easily auto-transferred into the surrounding air environment at relatively long distances during the natural evaporation of the liquids at room temperature. Such a natural transfer of the originally colloidal NPs into an aerosol state can easily lead to strong contamination of any biological system even if it is separated from the colloidal source of NPs by a gas phase space. We'll also report on an efficient transport of the colloidal NPs inside the vegetables through their root network.

To perform theses important illustrations, highly luminescent silicon carbide (SiC) NPs with cubic symmetry (3C) already successfully explored as fluorescent agents for living cell imaging [4] and therapeutic agents for preferential cancer cell killing [5] were used. The majority of our NPs have dimensions below 5 nm with the most probable size value being around 2.5 nm. Fresh thin onion epidermis and fibroblast animal cells (3T3) were used in our work as model bio-systems. The cells were exposed to the evaporating colloidal solutions at different distances in an open and confined airs. In another kind of experiments, onions were planted in water contaminated with the SiC NPs.

Typical fluorescence images of the onion epidermal cells exposed to the evaporating solutions without and with the SiC NPs are shown in Figures 1-a and 1-b, respectively. As one can see, no fluorescence is visible in the first case, while the cell structure of the fluorescent onion epidermis having large, well compacted, rectangular-like cells can be easily recognized in the second case. The similar results were obtained on animal fibroblast cells grown in DMEM solutions. Figure 2 illustrates dependence of the integrated luminosity per one cell on the vertical distance between the cell holder and the solution surface. In general, the higher the holder position is, the weaker the cell fluorescence intensity is, because the number of the NPs achieving the onion peels decreases drastically with the holder height. In addition, the curve behavior is strongly non-linear both for open and for confined air. However, the cell luminosity is generally higher in the case of the confined air in all range of the height values. Moreover, the smaller the NPs are, the higher the space is, that they can rich.

In conclusions, our presentation will illustrate the remarkable efficiency of the NP propagation in air and in living vegetables from aqueous colloidal solutions. Our results are very important for general understanding of the NP ability to contaminate air, water and food.

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Figures

Figure_1

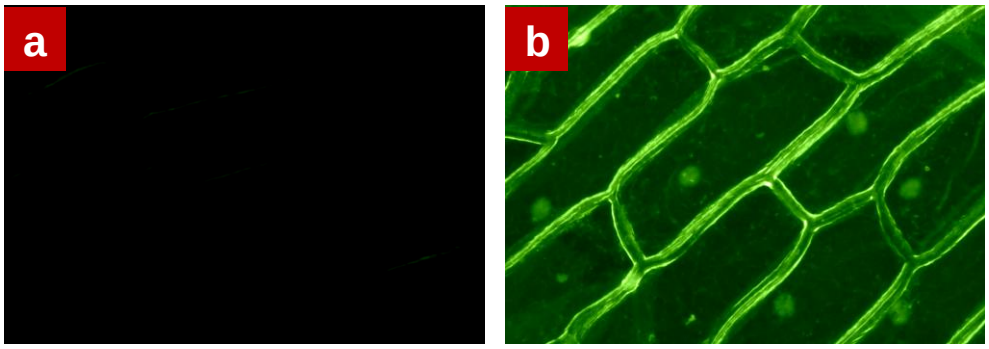

Figure_2

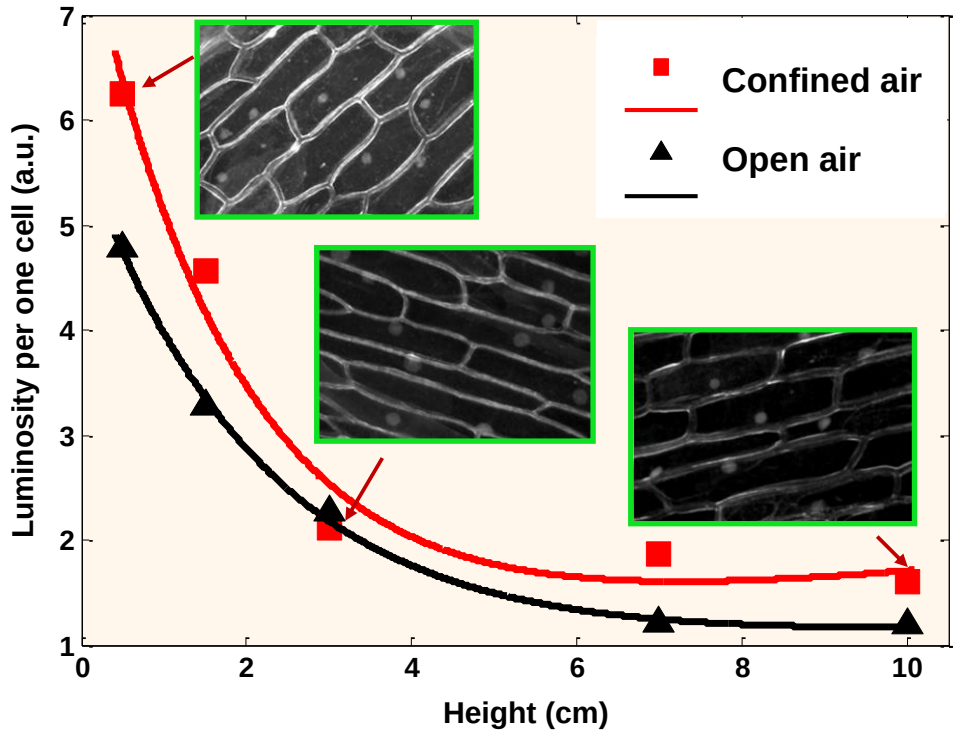

Figure captions:

Figure_1: Fluorescence microscopy images of onion epidermal cells exposed to the evaporating water solutions without (a) and with (b) the SiC NPs.

Figure_2: Dependence of the integrated luminosity per one cell on the vertical distance between the cell holder and the solution surface in open and confined airs.

Research lines in Hyperthermia at the Bioinstrumentation Laboratory of the Centre for Biomedical Technology

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The Bioinstrumentation Laboratory belongs to the Centre for Biomedical Technology (CTB) of the Technical University of Madrid and its main objective is to provide the scientific community with devices and techniques for the characterization of micro and nanostructures and consequently finding their best biomedical applications.

Hyperthermia (greek word for "overheating") is defined as the phenomenon that occurs when a body is exposed to an energy generating source that can produce a rise in temperature (42-45ºC) for a given time [1]. Specifically, the aim of the hyperthermia methods used in The Bioinstrumentation Laboratory is the development of thermal therapies, some of these using different kinds of nanoparticles, to kill cancer cells and reduce the damage on healthy tissues.

The optical hyperthermia is based on noble metal nanoparticles and laser irradiation. This kind of nanoparticles has an immense potential associated to the development of therapies for cancer on account of their Surface Plasmon Resonance (SPR) enhanced light scattering and absorption. In a short period of time, the absorbed light is converted into localized heat, so we can take advantage of these characteristics to heat up tumor cells in order to obtain the cellular death [2].

In this case, the laboratory has an optical hyperthermia device based on a continuous wave laser used to kill glioblastoma cell lines (1321N1) in the presence of gold nanorods (Figure 1a). The wavelength of the laser light is 808 nm because the penetration of the light in the tissue is deeper in the Near Infrared Region. The first optical hyperthermia results show that the laser irradiation produces cellular death in the experimental samples of glioblastoma cell lines using gold nanorods but is not able to decrease the cellular viability of cancer cells in samples without the suitable nanorods (Figure 1b) [3].

The generation of magnetic hyperthermia is performed through changes of the magnetic induction in magnetic nanoparticles (MNPs) that are embedded in viscous medium. The Figure 2 shows a schematic design of the AC induction hyperthermia device in magnetic fluids. The equipment has been manufactured at The Bioinstrumentation Laboratory. The first block implies two steps: the signal selection with frequency manipulation option from 9 KHz to 2MHz, and a linear output up to 1500W. The second block is where magnetic field is generated (∅ 5mm, 10 turns). Finally, the third block is a software control where the user can establish initial parameters, and also shows the temperature response of MNPs due to the magnetic field applied [4-8].

The Bioinstrumentation Laboratory in collaboration with the Mexican company MRI-DT have recently implemented a new research line on Nuclear Magnetic Resonance Hyperthermia, which is sustained on the patent US 7,423,429B2 owned by this company. This investigation is based on the use of clinical MRI equipment not only for diagnosis but for therapy [9]. This idea consists of two main facts: Magnetic Resonance Imaging can cause focal heating [10], and the differentiation in resonant frequency between healthy and cancer cells [11]. To produce only heating in cancer cells when the whole body is irradiated, it is necessary to determine the specific resonant frequency of the target, using the information contained in the spectra of the area of interest. Then, special RF pulse sequence is applied to produce fast excitation and relaxation mechanism that generates temperature increase of the tumor, causing cellular death or metabolism malfunction that stops cellular division.

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Figures

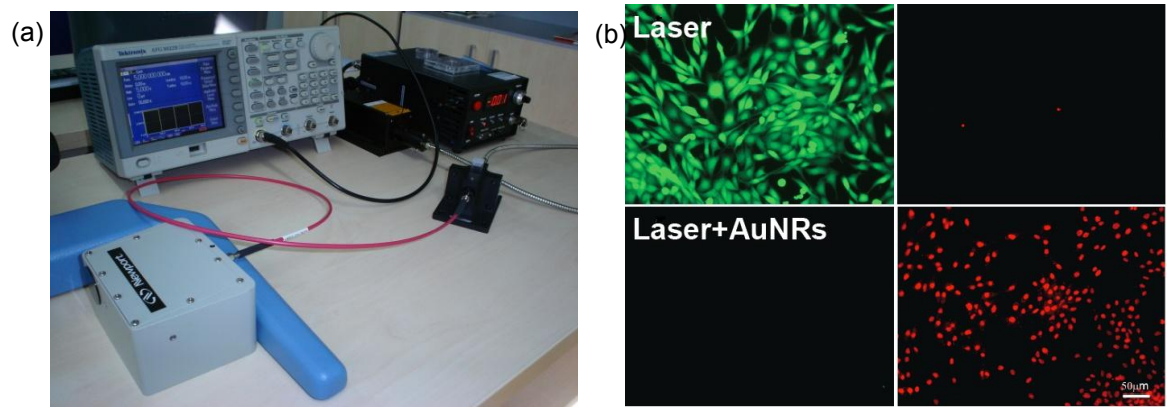

Figure 1. a) Optical Hyperthermia device, b) IP/Calcein essay in samples of glioblastoma cell lines and gold nanorods (24 h after the irradiation).

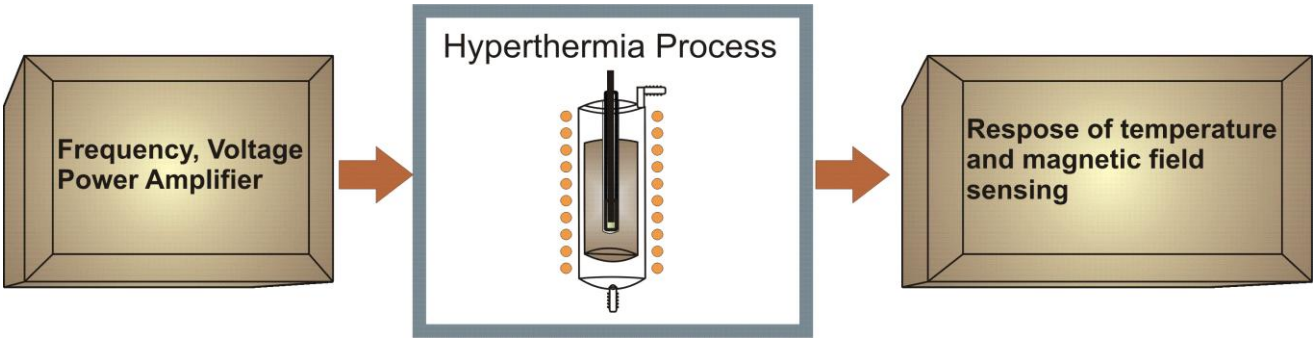

Figure 2. Schematic of the Magnetic Hyperthermia device

Quantitative measurements of waste water pollutants at very low concentration range using Surface Enhanced Raman Scattering, SERS.

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Laser Raman spectroscopy plays an increasing important role in nanobiotechnology and nanomaterials medicine and life science. Based on vibrational transitions, it has long been regarded as a valuable non destructive tool for the identification of chemical and biological samples as well as the elucidation of molecular structure. The problem appears when this technique is applied to the detection of very low concentration of analytes. Other methods are commonly used as HPLC or UV/Visible detection but they are time consuming and sample pre-treatment is normally required. Surface–Enhanced Raman spectroscopy (SERS) might be a more valuable detection method because it is a very sensitive technique that is manifested as an enhancement by many orders of magnitude of the intensity of Raman radiation by molecules in the immediate vicinity to nano-rough metal surfaces and nano-structured metal systems such as colloid clusters of noble metals[1]. When the localized surface plasmons of nanoscale roughness features on a silver or gold substrate are excited by visible or NIR light, strong electromagnetic fields are generated that increases the magnitude of the intensity of the inelastic scattering by orders of magnitude. It is in this way that SERS provides much better detection limits than Raman spectroscopy using standard Raman equipment.

According to the World Health Organization (WHO), the most dangerous threat for health of mankind emerging during the next years is polluted water. Under developed countries but also European countries already suffer from insufficient clean water supply. Many southern European countries will be affected due to increasing temperature and dryness caused by climatic changes so procure low quantities of fresh water by recycling of waste water should be the target. Water contaminated from industry and agriculture has to be efficiently treated to protect humans from being intoxicated with compounds or bacteria. It is well established in industrial wastewater treatment to use membranes for treatment of waste water containing high biodegradable organic compounds but are inefficient with respect to waters containing high amount of stress-inducing substances (olive oil industry), high colourity and low biodegradable organic compounds (textile industry).

In this context, to scrutinize the performance of pertinent membranes developed for the purification of relevant industrial wastewaters we have proceeded to a preliminary study applying quantitative SERS measurements of water solutions containing nanogram levels of Methylene Blue (methylthionine chloride), a heterocyclic aromatic dye used in the textile industry that causes severe central nervous system toxicity at plasma concentrations over 500 ng/mL[2]. We have utilized silver nanocolloids prepared according to modified Lee-Meisel method[3].

SERS measurements were based on the use of a "shaking cell"[4] in combination with the advantage of utilizing the right angle Raman light scattering collection geometry provided much higher sensitivity since this scattering geometry matches the scattering volume with the entrance slit of the spectrometer and improves the signal to noise ratio. The Raman spectra were excited with a water-cooled Ar^+ laser operating at 514.5 nm and were analyzed via a 64-cm focal length spectrometer (T-64000 JY - Horiba).

Representative SERS spectra from Methylene Blue at different concentrations are shown in Figure 1(A); the most intense Raman band centered at 1625 cm^{-1} is used for quantitative analysis by relating the peak intensity to concentration of Methylene Blue. Using Partial Least-Squares (PLS) analysis, a regression algorithm employing routines of the OPUS QUANT-2 Software (Bruker Optics) applied to a total of 11 spectra was used for quantitative analysis. Two ranks can be extracted, and the predicted versus known values of the concentration of the active agent for the 514.5 nm laser line shown in Fig. 1(B) allow for R^2 = 91% and RMSE = 15 ng/mL. For any practical purpose, these errors are in the same range as the threshold detection of Methylene Blue by the applied SERS method.

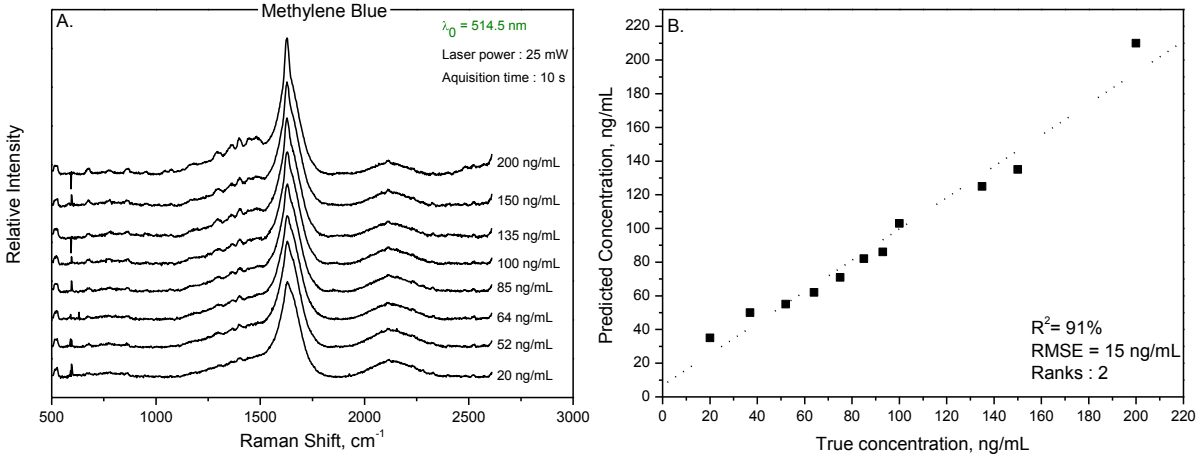

Figure 1. (A)Representative SERS spectra of methylene blue at different concentrations (left) and (B) The predicted correlated to the true values of Methylene Blue concentration quantitative results (right)

Future studies are oriented to test this nano-enhanced Raman scattering technique with a mixture of foulants from the textile industry. Similar quantitative SERS measurements at very low concentration level can be applied to test the efficiency of new nano-filtration membranes in the rejection of relevant low molecular weight organic pollutants in the cosmetics & pharmaceutical industry and in olive mill wastewaters, as well.

Acknowledgment.
Financial support was provided by the FP7 European Project BioNexGen, Contract Number 246039-2.

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A comparative toxicity study of a nanopreparation containing retinyl palmitate as the active agent and designed to treat skin conditions"

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Keywords: skin conditions, toxicity, nanopreparation, emulsion, 3T3, L929, HACAT

Photoaging is a premature skin condition caused by long-term exposure to the ultraviolet B radiations of the sun, and is frequently associated with skin cancer. Retinoids are natural and synthetic vitamin A derivatives. They are lipophilic molecules and penetrate the epidermis easily. Their ability to modulate genes involved in cellular differentiation and proliferation, makes them good candidates to treat and prevent photoaging.

The SkinTreat Project (EU FP7) aims to develop novel user-centered customized topical nano therapeutic strategies, and novel drug delivery systems, for skin diseases (Contact Dematitis, Photoaging and Psoriasis), based on personalized skin protocols.

In this current investigation we performed a comparative toxicity study on a novel nanopreparation based on polylactic-co-glycolic acid (PLGA) with and without retinyl palmitate as the active principle.
To assess for cytotoxicity we used a colorimetric approach based on the activity of the mitochondrial dehydrogenase enzyme (MTT test). Mouse fibroblast (3T3 and L929) as well as human keratinocytes (HACAT) were seeded in 96-well plates and incubated with the aforementioned nanopreparations for 24 hours. The mutagenic potential of both nanopreparations were studied using bacterial reverse mutation test (AMES). Cellular stress by means of ROS production (DCFDA) was also assessed upon nanopreparation exposure by flow cytometry.

Polymeric nanoparticle uptake studies for nanocarrier-based drug delivery in cancer treatment.

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Engineered nanoparticles (NPs) are a key point in new drug nanocarrier development for cancer treatment. Synthesized NPs must access the tumoral cell so the drug can be properly delivered and must not generate a toxic response that kills healthy cells. The goal of 7th Framework Program funded NANOTHER project is to develop these types of NPs to improve cancer treatment. Among many types of NPs synthesized in this project, poly(trimethylene carbonate)-b-poly(L-glutamic acid) (PTMC-b-PGA) polymersome nanoparticles were prepared. Rhodamine B was covalently attached to polymersome surface in order to track the system in the cell by specific fluorescence detection methods. HepG2 hepatocarcinoma cell line was used for the study. These cells grow in complete medium in the form of colonies of cells in a monolayer. The toxicity of PTMC-b-PGA NPs was assayed using Alamar blue. Apoptosis and necrosis studies were carried out by annexin V and propidium iodide stains using a flow cytometer. The polymersomes were non toxic at the concentrations assayed (0.1 and 0.5 mg/ml) at 24 and 72 h. The uptake rate and localization of the fluorescent nanoparticles were both studied using flow cytometry and confocal microscopy. The flow cytometry assays indicated that 5 hours after Red-PTMC-b-PGA NPs addition in cell medium, 50% of cells contained fluorescent NPs. However, more than 90% of cultured cells showed red fluorescence 24 hours after NPs addition. Confocal microscopy studies supported these data and also provided information of the cellular localization of the nanoparticles. In the case of this cell line, Rhodamine labelled polymersomes appeared to be located in the cytoplasm, in the proximity of the nucleus, inside small internal cellular vesicles. The data here presented together with previous results of doxorubicin (a well-known anticancer drug) high loading capacity [1] indicate that PTMC-b-PGA seems to be a good candidate system for drug delivery in cancer treatment.

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Real-time monitoring of ischemia with implantable potentiometric ion selective sensors in an array format

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Miniaturized, implantable chemical sensors, which can be employed for real-time monitoring of clinically important species, such as pH, O_2, and CO_2, Na^+, K^+ and Ca^{2+}; glucose; lactate… remain one of the great challenges in analytical and biomedical science. Ischemia is a shortage of the blood supply to an organ that causes severe tissue damage in a situation of lack of oxygen. Tissue damage from ischemia is critical for the development of further complications, multiple organ failure and death. Therefore, ischemia monitoring is very valuable during surgical procedures. When the tissue is under ischemia conditions, there is a decrease in the oxygen and glucose available to the tissue as well as a decrease in the removal of CO_2 from the tissue due to inadequate blood flow. In these conditions, the ions are not pumped properly and so intracellular and extracellular concentrations of important small molecules such as sodium, potassium and chloride shift, leading to abnormal ion concentration within the cells [1]. The device that our group is developing will be non-invasive, harmless, inexpensive, portable and fast in the response. This device will contain different sensors for ischemia detection such as pH and potassium sensors. This array will be integrated in and endoscope that will perform scarless surgery directly inside the stomach and the array will monitor in situ the evolution of the patient. The stomach in situ detection of ischemia allows the detection of the disruption of the gastrointestinal mucosa that plays a key role in the evolution of shock and it is the motor of multiple organ failure.

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Compact InP/ZnS nanocrstals through optimized aqueous phase transfer: high conservation of fluorescence and colloidal stability

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Semiconductor nanocrystals small enough to exhibit quantum confinement, also termed quantum dots, have shown to be very promising fluorophors for biological labeling owing to their size-dependent emission color, large absorption spectrum, high brightness and excellent photostability.[1] Most of the high quality quantum dots (e.g. CdSe, InP, InAs) are synthesized in organic solvents and are stablilized by surface ligands rendering them hydrophobic.[2] Therefore, in recent years a large number of different approaches for the aqueous phase transfer of quantum dots have been proposed, specially in view of biological application with these NCs. Among those, surface ligand exchange with small hydrophilic thiols has been shown to yield the lowest hydrodynamic diameter, on the order of 5-10 nm. Compact quantum dots are required for specific imaging applications (e.g. sentinel lymph node detection, study of synaptic signaling) and for maximizing renal excretion in *in-vivo* studies. Thiol-containing amino acids such as L-cysteine are of particular interest as capping ligands for hydrosoluble quantum dots as they exhibit low non-specific binding to serum proteins due to their zwitterionic character. However, cysteine is prone to dimer formation, yielding cistine, which limits the colloidal stability of the quantum dots.

We will demonstrate that the precise control of the pH value during aqueous phase transfer dramatically increases the colloidal stability of InP/ZnS quantum dots. While precipitation of the quantum dots in PBS buffer typically occurs within one day, DLS measurements show that no aggregation takes place even after several weeks in case of the correct choice of the pH during the transfer reaction. In addition to cysteine, various other bifunctional thiols have been tested. The pH has to be chosen in a range of 8-10 according to the pK_a value of the thiol function as only the thiolate ion exhibits strong binding to the quantum dot surface. The formation of disulfides has been prevented during phase transfer through addition of reducing agents, e.g. TCEP. Disulfides significantly diminish the fluorescence quantum yield (QY) of InP/ZnS quantum dots. To the contrary, in our procedure up to 90% of the initial QY is maintained. The obtained quantum dots emit at 650-720 nm with a QY of 15% at pH 7.4 and their hydrodynamic diameter is below 10 nm. The described procedure can equally be used for other types of nanoparticles, such as CdSe nanodots or nanorods, $CuInS_2$/ZnS quantum dots or $CuInSe_2$ nanoprisms.

Finally we will present the *in vitro* and *in vivo* behavior of the hydrosoluble quantum dots after surface functionalization with functional molecules (e.g. cell penetrating peptides).

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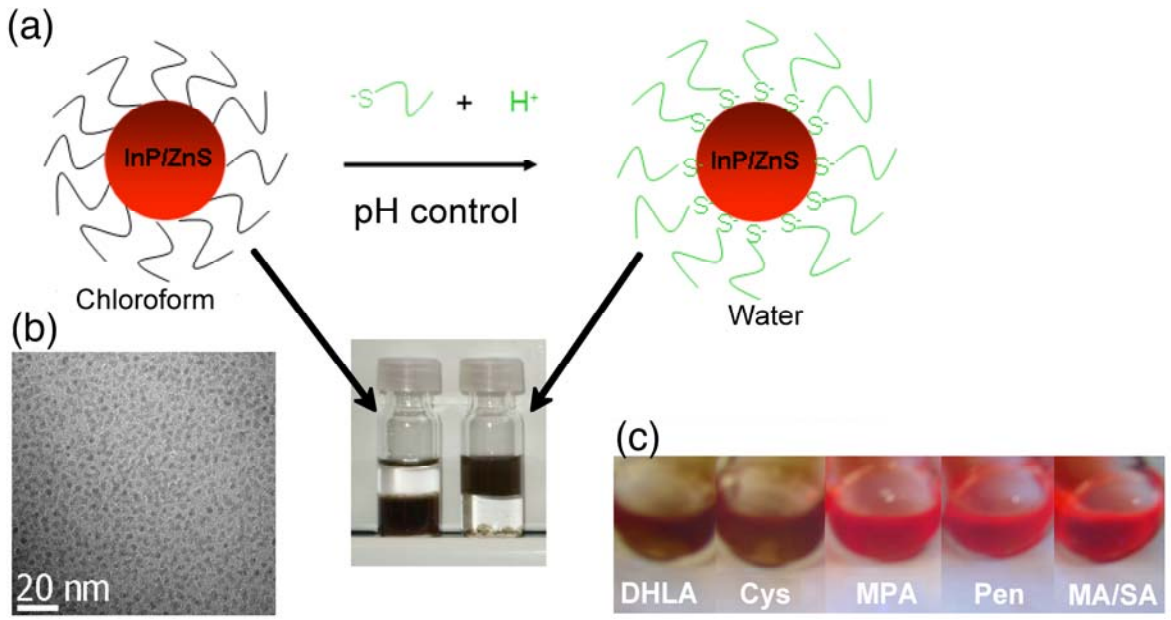

Figure 1: (a) Transfer of InP/ZnS quantum dots from the organic phase (chloroform, bottom) to the aqueous phase (DI water, top). (b) TEM image of the used quantum dots. (c) Photographs of samples transferred using different bifunctional thiols under UV light (DHLA: dihydrolipoic acid, Cys: L-cysteine, MPA: mercaptopropionic acid, Pen: D-penicillamine) compared to the sample in chloroform (MA/SA: myristic acid/stearic acid).

Detection of monoclonal antibodies using chemically modified graphite substrates

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This paper presents a novel sensor device based on chemically modified Highly Ordered Pyrolytic Graphite (HOPG). Biosensor diagnostics based on bio-functionalsised semiconductor devices are an important development in ultrasensitive sensors for early detection of disease biomarkers.

Electrochemical devices using chemically modified graphite (CMG) channels are excellent candidates for nano-biosensors[1] By attaching aniline to HOPG, via coupling with an aryl diazonium salt, the amino group of the aniline molecule has been used graft antibodies - (1) targeted against beta-actin and (2) targeted against 8-hydroxydeoxyguanosine (8-OHdG) - onto the HOPG surface.

Antibody attachment to graphitic surfaces has been verified using Laser Scanning Confocal Microscopy (LSCM) to detect attached quantum-dot labeled antibodies.

The current-voltage characteristics of virgin and chemically modified HOPG surfaces have been used to detect the presence of antibodies at nm concentrations.

This paper reports on a novel electrochemical method for chemical functionalisation of (HOPG) surfaces for the development of a generic biosensor technology. Following chemical functionalisation with an aryl amine linking molecule (see Fig. 1 and Fig. 2), any biomolecule with a carboxyl group can be attached to the HOPG surface.
The binding reaction has been validated using two antibodies i) an antibody targeted against the commonly expressed beta-actin molecule and ii) an antibody targeted against the prostate cancer biomarker, 8-hydroxydeoxyguanosine (8-OHdG). Detection of this biomarker using enzyme-linked immunosorbant assays (ELISAs) has been used to successfully differentiate patients with bladder and prostate cancer from healthy patients [2].
However, biomarkers are often present at very low concentrations and ELISAs have limited sensitivity. Detection of 8-OHdG using highly sensitive, label-free, electrochemical, graphene nano-biochips is an important development in early detection methods for cancer risk. Bio-functionalisation of surfaces with “bio-receptor” molecules capable of specific and selective binding with disease biomarkers such as 8-OHdG is an important enabling technology in the development of nanoscale diagnostic sensors.

Electrical characterization of the chemically modified HOPG surfaces shows that a decrease in current can be detected upon functionanalisation (Fig. 3). The amino group of the aniline molecule has been used graft antibodies – (1) targeted against beta-actin and (2) targeted against 8-hydroxydeoxyguanosine (8-OHdG) - onto the HOPG surface. Antibody attachment to graphitic surfaces has been verified using LSCM to detect quantum-dot labeled antibodies bound to the surface (Fig. 4). The method has been successfully verified using X-ray photoelectron spectroscopy (XPS), Atomic Force Microscopy. The effect of electrochemical functionalisation on the current-voltage characteristics of the HOPG device shows a reduction in the current upon chemical functionalisation of the HOPG (Fig. 3).

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Figures

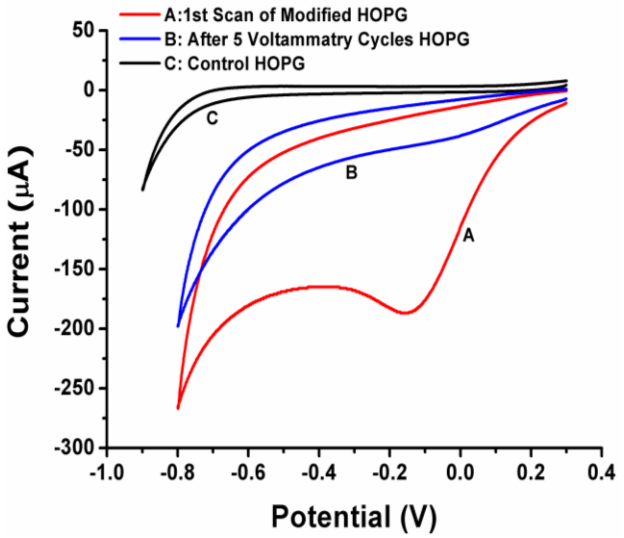
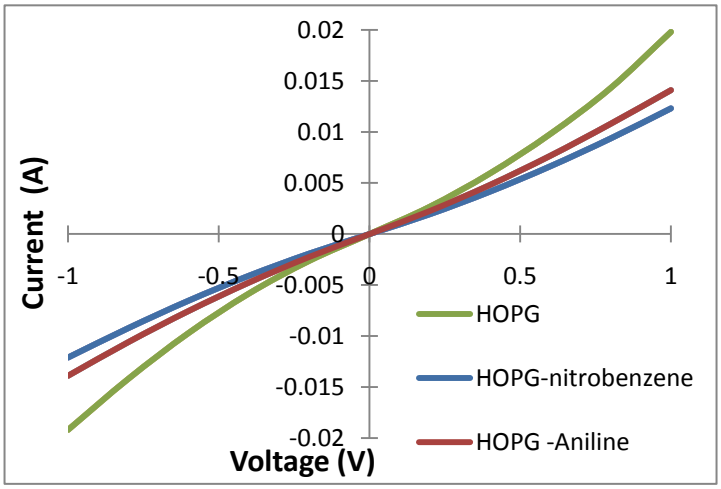


Fig. 1: *Cyclic voltammograms of a (A) during functionalisation of the HOPG electrode with the diazonium salt; (B) after 5 voltammatry cycles, the functionalisation is complete and the HOPG electrode is saturated (C) control HOPG electrode in non-aqueous electrolyte;.*



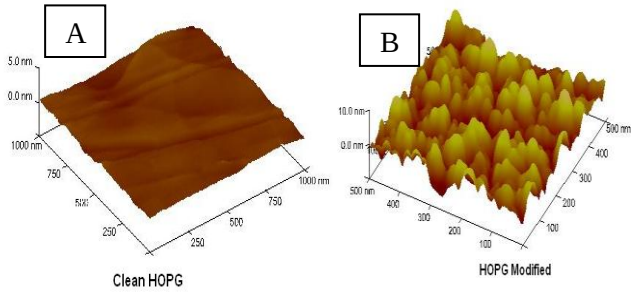


Fig. 2: AFM images of (A) control HOPG surface and (B) amine modified HOPG surface.

Fig. 3: I–V curves for blank HOPG and chemically modified HOPG substrates.

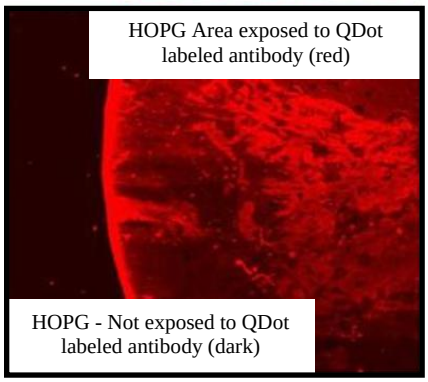


Fig. 4: Confocal microscopy images of aniline-modified HOPG, exposed to QDot labeled antibody in selected areas (red). Areas not exposed to the QDot antibody appear dark.

Breast cancer cell death induced by magnetic nanoparticles subjected to AC magnetic fields

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Iron oxide nanoparticle-based platforms have been investigated extensively for their potential applications on oncology and other biomedical fields.[1] Their magnetic properties have opened the possibility to develop more efficient and selective cancer diagnostic and therapeutic approaches than the current-ones. Coating these nanoparticles with polyester and polysaccharide materials renders biocompatibility, high charge levels and stabilisation against agglomerations and opsonisation under physiological conditions. This combination of properties favours cell internalization[2] and therefore promising perspectives on biomedical applications. Magnetic nanoparticles into a cell allow thermal treatment approach when subjected to AC magnetic fields at high frequencies (in the range of hundreds of kHz). The magnetic energy dissipation into heat and/or the Brownian motion of the nanoparticles into the cell may induce apoptotic mechanisms and consequently lead to cell death.

Here, we report on *in vitro* studies to determine the cell death mechanisms in cancer cells induced by internalized iron oxide nanoparticles subjected to AC magnetic fields up to 600 G in intensity and 500 kHz in frequency. We employ the cell line, in particular, human breast cancer (MDA-MB-231 cells). Iron oxide nanoparticles with sizes around 10 nm were synthesized by thermal decomposition method in organic media.[3] In a second step, the nanoparticles were coated with dimercaptosuccinic acid (DMSA) to remove the oleic acid in an aqueous solution where nanoparticles aggregate forming colloids with a hydrodynamic radius smaller than 100 nm.[4] Standard methyl thiazol tetrazolium bromide (MTT) assays were carried out in MDA-MB-231 cells incubated 24 h with DMSA (0.5 mg Fe/ml) and subsequently subjected under different exposure times to AC magnetic fields with different strength and frequency conditions. Cancer cells without internalized nanoparticles were also subjected to similar AC magnetic fields conditions as control. Optical microscopy was used to evaluate the effect of the applied AC magnetic fields on the morphology of the different samples.

MTT assays showed that cell survival was not affected by the nanoparticle internalization into the cells or by the electromagnetic field on cells without nanoparticles. As an example, Figure 1 shows an optical microscopy image of the magnetic nanoparticles internalized inside the MDA-MB 231 cells. However, the application of the alternating electromagnetic field to cells preincubated with DMSA-nanoparticles led to a significant cell death. Figure 2b shows the morphological changes observed by optical microscopy in these preincubated cells induced by application of an AC magnetic field at high frequency, in comparison with a control sample constituted by MDA-MB-231 cells (Fig. 2a). Furthermore, we have observed that cell death rate increases with magnetic field strength and frequency which might be due to a heat transfer effect from the iron oxide nanoparticles into the cells under application of AC magnetic fields.

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Figures

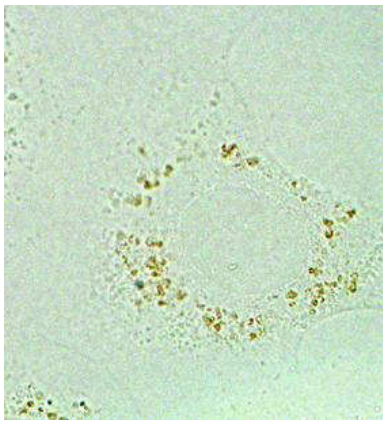

Figure 1. *Nanoparticles internalized inside living MDA-MB 231 cells after 24 h incubation with DMSA (0.5 mg Fe/ml). The intracellular pattern distribution consists in brown cytoplasmic spots of different sizes.*

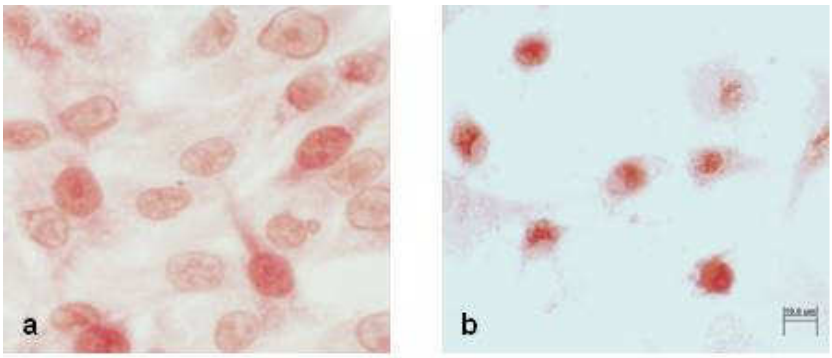

Figure 2. *MDA-MB 231 cells fixed and stained with neutral red. a) Control cells. b) Morphological changes induced by application of an AC magnetic field on cells preincubated with DMSA-nanoparticles.*

Gold Nanoparticle-Oligonucleotides conjugates for LSPR detection of Anabolic Androgenic Steroids

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In the last decade, there has been an increased interest on the application of nanoparticles to enhance the features of the biosensors or to improve their performance. Nanoparticle-based optical biosensors, like those based on localized surface plasmon resonance (LSPR), have been proved to be suitable for the quantitative detection of chemical and biological targets[1-3].

Short DNA sequences have demonstrated to be useful as tools to facilitate biomolecule immobilization on microarray and sensor surfaces. Thus, proteins, antibodies or haptens can be immobilized onto solid supports[4,5] avoiding problems related to denaturation or lose of the activity due to the immobilization. Similarly, colloidal metal nanoparticles of different size, shape and material can also be immobilized on different supports using the DNA-directed immobilization strategy (DDI). Thus, DNA-microarrays can be the base for the site-specific immobilization of gold nanoparticle-oligonucleotide bioconjugates functionalized with complementary strands, allowing the fabrication of novel nanostructured surface architectures[6,7]. This strategy can further be used as a *top down* approach for the site-selective DNA detection through the hybridization with the corresponding targets, but also, to detect proteins or other biomolecules using the bio-barcode assay[8].

In this communication, we will present the work performed to develop a nanostructured LSPR sensor transducer using the DNA hybridization to specifically address gold nanoparticles to defined spots of a glass support. This has allowed the construction of homogenous and biofunctional nanostructured surfaces useful for the detection of interesting target biomolecules using the LSPR physical principle. As a proof of concept, we have used this approach for the detection of small molecular weight analytes at low μg L^{-1} level. The presented strategy may open the doors for an easy way to construct encoded multiplexed LSPR sensor transducers using the DDI method in combination with nanoparticles showing distinct optical properties.

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Figures

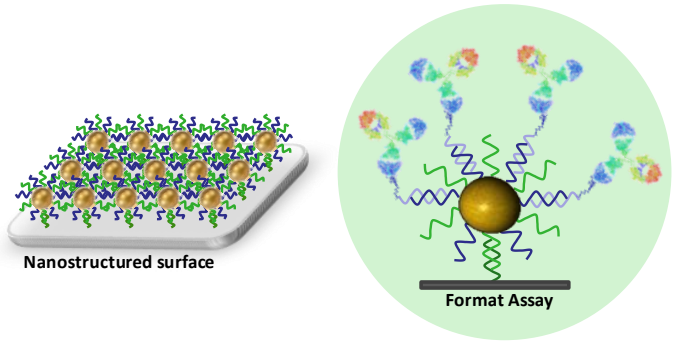

Figure 1: Scheme of this nanostructured surface and the format assay used to evaluate this platform as a LSPR transducer.

Synthesis and stabilization of dopamine embedded in amorphous TiO_2 matrix prepared by sol-gel method

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Parkinson's Disease is a debilitating, often fatal, neurological disorder that affects about 1% of the population over 50 years of age. It is characterized by tremor in the extremities, difficulty initiating voluntary movements, and rigidity. Dopamine (DA) (Scheme 1) is an important neurotransmitter in mammalian central nervous systems and low levels of dopamine have been found in patients with Parkinsons's disease [1]. The lost of dopaminergetic neurons in the substantia nigra is the primary cause of the Parkinson's disease [2].

Literature reports that dopamine is one of the major sources of reactive oxygen species (ROS) [3]. When exposed to the daylight, dopamine oxidizes very easy due to its chemical instability. DA contains an unstable catechol moiety with respect to its molecular structure, it can oxidize spontaneously in vitro, free radicals and quinones [4-6]. In addition, in the human substantia nigra, the oxidation products of DA may polymerize to form neuromelanin which may also be a highly cytotoxic substance [7]. Besides, a controlled release system to deliver the drug directly into the brain is of great interest for the treatment of the Parkinson's disease.

Amorphous TiO_2 matrix was synthesized by sol-gel method at room temperature in air atmosphere. Dopamine (DA) was encapsulated in a TiO_2 matrix to reduce its chemical instability and to retard its oxidation process. Two samples were prepared: one sample is TiO_2/DA and the second one was synthesized by adding 15C5 to protect the DA from oxidation process. Both samples show a red colour. The stabilization process to avoid the oxidation of the dopamine was followed by absorption spectra and IR spectroscopy. Oxidation processes of the DA can be identified by the presence of dopamine quinone and dopamine chrome whose infrared bands are reported in the literature. The TiO_2/DA/15C5 shows more stability than the TiO_2/DA. For TiO_2/DA/15C5 sample, the oxidation process is retarded by one month approximately, while for TiO_2/DA this process is retarded only seven days.

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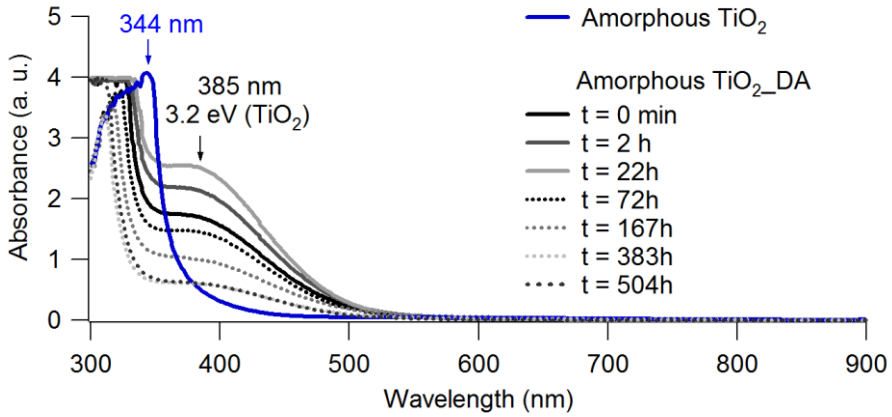

Figure 1. Optical absorption spectra of amorphous TiO_2/DA complex.

VACMON: Vaccine monitoring biosensor

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VACMON* is an ongoing project that stands for an impedimetric biosensor platform able to assess and quantify the immune response after a vaccination. The biosensor, based on hybrids biomolecule-nanoparticle, has as main objective to develop a portable device capable of providing real-time individualized profile of the immune response from a small blood sample. Both national and international public health programs, are working to reduce human papilomavirus (HPV) vaccination costs maintaining its effectiveness and safety. Taking into account the great negative impact caused by this virus, the quadrivalent vaccine against HPV has been chosen for the development of the biosensor device as a model for vaccine monitoring proof of concept.

Our device will be able to quantify the response of the immune system against HPV after vaccination detecting specifically the full-length of the external proteins that form the capside of the HPV (L1), proteins from the HPV types 6, 11, 16 and 18, which are the most incident and aggressive types. Thus, the biosensor will provide information on the maintenance of immunogenicity induced, as well as enabling the identification of people who present a pre-existing immunity, and therefore do not require vaccination or require a different schedule of vaccination.

The biosensor design consists in interdigitated gold microelectrodes design on glass wafers fabricated by photolithography and lift-off processes. Biomolecule-nanoparticle hybrids are covalently attached to the glass surface between the electrodes. Those hybrids consist on gold nanoparticles (Au-NP) functionalized with the most immunogenic regions of the L1 protein of each HPV type. Au-NP increase the active surface area, amplify the label-free impedimetric signal and, thus, enhancing the sensitivity of the biosensor. HPV specific antibodies from a blood sample recognize the L1 fragment attached to the biosensor thus acting as a barrier against the free displacement of ions between both electrodes, leading to a change in the electrical impedance. The two-step functionalization process and the possibility to use directly non-treated blood samples, converts this biosensor platform into a versatile system able to quantify the immune response to other vaccines.

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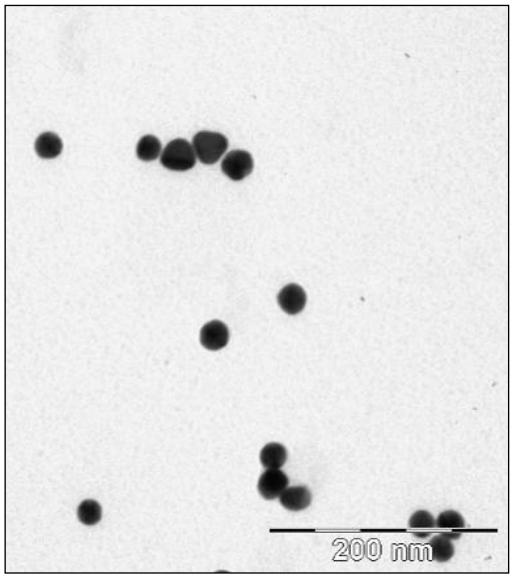

Fig.1: TEM image of Au-NP

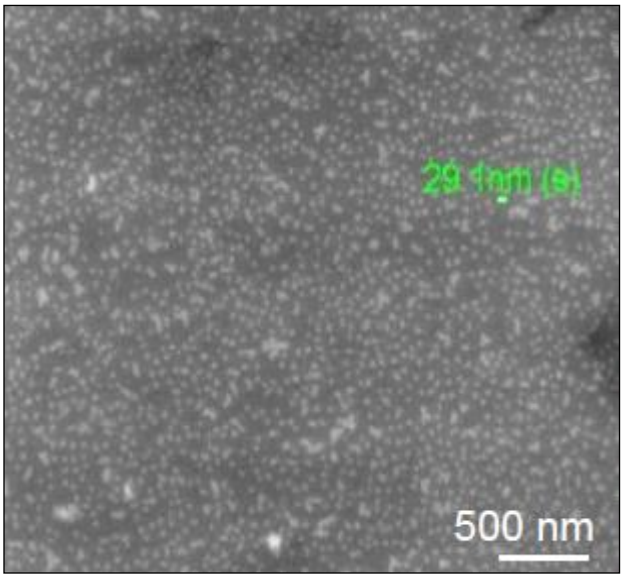

Fig.2: SEM image of Au-NP covalently attached to the biosensor surface.

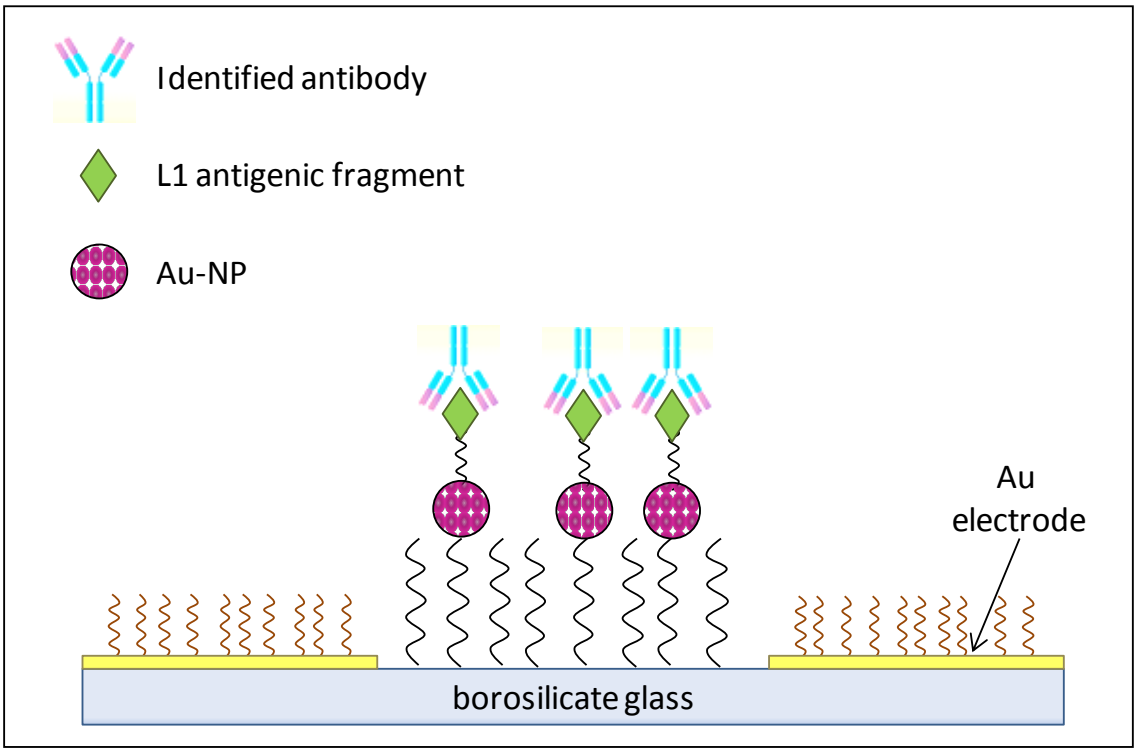

Fig.3: Schematic representation of the biosensor platform

Collagen-targeted growth factors for bone healing

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Reparation of bone defects and fractures is a major clinical and economic concern, being millions of bone grafts performed each year in the United States and the EU. Despite the acceptable bone induction properties of autologous bone grafts, the high morbidity of these approaches and limited amount of material that can be obtained from the donors is forcing the development of bone tissue engineering products for the restoration of damaged or lost bone. This search for alternatives led to the approval by the FDA of an absorbable collagen carrier combined with recombinant human bone morphogenetic protein-2 (rhBMP-2) for the treatment of certain bone diseases and fractures (INFUSE®, Medtronic, Minneapolis, MN, USA).

Osteoinductive growth factors have to be used in combination with a suitable osteoconductive carrier to retain them at the wound site, and permitting a slow release into the extracellular milieu. Although none of the today available carriers can be considered ideal, collagen is one of the most frequently used in experimental studies because of its versatility, high biocompatibility and low immunogenicity. Nevertheless, since most growth factors have a low natural affinity to collagen, these approaches require the use of very high doses of these osteoinductives, increasing the costs and decreasing the safety of these treatments.

In order to design cheaper and more safe delivery systems, we have developed a modified rhBMP-2 with an additional collagen-binding domain (CBD) derived from the von Willebrand factor. This rhBMP2-CBD demonstrated to have an improved affinity to absorbable collagen sponges (ACS) and the ability to induce ectopic bone formation at lower doses than native rhBMP-2 [1].

Since osteogenesis is a multimodal process, with many different growth factors and signaling routes involved, we are currently dedicated to the design of more complex and efficient alternatives to this system by including collagen-targeted angiogenic growth factors (rhFGF-2 [2]) and synthetic osteoinductive peptides with the aim of obtaining an “activated” ACS to improve osteogenesis in vivo.

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Figures

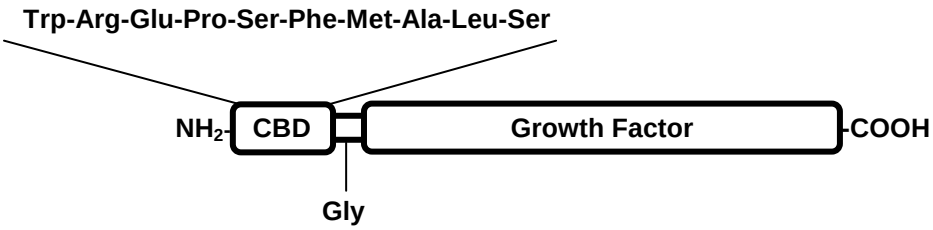

Fig. 1. Schematic representation of a collagen-targeted fusion protein construct.

NANOSCALE DRUG AND GENE DELIVERY SYSTEMS BASED ON NOVEL OLIGOELECTROLYTES AND NANOGELS

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Novel telechelic, block, comb-like and branched oligoelectrolytes including PEGylated ones of tailored molecular weight, narrow molecular weight distribution, and functionality possessing controlled solubility and surface activity were developed. The originality of the created approaches is based on the synthesis of functional surface-active oligomers containing end or side ditertiary peroxide fragments and their subsequent using for the obtaining block or graft oligoelectrolytes via radical solution polymerization. That provides molecular design and controlling the copolymer molecular structures, molecular weight characteristics and behavior in water media of various polarities, namely: conformational state and size of the micelle-like zones, rheology, pH and temperature responsivity, and biological compatibility or genuine physiological activity.

On the basis of such oligoelectrolytes biocompatible polymeric and polymer-mineral nanogels and nanoparticles with controlled size, porosity and functionality containing desired amount of radical forming ditertiary peroxide fragments were developed. Their synthesis is based on using surface-active mono – and poly functional unsaturated substances including peroxide monomers as well as coordinating metal complexes with functional oligoperoxide ligands as templates for nanogels and nanoparticle formation, curing and functionalization. Composite nanogels and nanoparticles comprise of polymeric (including fluorine containing ones) or siliceous, Fe_3O_4, Ni, Au, Ag core and functional reactive polymer shell.

There was shown by light scattering and SAXS techniques that size of the nanoscale drug delivery systems is in the range 20 – 100nm depending on oligoelectrolyte nature and content. Such novel functional oligoelectrolytes and nanoparticles possess ability to immobilize low molecular weight physiologically active substances (biocides, antibiotics including anticancer ones) via mechanisms of solubilization, formation of intermolecular complex, covalent binding etc and to form nanoscale water drug delivery systems. They can be labeled with luminescent, MRI and X-ray detectable markers. The availability of reactive functional fragments in their structures provides irreversible binding antibodies, lectins or saccharide-containing fragments possessing specific interaction with the cell membranes. The toxicity and genuine biological activity studied in the lab of Institute of Cell Biology witness their strong dependence on the molecular weight and molecular weight distribution as well as functionality and surface activity. Testing of oligoelectrolytes and nanoparticles *in vitro* and *in vivo* showed very low toxicity some of them and allowed to select the most promising ones as carriers for antimicrobial and anticancer drug delivery systems.

Water based nanoscale drug delivery systems consisting of novel oligoelectrolytes and nanogel carriers and immobilized chloramphenicol or ampicillins were successfully tested on microbial and fungi cultures. Study of anticancer drug (doxorubicin) delivery systems testified to their overcoming cell membranes and natural biological barriers and as a result high efficiency of the action on some tumor cells *in vitro* and *in vivo* and their low toxicity at the same time. This provides significant lowering the amount of anticancer drug. The study of the novel developed drug delivery systems is on the stage of the patenting.

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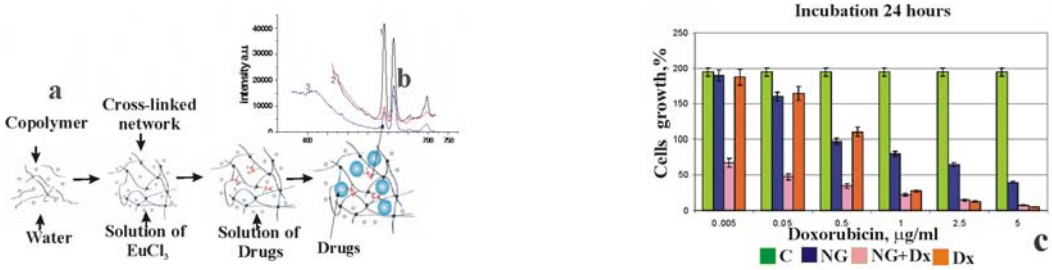

FIGURE. The scheme of nanogel formation and drug immobilization (a), the luminescence spectrum of nanogel labelled at different Eu^{3+} content (b) and diagram of mice leukemia L1210 cell treatment (c): C – control, NG – nanogel carrier, NG + Dx – immobilized drug, Dx – free doxorubicin

Positronium Annihilation Spectroscopy: A tool for the study of transport properties of self-assembled lipid biostructures

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The positron annihilation spectroscopy is well suited for the study of the size and density of nanometre scale voids in polymers [1] and molecular materials, including biostructures [2]. Relevant information about the free volume in biostructures, not measurable by other methods, can be obtained. Specially relevant is the information obtained from the pick-off annihilation of orto-Positronium (o-Ps), the S=1 state of a positron and a electron bound state. Its lifetime has been correlated with the rheological properties of lyotropic liquid crystalline phases of self-assembled lipid biostructures [3] and the transition between the gel-fluid phases has been observed [2]. These results will yield to a deeper understanding of the diffusion transport across the membrane of the cells. Experimental results are analyzed using a simple model developed by Tao and Eldrup that predicts a relationship between the Ps lifetime and the pore/void radius [4]. This model has been tested in organic liquids and is able to predict the pore/void radius, but it is only useful in materials with compact pores and it does not take into account the chemical effects due to the variation of the Ps interaction at different host materials.

Ps interaction in molecular materials is similar to other molecules: at large distances it feels an attractive force due to the dispersion interaction with the neutral molecules and at short distances it is repelled due to, mainly, the exchange-correlation interaction between the electrons in the molecule and the Ps [5]. On the other side, the wavefunction of both constituents, the electron and the positron, is always widely widespread inside the host material due to mainly the low mass of both (the mass of Ps is almost 2000 times smaller than H_2, the lightest molecule).

For a deeper understanding of the experimental data, theoretical modeling of the Ps distribution and annihilation properties is needed, in a similar way as for positrons in condensed matter [6]. But before the Ps-matter interaction potential needs to be better understood. This work presents the results on atom-Ps interaction using exact diagonalization and stochastic variational optimization method of correlated gaussian [7] basis.

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