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# *ABSTRACT COLLECTION*

# *LECTURES*

# Chemical functionalization of carbon nanotubes for applications in nanomedicine

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Carbon nanotubes (CNTs) have emerged as promising nanomaterials thanks to their unique properties, including high specific surface area and capacity to cross biological barriers. They open a wide variety of opportunities for applications in nanomedicine, such as therapy, diagnosis, imaging, neural interfaces, and tissue engineering.[1,2] To overcome insolubility issues, increase biocompatibility, and fully exploit their properties, functionalization of the nanotube surface is essential. In addition, derivatization of CNTs gives the possibility to impart specific properties by conjugation of bioactive molecules including drugs, imaging probes, and targeting ligands.

In this presentation, different strategies for the chemical modification of CNTs for biological and medical purposes will be presented.[3] Functional groups and biomolecules can be covalently grafted or non-covalently adsorbed on the nanotube surface. The inner cavity of CNTs can also be exploited for encapsulation of drugs, radioactive elements, or nanoparticles. Besides, the multi-functionalization of CNTs with a therapeutic molecule, a targeting ligand, and a fluorophore will be detailed with the purpose to use CNTs as multimodal drug delivery systems for the treatment of cancer.[4,5]

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## Carbon Nanotubes: Artificial Nanomaterials to Engineer Single Neurons and Neuronal Networks

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In modern neuroscience, therapeutic regenerative strategies (i.e., brain repair after damage) aim to guide and enhance the intrinsic capacity of the brain to reorganize by promoting plasticity mechanisms in a controlled fashion. Direct and specific interactions between synthetic materials and biological cell membranes may play a central role in this process and nerve tissue engineering has increasingly involved nanotechnology for the development of supermolecular architectures to sustain and promote neural regeneration following injury. The interaction between neurons and nanostructured materials is increasingly attracting interest, because it holds the potential of unexpected openings towards novel concepts for the design of smart devices based on nano(bio)materials properties. Ongoing efforts in this arena require the development of synthetic extracellular scaffolds able to provide unique micro-environments to tissue specific cell types. We used a multidisciplinary approach to investigate the impact of interfacing synthetic nanomaterials (carbon nanotubes) to neuronal networks.

## Nanomaterials: opportunity for medicine and emerging nanosafety issues

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The potential impact on health care of nanotechnology is immense and has ushered a new era aptly labeled as 'Nanomedicine' in view of the potential benefit in various diseases which are currently untreatable. Nanomedicine aims to develop novel and superior materials for diagnostic, therapeutic and preventive application and nanotoxicity provides for the necessary safety assessment of nano-products. To recognize the therapeutic value of a medicinal nano-product and avoid potential risks associated with its use are two-side of a coin, aimed at the achievement of the same goal, i.e., the improvement of human life.
Although it is prudent to investigate the adverse effects on health both during manufacture and use of nanomaterials, with adequate safe guards and standards emanating from nano-safety and toxicity studies, there is no reason to doubt the overriding benefit which nano-medicine will provide.[1]
This work will review in brief the nature of nano-materials and their unique properties which accounts for the significant research both in scientific institutions and industry for translation into new therapies embodied in the emerging field of nanomedicine. It will focus on nanomaterials already approved by FDA and on the most promising inorganic nanomaterials such nanoparticle contrast agents for magnetic resonance imaging (MRI), carbon nanotubes (CNTs) and boron nitride nanotubes (BNNTs).
Some open issues in nanotoxicology such as lack of appropriate guidelines, lack of standardization and unsuitableness of existing methodologies for risk assessment will be discussed. Finally, alternative solutions to replace the use of mammals with non-mammalian models for nanotoxicology assessment whenever possible, or to introduce the non-mammalian testing to prioritize in-vivo testing on mammals will be also considered.

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# Potential applications of polyoxometalates as inorganic nanodrugs and antibacterial agents

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Polyoxometalates (POMs) are discrete metal-oxides frameworks with general formula $[X_xM_mO_y]^{q-}$, in which structural and functional diversity can be generated by an appropriate choice of metal addenda (M = Mo(VI), W(VI) etc), hetero atoms (X), or upon decoration with organic pendants.[1] The biological effects of POMs are mainly associated to their unique redox properties, sometimes resulting in bio-mimetic activities. In particular, the ability to oxidize a number of biochemical substrates and to form reduced oxygenated species (ROS) has been exploited to kill cancer cells and bacteria. In addition, their polyanionic surface allows the formation of adducts with macromolecules, through electrostatic/hydrogen bond interactions. This behaviour has shown to be useful to denaturate proteins and inhibit enzymes, leading to antiviral and antitumoral activities.

The lecture will thus present:

(i) Examples about the redox activity of selected POMs;
(ii) An overview on the interaction of POMs with extracellular and intracellular biological targets;[2]
(iii) Recent results focusing on delivery strategies and cell localization of fluorescent hybrid POMs (Figure 1);[3]
(iv) Recent results about the use of nano aggregates based on POMs encapsulated into chitosan, exhibiting synergic activity of the two components against *E. Coli*.[4]

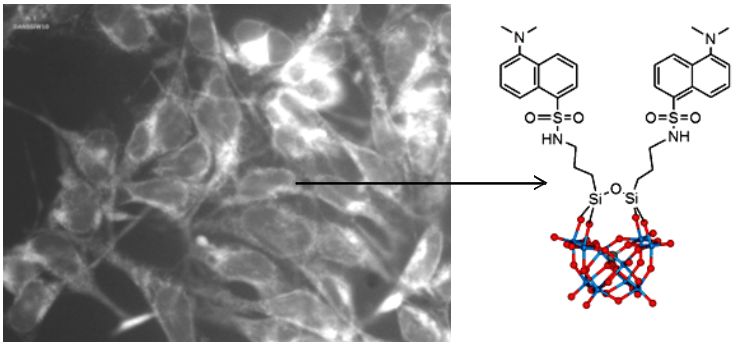


**Figure 1:** Fluorescence microscopy of HEK cells incubated with a dansyl-tagged POM.

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# Self-organized silica nanoparticles as paradigm of nano-bio interactions and drug delivery agents

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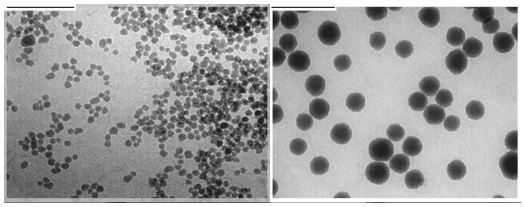

**Figure 1.** TEM images of silica nanoparticles (left, 20-nm diameter; right, 70-nm diameter).

Over the last few years, silica nanoparticles have attracted a great deal of interest as materials for biomedical applications.[1] At first glance, this may appear quite surprising. Unlike other nanomaterials, silica nanoparticles do not have any nanosize-related properties, nor are they easily biodegraded. So, what makes silica nanoparticles so attractive? The answer lies in their highly cross-linked polymeric nature. Being of polymeric structure they can accommodate in their interior (either in the silica matrix or within pores) active molecules, such as drugs, dyes and photosensitizers. The loading capacity of silica nanoparticles is hence much higher than that achievable with other nanomaterials which allow only surface grafting, and this enables their use for drug delivery applications. On the other hand, their highly cross-linked nature renders silica nanoparticles much more rigid than other polymeric nanoparticles. This makes it possible to process them into very complex structures, including mesoporous particles, nanoshells and multi-shell particles.

Such versatility has opened the way to the realization of several sophisticated systems, such as gated porous particles for controlled release, multimodal imaging and delivery agents, chemical sensors. Notably, silica does not need to be made only by silicon oxide. Using organosilane precursors in the formation of the silica network, organically modified silica (ORMOSIL) materials can be obtained. Here, the possibilities for tuning the nanoparticles properties, either of the surface and of the bulk phase, or by changing the nature of the organic moiety introduced, are very broad.

Silica nanoparticles are also the first nanotecnology that entered everyday life of human kind and still the major player in industrial applications. Indeed, many years before nanotechnology established itself as a scientific discipline, silica nanoparticles were already being produced and used, being their preparation reported for the first time in the nineteenth century and commercial production started in 1933.[2] Since then, colloidal silica particles have been used in a wide range of applications including, to cite a few ones, investment casting, silicon wafer polishing, composite materials, beverage clarification. From this point of view they represent and interesting candidate to evaluate the potential issues arising from the exposure of biological entities and organisms to nanomateriales.

In this lecture, we will shortly discuss the lessons learned by silica nanoparticles on the interaction between nanoparticles and biological entities.

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## Engineered gold nanoparticles for diagnosis, imaging, and therapy

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Alcoholic solutions of gold colloids were used for medical purposes since the fifth and fourth centuries B.C. as documented in tracts by Chinese, Arabic, and Indian scientists. Science has made huge progresses in understanding the physical/chemical properties of gold nanoparticles (NPs) and in the development of synthetic procedures for the fine tuning of their size, size dispersion, and shape; the control of these aspects is instrumental to fully exploit their optical, magnetic, and electronic features.[1] Thanks to these achievements and to their stability, with respect to NPs composed of other elements, gold NPs are the most studied type of NPs. Moreover, gold NPs are robust platforms for the grafting of organic ligands which provide stabilization against aggregation, determine the properties of the NP surface in contact with the biological media and are responsible for NPs manifold functional activities.[2] In this respect impressive results have been obtained in order to address NPs specifically to diseased cells by using targeting elements on the NPs surface, enabling selective delivery of their cargo (drugs or nucleic acids).[3] Important achievements have been reported on the use of gold NPs in diagnosis for the detection/identification of specific proteins.[3,4] Moreover, properly modified gold NPs have been successfully applied to a variety of imaging techniques.[5] Particularly appealing are NPs capable of transporting and delivering bioactive molecules, including therapeutic agents and imaging contrast enhancers, to target tissues or for active triggered release of their payload at the disease site. However, in spite of the incredibly large amount of research work in the field, only one example exists of gold NPs that has been approved for clinical studies.[6] More surprising is the fact that still nowadays solutions of colloidal gold are commercialized, mainly via the web, claiming anti-inflammatory properties, enhanced moods, improved cognitive functions, rejuvenating properties. In this lecture, an overview of the different strategies to functionalize NPs with a variety of ligands will be presented and some case in point from the plethora of examples reported in the literature and from our group will be discussed.

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# Photothermal therapies and drug release devices based on laser-activated plasmonic nanoparticles and nanocomposites

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Stimuli-responsive polymeric biomaterials have attracted much attention for their prospective application in several fields including biomedicine, biotechnology and biosensing. As a rule of thumb a stimuli-responsive system is capable of undergoing conformational and chemical changes on receiving an environmental signal. Exemplary stimuli include temperature, pH, light, ultrasounds, magnetic fields, ionic and supramolecular interactions, and redox potential. As a consequence of their action several alterations in relevant material properties such as dissolution or formation, modifications in size and in shape, and enhanced or reduced physical and chemical characteristics can occur.

Here we present two examples of laser-activated biomaterials including plasmonic nanoparticles absorbing near-infrared (NIR) light, that we have recently developed and characterized as viable solutions to critical issues in tissue repair and drug delivery applications.

Laser-assisted tissue repair or laser welding has been proposed to close chronic accidental and surgical wounds. Typically, laser light is delivered 1) to a wound site to be repaired which has been stained with an exogenous optical absorber or 2) to a photoresponsive medical dressing (e.g. patches, stents, etc) placed in intimate contact with the tissue to be repaired, in order to produce a photothermal effect. The endogenous tissue or the externally applied dressing respond to the thermal stimulus producing different chemostructural modifications such as denaturation and fusion, which can mediate the repair of the wound.

An exemplary application is corneal laser welding,[1-3] which is obtained by staining the cut edges of a stromal tissue with the photosensitizer Indocyanine green and by irradiating them with a near-infrared laser light to produce collagen denaturation and reorganization of the noncollagenous components, which can ultimately sustain wound closure and tissue fusion.

We have recently engineered an hybrid bioadhesive consisting in a chitosan film doped with gold nanorods (GNRs) that can be activated by NIR laser light at 810 nm emitted by a diode laser.[4] These films (0.8 cm diameter, 40 μm thickness) are insoluble, flexible, resistant and stable in a physiological environment. The use of GNRs provides amplified optical absorbance of the laser light due to efficient plasmon bands in the NIR window, where tissue components and chitosan are mostly transparent. Upon laser irradiation a well-localized photothermal effect can thus be produced in the film, which is in turn stimulated to produce adhesion with a proximal tissue surface (e.g. arterial wall, tendon, lens capsule). The excellent biocompatibility and biodegradability of chitosan make it a preferred choice for such biomedical applications as wound dressing, tissue engineering and drug delivery.

Capitalizing on this previous experience, we have succeeded in fabricating an implantable device for on demand chemical release in the form of a light-activated sponge-like nanocomposite scaffold.[5-6]

The sponge consists of a porous chitosan scaffold containing a dispersion of GNRs, which acts as an absorber of the incoming laser light, and of thermosensitive polymeric micelles, which serve as a reservoir for the drug molecules to be released. The photothermal response of the nanoparticles contained inside the sponge triggers a contraction in proximal micelles, thus promoting the expulsion of the drug that in turn is released from the sponge to the external environment. We proved the possibility of regulating the temperature generated in the sponge by laser illumination with a continuous wave diode laser light through a variation in the laser intensity (in the 0.3 - 0.5 W cm-2 range) and with a linear relationship within the temperature range of interest for the activation of the release (40 – 45 °C).

The peculiar physiochemical and structural properties of the nanocomposite sponges impart a number of interesting features to this drug release system, including the possibility of spatially confining the therapeutic treatment, as well as of precise control of the amount of released drug as a function of duration and power of the excitation light.

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# Design of Peptide and Peptidomimetic Systems as Selective Delivery of Nanostructures for Diagnosis and Therapy

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A challenge for drug therapy research is to increase therapeutic or diagnostic efficacy of the administered drug and reduce potential toxic side effects on non-target organs. Active targeted drug delivery is appealing for application in a variety of diseases, such as cardiovascular diseases and diabetes. However, the area of main interest for the application of these methods is in oncology where concentration of the drug in tumor cells is a crucial issue. One approach is based on utilizing nanoparticles that have been externally modified with bioactive molecules capable of selectively recognizing targets present in cancer. Cell surface receptors with low molecular weight and high affinity versus endogenous ligands, such as somatostatine receptors and others G protein coupled receptors (GPCRs), may perform as potential targets for radiolabelled compounds for diagnosis and treatment or chemiotherapeutics carrying supramolecular aggregates.[1]

Different systems are used to provide targeting capabilities and include monoclonal antibodies, receptor-specific peptides or proteins, nucleic acids (DNA/RNA aptamers), small molecules, and even vitamins or carbohydrates. All class show advantages and hazards. Synthetic peptides are a class of small ligands that have great potential for such applications. They offer the advantage of providing infinite sequence/structure possibilities that can potentially be designed to bind any cancer related target and are virtually non-immunogenic. Furthermore, receptor-targeting peptides have shown a high level of internalization within tumor cells via receptor-mediated endocytosis. Such a feature of these systems may be of value in facilitating intracellular delivery of the intended payload. The drawbacks related to the use of these compounds are the relatively lower target affinities and the metabolic instability. Studies have been devoted to overcome these problems. The criteria and the design of peptide analogs able to act as carrier contrast agent for imaging will be presented.[2]

In the second part will be presented the case of modified supramolecular aggregates for selective delivery of contrast agents and/or drugs. A new class of peptide-derivatized nanoparticles: Naposomes will be examined.[3] These nanoparticles are based on the co-aggregation of two different amphiphilic monomers that give aggregates of different shapes and sizes (micelles, vesicles and liposomes) with diameters ranging between 10 and 300 nm. Structural properties and *in vitro* and *in vivo* behaviors are discussed. For the high relaxitivity values and to detect for the presence of a surface-exposed peptide, these supramolecular aggregates are very promising candidates as target-selective MRI contrast agents and drug delivery carriers.

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## "Smart" nanocarriers in drug delivery

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"Smart" nanocarriers are colloidal systems designed to **1)** carry out sequential and "logic" operations to yield site-selective accumulation and **2)** to undergo programmed drug release. Colloidal carriers interact massively with the surrounding environment, namely endothelium vessels, cells and blood proteins, which causes a rapid removal from the circulation. By bearing this issue in mind, the design of "smart" nanocarriers, in first instance, should be guided by the principles that dictate the "stealth" properties of drug nanocarriers and the performance of the surface coating in controlling the opsonins/macrophages interaction with the colloidal system.[1]

Many efforts have been done to obtain "smart" nanocarriers for cancer treatment by using amphiphilic copolymers able to self-assemble into micelles or vesicles. These carriers have been engineered for the delivery of oligonucleotides and water-insoluble anticancer drugs to overcome the poor drug solubility, protect therapeutics from degradation and provide for drug release by diffusion or molecular displacement. Size and surface properties of these carriers can be tailored to reduce RES removal and prolong permanence in the bloodstream for passive accumulation in solid tumors by the EPR effect, thus minimizing off-site effects. A series of "smart" carriers has been proposed that respond with sharp morphological rearrangement to the environment of the tumor tissue, such as low extracellular pH, redox potential alteration, peculiar enzymatic pool and increased temperature.

pH-sensitive nanovectors for cancer therapy can be designed to passively accumulate in solid tumor, where their pH responsiveness traduces into localized drug release and/or interaction with cell membrane yielding cell penetration. According to the tumor and blood conditions, systemically administered pH-responsive formulations need to be programmed for response in the 7.4-6.5 and 7.4-5.0 pH intervals to guarantee extracellular and intracellular drug release, respectively.[2]

A class of stimuli sensitive self-assembling materials was obtained by copolymerization of PEG-methacrylate (PEGMA) and 2-(methacryloyloxy)ethyl-3-chloro-4-hydroxybenzoate (MCH) as the pH-responsive unit. The copolymer hydrophilic/hydrophobic balance controlled the association into micelles or polymersomes. The carrier can efficiently load cationic anticancer drugs and release them under acidic conditions. The pH controlled drug release reduced the cytotoxicity of loaded anticancer drug at pH 7.4 while under slightly acidic conditions, a 10-fold increase in cytotoxicity was observed. Tamoxifen half-life in blood was remarkably prolonged after encapsulation in the micelles.[3]

A second class of pH responsive material was investigated for tumor targeting and intracellular delivery of siRNA. A poly(gliceroyl-methacrylate-*co*-C6-imidazoyl-methacrylate)-mPEG tri-block copolymer was synthesized by RAFT polymerization and terminated with folic acid for cancer cell targeting. Dynamic light scattering and transmission electron microscopy indicate that vesicles with diameters of 100-200 nm are formed under the pH-ranges where the weakly basic side-chains of the central block of the copolymer are deprotonated (pH 7.4) and rapidly dissociates under conditions that mimic the endosomal compartment (pH 5). The vesicles act as pH-responsive containers as was shown by DNA encapsulation and release studies and possess endosomolitic activity. Biological studies indicated that active targeting of the carrier was achieved by folate receptor mediated uptake in human cancer cells.

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## Liposomes in drug delivery

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Nanocarriers have been investigated for many years to modify pharmacokinetics and biodistribution of various active molecules. In the cancer domain, one of the biggest challenges still remains the improvement of the therapeutic index, often too low, for the majority of antitumor drugs. The application of nanotechnologies for the treatment and the diagnosis of cancers are nowadays currently developed, or under development, and liposomes[1] play an important role in the history of nanodevices. Because of their high degree of biocompatibility, liposomes have been used to improve pharmacological profiles of various anticancer drugs otherwise discarded because of their low water solubility, poor bioavailability or either fragile and subjected to rapid biotransformations. This presentation introduces an overview of liposomal formulations, techniques of preparation, their characterisation and evolution of their structures.

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# Application of magnetic nanoparticles in nano medicine: Magnetic Fluid Hyperthermia

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Research in the biomedical science has been recently focused on magnetic nanoparticles (MNP) due to the rapidly increasing number and variety of their applications which includes drug targeting, diagnostics, molecular biology, cell separation and purification, and magnetic hyperthermia (MHF). Moreover, the possibility of combining more than one of these functionalities in a single biovector has a huge potential in order to implement the therapeutic efficacy while reducing the side effects. Particularly, the MNP capability of working as contrast agents for MRI and as nanoseeds for MFH has highly contributed to the development of a novel research domain in biomedicine, *theranostics*, aimed to simultaneously track the path, deposition and action of the carriers inside human body and cancer cells.

Due to the energy losses produced during the MNP magnetization reversal process, a magnetic ac field can induce remarkable heating effects which depend on the material design and increase with the field frequency and amplitude. Biological systems, however, impose severe limitations to the allowed values for these parameters and to their product. Besides to this constraint, it is always desirable to achieve the temperature enhancement needed for a special application with as low as possible amount of MNP, particularly in applications where target concentration is very low as in antibody targeting of tumours and metastasis treatments. An effective material designing and development then requires a thorough investigation of the many structural and magnetic parameters influencing magnetothermic activity at biologically compatible applied ac magnetic fields. In addition to a full control of the MNP core, size, shape, chemical composition, degree of aggregation and surface state, the optimization of MNP as nanoseeds for magnetic thermotherapy requires a proper biocompatibility to ensure the circulation within the living organism and an effective vectorization to direct them towards the desired target.

Despite these complex limitations, the exciting perspective to realize theranostic agents able to pursue an effective *find, fight* and *follow* strategy for cancer therapy has promoted in the last years a world wide production of more and more sophisticated nanosystems, most of them based on smart solutions. To compete in this emerging field, therefore, a deep understanding of the material properties and the mechanisms that underlie the hyperthermia application, is now essential.

In this contribution we will present a brief overview of the basic concepts of nanomagnetism with particular attention to those aspects useful to introduce the heating release mechanisms. We will then discuss the magnetic properties and hyperthermic efficacy of a selected set of nanosystems we investigated in last years at the Laboratory of Molecular Magnetism, in Florence, including highly monodisperse ferrite particles with average size of few nanometers embedded in different chemical environments and coated by properly designed grafting molecules. The characterisation of the physical properties of the nanosystems was mainly focused to establish the correlation between synthetic parameters, structural features (size, crystallinity, composition) and magnetic properties (magnetic moment, magneto-crystalline anisotropy) with particular attention to those determining the hyperthermic behaviour. The understanding of such relations allows to attain the capability of tailoring the properties of the nanomaterials and to select the best promising products for the proposed biomedical applications.

# Magnetic Nanoparticles as contrast agents for MRI

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Signal Intensity in Magnetic Resonance Imaging depends on a number of parameters including proton density, relaxation times (T1,T2 and T2*), water diffusion, blood flow, blood oxygenation. This multi-parametric dependence of Signal Intensity is the base for the excellent soft-tissue contrast of the technique and for its diagnostic power. Nevertheless, since the beginning, it became clear that MRI contrast agents (MRI CAs) could play a vital role in diagnosing diseases. MRI CAs are exogenous chemicals with the property of affecting proton relaxation times (T1, T2, T2*) of tissues. Attention was initially devoted to paramagnetic ions (mainly Gd) that due to their toxicity were complexed to suitable chelating agents, like Gd-DTPA (Magnevist®, Schering). This first generation of contrast agents, that is referred to as “low molecular weight” or “extravascular” contrast agents, was constituted by low molecular weight complexes which, after their injection in the blood flow, rapidly diffuse in the extravascular-extracellular space and enhance signal intensity of tissues mainly in dependence of vascular permeability.[1] They are in fact widely used in the diagnosis of Central Nervous System pathologies characterized by alterations of Blood-Brain-Barrier permeability. The need for CAs which remain confined in the vascular space for relatively long time intervals (blood pool contrast agents) has initially driven the research activity in this field toward macromolecular contrast agents (e.g. Gd-DTPA-Albumin[2]) or magnetic nanoparticles (e.g. MION, Monocrystalline Iron-Oxide Nanocompound, SPIO, Superparamagnetic Iron-Oxide Nanoparticles[3]). Nowadays, Magnetic nanoparticles (NPs) are viewed not only as MRI CAs but as nanocarriers that can join diagnostic and therapeutic properties, posses multimodal diagnostic capability and target-specific selectivity. Most of magnetic NPs are based on Iron-oxides as magnetically active moiety although other ions have been considered. MRI CAs can be classified depending on their effect on MR Signal Intensity as positive or negative contrast agent: a positive contrast agent will increase, while a negative one will decrease, the signal intensity of the target tissue. Positive or negative contrast agents are referred also as T1- or T2-relaxing CAs since they affect mainly T1 and T2, respectively.
In this lesson I will provide an overview of magnetic NPs currently under investigation as MRI contrast agents. In the first part, I will introduce the basic principles of the magnetism of magnetic NPs as well as the principles of MRI to explain the impact of magnetic NPs on MR Images. In the second part, I will show some relevant biomedical applications.

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# Surface engineered iron oxide nanoparticles: synthetic routes, application and perspectives

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Superparamagnetic iron oxide nanoparticles (SPIONs) modified with organic coatings have attracted enormous attention for their potential use in the biomedical field for both diagnostic and therapeutic applications.[1] Their most attractive aspect is the intrinsic multifunctionality due to the combination of superparamagnetic properties of the iron core with biocompatibility and specific biological activity provided by the organic coatings. Since the hybrid nature of the surface plays a pivotal role in determining the interaction between the SPION and its biological target, the quality of the surface functionalization is crucial to successful applications. For this reason, the present decade has seen a surge of interest in functionalization studies of magnetic metal oxides with specific coatings usually based either on biocompatible polymeric films (such as dextrane) or on molecular monolayers (SAM) whose stability is related to the anchoring properties of the specific tethering group [–COOH, $–SO_3H$, $–Si(OR)_3$, $–SiCl_3$ and $–PO_3H_2$]. In this context, a comparison of the synthetic strategies for the functionalization of iron oxides will be presented, focusing, in particular, on phosphonic-based SAMs which were proven to combine excellent thermal and hydrolytic stability with simple and efficient synthetic routes.[2] Since monolayer-based coatings are usually adopted as molecular linkers between the surface and the active molecule, the various chemical routes[3,4] (such as nucleophilic substitutions, 1,3-cicloadditions) adopted to bond the active molecule (molecular receptors, luminescent probes, drugs etc..) on the monolayer will be also described. A key point in the developing of these synthetic strategies is the precise characterization of the resulting hybrid material which can be achieved only combining chemical and structural characterizations (XRD, FTIR, SEM, TEM, XPS and SIMS) with specific approaches for the evaluation of functional properties (magnetic and hypertermic performances, colloidal stabilities, biocompatibility, drug load capabilities etc…). Through the illustration of some examples, the potentiality of these multi-steps anchoring routes for the preparation of multifunctional system will be discussed.

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# On the use of gold nano-particles to increase the sensitivity of protein detection by means of microbalances: Counting protein on surfaces one by one, selectively!

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Micro-resonator sensors have achieved a large diffusion in basic as well as in biological and medical applications because of their great sensitivity and versatility.[1] Typically, the shift in the resonance frequency induced by the mass of adsorbed analyte is measured, with resolution down to the zeptogram range.[2] Unfortunately, those performances strongly degrade in wet environment, and therefore the application of microresonators to real time biological essays is still difficult. Two approaches have been generally proposed. The first, dip and dry, also at microfluidic chip level,[3] where resonators are first immersed in the analyte solution and later dried for performing the measure. The second, channel resonators,[4] where microchannels are integrated within the resonator, so that it can be operated in vacuum ambient, with negligible performance degradation, at a cost of complex fabrication and operation procedures. We proposed a third route based on the development of micromechanical sensors based on silicon micropillars, shown in Fig. 1.[5,6] Because of the small sensitive area, diffusion limited reactions are produced at a three order of magnitude faster rate.[7] Moreover, using a carefully designed geometry is possible to create a superhydrophobic structure in which only the top of the pillars is wetted by the analyte solution. In these conditions the resonance peak preserve the high quality factor observed in air and read out can be obtained in real time.[8]

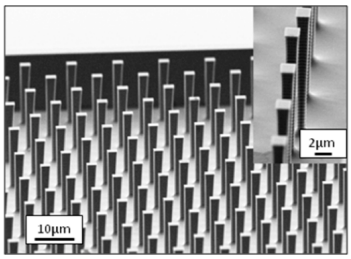


**Figure 1.** SEM image of an array of pillar sensors

After an introduction to mechanical sensors in general and microcantilevers in particular, the recent trends of nanosensors applications in nano-medicine and the latest achievements in the design and developments of nanomechanical sensors will be discussed. Finally the proposal of a nanomechanical sandwich assay in which nanoparticles are used as protein mass amplifiers (see Fig. 2) to achieve single protein sensitivity will be illustrated.

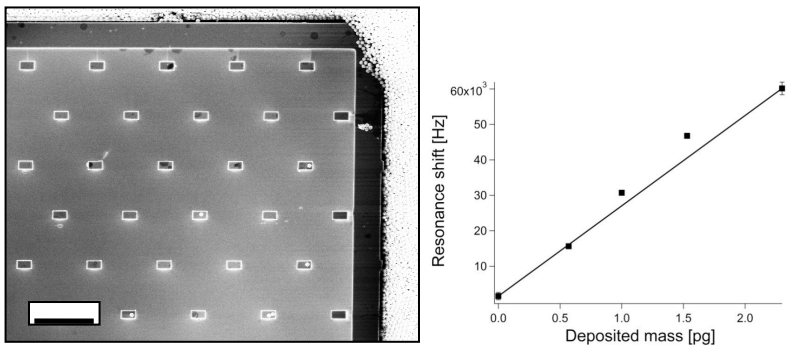


**Figure 2.** (a) SEM image o fan array of pillar sensors with a discrete number of nanoparticles on top. (b) discretized frequency shifts induced by single particle deposition.

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# POSTERS

# The development of the SCARLETcell code for radiation dosimetry and therapeutic efficacy calculations at the cellular level

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The "cell" module of the SCARLETcell code was developed to predict the therapeutic efficacy of different radionuclide-filled Carbon Nanotube (CNT) systems through radiodosimetry calculations on the single-cell level based on the mathematical formalism recommended by the Medical Internal Radiation Dose (MIRD) International Committee.[1] The code breaks the cell down into subcellular compartments (the nucleus, cytoplasm, cell surface, and entire cell), and computes the distribution of radioactive decays, radiation dose, as well as the cell-kill probability. The use of SCARLETcell provides calculations for the comparison of administered activities, radionuclide selection, and acceptable limits for cell-kill efficacy.

In the current version of the SCARLETcell code the S-Values (dose in the target region per decay in the source region) for the various subcellular compartments were computed using the convolution integral method[1]. This method uses the Cole-Howell stopping power expression, the geometric factor for each source-target combination, and a particular radionuclide emission spectrum for a spherical cell. In future versions of SCARLETcell, more accurate calculations of the S-Values will be obtained using Monte Carlo simulations and Dose Point Kernel approximations.[2]

The cell-kill probability is calculated to determine the therapeutic efficacy of the administered activity in the cell. In SCARLETcell, the Linear-Quadratic (L-Q) model is presently used to relate the dose to the nucleus to the cell survival. This model considers the dose-rate effect through two empirically determined values of α and β. These values represent irreparable lethal damage caused by a single electron track (α) and uncorrelated sublethal damage (β) respectively. The efficacy results depend upon the amount of administered activity as well as the selected radionuclide. Currently, SCARLETcell considers six Auger electron emitting radionuclides. Although the program can be applied to other types of radiation, low-energy electron emitters are particular sensitive to the intracellular CNT kinetics.
The SCARLETcell program is used to analyze data from a case study of multi-walled CNTs distributed in MCF-7. A comparison of I-125 and Tc-99m shows that with the administration of 1 Bequerel of activity in the cell, the probability of cell-kill differs by over 10 percent (99.9 % and 87.9 %). When only 0.1 Bequerel of activity is administered, the cell-kill probability using I-125 is 50.0 % while the Tc-99m is only 19.0 %. This demonstrates the importance of carefully choosing the radionuclide with respect to its associated half-life and energy emission spectrum.

The research leading to these results received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n° 290023.

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# Assessment of candidate drugs and cell lines for nanoparticle specific tumour targeting and therapeutic efficacy

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In most cases killing cancer cells is easy and not the only challenge facing oncology. If this was the case there would be many more cancer survivors. The difficulty is that the most effective chemotherapeutics, like doxorubicin (DOX) can cause the cancer cells to become resistant to the chemotherapy.[1] Also, the more DOX you give, the more it induces cardiotoxicity.[2] Curcumin is a natural supplement that has been shown to increase the effectiveness of DOX by reducing multidrug resistance. In addition, curcumin protects the heart from DOX damage.[3] In this study we investigate the cellular drug uptake by flow cytometry and fluorescence microscopy (Figure 1A) where a clear dose dependence uptake was observed and a higher uptake of curcumin was detected when incubated in combination with DOX. Moreover, we studied the cytotoxicity of these two drugs in combination and separately by MTT assay to evaluate if a co-treatment could enhance the therapeutic efficacy (Figure 1B). In this study the optimal concentrations to be used in a nanocapsule formulation were confirmed.

The specific delivery of anticancer drugs to cancer cells has important implications for diagnosis and therapy. Before using a specific nanocarrier to target cells, the expression of the targeting receptor has to be investigated. In this study we validated the expression of two receptors, the folic acid receptor (FOLR) and vasoactive intestinal peptide receptor (VIPR) in different cancer cell lines. This data provides us information to formulate a nanocarrier with an optimal drug concentration and an efficient targeting ligand to treat cancer cells.

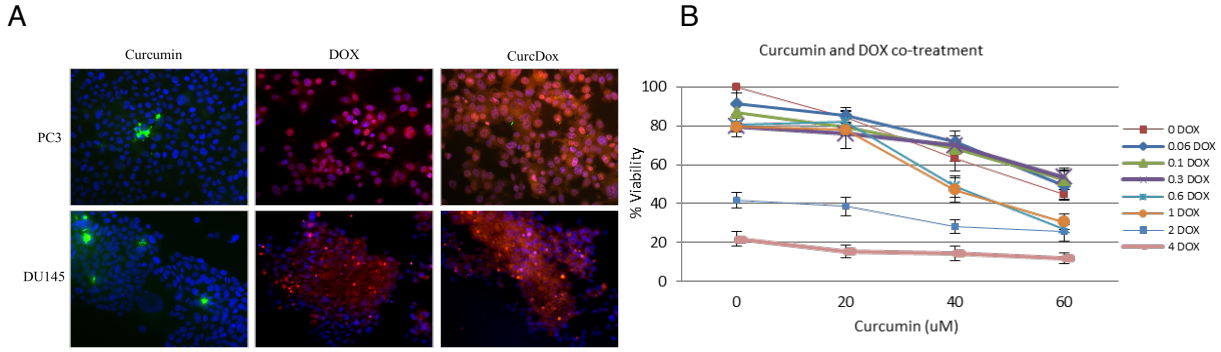


**Figure 1.** (A) Drug Uptake by fluorescence microscopy in prostate cancer cell lines (PC3 and DU145). (B) MTT Assay after 72 h incubation with DOX and curcumin on PC3 cells.

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# Study of Dispersibility of Carbon Nanotubes

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Due to the strong van der Waals interactions, as-synthesized carbon nanotubes (CNTs) form bundled structures, which lead to poor solubility and poor dispersibility in solvent media.[1] Besides, as a result of the strong van der Waals interactions, the quality of CNTs properties decreases and also, their chemical treatment is more complicated. To improve the dispersibility, CNTs were treated in organic solvents by covalent attachment[2], dimethylformamide[3] (DMF), 1-Methyl-2 pyrrolidinone[4] (NMP) or the bundles were exfoliated using sodium dodecyl sulfate[5] (SDS), block copolymer dispersants[6] and many other attempts were realized.

To obtain homogenous CNT samples, a good dispersion of CNTs is important. In our study we attempt to homogenize CNTs by a two-step homogenization process. During the first step, CNTs are mechanically homogenized with mortar and pestle and in the second step, CNTs are dispersed in NMP or DMF by ultrasonication. We studied dispersibility of CNTs in ethanol, isopropanol, DMF, NMP and1 Cyclohexyl-2-pyrrolidinone. We analyzed different concentrations of CNTs in dispersion (0.01 mg/mL, 0.02 mg/mL, 0.05 mg/mL, 0.5 mg/mL, 1 mg/mL, 4 mg/ml). We also studied the effect of sonication time (2 min, 5 min, 15 min, 20 min, 40 min, 60 min).

The homogenized CNTs samples were analyzed by Raman spectroscopy and thermogravimetric analysis. In particular, to evaluate the quality of homogenization of the sample, the radial breathing mode (frequency between 100–400cm$^{-1}$) in the Raman spectra were examinated by measuring several spots in the same sample.

We found that the used solvents remain in CNT sample, changing the CNTs characteristics. Therefore we conclude that the use of organic solvent as NMP or DMF should be avoided.

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# Purification and filling of single walled carbon nanotubes for biomedical applications

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Carbon nanotubes (CNTs) can be employed as nanocarriers in biomedicine. However, to increase their biocompatibility high purity and specific size are desired. Many methods have been developed to achieve an effective purification and to decrease the length of the nanotubes. The nanotubes can be then filled with a chosen payload and externally functionalized.[1] Suitable functionalization improves their biocompatibility,[2] pharmacokinetics and allows modulate the biodistribution of tailored carbon nanotubes in living organisms.

Here we report on the steam treatment[3] of single walled carbon nanotubes (SWCNTs) followed by HCl purification and their filling with chosen payloads. As-produced SWCNTs have lengths varying from hundreds of nanometers up to several microns. Potential use of CNTs for biomedicine for targeted delivery requires shorter structures.[4] Steam treated nanotubes were next filled using molten filling method with selected payloads.

Each step of preparation of nanocapsules (filled carbon nanotubes) was monitored by scanning transmission electron microscopy (STEM). The length of SWCNTs after steam treatment was measured with Digital Micrograph software from scanning electron microscopy (SEM) images. Efficiency of purification was examined by thermogravimetric analysis (TGA), which confirmed decrease of metal impurities after steam and HCl treatment. The length of the nanotubes decreases with time of steam exposition. Transmission electron microscopy (TEM) additionally confirmed that SWCNTs were indeed filled with the chosen payloads and external material was removed. Further, they will be used for targeted delivery of radioactivity in nanomedicine.

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# Functionalization of filled CNTs by [2+1] cycloaddition for the targeted delivery of radioactivity

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This research work is embedded within the ITN program RADDEL dealing with the targeted delivery of radioactivity. The general purpose is the development of carbon nanocapsules, closed-ended filled carbon nanotubes (CNTs) sealing radioactive material in their interior, and their external decoration with biomolecules to render them biocompatible, and for targeting purposes. Our objective within this framework is focused on the development of methodologies for the covalent organic functionalization of different types of CNTs, with the aim to generate mono-or multi-functionalized CNTs and to preserve their closed end, in order to avoid leakage of the internal radionuclide. In particular, we focus our investigations on [2+1] cycloaddition reactions, leading to the formation of 3-membered rings on the CNT sidewall: the Bingel reaction,[1] which forms a cyclopropane ring, and the nitrene reaction,[2] which introduces an aziridine ring on the nanotube surface (**Figure 1**).

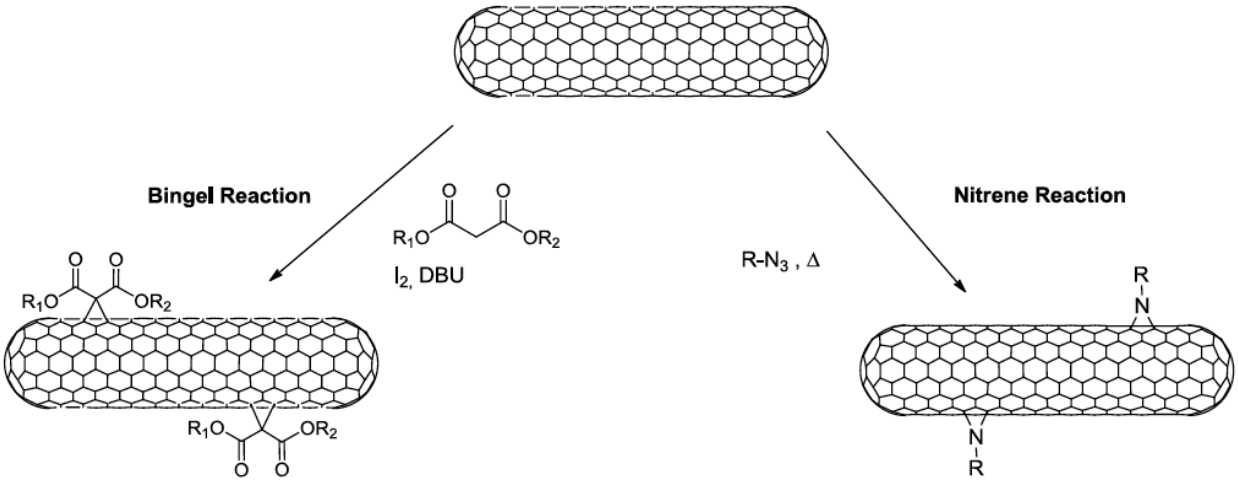


**Figure 1.** Representative scheme of the Bingel reaction and the cycloaddition of nitrene on CNTs.

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# Side-wall covalent chemical functionalization of carbon nanotubes for biomedical aplication

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Carbon nanotubes (CNTs) offers feasible and interesting applications in materials science and medicinal chemistry.[1] In the last two decades, research in the CNTs field has attracted great interest to provide chemical affinity to the biological matrices. Various strategies have been applied to render carbon nanotubes more compatible in physiological conditions. The most promising approach is the functionalization of CNTs' surface via covalent bonds or hydrophobic interactions between hydrophilic molecules and CNTs.[2] Both strategies remarkably improve the water dispersibility of the nanotubes, and at the same time offer a flexible platform for further derivatizations. So it is possible to design CNTs for specific applications as fluorescent probes, MRI contrast agents, and drug delivery systems.[3]

Covalently functionalized CNTs have a promising future as new delivery systems of radioactivity in tumor treatment.[4]

In our group, we are currently investigating new approaches in the CNTs functionalization, to improve their dispersibility and solubility in polar solvents and aqueous media as well as to increase the biocompatibility.

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# Electron Microscopy Studies of Carbon Nanocapsules for Targeted Delivery of Radioactivity

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Functional carbon nanotubes (CNTs) are attracting increased attention due to their potential use for biomedical applications, including in vivo imaging, tumour targeting and drug delivery systems.[1] An intrinsic characteristic of carbon nanotubes is that their inner cavity can be filled with a chosen material whilst the outer surface can be modified to improve their dispersability and biocompatibility.

Electron microscopy techniques are an essential tool for the characterisation of these carbon based nanomaterials. We have used scanning electron microscopy (SEM), high resolution transmission electron microscopy (HRTEM), scanning transmission electron microscopy (STEM) along with analytical tools such as energy-dispersive X-ray spectroscopy (EDS) and electron energy-loss spectroscopy (EELS) to characterize these samples at different stages. Among others, we have studied sample purity, length distribution of carbon nanotubes, presence/absence of filling material, formation of nanocapsules with closed/sealed ends, detection of functional groups on the external surface, simultaneous detection of the filling and external functionalities and the interaction of the carbon nanotubes with cells.

Herein we will present our results on the use of electron microscopy techniques for the characterisation of functional carbon nanotubes.

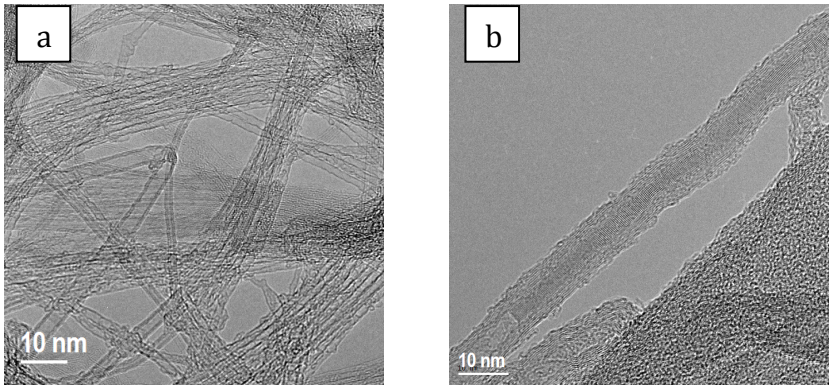


**Figure 1.** TEM image of a) purified single-walled carbon nanotubes, b) filled multi-walled carbon nanotubes

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# Filling of single-walled and multi-walled carbon nanotubes with inorganic payloads

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Single-walled carbon nanotubes (SWCNT) are made of one graphene sheet rolled-up into a cylinder, while multi-walled carbon nanotubes (MWCNT) consist of several graphene sheets rolled-up into concentric cylinders. The inner hollow cavity of carbon nanotubes can be filled with a large variety of materials.[1] Such hybrids have potential for both *in-vivo* and *in-vitro* application in diagnosis and therapy.[2]

Samples of as-made single-walled and multi-walled carbon nanotubes have been treated in a high temperature furnace using a mild-oxidizing agent - water steam, combined with argon.[3] Purified and shortened nanotubes are produced this way, assuring that the ends of the nanotubes are opened which is essential for the subsequent filling with different materials. Both single-walled and multi-walled carbon nanotubes have been filled with different inorganic payloads by either solution or melting filling.

The prepared samples have been characterized at different stages by T/SEM, TEM, TGA and/or EDX. The advantage of melting filling compared to solution filling is that after annealing single-walled carbon nanotubes at high temperatures, the ends of the nanotubes close while cooling. Therefore, this results in the confinement of the payload inside the nanotubes, producing this way what we refer to as carbon nanocapsules.[4] The formation of such nanocapsules allows the removal of the external (not encapsulated) material whilst preserving the encapsulated compound. UV/VIS can be used to monitor the presence of sodium iodide in the water after washing sodium iodide filled single-walled carbon nanotubes, directly proving the efficiency of the protocol used for washing the samples and confirming the absence of external material at the protocol endpoint.

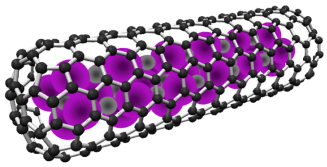

**Figure 1.** Schematic representation of a single-walled carbon nanotube filled with sodium iodide

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**Acknowledgements**

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## Multi-Walled Carbon Nanotubes with different lengths decorated with SPION

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Nanomaterials such as Multi-walled carbon nanotubes (MWNTs) can be used as imaging agents for biomedical imaging. Their large surface area facilitates the incorporation of Magnetic Resonance Imaging agents, like Superparamagnetic Iron Oxide Nanoparticles (SPION).[1]
As-prepared MWNTs contain impurities such as catalytic metal particles, amorphous carbon and graphitic particles which have been shown to be responsible for the initially attributed toxicity to CNTs.[2] Steam treatment process[3] uses a mild oxidizing agent, to obtain high quality samples. Apart from that, during the process MWNTs are shortened, thus being a method to obtain different length of MWNTs.[4]
Herein we present a novel approach to synthesize magnetic MWNTs for its use as MRI contrast agents. Two different length of MWNTs have been used and have been decorated with γ-$Fe_2O_3$ nanoparticles (NPs) using a solution of an iron salt as a precursor. Sample characterisation by Transmission Electron Microscopy and Thermogravimetric Analysis allows the structural study of the resulting hybrid. X-Ray Diffraction pattern and X-ray Photoelectron Spectroscopy are used to determine the composition of the nanoparticles. Moreover, the superparamagnetic properties of the nanoparticles are analysed carrying out magnetic measurements with SQUID. Cell culture work, including in vitro studies with J774 cell line have been done to study the uptake of the hybrid. In addition, the toxicity of the hybrid is assessed with the modified LDH assay.[5]

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# Nanostructures functionalization with squaraines: potential use in photodynamic therapy

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Squaraines are a class of organic dyes based on a squaric acid core. These molecules show an intense and sharp fluorescence and the capability to generate reactive oxygen species (ROS) including singlet oxygen ($^1O_2$). For these properties squaraine dyes have been used for imaging and photodynamic therapy (PDT) applications. PDT is a noninvasive method of treating malignant tumors and age-related macular degeneration and is particularly promising in the treatment of multidrug-resistant (MDR) tumors alternative to the local heating. The dye is excited with light between 650 and 800 nm, low absorptivity region in typical mammalian tissues, generating ROS and thus irreversibly damaging tumor cells.

Through the enhanced permeability and retention (EPR) effect, nanostructured materials upon systemic injection can accumulate in tumor tissues by escaping through the abnormally leaky tumor blood vessels,[1] making them useful for drug delivery applications. A myriad number of nano drug delivery systems including polymer micelles, liposomes, polymer conjugates, carbon nanotubes, dendrimers and nanoparticles have been widely studied.[2]

This work is focused on of the conjugation of squaraine dyes with two different types of nanostructures: single wall carbon nanotubes (SWCNTs) and halloysite.

SWCNTs have been deeply studied as carrier material. They can effectively shuttle various bio-molecules into cells including drugs, contrast agents, peptides, plasmid DNA and small interfering RNA (siRNA) via endocytosis.[3]

The results obtained with SWCNTs have been compared with a halloysite ($Al_2Si_2O_5(OH)_4 \cdot 2H_2O$), a natural, two-layered, aluminosilicate nanotube, with a predominantly hollow tubular structure in the submicron range and chemically similar to kaolin.[4]

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# Fluorescent Probes for Carbon NanoTubes-Based Drug Delivery System

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This work is part of a project aimed at the production of fluorescent labeled CNTs for drug delivery (figure 1). We are developing the synthesis and the characterization of new derivatives of a class of fluorescent probes known as “Bodipy”. A fluorescent probe bonded to a drug delivery system allows, through spectrofluorimetric analysis, its detection inside cells. The core of the synthesized fluorescent molecule is a boron dipyrazine complex (Bodipy, figure 2). This compound was chosen for its great stability and versatility. The bodipy core was functionalized with a group (azide) able to give a coupling reaction with the surface of the carbon nanotube. Moreover to tune the absoption and fluorescence wavelength, the bodipy core was decorated with different types of aromatic molecules. This way, the azido-bodipy, the bis-phenyl, the mono-pyridyl and the bis-pyridyl adducts were obtained. A Bodipy decorated CNT was also obtained. Currently, the decoration of multi-walled carbon nanotubes (MWCNT) with other Bodipy fluorescent derivatives is undergoing in our laboratories.

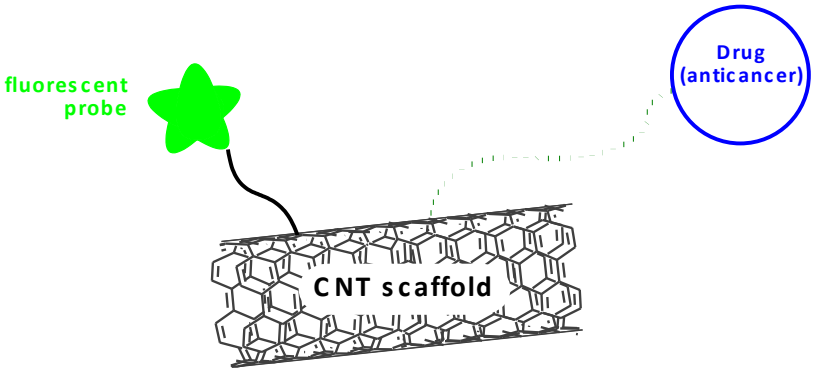


**Figure 1.** Scheme of the drug delivery system

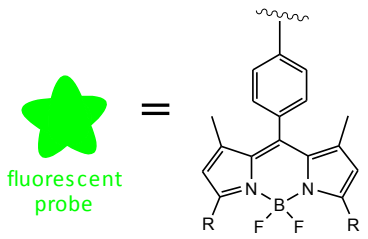


**Figure 2.** Scheme of the fluorescent bodipy core

## Nanocomposite scaffolds based on carbon nanostructures and polysaccharides

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Several natural polymers are now available for tissue engineering applications, due to their biocompatibility and bioactive properties. However, biomaterials based on these polymers often display poor mechanical properties: for this reason nanocomposite reinforcements are being widely investigated with respect to both structural and biological features. In this frame Carbon Nanostructures (CNSs) have been receiving increasing attention during the last years for their unique physical and chemical characteristics that made these structures good candidate for their use for neurological and bone tissue engineering applications.[1] Although the current scientific data have shown conflicting results about potential nanotoxicity of CNSs, many studies are pointing out the biocompatibility of several forms of CNSs (especially in functionalized form) and their ability to support growth and proliferation of cells like neurons and osteoblasts.[2,3] Carbon Nanotubes (CNTs) possess exceptional mechanical, thermal, and electrical properties, facilitating their use as reinforcements or additives in various materials to improve the properties of the materials.[4] The use of CNTs in combination with bioactive biopolymers aims at the development of novel biocompatible nanocomposites in which biopolymers are implemented by physical and biological properties of CNTs.[5] The aim of this work is to develop new nanocomposites biomaterials based on natural polysaccharides such as alginate, chitosan and hyaluronic acid, and functionalized CNTs, with potential application in the fields of bone tissue regeneration and neuronal growth. Attention will be focused to the mechanical characterization of the nanocomposites and on the effect on the cellular viability and toxicity of CNTs and nanocomposites.

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# Functionalized bioactive glasses as stimuli-responsive biomaterials for treatment of bone diseases

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Controlled release systems have grown considerable success, due to their advantages compared to conventional dosage forms in terms of efficiency, toxicity and patient compliance. Significant challenges remain in the development of stimuli responsive materials, that change properties in response to local environmental stimuli, such as pH variation. These "intelligent" devices enable the delivery of drugs at the target site by exploiting physiologic conditions, and its release in situ when the therapeutic effects are needed.[1,2]

Osteosarcoma, the most common primary bone cancer and a frequent cause of morbidity and mortality in pediatric oncology, is a malignant tumor of connective tissue origin within which the tumor cells produce bone or osteoid, as well as cartilage matrix and fibrous tissue. Standard therapy for osteosarcoma consists of chemotherapy, with surgery as the preferred means of local control.[3a,b]

Bioactive glasses represent a vast class of inorganic biomaterials widely employed for the realisation of prostheses in orthopaedic and dentistry fields. Thanks to their ability to bond to bone tissue through the formation of an hydroxycarbonate apatite layer, this class of materials can be used as bone filler to replace damaged bone tissues.

Our purpose is the development of a bioactive glass to be used as prosthetic material, which is able to release an anti-cancer drug (ie doxorubicin, one of the most active agents against osteosarcoma) directly at the tumor site, thus obtaining a specific activity only on the malignant cells, and avoid both systemic and local toxicity of the drug. Here we report the covalent functionalization of the bioactive glass through a pH-sensitive bond, namely a maleyl-amide bond, and its conjugation with cysteamine (a simple and non-toxic model for doxorubicin). When exposed to the acidic environment typical of osteosarcoma cells, compared to the physiological pH characteristic of normal tissue, this biomaterial can favour the drug release at the target site. The bioactive glass synthesis (in terms of using also 3-(Aminopropyl)triethoxysilane as source of silicon atoms), its functionalization (with maleic and cis-aconitic anhydrides) and conjugation (with cysteamine), and physico-chemical characterization (by Powder X-ray diffraction, Raman and FTIR spectroscopies, $N_2$ adsorption and thermogravimetric analysis) at the various step of the preparation procedure are reported. Preliminary drug delivery tests, in simulated biological fluids at different pH, were performed. Raman spectroscopy demonstrated the disappearance of cysteamine after 3 days of soaking in acidic buffer (pH = 4.5), whereas in physiological conditions (pH = 7.4) the characteristic signals are still present.

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# Stealth silica nanoparticles for theranostic applications

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Silica nanoparticles (SNPs) of controllable size, shape and porosity had shown to be a useful platform for different uses. In recent years our group studied and developed SNP-based systems, functionalizing, loading and embedding SNPs for different purposes.[1]

As far as biomedical applications are concerned, the surface modification of SNPs with molecules for active targeting has turned out to be a desirable step. For this purpose we selected Boletus Edulis Lectin (BEL), since it shows T-antigen recognition capabilities together with antiproliferation activity.[2] Systems composed of silica functionalized with lectin molecules have been already described in literature, for chromatography applications or assay probes. Nonetheless, to the best of our knowledge, no one has ever performed the grafting of lectins onto SNPs obtaining a functional system to be used in nanomedicine.

The aim of our research is the creation of a theranostic system, composed of SNPs suitably tailored with BEL, and carrying contrast agents and/or therapeutic phases. The tethering of PEG molecules on the nanoparticles surface will be performed in order to impart stealth properties, steric stabilization to the system and reduce citotoxicity.

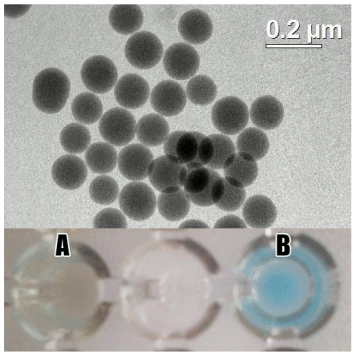


**Figure 1.** TEM micrograph of SNPs (upper part) and photograph of Coomassie colored wells of nanoparticle before (A) and after (B) lectins surface grafting.

Herein we present the first results of this research. The grafting of BEL has been attempted *via* two bioconjugation routes: the first relies on *in situ* reduction of the Schiff base generated from the reaction between protein's amine groups and aldehydic functionalities on previously modified silica surface. The second protocol is the well-established reaction involving EDC coupling, eventually in presence of NHS[3]. Coomassie Brilliant blue assay was performed to have a qualitative indication of the success of bioconjugation protocols. A surface coverage of 8,72 nmol of lectins per mg of SNPs was estimated from spectrofluorimetric assays recording the emission of protein's tryptophans on modified SNPs, having a diameter of around 150 nm. The permanence of the diameter of the particles after their superficial modification was confirmed by DLS measurements, while ζ-potential analyses showed a slightly negative potential in both values measured before and after modification.

At the same time, PEGylation was successfully performed on pristine SNPs with a co-condensation process, which allowed to obtain fairly monodisperse SNPs covered with a polymer layer, as confirmed by TEM observations, IR spectroscopy and cell viability tests.

Although some issues have been encountered, like the presence of some aggregates during the grafting of lectins and a partial loss of the activity of BEL, we foresee the possibility of combining the two procedures creating an efficient and multi-purpose theranostic nano-carrier.

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# Fluorescent hybrid polyoxometalates: sensing applications and cell penetration studies

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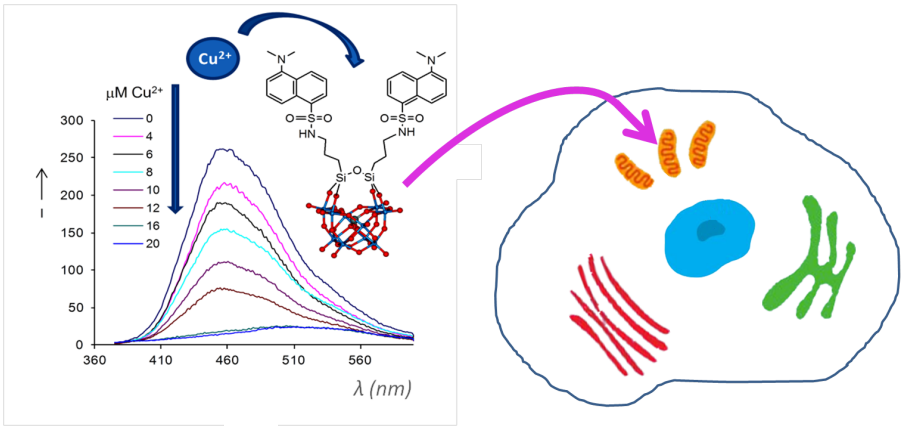
In this communication, we report the synthesis of hybrid polyoxotungstate derivatives containing luminescent chromophores (dansyl, fluorescein, pyrene), grafted as phosphonate or silane derivatives. Bis-decorated molecular hybrids have been isolated and characterized in solution and at the solid state by multinuclear NMR, ESI-MS, UV-Vis, fluorimetry and FT-IR. The sensing capabilities of the fluorophore-tagged polyoxometalates (POMs) towards metal ions and organic molecules have been demonstrated by fluorescence spectroscopy (Figure 1 A). Furthermore, the assembly behavior and the stability of the luminescent hybrid POMs in physiological conditions have been investigated by means of dynamic light scattering (DLS), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The analysis have shown the formation of spherical aggregates (100 - 200 nm).
Due to the potential applications of POMs in medicine (many POMs exhibit antiviral, antitumoral and antibiotic activity), their association to organic domains has been shown to be of interest to improve targeting and delivery strategies. Herein, the luminescent POMs have been exploited for *in vitro* fluorescence imaging. They have been tracked in the cells, showing their localization in different subcellular regions (including mitochondria, Figure 1B).




A B

**Figure 1. A** Interaction of a bis-dansylated POM with $Cu^{2+}$ (emission ($\lambda_{exc}$ = 324 nm) spectra); **B** Schematic representation of the interaction of a bis-dansylated POM with cellular portions.

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# Artificial Catalase strategies against oxidative stress

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Reactive oxygen species (ROS) are involved in oxidative stress within cellular environment and also play a crucial role in neurodegenerative (e.g. Alzheimer and Parkinson) diseases.
Sacrificial antioxidant molecules may be useful to control ROS, however, due their low selectivity, very high doses are required to reduce the oxidative damages.[1]
Herein, we present the use of robust coordination complexes or inorganic catalytic species with catalase activity, to contrast ROS injuries at catalytic doses. In the first case, we are studying a dinuclear manganese(II) complex,[2] conjugated with a rhodamine derivative, in which the cationic dye is expected to target mitochondria through electrostatic interaction, while maintaining catalase mimicking. In the second case, we are using a polyoxometalate with formula $Na_{10}[Ru_4O_4(OH)_2(H_2O)_4(\gamma\text{-}SiW_{10}O_{36})_2]$ (RuPOM). This POM has a tetranuclear core of ruthenium (IV) which is able to catalyze $H_2O_2$ dismutation (Figure 1a).[3] RuPOM is also useful to prevent the aggregation of Aβ amyloid, a peptide involved in Alzheimer disease. TEM and CD analysis have indeed confirmed the ability of RuPOM to block amyloid fibrillation. RuPOM could thus be useful to contrast the formation of such toxic fibrils (Figure 1b) as well as of ROS, whose production is catalyzed by the transition metals (e.g $Cu^{2+}$, $Fe^{3+}$), localized within the amyloid plaques.[4]

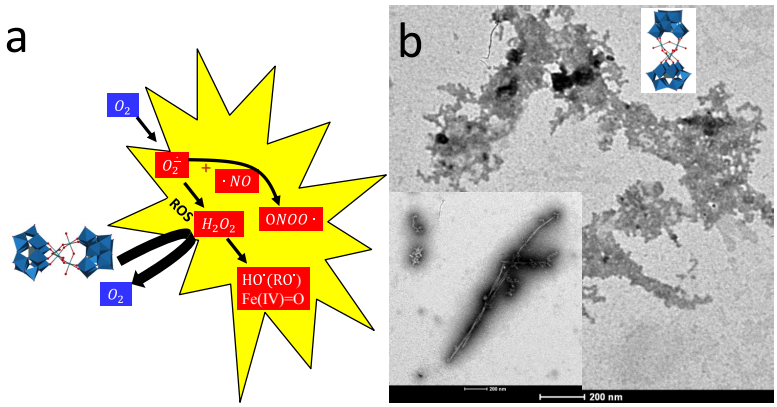


**Figure 1.** a) Action of the catalytic systems (RuPOM) against ROS;
b) TEM images of Aβ amyloid, with and without (inset) RuPOM.

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# Selective recognition of biological targets by biotinylated polyoxometalates

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The development of nanomedicinal agents, able to selectively target tumor cells is a challenging task which has raised great interest among the scientific community in the last decade. To this aim, the active drug and the targeting component should be combined into a single nanodimensional system to be delivered into the cells.

Polyoxometalates (POMs) are nano-sized polyanionic metal oxides, which have shown interesting antiviral and anticancer properties.[1] The possibility to covalently functionalize POMs with organic pendants and to tune their solubility by suitable counteraction, allows their conjugation with small biomolecules,[2] which may be useful to address the inorganic cluster inside the cells, while increasing their stability in physiological conditions. Hybrid POMs may thus represent ideal candidates for the design of smart cancer-targeting devices.

To this aim, in this communication we present the synthesis of a hybrid derivative, consisting in a vacant polyoxotungstate functionalized with biotin, a water-soluble vitamin, whose receptors are over expressed on the membrane of tumor cells (Figure 1). The interaction of such POM-conjugate with avidin, the native binding protein of biotin, was investigated via UV-spectrometry titrations, while the affinity between the two components was evaluated through surface plasmon resonance (SPR) analysis. Both experiments have confirmed the stability of the derivative in physiological conditions and the capability of the targeting molecule to interact with its receptor.

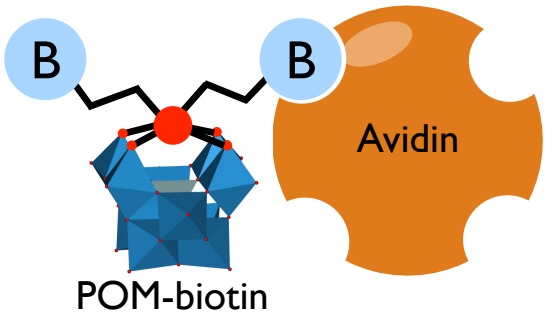


**Figure 1.** Scheme showing targeting of avidin by the biotinylated-POM

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# New frontiers in burn healing: investigation of the chemistry and distribution of silver nanoparticles

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Considering the enormous interest in the application of silver nanoparticles (Ag NPs) in wound therapy and the increasing widespread use/ misuse in cosmetics and sanitary products, clarification about their safety and biocompatibility *in vivo* are urgently required.

Acticoat™ Flex 3 and Flex 7 (Smith & Nephew, Milan, Italy) are wound dressings containing metallic silver in nanoparticulate form, that are commonly used in burn centers worldwide due to their well demonstrated broad spectrum antimicrobial properties and their contribution to wound healing.

In this research work four skin biopsies in duplicate were taken from a patient treated with a Ag NPs dressing at different times during the healing process. The samples were subjected to histological analysis to evaluate the tissue structure during the course of the healing process whilst transmission electron microscopy (TEM) analyses were carried out to determine the Ag NPs subcellular localization. The depth profiles of the Ag concentrations were determined along the duplicates of the skin biopsies by inductively coupled plasma mass spectrometry (ICP-MS) analysis with a spatial resolution of 1 mm.

ICP-MS results showed that in the healed sample most of the silver remained in the surface layers, whereas in the unhealed sample, the silver penetrated more deeply. The cumulative Ag concentration in skin samples seems to correlate with the severity of the wound as well as with the number of dressings applied.

TEM analysis showed nanoparticles present in the healed sample whilst their presence was less obvious in unhealed samples. In the healed skin sample, Ag NPs are released in the dermis as aggregates, enter into fibroblasts using endocytic vesicles and are released in the cytoplasm with no signs of cell death.

For the first time, the chemistry and the distribution of Ag NPs were investigated in real samples taken from burn patients and in not skin models.
Thanks to these *in vivo* studies we are able to conclude that a Ag NPs -based dressing does not create an obstacle to the recovery of severe partial thickness burns, allowing the reorganization of a normal skin structure.

## Gold-Iron Nanoparticles: Evaluation of a New Multimodal Imaging Tool for Future Theranostic Application in Cancer Management

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Cancer is the second cause of death in the modern society and early diagnosis is crucial to increase survival and a better management of the disease. In recent years, nanoparticles and other nanosystems have been extensively investigated as delivery tools for imaging and therapeutic applications, since nanosystems could be used for tumor treatments when loaded with cytotoxic molecules and as diagnostic tools when loaded with tracers for fluorescence, radioactive, MRI or Raman spectroscopy imaging.
We have evaluated a composite metal nanosystem, i.e. nanoparticles made with an alloy of gold and iron (Au-Fe NPs), which offers a double imaging modality for cancer management: they enable imaging approaches based on both magnetic resonance imaging (MRI, thanks to iron) and Raman spectroscopy (SERS, thanks to gold) techniques. Our Au-Fe NPs showed a good activity in both SERS and MRI analysis and a good biocompatibility on “in vitro” assays: no signs of toxicity or apoptosis were detected on human and mouse tumor cells, and also no effects on clonogenicity were observed. Their double imaging features were investigated in an “in vivo” experiment, with promising results. Finally, thermal ablation therapy experiments after laser excitation are underway.

# Zwitterion-Coated Iron Oxide Nanoparticles as contrast agents for MRI applications

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Iron Oxide Nanoparticles (IONPs) have received enormous attention in various research areas because of their unique magnetic properties, facile surface modification and biocompatibility.[1] Especially for the field of nanobiotechnology, IONPs have emerged as promising tools with increasing applications in magnetic resonance imaging (MRI),[2] drug delivery[3] and hyperthermia therapy.[4] Among them MRI is one of the key application because functionalized IONPs have widely served as $T_2$ contrast agents that show high efficiency in enhancing soft tissue images or in cells labeling. In this regard tremendous efforts have been made in fabricating stable colloidal IONPs solutions with superior magnetic properties, good dispersibility, and biocompatibility.

We prepared monodisperse IONPs *via* a solution-phase method based on the thermal decomposition of $Fe(CO)_5$ in the presence of oleic acid (OA) Fig. 1a.[5] Subsequently the native OA ligands were replaced by zwitterionic dopamine sulfonate (ZDS) following a procedure reported by Wei H *et al.*, that gives stable colloidal solution of IONPs (Fig. 1b) highly dispersible in water.[6] To test the MRI contrast enhancing capability *in vivo*, IONPs were intravenously administered into CD1 male mice weighing 30 g at the dosage of 1mg Fe/kg. *T*2*-weighted MRI was performed before and after injection. As shown in Fig. 1d, hypointensities induced by IONPs can be readily observed in liver at 15 min after injection. The contrast in liver began to decrease after 1d of injection because of the liver clearance. The contrast enhancement indicated the accumulation of IONPs in this organ, demonstrating that IONPs can be potentially used as effective *T*2*-weighted MRI contrast agents.

In addition, we performed preliminary investigation of IONP uptake *in vitro*, that are particularly relevant for future cell tracking studies. Different doses of IONPs were incubated with BV-2 microglial cells for 6h. We found that the amount of iron uptake is dose dependent (Fig. 1c) and does not affect cell viability, even at higher doses, likely needed for efficient *in vivo* tracking. Further studies are in progress to optimize MRI detection of myeloid cells.

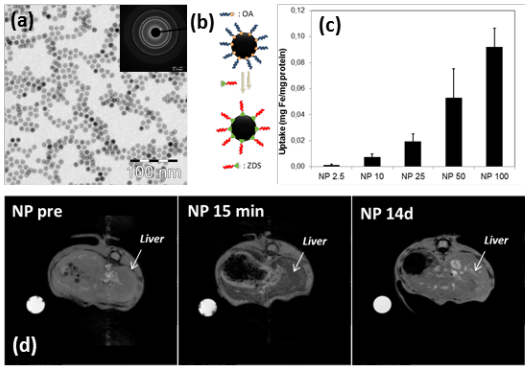


**Figure 1.** a) TEM image of IONPs b) ligand exchange scheme (c) Dose-dependent iron uptake of BV-2 microglial cells after 6 h exposure d) *In vivo* mouse T2*-weighted MR images at different time point after IONPs administration.

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# Labeling of human neural stem cells derived from foetal spinal cord (CB660SP) with carboxymethyl-dextran iron oxide nanoparticles

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A protocol to label *in vitro* human neural precursor cells with commercial dextran-coated superparamagnetic iron oxide nanoparticles (Sinerem and Endorem) has been described[1] and it was demonstrated that labeled cells can be effectively tracked *in vivo* by magnetic resonance imaging (MRI). In order to obtain a large number of labeled cells and to achieve an high nanoparticle (NP) loading needed for efficient tracking, cells are treated with iron oxide NPs at high concentration. However, this high NP loading in cells must not adversely affect cell survival, self-renewal and proliferation capacity.

We investigated the uptake of iron NPs coated with carboxymethyl-dextran by human foetal spinal cord neural stem cell line (CB660SP) in monolayers culture[2]. We prepared monodisperse iron oxide NPs coated with oleic acid by the solvo-thermal decomposition of $Fe(CO)_5$ in the presence of oleic acid[3]. Then, oleic acid was first exchanged with oleylamine in toluene and then oleylamine was replaced by carboxymethyl-dextran. This procedure gave iron oxide NPs coated with carboxymethyl-dextran (CMD-NPs) that are dispersible in water and retain the narrow size distribution of as-synthesized NPs.

CB660SP cells were treated with CMD-NPs for 30 minutes at different NP doses (25, 50 e 100 $\mu$g Fe/ml). NP uptake, as visualized by Prussian blue (PB) staining, resulted to be dose dependent.

The cytotoxic effect was assessed using the MTT assay: the cell viability after incubation with NPs was 70%. The oxidative stress, possible consequence of exposure of cells to NPs[4], was evaluated by staining with MitoSoxRed, indicator for specific visualization of mitochondrial superoxide in live cells. We have observed an increased mitochondrial production of reactive oxygen species (ROS) induced by NPs exposure.

We have thus shown that CB660SP cells can be efficiently labeled by CMD-NPs.

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CB660SP cell line is kindly provided by Prof. Austin Smith, University of Cambridge, UK to Dr. Ida Biunno. She allowed us to use it, so we gratefully acknowledge both.

# Manganese oxide nanoparticles with anisotropic shapes

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Manganese(II) oxide (MnO) nanoparticles (NPs) with anisotropic shape can represent a useful building block on the way to the fabrication of NPs for *in vivo* diagnostic application, e.g., $T_1$ agents for MRI. In this work, MnO NPs have been synthesized by the high-temperature decomposition of a manganese(II) carboxylate precursor $(R_1COO)_2Mn$ in octadecene, in the presence of sulphur and (possibly) free fatty acid $R_2COOH$ as a surfactant. $R_1$ and $R_2$ independently are oleyl (Ol) or stearyl (St) residues.

MnO NPs are obtained even in the presence of sulphur provided that the S:Mn ratio is lower than 0.5.[1] The keypoint is that the MnO NPs prepared with S:Mn = 0.5 have peculiar anisotropic concave shapes which have lower symmetry than the cubic lattice of MnO (rocksalt structure). These shapes can be controlled by changing the type of $R_1$ and $R_2$ fatty acid residue and by varying the surfactant:precursor molar ratio. When $R_1 = R_2 =$ Ol, we obtained multipode NPs comprising $n = 1$ to 6 oval lobes, which can make up concave shapes (Fig. 1 left). When $R_1 = R_2 =$ St, we obtained different multipode NPs comprising $n = 1$ to 4 jagged-edged linear branches, namely, rods, T's and crosses (Fig. 1 right). We also explored cases where $R_1 \neq R_2$. The overall NP shape (lobed *vs.* branched) is governed by $R_1$, the type of fatty acid in the precursor. This can be explained by assuming that the seed formation and the initial growth mainly involve $[R_1COOMn]^+$ and $(R_1COO)_2Mn$ species, without significant involvement of free $R_2COOH$. The influence of the surfactant/precursor ratio on the NP shape has been studied in the $R_1 = R_2$ case with molar ratio ranging from 0:1 to 4:1. The lowest molar ratios produce NPs with convex shape (spherical, octahedral, irregular), conversely higher molar ratios give NPs with well-defined and anisotropic shape. On increasing the surfactant concentration, we observed a progressive development of the concave shapes, as judged by the outgrowth and elongation of the lobes/branches, which are well developed at a 1:4 ratio. HRTEM showed that both $R_1 = R_2 =$ Ol and $R_1 = R_2 =$ St multipodes are single crystals (Fig. 2), grown as such from a single seed or developed by oriented attachment[2] of smaller crystals. However, the progressive development of the concave shapes with increasing surfactant/precursor ratio strongly suggest that such shapes are the outcome of a broken-symmetry growth from a single seed.

The magnetic behaviour of $R_1 = R_2 =$ St ($R_1$:$R_2$ = 2:1) multipodes was investigated by measuring the hysteresis loop and the ZFC/FC magnetization. At 5 K the *M-H* curve opens up showing that a ferro(i)-magnetic phase different from MnO is present. The (Z)FC curves show the transition of such phase from the paramagnetic to the ferro(i)magnetic regime at ~40 K, suggesting the presence of $Mn_3O_4$ formed by oxidation by air. No $Mn_3O_4$ has been detected in NPs kept under argon for several months. Magnetic properties of such NPs are currently under study. This is an important issue because the presence of $Mn_3O_4$ decreases the specific relaxivity ($r_1$) of MnO NPs.[3]

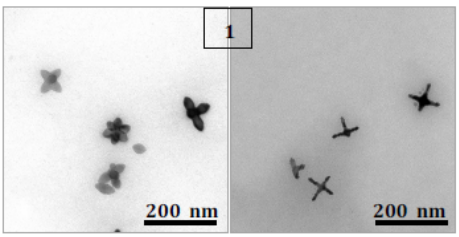


**Figure 1.** *TEM images Left) NPs with Oleate surf/prec 4:1; right) NPs with Stearate 1:1.*

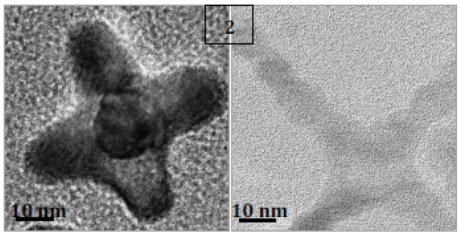


**Figure 2.** *HRTEM images. Left) NPs with Oleate surf:prec 4:1; right) NPs with Stearate 1:1.*

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# Decoration of iron oxide magnetic nanoparticles (IOMNPs) with a mimetic of α-Tn antigen for cancer immunotherapy and general strategies for MNPs functionalization

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Iron oxide magnetic nanoparticles (IOMNPs) represent a useful platform for many applications in biomedicine. The main uses are as contrast agent in magnetic resonance imaging, as heat mediators for magnetic fluid hyperthermia, exploiting the capability of dissipating heat when an alternating magnetic field is applied, and as carrier for drug delivery.[1] However, the most attractive property in the field of biomedicine is the possibility of gather all of these functionalities in a single nanosystem, so as to realize the so-called theranostic approach.
Within this context we describe here the functionalization of ferrimagnetic biocompatible IOMNPs with a rigid mimetic of the α-Tn antigen[2] for potential immunotherapy application (Fig.1). α-Tn antigen is a tumour associated antigen and represents one of the saccharidic moieties of mucin-like proteins. Alterations in these kind of proteins lead to the development of cancer and influence cellular growth, differentiation, transformation, adhesion and immune surveillance.[3]
Binding biologically active molecules in a multivalent manner onto the IOMNPs is important to amplify the immune response. For this purpose is fundamental the development of "functionalization protocols" in order to obtain water soluble nanosystems stable in physiological conditions capable to correctly expose the active molecules towards the external environment. We give here a brief overview of the methodology developed to prepare these water soluble, stable and biocompatible nanosystems.

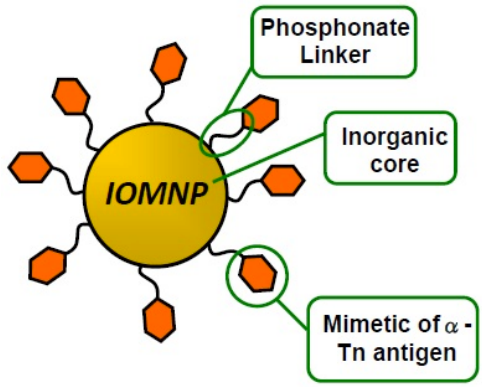


**Figure 1**. Decoration of IOMNPs.

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# Nanocarrier for Rifampicin

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Necessity to increase the therapeutic potential of molecules of known drugs, has stimulated numerous studies to get around the factors that limit this potential. Limitations like solubility and stability in physiological environment may decrease considerably adsorption and biodistribution of the drug. In fact, this can induce the use of higher doses to achieve the desired therapeutic effect. Delivery to target specific and controlled release of the drug may be an option to circumvent these limitations, which are even more evident in the prolonged use of chemotherapy like for treatment of tuberculosis.[1,2]

In the 70s rifampin (RIF) was introduced to combat Mycobacterium tuberculosis as an antibiotic of broad-spectrum action. However, when RIF is administered orally can undergo hydrolysis in stomach pH, so be poorly adsorbed from the intestinal tract. A strategy to expand the therapeutic potential of RIF is a vectorization to target organ or protection of this molecule via incorporation a system loader.[1-4]

In this context, this work presents a magnetic nanoparticle system (MNPS) for transport of RIF. For this incorporation, a fixed volume of MNPS previously synthesized was added to different amounts of RIF and then separated for quantification of the drug not incorporated. Through analyses of infrared vibrational spectroscopy (IR), zeta potential, hydrodynamic diameter and X-ray diffraction were performed for characterization of MNPS and RIF incorporated to MNPS (RIF-MNPS). The results suggest that the RIF can be adsorbed to the nanoparticles.

The incorporation of RIF in the MNPS, suggest another perspective pharmaceutical formulations, aqueous colloidal dispersion, for this drug, where around eighty percent of drug was incorporated in the nanoparticulate system.

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## Ferritin based multifunctional nanoparticles for Magnetic Fluid Hyperthermia

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Magnetic nanoparticles (MNPs) are the building-blocks for developing innovative nanodevices with multi-fold therapeutic and diagnostic activities, including magnetic fluid hyperthermia (MFH), contrast agents for Magnetic Resonance Imaging (CA-MRI) and targeting of tumor cells. Such innovative anticancer materials can be realized through the proper functionalization of the magnetic core with a biocompatible shell, which can include one or more biologically active molecules, like drugs, antibodies or small peptides. In this framework, iron oxide MNPs mineralized within the internal cavity of the human variant of ferritin (HFt), can represent a viable platform to achieve this goal as they offer multiple advantages: HFt protein has the appropriate size to freely circulate in the body and is naturally tailored for iron sequestration and NPs incorporation. Moreover, the biocompatibility of iron oxide HFt-NPs has already been demonstrated. However, the main constraint of HFt-based MNPs is that their size cannot exceed the protein shell inner diameter (ca. 8 nm). This size is large enough for MRI application, but it is too small for MFH, as theoretical and experimental studies demonstrated that the maximum MFH efficiency is reached for magnetite NPs of $d$=16-18 nm, while very poor effects are expected for $d < 10$ nm.[1] Such limitation for the use of HFt-NPs in MFH can be overcome through the controlled doping of the core with small amount of Co(II). In fact, the presence of Co(II) inside the spinel lattice significantly enhance the magnetic anisotropy constant of the material,[2] a parameter which has a great influence on hyperthermic efficiency.

To this aim, highly monodisperse Co doped iron oxides NPs with average size of 7 nm are mineralized inside a genetically modified variant of HFt, carrying several copies of $\alpha$-melanocyte-stimulating hormone peptide, which has already been demonstrated to have excellent targeting properties towards melanoma cells whit high selectivity.[3] HFt are also conjugated to polyethylene glycol molecules to increase their *in vivo* stability. The investigation of hyperthermic properties of HFt-NPs shows that a Co doping of 5% is enough to strongly enhance the magnetic anisotropy and thus the hyperthermic efficiency with respect to the undoped sample. *In vitro* tests performed on B16 melanoma cell lines demonstrate a strong reduction of the cell viability after the treatment with Co doped HFt-NPs and the exposition to the alternate magnetic field. Clear indications of an advanced stage of apoptotic process are also observed from immunocytochemistry analysis. The obtained data suggest this system represents a promising candidate for the development of a novel protein based-theranostic nanoplatform.

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# Polyhedral iron oxide core-shell nanoparticles in a biodegradable polymeric matrix: Preparation, characterization and application in magnetic particle hyperthermia and drug delivery

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Nanotechnology is at the leading edge of rapidly developing new therapeutic and diagnostic schemes in diverse areas of biomedicine. Different materials from natural to synthetic polymers as well as inorganic materials with variable structural and physical properties are used as building blocks of biomaterials. Recently, a new term 'theranostics' is used in order to encompass two distinct definitions which is the combination of therapeutic and diagnostic agents on a single platform. The development of theranostic nanoparticles is emerging as a new form of "smart" nano-materials that may simultaneously monitor and treat diseases.[1]

The aim of the present study is to characterize the polyhedral iron oxide nanoparticles (IOs) and their magnetic properties that can then be used for the encapsulation of the Paclitaxel drug using two different polymer matrices such as PPSu and its block copolymer mPEG-PPSu-mPEG. Both have been chosen because of their excellent biocompatibility and biodegradability and also because they have melting point temperatures close to the body temperature (Tm=42°C and Tm=44°C). This is very essential in case these IOs will be used for combinatory cancer treatment with hyperthermia and drug release and therefore the drug release was studied at 37°C and at 42°C. The encapsulation of iron oxide nanoparticles into a polymer matrix is confirmed by transmission electron microscopy and further corroborated by high angle annular dark field scanning transmission electron microscopy (HAADF-STEM). Energy dispersive X-ray spectroscopy mapping allowed us to determine the presence of the different material ingredients in a quantitative way (Figure 1). The high heat capacity, which can be maintained in the nanovehicles of IOs encapsulated in the polymeric matrix, is sufficient to provoke damage of the cancer cells. Therefore, this nanosystem, in which polyhedral magnetic nanoparticles are incorporated in a biocompatible and biodegradable polymeric matrix, can be used as a multifunctional magnetic particle hyperthermia agent together with heat-assisted drug-delivery addressing directly the current theranostic trends.

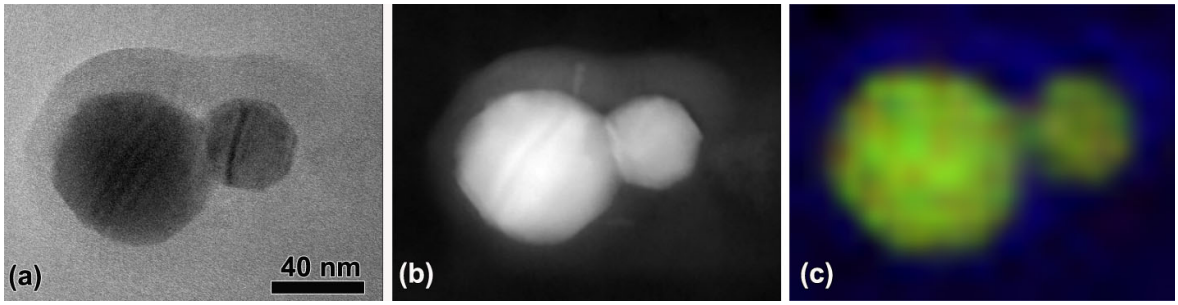


**Figure 1.** (a) Bright field TEM image of mPEG PPSu-mPEG -IOs (b) HAADF- STEM image of the particles of Figure (a) and (c)HAADF-STEM EDX mapping (C- blue, Fe - green, O-red) of mPEG- PPSu- mPEG –IOs. The scale bar stands for all images.

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# Polyethylene glycol: conjugation with eugenol and ibuprofen

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Polyethylene glycol (PEG) is a synthetic polyether polymer (HO-$(CH_2CH_2O)_n$-$CH_2CH_2OH$) easily available, soluble in both water and organic solvents and biocompatible. PEG is used as inert polymeric support in organic synthesis and as conjugating agent of bioactive molecules of pharmacological interest.[1] A new bi-functional derivative of PEG with linkers bearing different reactive terminal amino groups has been prepared.[2] Commercial PEG has been orthogonally protected to allow the synthesis of a mixed conjugate, to link different bioactive molecules on the same polymeric support. Ibuprofen has been widely used to treat inflammatory disease. However, side effects, such as ulcerogenic action have been reported both in experimental animals and in clinical use. Eugenol, a volatile compound extracted from *Eugenia cariophillata*, has good anti-oxidative, analgesic, antipyretic and anti-inflammatory activity, was chosen on the basis of conjugating two drugs having different pharmacological activities to make a mutual prodrug with synergistic and anti-inflammatory effects and reduced GI irritation.[3,4] Eugenol (EU) and ibuprofen (IBU) has been covalently bound to bi-functionalized PEG, used as molecular carrier of drugs, and then the release kinetics of the two bioactive molecules were studied *in vitro* at physiological pH, in an artificial gastric juice at pH 1.2 and simulated extra-cellular fluid at pH 7.4, and in plasma (figure 1). The aim of the present conjugation is to obtain get an amphiphilic compound; the study *in vitro* showed that the conjugate is susceptible to hydrolysis by plasmatic enzymes.

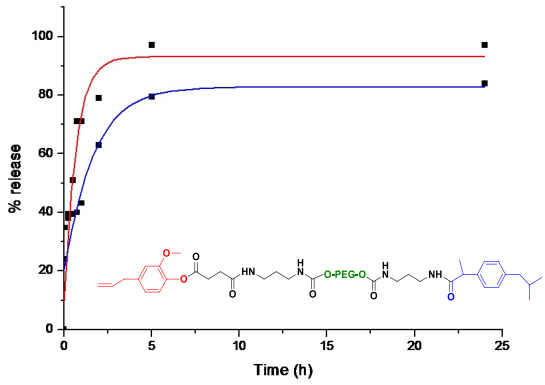


**Figure 1.** Release of EU (red) and IBU (blue) from the mixed conjugate in mouse plasma at 37°C.

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# α-ELASTIN-g-PLGA nanocarriers as innovative devices for restenosis treatment

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Dysfunctions of vascular healing process can produce a phenomenon known as restenosis, due to an intimal hyperplasia because of an abnormal vascular smooth muscle cells (VSMCs) migration and proliferation, leading to the reobstruction of vessel where a tissue engineered vascular graft (TEVG) or a stent has been implanted. With the aim to prevent this phenomenon, several antiproliferative and antiinflammatory drugs are currently in therapeutic use to reduce proliferation of VSMCs after a reconstructive surgery or they are loaded into stents to prevent restenosis. Moreover, elastin is known to maintain contractile phenotype of VSMCs inhibiting their migration and proliferation[1] and it reduces restenosis after a parenteral administration in a pig model. Therefore, exploiting this biological role of α-elastin together with the activity of antiinflammatory drugs, aim of this research was to produce appropriate nanoparticles for potential treatment of restenosis, based on a graft copolymer of α-elastin with poly(lactic-co-glycolic) acid (PLGA), containing dexamethasone dipropionate. In particular, nanoparticles of α-elastin-g-PLGA with a mean size of 200 nm (Figure 1a) were produced and loaded with dexamethasone dipropionate (10 % w/w), chosen as a model drug that inhibits proliferation of VSMCs. These nanoparticles were able to sustain the drug release for more than 24 hours and showed a pronounced sensibility to elastase. Drug unloaded nanoparticles stimulated the differentiation of human umbilical artery smooth muscle cells (HUASMCs) to a contractile phenotype as demonstrated by immunofluorescence, flow cytofluorimetric and western-blotting analyses. Finally, drug loaded nanoparticles efficiently reduced viability of HUASMCs as evidenced by cell viability assay. Therefore, α-elastin-g-PLGA nanoparticles show an intrinsic ability to promote the differentiation to the contractile phenotype (Figure 1b) associated with the anti-proliferative effect of released drug that, thanks to the entrapment into the nanoparticles can be administered in aqueous medium without the use of organic solvent. The obtained results can be considered as positive preliminary data for a further design of innovative devices for restenosis treatment.

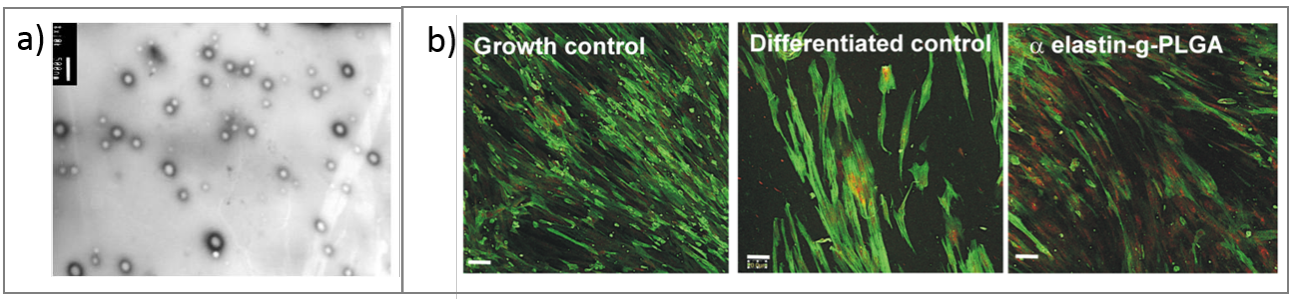


**Figure 1.** a) TEM picture of drug unloaded α-elastin-g-PLGA nanoparticles. b) Morphological analysis by LSCM: HUASMC non-treated (growth control) and treated with differentiation medium (differentiated control) or α-elastin-g-PLGA nanoparticles (α-elastin-g-PLGA)

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# A novel approach to monitor intracellular degradation kinetics of poly(lactide-co-glycolide) nanoparticles via flow cytometry

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The intracellular degradation of poly(lactide-co-glycolide) (PLGA) nanoparticle (NPs) is studied by means of flow cytometry (FACS). NPs are prepared with PLGA of two different ratios of the D,L-lactide and glycolide blocks: 85:15 and 65:35. PLGA molecules are labelled with rhodamine B. Flow cytometry is used first to follow the degradation of PLGA NPs in PBS over time by measuring the decrease in fluorescence per particle. PLGA NPs 85:15 progressively degrade during the first 10 days and remain constant afterwards. The PLGA NPs 65:35 remain unaltered, showing no changes in fluorescence intensity. FACS data are confirmed by Transmission Electron Microscopy and Dynamic Light Scattering measurements. Intracellular degradation of PLGA 85:15 is measured by the increase in the fluorescence intensity in the cell population with time due to the liberation of rhodamine B labelled PLGA molecules from the NPs in the cell interior where the rhodamine display an increased quantum yield. The fluorescence intensity from PLGA 85:15 NPs increases up to 24 hours, remaining constant thereafter. No change in the fluorescence of PLGA 65:35 NPs is observed over 4 days. The intracellular behaviour of the PLGA NPs is confirmed by Confocal Raman Microscopy.

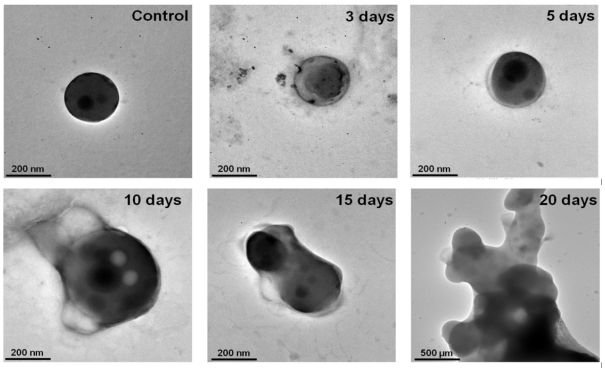


**Fig. 1.** Morphology and size change of PLGA15 NPs in PBS at 37 ºC monitored over time by TEM.

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# Design of peptide-functionalised nanoparticles for selective drug delivery

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## Hypotesis and Purpose

To exploit the agent YHWYGYTPQNVI (GE11),[1] a dodecapeptide that has been demonstrated to selectively recognise the epidermal growth factor receptor (EGFR), in order to realize selective nanosized drug delivery systems by combining passive and active cell targeting.
To set-up chemical and technological protocols to obtain a GE11-poly(lactide-co-glycolide) conjugate (GE11-PLGA) as “smart” nanoparticulate platform for drug delivery.

## Methods

A model tetrapeptide (FQPV, Mw 489.57 g/mol) and GE11 were synthesized by standard fluorenyl-9-methoxycarbonyl (Fmoc) protocol using a Biotage Initiator SP Wave synthesizer. They were purified by semi-preparative HPLC using an AKTA Basic100 instrument and their purity determined by analytical RP-HPLC. The peptides identity and molecular weight were confirmed by MALDI TOF mass spectrometry (Bruker Microflex LT Spectrometer). Both GE11 and FQPV peptides were obtained in good yield (35% and 57%, respectively) and their purity was shown to be > 95%.
FQPV was used to set up the best protocol for chemical conjugation of peptides to PLGA (7525 DLG 3A, Mw 35,000 Da, Lakeshore Biomaterials, USA) (FQPV-PLGA), using carbodiimmide chemistry. The coupling between the two peptides and PLGA was proved by $^{1}$H NMR spectra.
FQPV-PLGA nanoparticles were prepared by suitably set up nanoprecipitation technique.[2] The nanoparticles were recovered by high speed ultracentrifugation and were characterized for their morphology (TEM), particle size and Z-potential (NICOMP 380 ZLS apparatus).

## Results and future perspectives

Placebo PLGA nanoparticles showed homogeneous size distribution and suitable dimensions. Zeta potential was -4.27 mV. FQPV-PLGA nanoparticles didn’t show significant differences in terms of dimensions and surface charge. TEM images confirmed nanoparticle size distribution and revealed regular spherical shape.
GE11-PLGA nanoparticles are currently in course of preparation. We are also labeling a GE11 peptide sample with carboxyfluorescein in order to quantify, by fluorescence spectroscopy, the amount of peptide covalently bound to the polymer.
Finally we are evaluating alternative polymeric scaffolds for nanoparticle preparation, in particular poly-γ-glutamic acid, a water soluble and atoxic bacterial polymer.

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## Pharmaceutical Nanotechnology: Targeting the Central Nervous System

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In the last years, the application of "nanotechnology" to the field of "medicine" surely represented the most innovative strategy to cope with diseases and it could be named as nanomedicine applied to difficult-to-treat diseases. In particular, delivering agents to neurological and neurodegenerative diseases, an represent a stimulating issue, as the pharmaceutical treatment of Central Nervous System (CNS) disorders is the second largest area of therapy, following cardiovascular diseases.

Nowadays, non-invasive drug delivery systems for CNS are actively studied. In fact, the development of new delivery systems (nanoparticles and liposomes) started with the discovery that properly surface-engineered colloidal vectors, with a diameter around 200 nm, were shown to be able to cross the Blood-Brain Barrier without apparent damage, and to deliver drugs or genetic materials into the brain.

A particular focus has been devoted by our research group to peptide-modified NPs (g7-NPs) able to target the CNS. In vivo evidences showed the efficacy of g7-NPs in CNS targeting after different routes of administration (i.v./i.p./oral/nasal), their pathways and mechanisms for BBB crossing and their CNS localization. In particular, the brain localization and the multi-modal pathways for BBB crossing highlighted the endocytosis as preferential pathway which was studied in depth in vitro and in vivo. Moreover, the fate, the dynamics and trafficking of modified NPs were assessed by in vivo and in vitro experiments, together with highlights on cell/area tropisms and distribution in dose- and time- response.

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## Lignin stabilised Pd and Pt nanoparticles in catalytic reduction reactions

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Chemo-selective catalytic hydrogenation of multi-unsaturated molecules is a demanding task for the flavour, fragrance and, above all, pharmaceutical industries. Metal nanoparticles (NPs) are good candidates for this purpose.[1] From the synthetic point of view, the selectivity to the C=C vs. C=O reduction group can often be controlled by several parameters: among them the nature of the metal and particle shape and size represent the most important tool to keep into consideration.
Pd an Pt NPs, synthesised by a simple and green way like low T and P, water solution and employing as stabilizing/reducing agent lignin, a widely available by-product of wood and paper industry, showed interesting catalytic properties since they can act as both oxidant and reductant.[2]
Starting from this idea, we planned to investigate other reduction reactions of mono and di-functionalized olefin and/or carbonyl derivatives employing the above-mentioned Pd and Pt NPs of different lignins.
In the present investigation, Pt and Pd lignin nanoparticles showed good catalytic activity towards double bond hydrogenations, always in mild conditions. When other reducible functions (C=O) are present, Pd nanoparticles showed a good chemoselectivity allowing the reduction of double bond and leaving unchanged the carbonyl moiety.
An useful application of our catalytic system is the reduction of 2,2,6-trimethyl-1,4-cyclohexenedione to get selectively 2,2,6-trimethyl-1,4-cyclohexanedione, a key intermediate in the synthesis of optically active xanthophylls, xanthoxin and zeaxanthins[3,4] as well as aroma constituent of tobacco and saffron.[5]
2,2,6-Trimethyl-1,4-cyclohexenedione was tested in the presence of Pt NPs and Pd NPs, the conversion, in both cases, was quantitative but the selectivity in the Pt catalyzed reduction was 50% (we obtained 50% of 2,2,6-trimethyl-1,4-cyclohexanedione and 50% of 2,2,6-trimethyl-1-hydroxycyclohexenone) while the selectivity in the reduction Pd catalyzed was 100% (we obtained quantitative reduction and selectivity of the C=C double bond).
An increase of reaction time (12 h), or Pd NPs amount, or $H_2$ pressure led to a decrease of selectivity.

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# $TiO_2$-Lignin Nanoparticles for potential healthcare applications

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It is well recognized that UV light in the range 280–400 nm is responsible for the majority of photodamage to the skin such as sunburns (UVB) and melanoma (UVA). Inorganic particles, such as titanium dioxide, have been introduced in sunscreen formulations in order to reflect and scatter UV radiation. However, when $TiO_2$ is photoactivated by UV light, it promotes electrons which react with oxygen to form superoxide and hydroxyl radicals.[1] Lignin is the major constituent of wood; it is a bio-mass renewable by-product and it is biodegradable and environmentally compatible.[2] Recent studies have revealed the efficacy of lignin as reducing and stabilizing polymer,[3] natural antioxidant and solar protectant[4]. Since the photocatalytic activity is the culprit in all the damage scenarios, the aim of the work is to reduce the photocatalytic activity of $TiO_2$, blocking the emission of the surface electrons and preserving its UV filter properties.

We synthesized new $TiO_2$ nanoparticles (NPs) coated with lignin because could be potentially harmless for humans, as well as benign for the environment. The NPs have been characterized by TEM (Fig.1); the sizes of clusters are about 90-100 nm. The synthesis strategies were performed with *Frangville et al.* methods, opportunely modified. In brief, $TiO_2$-Lignin NPs have been achieved by co-precipitation of $TiO_2$-Lignin from an ethylene glycol solution (method a) and from a high-pH aqueous solution (method b), using ultrasonic probe and vortex homogenizer. We tested the decrease of NPs photoxidative power, using the photoxidation of isopropanol to acetone. These new NPs could be a potential good solution to prevent $TiO_2$ cytotoxicity in sunscreen formulations to protect human health and could be applied not only for inorganic filters but also to increase the photostability of organic sunscreens.

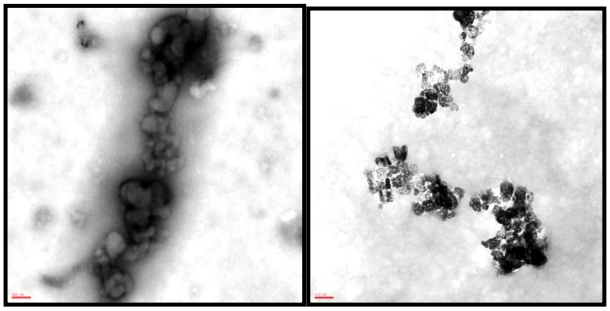

**Figure 1.** TEM images of $TiO_2$-Lignin NPs (a-b)

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# Chemotherapeutic-loaded anti-CD20 biodegradable nanoparticles application in the treatment of B-cell disorders

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B-cell malignancies are a heterogeneous group of clinical conditions including highly variable clinical courses which span between indolent diseases, such as Chronic Lymphocytic Leukemia (CLL), and highly aggressive lymphoproliferative disorders such as Burkitt Lymphoma[1]. B-cell disorder treatments take advantage of both dose-intensive chemotherapeutic regimens and immunotherapy[2]. Unfortunately, they may lead to insufficient tumor distribution of therapeutic agents and cause several adverse effects[3]. We propose a novel therapeutic approach for the treatment of Chronic Lymphocytic Leukemia (CLL) and Non-Hodgkin lymphoma in which high doses of chemotherapeutic drugs (Fludarabine, Hydroxychloroquine and Chlorambucil) were loaded in biodegradable nanoparticles (BNPs) coated with an anti-CD20 antibody. BNPs' binding on the CLL cell line MEC-1 and Burkitt lymphoma cell line BJAB was demonstrated using cytometric analysis, confocal microscopy and transmission electron microscopy. This last analysis also suggested BNPs' internalization in tumor B-cell through a process different from endocytosis.
The importance of anti-CD20 antibody in inducing BNPs' homing was demonstrated through in vivo biodistribution's studies using a localized model of B-cell disorder. This model was created injecting subcutaneously MEC-1 cells into the flank of SCID mice and biodistribution analysis was performed using targeted and untargeted nanoparticles. Anti-CD20 BNPs demonstrated their ability to specifically target CD20-expressing tumor B-cells, with a pick after 72 hours. On the contrary, untargeted BNPs were not able to bind tumor B cells, confirming the importance of anti-CD20 antibody in inducing BNPs' homing. In this analysis it was also evident that the liver is the main site of BNPs' elimination while in the other organs the presence of fluorescent nanoparticles was very low.
For what concerns therapeutic effects, BNPs with anti-CD20 antibody in the shell and chemotherapeutic drugs in the core were able to kill up 90% of tumor B-cells in vitro. The same amounts of free drugs showed comparable effects, suggesting how chemotherapeutic agents maintain killing properties even if they are encapsulated into BNPs. On the other hand, BNPs with just anti-CD20 antibody in the core showed very low cytotoxic effect, confirming BNPs' safety.
Finally, in vivo studies demonstrated both the chemotherapeutic drugs-loaded anti-CD20 BNPs' therapeutic effect in a human-SCID model of disseminated B-cell leukemia and BNPs' ability to completely abrogate the chemotherapeutic agents' toxic effects[4].

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# High sensitivity detection of DNA/miRNA targets based on AFM Nanografting arrays

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Techniques to detect and quantify DNA and microRNA (miRNA) molecules have a crucial role for genomic research. Significant label free[1] and real-time[2] studies reported that miRNAs are expressed differently in patients having cancer and different level of heart failure,[3] and could be therefore useful as clinical biomarkers for disease diagnosis. However, miRNAs detection is still a quite challenging task, due to their easy degradation and non-compatibility with conventional amplification schemes.[4] Novel routes based on nanotechnology to improve the sensitivity of miRNA detection are therefore desirable. We propose here to use Atomic Force Microscopy (AFM) based nanografting to produce DNA nanoarrays with variable molecular density capable of rapid and accurate detection of DNA/miRNA targets, through the measurement, via AFM, of the different nanomechanical response of ssDNA and DNA/miRNA hybridized nanopatches. With this method we plan to measure differential expressions of picomolar-level target miRNAs from cell lysate in a micrometer sized area.

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# Transforming blue copper Azurin into a $Cu^{2+}$ ion biosensor

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Copper is essential for living organisms because it is present as metal cofactor in many enzymes that are crucial for the cell's life. Some of these enzymes are involved in the organism's damage protection caused by the partially reduced oxygen species (PROS), but, when the copper ion is present in high amount into the cell, it gives toxicity reacting with $O_2$, generating itself PROS and subsequently inducing DNA damage, lipid peroxidation, protein modification and other effects, all symptomatic for numerous diseases, involving cancer, cardiovascular disease, diabetes, atherosclerosis and neurological disorders.[1] For this reason it is important to detect the level of $Cu^{2+}$ in biological samples. The present study has been performed using electrochemical and fluorimetric investigation of the recombinant wt and R129W mutant of Azurin from *Psudomonas aeruginosa*, which is a small and very stable protein, even at the high ionic concentrations of the cytoplasm. The protein is characterized by a hydrophobic patch[2] near the electron transfer center able to interact with hydrophobic surfaces such as methyl-terminated alkanethiolate SAM organized on gold surfaces or nanoparticles; furthermore it has one tryptophan residue, Trp48, pointing, with its side chain, to the center of the hydrophobic core of the protein.[3] The detection of copper ions in solution has been determined adding a 6-His tag to the C terminus of the protein, which is located at the opposite face without interfering with the hydrophobic region. In this way, once the protein is immobilized on the SAM (in our case formed by decanethiol DT), it will have the tag facing the solution. As it is known that the histidine is a good ligand for $Cu^{2+}$, the quantitative determination of copper bound to the tag was then performed using electrochemical techniques (CV or SW) or measuring the intrinsic fluorescence, characterizing fluctuations of intensity of the band at 350 nm typical of the exposed Trp129.

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# LECTURES

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