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SECOND WORKSHOP ON NANOMEDICINE UAB-CEI

Abstracts compilation

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UAB CEI WORKSHOP ON NANOMEDICINE

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Aggresomes: a new type of nanoparticles with putative therapeutic applications in nanomedicine?

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With the unstoppable growing of nanobiotechnology in recent years, new drug delivery systems are receiving growing attention. Among them, nanoparticles are emerging as potential candidates to deliver therapeutic agents into a tissue or cell type. Many recombinant proteins produced in bacteria spontaneously aggregate as insoluble clusters named inclusion bodies (IBs). IBs contain functional proteins, are biocompatible, internalized by mammalian cells, and promote their recovery from diverse stresses. Thus, IBs have been proposed as a new platform, named “nanopills”, for drug release in advanced cell therapies. Protein packaging into nanoparticles is not exclusive of prokaryotic systems. Aggresomes are protein-based aggregates found in mammalian cells proposed as a cellular response to misfolded proteins.

In this work, aggresomes have been explored as a putative, new type of nanopills with potential therapeutic applications. For that, we have transfected mammalian cells to produce a human α-galactosidase A (GLA), a lysosomal enzyme used in enzyme replacement therapy in Fabry disease. Our results indicate that ~40% of the expressed GLA accumulates into aggresomes. This packaged GLA is enzymatically active, and shows an excellent, improved thermal stability. Moreover, GLA aggresomes are able to reduce globotetraosylceramide (Gb3) levels in mice endothelial GLA-deficient cells, being their efficacy ~50% of that of the commercial therapeutic compound.

Eventhough these results are preliminary and further work needs to be done to elucidate aspects like biocompatibility, toxicity or *in vivo* assays, aggresomes seem to have the potential to deliver therapeutic proteins to specific targets, as a new type of self-assembling nanopills.

DUAL MRI AND SPECT BIOMEDICAL IMAGING WITH MAGNETICALLY DECORATED CARBON NANOTUBES

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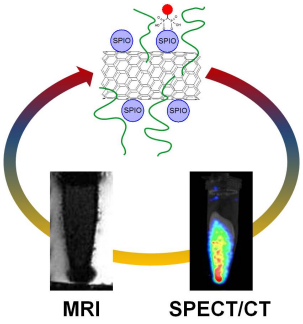
Carbon nanotubes (CNTs) are promising nanomaterials to be used for drug delivery as well as biomedical imaging. The present study developed radio-labelled iron oxide decorated multi-walled CNTs (MWNTs) as dual magnetic resonance (MR) and single photon emission computed tomography (SPECT) imaging agents. Superparamagnetic iron oxide nanoparticles (SPION) were grafted onto MWNTs. Further comprehensive examinations including high resolution transmission electron microscopy (HRTEM), fast Fourier transform simulations (FFT), X-ray differaction (XRD) and X-ray photoelectron spectroscopy (XPS) assured the conformation of prepared SPION as γ-Fe_2O_3. High r_2 relaxivities were obtained in both phantom and *in vivo* MRI compared to the clinically approved SPION Endorem®. The hybrids were successfully radio-labelled with technetium-99m through a functionalized bisphosphonate and enabled SPECT/CT imaging and γ-scintigraphy to quantitatively analyze the biodistribution. No abnormality was found by histological examination. TEM images of liver and spleen tissues showed the co-localization of SPION and MWNT within the same intracellular vesicles, indicating the *in vivo* stability of the hybrids after intravenous injection. The results demonstrated the capability of the present SPION-MWNT hybrids as dual MRI and SPECT contrast agents for *in vivo* use.



Dual SPECT/MR imaging of SPION-MWNT hybrids phantoms.

NANOVESICLE-BIOACTIVE CONJUGATES PREPARED IN ONE STEP BY A COMPRESSED FLUID-BASED SCALABLE METHOD

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In the past 30 years there has been an explosive growth in the number of micro- and nano-particulate molecular materials as drug nanocarriers for the improvement of the pharmacological properties of therapeutic actives [1]. In particular, small unilamellar vesicles (SUVs) have gained a lot of attention in the drug delivery field because of their size (<200 nm) and their easy surface functionalization with multiple functional molecular units (i.e. targeting ligands, polyethylene glycol (PEG)) [2]. Despite their versatility, a high degree of structural homogeneity is crucial for an optimal performance of vesicles as drug delivery carriers. Thus, the formation stage of these supramolecular entities must be tightly controlled in order to achieve a homogeneous assembling of the lipids and other components constituting the vesicular membrane [3].

In this communication will be shown the potential of a new methodology based on the use of compressed CO_2, which we named DELOS-susp, for the preparation of vesicular systems with outstanding vesicle to vesicle homogeneity regarding size, morphology and membrane lipid supramolecular organization [4,5]. Special emphasis will be given to the scalability and regulatory aspects of this new synthetic route [6]. Recent results will be presented to illustrate the suitability of this platform for the one-step preparation of SUVs functionalized with peptides, proteins and biocompatible polymers [7].

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A sponge like organization of bacterial IBs supports the sustained release of protein drugs in regenerative medicine

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In the last years, bacterial inclusion bodies (IBs) have been described and deeply characterized as non-enveloped, porous, hydrated, mechanically stable and biologically active protein-based particles, mainly constituted by functional proteins. All these features have increased the interest in the use, exploration and further adaptation of IB as nanostructured functional materials. In biomedicine IBs are particularly interesting, due to the broad applicability in tissue engineering and in protein-based medicines for intracellular delivery by mimicking protein hormone secretion. Samir K. Maji (*1*) et al have recently published that in the case of many protein hormones, these are accumulate in secretory granules in form or amyloids. However, the mechanism by which the endocrine system slowly releases the necessary protein from amyloid blocks is still unsolved. Interestingly, IBs spontaneously internalized by mammalian cells release sufficient amounts of functional protein to render a potent biological effect without losing their mechanical integrity, what could be a good model to generically investigate protein release from amyloids.

As the supramolecular organization of IB polypeptides still remains unsolved, in this work we have determined the material in VP1GFP IBs (produced in different *E.coli* genetic backgrounds lacking main chaperones and proteases of the protein quality control network) that remained resistant to Proteinase K digestion by using a time course approach. Data show that IBs are formed by different protein populations with distinguishable conformationals states (three distinguishable populations: proteinase K-sensible, with intermediate resistance and a core proteinase K-resistant) and the ratio in which they are found are clearly influenced by the cell's genetic background.

In order to test the architecture of the proteinase K-resistant core, the size, the activity (fluorescence) and the appearance of the remaining protein were monitored during protein digestion kinetics. Data show that IB size remains constant after protease digestion; however, fluorescence progressively declines during the proteolytic attack. In this context, confocal and cryo-TEM microscopy images confirm that the digestion indeed ablated the protein activity but there are no effects on the IB size. Interestingly, cryo-TEM microscopy images revealed a notable loss of IB density after being treated with proteinase K. Finally, to check if IB skeleton was responsible for the mechanical stability in the whole particle, we have also tested the partially digested IB for their potential as scaffolds to mechanically stimulate mammalian cell proliferation when used as nanotopologies. Experiments have evidenced that IBs treated with proteinase K ameliorate identically to IBs non treated mammalian cell proliferation, confirming that IB integrity is fundamentally supported by the proteinase K-resistant core.

To sum up, the study proposed a sponge-like organization of IBs formed by a proteinase K-resistant core recovered by proteinase K-sensitive and functional protein.

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Iron oxide nanoparticle-based approach to promote angiogenesis in brain

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Endothelial progenitor cells (EPCs) constitute a new model for angiogenesis, endothelial regeneration and vessels repair.[1] In recent years stem cell labeling with superparamagnetic iron oxide nanoparticles (SPIONs) has been used as strategy for cellular therapy and tissue repair, as in central nervous system diseases. Our project aims to develop highly magnetized functional EPCs which can be accumulated in damaged brain areas by using an external magnetic field to induce angiogenesis and tissue repair.

Citrate coated SPIONs were synthesized through thermal decomposition route with a γ-Fe_2O_3 core of 6 ±1 nm in diameter and subsequent transfer in water with anionic surfactants. We have tested citrate coated SPIONs stability in different media, using PBS 1X, EGM-2 (endothelial growth medium supplemented with 10% FBS). To control particle aggregation extra sodium citrate was added in EGM-2 at concentrations 0.2 mM, 5 mM and 10 mM. Internalization of SPIONs into endothelial cells was investigated by TEM microscopy: differences in size and number of vacuoles have been observed depending on particle aggregation conditions. Seven-fold more efficient uptake has been found for systems with a certain nanoparticle aggregation which results in an enhancement of MRI contrast without compromising cell viability. [2] Moreover, our results show that magnetized outgrowth EPCs were fully functional since they shaped vessel-like structures as non-magnetized cells. Finally a preliminary *in vivo* cell tracking demonstrates that magnetized EPCs can be guided to cortical areas of the brain by an external magnetic field as confirmed by MRI images.[3]

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Synthesis of new nanoscale MOFs for contrast agent applications

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Because of its noninvasive character and its sub-millimeter spatial resolution, Magnetic Resonance Imaging (MRI) is one of the most powerful diagnosis tools in medical science. Based on the detection of nuclear spin reorientations under a magnetic field, MRI has demonstrated to be very effective not only for the assessment of anatomical changes but also for monitoring of organ functions. However, it was also found that in some cases (*e.g.* gastrointestinal tract or cerebral area) the sensitivity of MRI is not sufficient. In these cases, the use of a contrast agent (CA) to enhance the image contrast is necessary. Today, CAs are used in 35 % of MRI scans. They act shortening the T1 and or T2 relaxation times of water protons, enhancing contrast between the diseased and normal tissue. To date, the major family of CAs are chelates of the highly paramagnetic Gd(III) ion, which are extensively employed in the clinical setting. However, some limitations still persist due to the low sensitivity, lack of selectivity, and low retention time that make them effective only in areas of high accumulation. To solve these problems, a common strategy consists on using nanostructures containing Gd(III) ions that provide increased *in vivo* circulation times and higher concentrations of Gd(III) ions per CA unit, which if targeted, yield superior MRI relaxativities. For example, Gd(III) chelates have been introduced in a variety of nanoparticle-based templates, such as nanoparticles, dendrimers, viral capsids, proteins, mesoporous silica, liposomes and zeolites.

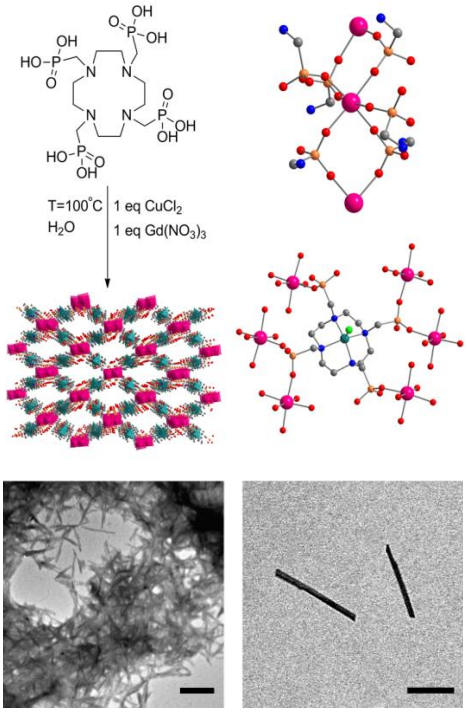

Figure 1. (Above). Metal-organic structure resulting from the reaction of DOTP, Cu(II) and Gd(III) obtained by single crystal X-ray analysis. (Down). TEM images of the nanostructured version of this MOF. Scale bar 100 nm.

Resulting from the combination of multitopic organic ligands with inorganic cores, Metal-Organic Frameworks (MOFs) can be also excellent candidates to incorporate Gd(III) ions into extended structures.For instance, Lin *et al.* have used this strategy to create three dimensional (3D) MOFs containing high concentration of Gd(III) ions, which in turn have shown exceptional relaxativities rates[1]. To create

new Gd(III)-based MOFs that could be used for MRI, here we present a new supramolecular approach that consists on using cyclen-derivate ligands (commonly used as chelating agents to design molecular CAs) to create novel MOF-based structures with promising CA properties, controllable sizes and high stabilities. These ligands present two differentiated coordination sites: i) the nitrogenated core, and ii) the pendent arms that can be functionalized with carboxylate, phosphate or N-derivative groups. These two coordination sites can serve to create bimetallic structures that incorporates Gd(III) ions, and therefore, that can act as novel multimodal contrast agents. Following this approach, in this poster we show the first synthesized MOF made of Gd(III) and Cu(II) metal ions and the cyclen-derivative ligand DOTP (**Fig. 1**). The obtained MOF presents a 3-D porous structure in which the Cu(II) ions are placed in the center of DOTP, coordinated by the four nitrogen atoms and a chlorine, whereas Gd(III) ions expand the structure through phosphate coordination. Significantly, this new MOF can also be synthesized at the nanoscale in the form of nanowires of less than 100 nm in length and 10 nm in diameter. These nanowires present an exceptional stability and dispersability in physiological media. In addition, they show very low toxicity and promising CA properties, making them potential candidates for a future use in MRI.

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Siusplau

Development of a multiplexed fluorescent microarray for the cardiovascular biomarkers detection

Glòria Colom, J.-Pablo Salvador, M.-Pilar Marco

Summary

Cardiovascular diseases are one of the major cause of death in the first world. In this communication, preliminary data of the performance of a multiplexed fluorescent microarray for the detection and quantification of several cardiac biomarkers will be presented.

Abstract

Cardiovascular diseases (CVDs) are the main cause of death in the world and in Europe. Although in vitro diagnostic (IVD) for Acute Myocardial Infarction (AMI) relies on well-established biomarkers[1,2], it is evident the need of a diagnostic platform combining distinct biomarkers which would provide a more complete information of the progression of the disease, the prognosis or a more accurate stratification of the patients to provide a more personalized medicine. Ongoing clinical studies[3-6] have proposed different biomarkers for a wide monitoring of different cardiovascular diseases, from early stages such as inflammation to heart failure. Accordingly from these studies, cardiac Troponin I (cTnI), C-reactive protein (CRP), N-terminal pro-Brain Natriuretic Peptide (NTproBNP), Cystatin C (CysC) and Heart Fatty Acid Binding Protein (HFABP) have been identified as priority biomarkers to assist clinicians in this respect and therefore for a better diagnosis and prognosis of CVDs.

Antibodies for cTnI and NTproBNP produced and characterized in our group and other commercial immunoreagents for CRP, Cystatin C and HFABP, have been combined and used to develop a multiplexed microarray device able to analyze simultaneously these biomarkers in plasma and serum samples. Protein microarrays provide high analytical resolution, detection sensitivities and sample throughput.

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Polymeric nanoparticles by nano-emulsion templating for biomedical applications

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Nano-emulsions are dispersions of two immiscible liquids (e.g. oil, O and water,W) stabilized with a surfactant (S) monolayer. Nano-emulsion droplet size normally falls in the range 20-200nm and due to the small droplet size they are stable against sedimentation and their aspect is transparent to translucent. Nano-emulsions can be prepared by different methods but, in the lasts years, the low-energy methods are focusing great interest. These methods allow obtaining droplets with smaller size and lower polydispersity than high-energy methods. In addition, the energy input is considerably reduced and, as consequence, the final cost of the process. Low-energy emulsification methods are based on the use of the chemical energy stored in the system which is released during emulsification. The characteristic properties of nano-emulsions (size, stability, safety), make them appropriate candidates as templates for nanoparticle fabrication.

The main objective of our work is to apply these procedures to develop nanocarriers for biomedical applications, focusing in drug-delivery. Due to the big versatility of these methodologies, different materials have been obtained depending on the final application. This contribution is an overview of the main achievements of our group on this topic.

Characterization of gold and magnetic nanoparticles for potential biomedical applications in diagnosis and therapy:

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Nanomaterials have acquired considerable interest due to the wide variety of applications that they have in the field of biomedicine. Nanomaterials used in biomedicine both magnetic nanoparticles (MNPs) and gold nanorods (GNRs), have a special interest in terms of their physical characteristics.

The objectives of this study are: i) the development of contrast agents based on biofunctionalized magnetic nanoparticles for early diagnosis of Alzheimer's disease (AD) by MRI and ii) the induction of tumor cell death by hyperthermic therapy based on biofunctionalized gold nanorods,

MNPs biofunctionalized with antibodies anti-ferritin were used to detect the onset and progression of AD using a transgenic mice model of this disease (5xFAD mice). We previously confirmed the presence of iron in higher concentrations in 5xFAD mouse brain compared to control mice using an antibody anti-ferritin. A new nanoconjugate to detect iron accumulation in 5xFAD brain sections was synthesized using MNPs and the anti-ferritin antibody. The ability of the nanoconjugate MNPs-anti-Ferritin to accumulate in 5XFAD mice compared to control mice was first tested in brain sections and then by MRI in mice previously injected intravenously with the biofunctionalized MNPs. Analysis of brain sections incubated with MNPs-anti ferritin showed a high affinity of the nanoconjugate in 5XFAD mice compared to control mice. Ex vivo MRI was performed in 5xFAD and control brains, previously injected with the nanoconjugate and fixed 6h after the injection. The region of interest-based quantitative measurement of T2* values showed that MNPs-anti-ferritin injected 5xFAD mice had significantly reduced T2* values in thalamus and subiculum, where accumulation of ferritin and iron has been demonstrated.

Epidermal growth factor receptor (EGFR) is a cell surface receptor that contributes to the regulation of cell proliferation. Overexpression of the receptor is associated with several types of cancer including breast cancer, melanoma, and brain glioblastoma, leading to its use as a common indicator of degree of tumoral activity. GNRs were conjugated with anti-epidermal growth factor receptor (anti-EGFR) antibodies to induce cell death after laser irradiation. Two cell lines showing high and low EGFR expression (U373 and MC3T3-E1, respectively) were assayed to test the ability of the GNRs-EGFR nanoconjugate to induce cell death after laser irradiation. The rate of cell death after laser irradiation in the presence of the biofunctionalized nanoconjugate was higher in U373-MG cells than in MC3T3-E1 cells, demonstrating the efficiency of this nanoconjugate to bind to and eliminate cells expressing EGFR differentially in their membranes after laser irradiation.

QUATSOMES: VESICLES FORMED BY SELF-ASSEMBLY OF STEROLS AND QUATERNARY AMMONIUM SURFACTANTS

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There is a large interest in finding non-lipid building-blocks or tectons, which self-assemble into stable vesicles, and which satisfy the quality standards required in pharmaceutical formulations.[1, 2]
Here we show the ability of quaternary ammonium surfactants and sterols to self-assemble forming stable amphiphilic bimolecular building-blocks with the appropriate structural characteristics to form, in aqueous phases, closed bilayers, which we named Quatsomes. When prepared by using compressed fluids, these colloidal structures are stable for periods as long as several years, their morphology do not change upon rising temperature or dilution, and show outstanding vesicle to vesicle homogeneity regarding size, lamellarity and membrane supramolecular organization.[3, 4] Phase behavior analysis of different aqueous mixtures of the quaternary ammonium surfactant CTAB and cholesterol (Chol), using optical density, quasy-elastic light scattering and cryo-TEM, have shown that a pure vesicular phase is only formed at equimolar proportions of both components, whereas coexistence of vesicular structures with other types of colloidal and crystalline phases is observed when one moves away from the equimolar ratio.[5] Molecular dynamic simulations with atomistic detail revealed that the cholesterol and CTAB pair works as a unique supramolecular architecture for the formation of more complex colloidal phases such as vesicles. This bimolecular synthon can be considered, to a good extend, as a single entity which self-assembles in particularly stable vesicles. The remarkable structural and thermodynamic properties of a Chol/CTAB bilayer at 1:1 molar ratio predicted from MD simulations provide a theoretical support to justify the experimental high thermal stability and the exceptional morphological properties attributed to vesicles of such composition obtained following in-solution preparation routes in comparison to vesicles prepared by procedures involving a solvent-free stage.

Much functionality can be implemented simultaneously in quatsomes, either by covalent attachment to sterol like molecules, by electrostatic interaction with the cationic ammonium head of surfactant units or by hydrophobic interaction with the bilayer. These possibilities open a broad range of applications in pharmacy,[6, 7] cosmetics and materials synthesis.

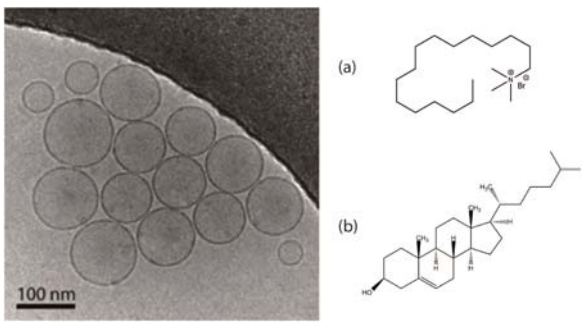

Figure 1. Cryo-TEM image of quatsomes formed by the self-assembling of (a) CTAB and (b) cholesterol molecules.

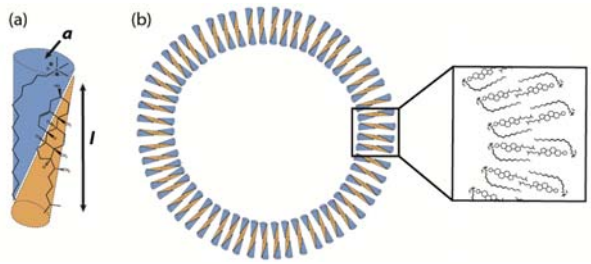

Figure 2. Schematic illustration of the formation of (a) a Chol/CTAB bimolecular amphiphile and (b) their self-assembling to form quatsomes.

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OEG-dendrons synthesized by click chemistry and applications in medical imaging

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Dendrimers are a class of globular highly branched macromolecules with precise architecture. They consist of a multivalent surface with functional group, a core unit where branching starts, and the interior is made of branching units and the void space in between the branched. Interesting properties of dendrimers are monodispersity, multivalency and a globular geometry.
Click chemistry is a 'set of powerful, highly reliable, and selective reactions for the rapid synthesis of useful new compounds and combinatorial libraries'. The most commonly used click reaction is the copper-catalyzed azide-alkyne cycloaddition (CuAAC). Click chemistry is a powerful tool for the construction and functionalization of dendrimers.

The principle objective of our work is the use of click chemistry for the synthesis of higher generation dendrons and exploring the possible use of these dendrons for biomedical applications.

The first generation dendrons which were synthesized in this work consist of two distinct parts: 1) a core unit derived from the acid diethylene triamine pentaacetic acid (DTPA); 2) monodisperse chains of oligoethylene glycol (OEG) of exact length which are coupled to the DTPA core unit by amide bond formation. The second and third generation dendrons were obtained in two steps: 1) conversion of the surface functional groups to azides; 2) coupling the azide building block unit to the azides of the core unit through CuAAC.

Apart from the synthesis of the dendrons using click chemistry, the present work also describes some biomedical applications of the mentioned dendrons. The core unit derived from DTPA is orthogonally protected and this allows functionalizing the dendrons with distinct moieties. Furthermore, the DTPA derivative endows the dendrons the intrinsic capability to chelate metal ions. The chelation depends on the type of complexated metal ion and also on the functional group in the focal point of the dendron. The metals which can be chelated include gadolinium, terbium and indium, all of which are interesting for medical imaging purposes. Chelating gadolinium with dendrons increase the relaxivity induced by the gadolinium ion because the size of the dendrons slows down the rotation of the metal center. Also, the relaxivity is increased due to the hydrophilic character of the dendrons.
Combining the ability to chelate with the multivalency of the dendrons several multimodal platforms for medical imaging were constructed. The platforms were functionalized with targeting peptides and a fluorophore and the DTPA derived core unit carried an isotope of indium. Internalization assays demonstrated that the peptides were able to direct the platforms towards the targeted cells and other preliminary *in vivo* experiments indicated that the constructs accumulated in the tumors as shown in fluorescence and SPECT imaging.

In conclusion, it has been demonstrated that click chemistry is a powerful tool for the synthesis and versatile applications of OEG-based dendrons.

SELF-ASSEMBLED POLYELECTROLYTE COMPLEXES AS NANOCARRIERS FOR ENZYME REPLACEMENT THERAPY IN THE TREATMENT OF FABRY DESEASE

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Fabry disease is an X-linked recessive disorder caused by a deficiency of lysosomal hydrolase α-galactosidase A (GLA). Current enzyme replacement therapy (ERT), with exogenously administered recombinant enzyme, has a limited treatment efficacy because the enzyme may be cleared from the blood by the liver and the spleen, due to the lack of effective protein delivery systems that allow the controlled release of GLA into the lysosomes. We have recently developed functional trimethyl chitosan (TMC)-based polyelectrolyte complexes (PECs) through self-assembly and ionotropic gelation, able to release the enzyme at acidic pH. These PEC nanoparticles, with average size smaller than 200 nm and with low polydispersity (PDI<0.2), were stable and active under physiological conditions, and were efficiently internalized by human endothelial cells and mostly accumulated in lysosomal compartments. In order to increase therapeutic efficiency, PECs were functionalized with an RGD peptide (RGD-PECs), capable of recognizing αVβ3 integrins on the surface of endothelial cells, and freeze-dried, to avoid stability problems. An adequate freeze-drying protocol was found by using slow freezing, otherwise severe structural alterations were observed. Produced PECs and RDG-PECs were found to be non-toxic in HMEC-1 and HeLa cell lines, and no significant haemolysis was observed when PECs and RGD-PECs were tested with mouse blood cell fractions. *In vitro* GLA activity of PECs was tested in primary cultures of mouse aortic endothelial cells derived from GLA defective mice. In these cultures, PECs and RGD-PECs showed to have similar or even better activity reducing intracellular Gb3 deposits than the recombinant enzyme currently used in the clinics. Although addition of the RGD moiety does not seem to add any advantage to GLA PECs in terms of in vitro cell activity, its putative role ameliorating cell internalization or *in vivo* biodistribution of PECs remains to be elucidated.

Giannotti MI et al. (**2011**) pH-responsive polysaccharide-based polyelectrolyte complexes as nanocarriers for lysosomal delivery of therapeutic proteins. *Biomacromolecules*. 12(7), 2524.

Patent: *Polyelectrolyte complex, process for its manufacture and use thereof*. Publication number: **WO2012/085888**

INITIAL STUDIES TO EVALUATE THE INTERACTION BETWEEN IRON OXIDE NANOPARTICLES AND CAENORHABDITIS ELEGANS

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Caenorhabditis elegans (*C. elegans*) is a 1-mm long free-living soil nematode widely used in biomedicine as a model organism. Its main attributes as an experimental system include simplicity, transparency, short life cycle, sequenced genome and small body size that together with the ease of cultivation in the lab make of *C. elegans* a promising animal model to evaluate nanoparticles *in vivo*.

Our final aim is to use *C. elegans* as a first screening in the manufacturing lab of nanoparticles with potential biomedical applications in order to validate their use, to optimize their design, and to study their toxicity.

In the present work, our specific objectives were to develop an appropriate test media for the NP-*C. elegans*. As an initial system, we have used superparamagnetic iron oxide nanoparticles (SPIONs). We have then evaluated their interaction with *C. elegans*.

We assessed the stability of the nanoparticles in the *C. elegans* media by Dynamic Light Scattering. We developed different test media in which nanoparticles are more stable, and validated the tolerance of *C. elegans* to such media in a 24-hour assay. Nanoparticles uptake by the *C. elegans* was evaluated by magnetometry, from which we quantified the iron content of worms treated with 500 μg/ml SPIONs for 24 hours, and found a value of 119 pg Fe per worm. To study the localization of SPIONs within the body of the worm, we stained both treated and control worms with Perls' Prussian blue.

Next steps of this work will include studying the cuticle of the worm by electronic microscopy imaging, studying the time-dependence of the iron uptake by the treated worms, and the evaluation of different types of nanoparticles.

SYNTHESIS AND PURIFICATION OF SINGLE WALLED CARBON NANOCARRIERS

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Carbon nanotubes (CNTs) are being studied for both diagnosis and treatment of cancer. Structures employed in nanomedicine should be characterized by high purity, specific size and good biocompatibility [1]. Therefore it is essential to purify and shorten as-made carbon nanotubes for biomedical application. Carbon nanotubes can be then filled with a chosen payload and externally functionalized [2].

Here we report on the steam treatment of single walled carbon nanotubes (SWCNTs) followed by HCl purification [3] and their filling with selected payloads. Each step of preparation of nanocapsules (filled carbon nanotubes) was monitored by scanning transmission electron microscopy (STEM). Efficiency of purification was examined by thermogravimetric analysis (TGA). The effects of the duration of steam treatment and HCl exposition on the resulting SWCNTs was studied by Raman and near-infrared (NIR) spectroscopies. Analysis of the data showed that the purification is effective. The length of the nanotubes decreases with time of steam exposition, with continuous decrease of defects. The HCl treatment seems does not alter their structure. SWCNTs were indeed filled with the chosen payloads and will be used for targeted delivery of radioactivity in nanomedicine.

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Microfluidic Transwell Platform to Recreate Physiological Conditions and Epithelial Structure of Renal Proximal Tubule

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Excreted urine results from a highly regulated process, in which, initial blood filtration passes through several reabsorption processes to recover useful elements and discard toxins and metabolic residues. Study renal epithelium in *"physiological like"* conditions is hard to achieve by *in vitro* classical methods and finally requires the use of animal experimentation even in early steps of research. In this work we present a microfluidic "*transwell"* device for the creation of a biomimetic cell culture platform for renal proximal tubule cells. Devices have been designed to obtain two microchambers separated by a permeable membrane. Each microchamber has independent microfluidic channels, allowing the use of different liquids passing through the microchambers at the same time, recreating blood and urine in kidney. First microfluidic chips have been successfully fabricated by SU-8 technology [1], where shear stress values near to physiological proximal tube levels can be obtained. Further experimental work includes cell culture validation and functionality assays, results will be presented in the congress. The use of the device presented in this work could therefore decrease and delay the use of animal experimentation, which would have a big ethical and economic impact with respect to nowadays technologies.

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MIXED METALLOPHOSPHOLIPID-NANOVESICLES AS CO RELEASING AGENTS

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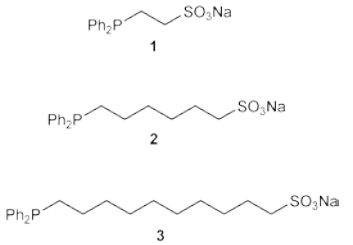
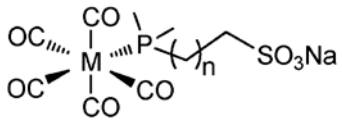
We have developed a new kind of metallosurfactants (surfactants that contain a metal atom in the molecular structure) with very few precedents in the literature because the metal atom is located in a characteristic hydrophobic environment. They were prepared from surfactant phosphines (**1**, **2**, **3**), allowing to modulate the properties of metallosurfactants by means of the length of the hydrocarbon chain.[1]

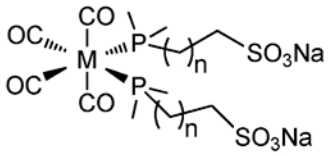
On the other hand, in recent years has been corroborated that CO plays an important role as a signal messenger in mammals. At certain levels, CO is a useful therapeutic agent with beneficial effects as anti-inflammatory, for cardiovascular diseases and also in organ transplantation.[2] It should be emphasized that metal carbonyls are promising compounds as CO releasing molecules, and particularly, molybdenum carbonyls, because they can decompose in living systems releasing carbon monoxide. In a recent communication, we reported the amphiphilic molybdenum carbonyl complexes **4**-**9**, which exhibit molecular self-assembly in water, forming micelles and/or vesicles.[3] The combination of all these properties makes these compounds particularly attractive as potential therapeutic agents.

Our current studies are focused to prepare mixed systems constituted by phospholipids and one of the metal carbonyl complexes **4**-**9**. These new nanostructured systems can form supramolecular arrangements kinetically stabilized. Recent studies confirm the viability of this approach.





4 (n=1), **5** (n=5), **6** (n=9)



7 (n=1), **8** (n=5), **9** (n=9)

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CARBON NANOCAPSULES CONTAINING SODIUM IODIDE

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Carbon nanotubes hold a promising future implementation in nanomedicine in the field of diagnosis and therapy. Potential *in-vivo* and *in-vitro* applications have been studied. [1] Advantages of using carbon nanotubes for biomedical applications lie in their low toxicity, availability of the inner cavity for sample holding and protection, availability of the outer wall for functionalization and biocompatibility. Sodium iodide is available as a radioactive salt that can be used in diagnosis and treatment of cancer.

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. [2] Purified and shortened nanotubes are produced in this way, assuring that the ends of the nanotubes are opened which is essential for the subsequent filling with sodium iodide.

Here we report on the filling of both single-walled and multi-walled carbon nanotubes with sodium iodide. The encapsulation efficiency of this material is investigated, along with the washing ability of the external, non-encapsulated, sodium iodide. Different techniques for the analysis of sodium iodide content in the samples have been explored.

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ON-CHIP MAGNETO-IMMUNOASSAY FOR ALZHEIMER'S BIOMARKER ELECTROCHEMICAL DETECTION BY USING QDS AS LABELS.

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Dementia is a cardinal health problem in developed countries which affects over 25 million people worldwide. The most frequent cause of dementia is Alzheimer's disease (AD), which results in a progressive loss of cognitive function and affects one in eight adults aged 65 years of age or older [1].

Apolipoprotein E (ApoE) is a potential biomarker of AD which can provide objective information for clinical diagnosis and its early detection [2]. Electrochemical detection of cadmium-selenide/zinc-sulfide (CdSe@ZnS) quantum dots (QDs) as labelling carriers in an assay for apolipoprotein E (ApoE) detection has been evaluated. The immunoassay was performed by using tosylactivated magnetic beads (2.8μm of diameter) as preconcentration platform into a flexible hybrid polydimethylsiloxane (PDMS)-polycarbonate (PC) microfluidic chip with integrated screen printed electrodes (SPE). All the conjugation steps were performed in chip and in flow mode. The sensitive electrochemical detection was achieved by square wave anodic stripping voltammetry. ApoE was evaluated for its potential as biomarker for Alzheimer's disease detection. For this set-up, the achieved limit of detection (LOD) was ~12.5ng mL^{-1} with a linear range between 0 to 200ng mL^{-1}. Finally, dilutions from human plasma were assayed with high accuracy respect to the calibration curve. According to the proposed microfluidic set-up, the original concentration of ApoE in the human plasma sample was measurement at ~80 ± 4.6 μg mL^{-1}, comparable with standard determination methods.

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OEG BASED DENDRONS AS ANTITUMORAL DRUG DELIVERY SYSTEMS.

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We have designed and synthesized a new type of dendrons composed by a nucleus of diethylenetriaminepentaacetic acid (DTPA) and oligoethylene glycol (OEG) branches [1]. OEG presents suitable characteristics for drug delivery due its solubility powering water, high biodisponibility, and low immunogenicity and toxicity. Our DTPA core has four equivalent positions and one differentiated (focal point). OEG branches are incorporated on these four equivalent positions and a wide number of different functional groups can be introduced at the dendron's surface, allowing the conjugation of large diversity of drugs. The focal point can be used to link targeting molecules, such as peptides, or chromophores.

In general G1 and G2 dendrons are non toxic, haemocompatible and non immunogenic. Only in the case of G2 dendron with free amines on the surface we have detected some citotoxicity in some cell lines.

5-Fluorouracil (5FU) is the antitumoral drug chosen to be administrated with our dendron system. 5FU is an antimetabolite drug that is widely used for the treatment of cancer, particularly for advanced colorectal cancer. The main problem of this drug is the resistance and the low effectiveness that shown (less of 20% of the administrated drug reaches the target). Clinically this effectiveness is improved with the combined administration with other drugs, like irinotecan or oxaliplatin. The development of an adequate linker to conjugate 5FU to our dendrons or other polymeric systems have been developed to improve its half life time in plasma and the drug release on cancer cells. Several linkers with diverse chemical nature have been explored and their stability at different pHs, plasma and their IC50 values in colorectal cancer cells (HCT-116) considered to select the linker to be used to conjugate the drug to the dendrimer platform. We have synthesized 5FU-dendrimer conjugate and performed a preliminary biodistribution assay. At this moment *in vivo* efficacy are ongoing.

Nowadays we are working on the conjugate of G1 with 7-ethyl-10-camptothecin (SN38). SN38 is the active ingredient of irinotecan. Despite SN38 is 100- to 1000-fold more potent in vitro than Irinotecan, its poor solubility causes it cannot use for therapeutic applications. We expect to increase its solubility by the conjugation to our dendrons.

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DESIGN AND DEVELOPMENT OF MICROFLUIDIC DEVICES WITH INTERNAL SCAFFOLDS FOR 3D CELL CULTURE

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This work presents the design and fabrication of SU-8 based microfluidic chips for cell culture applications. The novelty of the system relies on the integration of 3D scaffolds, which allow the study of 3D cell growing under microfluidic control conditions. Due to the SU-8 transparency and that is a polymer, these chips are compatible with optical inspection and NMR. Furthermore, SU-8 based cell culture microfluidic devices hold the potential to allow the constructions of advanced biomimetic systems. Its capability to build 3D microfluidic networks and the availability of monolithical integration strategies for microsensors and microactuators make SU-8 technology a promising route for next generation of cell culture microfluidic devices. We have designed a microfluidic chip based on SU-8 technology. The design includes a culture chamber where the scaffold is located and lateral microchannels. The aim of these microchannels is the correct perfusion of media through the culture chamber to keep the cultured cell with the proper level of nutrients and oxygen. First devices have been successfully fabricated, obtaining a chip with a culture chamber of 300 μm height and the correct insertion of the scaffold on it. The scaffold material is completely compatible with the different microfluidic fabrication steps that should support temperatures of 90ºC with no degradation. Biological assays and the corresponding results will be also presented.

ANTIBODY MICROARRAYS REPORTED BY QUANTUM DOTS NANOCRYSTALS FOR ALZHEIMER BIOMARKER SCREENING

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We have developed a highly sensitive biosensing system for biomarker screening based on antibody microarrays as selective biomarker probes and quantum dots (QDs) as reporters of the performed sandwich immunocomplexes. Apolipoprotein E, a potential Alzheimer biomarker,[1] was chosen as a target. Showing a limit of detection around ~60 pg mL, we have demonstrated that the proposed QD microarrays are able to outperform other kinds of biomarker screening approaches such as the enzyme-linked immunosorbent assay (ELISA) and microarrays reported by organic dyes (particularly, Alexa 647).[2] As a potential diagnosis tool, this approach might be extended to other biomarkers as well as new multiplexed assays.

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TARGETING TUMORS WITH WASP VENOM

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The use of venoms in cancer therapy continues to be a challenge. These natural weapons have been widely studied for the treatment of several immune-related diseases, and have recently entered preclinical phases for cancer treatment.[1] However, the high toxicity of these potential therapeutic peptides caused by non-specific cellular lytic activity and their rapid degradation in blood make them of limited use in cancer therapy. These peptides are between 10-50 residues in length, and they show amphipathic properties. They have a propensity to interact with membranes, oligomerizing on the surface so as to form transient pores, thus causing cell death. Since free cytolytic peptides are not able to elicit a therapeutic benefit at a safe dose, they have to be targeted and delivered as pro-cytotoxics.

Here we present a peptide-polymer design strategy to obtain pro-cytotoxic systems based on lytic peptides conjugated to PGA polymer through specific cleavage sites that are sensitive to be cut by overexpressed tumor proteases, such as MMP2 or cathepsin B. The potent cytotoxic peptides are inactive when conjugated to the polymer and then become active again once released through the tumor proteases. This strategy is thought to prevent the side effects that occur *in vivo*. Furthermore, this pro-cytotoxic carrier was decorated with peptides in order to specifically target tumor cells. In this way, the system would improve *in vivo* the maximum tolerated dose and the pharmacokinetic parameters of cytotoxic peptides.

After facing the necessity of modifying the innocuous PGA to overcome solubility problems, we successfully demonstrated how a simple targeted polymeric cytotoxic carrier could be potentially useful in clinical use for delivering lytic peptides. Further studies are ongoing to test the harmlessness and efficacy of our system *in vivo*.

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MULTIFUNCTIONAL COORDINATION POLYMERIC NANOPARTICLES. AN ALTERNATIVE TO CLASSICAL NANOPLATFORMS

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Coordination polymers are a fascinating family of materials created from supramolecular assembly of metal ions and organic ligands that act as building blocks to generate a superstructure with genuine and highly tailorable properties [1]. These systems present the combined advantages of the classical metal nanoparticles and the polymeric nanoparticles based on pure organic materials for the application in medical therapy (drug delivery) and diagnosis (bioimaging) [2].

Our research group has focused in the synthesis of nanoscale coordination polymeric particles (CPPs). These nano-objects are able to encapsulate a wide variety of sustaces (drugs, metal nanoparticles, quantum dots, etc…) [3], and act as smart response materials [4]. The recent results include the optimization of encapsulation properties of these materials and the surface functionalization to achieve new elegant biocompatible multifunctional platforms with interesting applications in medicine for drug delivery, bioimaging and targeting directionality for specific recognition. Moreover, the rational design of the new nanoplatforms has afforded new materials that exhibit smart responses against different external stimuli such as temperature, pH, redox environments. Complementary studies of stability and degradation processes have afford interesting results concerning the suitability of these nanoparticles to be used in drug delivery control release. The precise design of the nanoparticles (size, shape and composition) allow to control the drug release profile and adequate them to a specific therapy actuation [5].

The preliminary results indicate that these new multifunctional platforms open a new wide variety of possibilities to be used in therapy and diagnosis due to their high stability, low toxicity and high cellular uptake.

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COMPARISON OF PROTOCOLS FOR THE IMMOBILIZATION OF DNA APTAMER ONTO GRAPHITE-EPOXY COMPOSITE ELECTRODES

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This works presents the study and comparison of different protocols for the immobilization of a DNA aptamer onto a graphite-epoxy composite electrode, in search of the most practical labeless impedimetric aptasensor. The immobilization protocols tested included: physical adsorption, avidin-biotin affinity interaction, amide covalent bond via electrochemical activation and via electrochemical grafting using 4-carboxybenzenediazonium coupling. The impedance-based detection principle relied on the changes of the interfacial properties of the sensing surface which were probed in the presence of the reversible redox couple $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ using EIS measurements. Different thrombin impedimetric aptasensors were therefore assayed, whereas the increase of the interfacial charge tranfer resistance (Rct) was noticed after the aptamer-thrombin interaction. Physical adsorption showed the lowest detection limit (4.5 pM), while avidin-biotin interaction allowed the highest selectivity and reproducibility (4.9%RSD in the pM range).

CHIRAL POLYFUNCTIONAL CYCLOBUTANE PLATFORMS: SYNTHESIS AND APPLICATION TO MAGNETIC RESONANCE CONTRAST AGENTS DEVELOPMENT

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Due to the synthetic versatility of 1,3-difunctional cyclobutanes of amino acids and the advantages of the use of the cyclobutane moiety as a steric restriction element, our research group has conducted several studies focused on the use of these compounds. Considering the need for contrast agents that provide high efficiency and selectivity and making use of the steric restrictions given by the cyclobutane motif, a project on the synthesis of contrast agents for magnetic resonance imaging (MRI) has been started.

The structural versatility of these platforms and preliminary results *in vitro*, demonstrate the potential usefulness of compounds **1** - **4** (Figure 1), which are cyclobutane triamines conjugated to Gd-DOTA, as contrast agents in MRI. Longitudinal and transverse relaxation rates (R_1 and R_2), observed *in vitro* for compounds **1**, **2**, **3**, and **4** show an influence of the radicals X^1 and X^2 on the Gd-DOTA unit. This is possibly due to the action of these radicals on the interchange process of water molecules, which is a factor influencing relaxivity. Values detected at 7 Tesla show that **4** has a better behavior *in vitro* when compared to Dotarem, a commercial product used as a standard. On the other hand, theoretical calculations show that the chirality and substitution of the cyclobutane platform could be an important factor to promote better contrast.

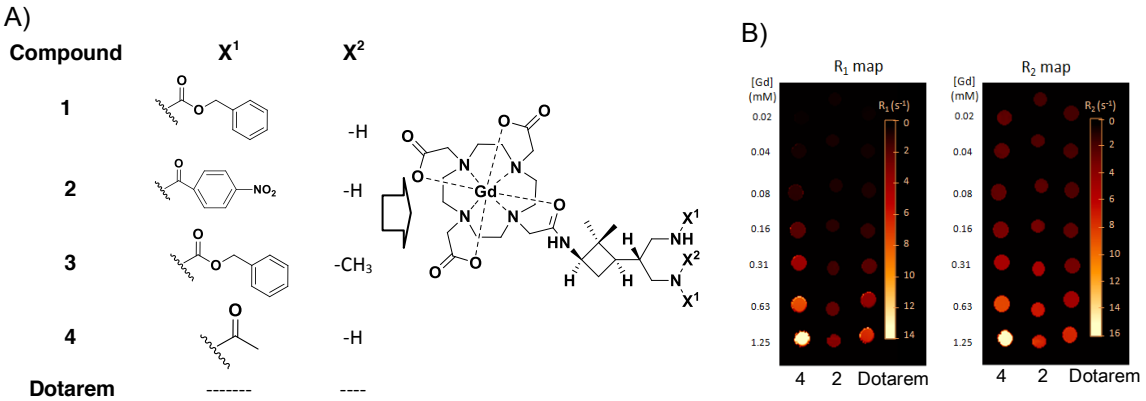

Figure 1. A) Gd-DOTA conjugated to cyclobutane triamines; B) R_1 and R_2 map of phantoms of compounds **4**, **2** and DOTAREM at various concentrations.

A Novel Immunochemical Approach for the Diagnosis of Infectious Diseases Caused by Pseudomonas aeruginosa

Carme Pastells, Núria Pascual, F. Sanchez-Baeza and M.-Pilar Marco

Pseudomonas aeruginosa (PA) is an opportunistic pathogen producing many virulence factors and has the capacity of biofilm formation which increases the difficulties to eradicate these infections and generates antibacterial resistance. PA is mainly related with respiratory trac infections being sepsis one of the most complicated situations, where rapid and reliable detection of the pathogen is crucial to guarantee patients survival. Unfortunately, currently pathogensidentifications standard methodology is based on blood culture, which presents several limitations such as low sensitivity and time-consuming, implies a delay of 48-72 hours. Immunochemical detection could overcome these limitations, as it is sensitive, reliable and fast. At present work, novel antibodies against an specific determinant of PA have been developed and an specific, robust and reproducible ELISA has been established. The ELISA technique is easy to implement in a laboratory, cost-efective, high-throughput screening capabilities and at the same time is a useful tool for the characterization of the immunoreactives for their further implementation on a point-of-care device.

Biological properties and characterisation of novel self-assembling CD44-targeted protein-only nanoparticles

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CD44 is a multifunctional cell surface receptor involved in cell proliferation and differentiation and it is the most frequent molecular marker in a large variety of tumor types. Many evidences strongly support that an alteration on CD44 expression promotes tumor cell survival and aggressiveness and induces tumorigenesis and metastasis. Thus, the tumorigenic and metastatic potential of CSCs have been associated to CD44 expression, representing an appealing target for drug delivery in nanomedicines of cancer. In a drug delivery context, many nanoparticles among assorted carrier types have been proposed for specifically targeting CD44 expressing cells being most of them formulated by the conjugation with hyaluronic acid; however, it has been described that not all CD44 + cells constitutively bind hyaluronic acid (HA). Along these lines, proteins binding CD44 represent a potential alternative to HA allowing, through conventional protein enginnering, functionalized and adaptable nanocarriers.

Taking these alternative nanoparticles into consideration, we have explored several protein-based ligands of CD44 as the basis for the construction of multifunctional, cell penetrating polypeptides targeted to CD44+ cells. Two of the tested ligands drive the formation of self-assembling, fully biocompatible protein-only nanoparticles that efficiently bind and internalize target cells upon exposure, in form of stable ring-shaped entities. Such particulate protein organization confers added value properties to the constructs favouring cellular penetrability, what opens a plethora of possibilities for the rational design of protein-based, fully biocompatible nanomedicines.

Polymer-drug Conjugates based on Polyglutamic Acid and 5-Fluorouracil for the treatment of advanced Colorectal Cancer

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Although local colorectal cancer (CRC) is easily treated with surgery and conventional chemotherapy, advanced or metastatic CRC still shows very high mortality rates. Current chemotherapeutic treatment involves high doses of cytotoxic drugs, particularly adjuvant combinations of 5-fluorouracil (5FU) and Irinotecan (prodrug of SN38). However, these treatments cause many side effects to the patients. The use of polymer-drug conjugates (PDC) has attracted great attention in the field of controlled drug delivery for cancer treatment, improving the ratio of cytotoxic drugs in tumour tissues, taking advantage of the enhanced permeability and retention (EPR) effect [1].

Our efforts are directed towards the synthesis and preclinical evaluation of new PDC based on polyglutamic acid (PGA) to achieve a targeted and combined release of 5FU and SN38. It is known that levels of matrix metalloproteinase 7 (MMP7) increases as CRC progress. Due to the overexpression of MMP7 in CRC tissues, an MMP7-sensitive PDC has been designed to get a targeted release of the drug, by using a MMP7 sensitive peptide sequence (RPLALWRS [2]) linked between the drug and the polymeric carrier.

Different PDC have been synthesized for single or combined therapy of 5FU and SN38. Drug loading, stability in plasma and at different pHs, diverse physical parameters such as size and Z-potential have been studied for each PDC. The *in vitro* efficacy of each PDC has been determined by MMT experiments with HT-29 and HCT-116 CRC cell lines. *In vivo* efficacy and biodistribution assays for PGA-5FU PDC system are currently ongoing.

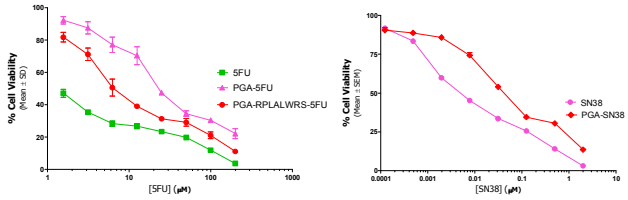

Figure 1. Cell viability of different PDC synthesized in HCT-116 cell line

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Controlling multivalency and multimodality: Up to pentamodal dendritic platforms based on diethylenetriaminepentaacetic acid (DTPA) cores.

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During the last few years, there has been an exponential growth in the field of biomedical research. Innovative techniques from many disciplines such as chemistry, biology, nanotechnologies and chemical biology contributed to achieve these advances. As a consequence, new strategies for therapy, diagnostics, theranostics (combination of both) have been developed. To make further progress, new tools are required in order to facilitate combination therapies [1], or to prepare better imaging agents and better drug delivery vehicles. From a chemical point of view, the generation of new well-defined multimodal molecules that allow the incorporation of several functionalities, such as drugs, imaging agents or targeting molecules, onto the same scaffold in a precise and controlled manner is particularly relevant [2].

Our laboratory has spent recent years working on the preparation of dendritic-type structures based on oligoethylene glycol (OEG) moieties [3]. Herein we describe a highly versatile synthetic strategy to generate multimodal and multivalent dendritic platforms, based on a diethylenetriaminepentaacetic acid (DTPA) core and short OEG branches. Following straightforward chemical methods and easy purification steps (extraction and precipitation) it has been possible to prepare compounds with different functionalization patterns, ranging from mono- to pentamodal. To assess the versatility of this methodology several compound have been prepared containing molecules of distinct natures, such as peptides, fluorescent probes, oligoethylene glycol from different sizes, or aliphatic chains.

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Cis-γ-amino-L-proline peptides as an example of cell-penetrating peptides.

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The discovery of cell-penetrating peptides (CPPs) is a promising breakthrough to achieve non-invasive delivery of non-permeable biomolecules in the intracellular compartment [1]. CCPs with non-natural amino acids in their sequences (**β**-amino acids or **γ**-amino acids) have the main advantage that they show higher stability to proteolytic degradation [2]. Our group designed and synthesized two discrete γ-peptide libraries based on (2*S*, 4*S*)-4-amino-L-proline, all compounds having a well-defined secondary structure [3, 4]. The libraries were synthesized on solid-phase, using one protected monomer that can be easily functionalized by modification of the **α**–amino side chain. These peptides can mimic protein interactions and consequently their suitability for diverse therapeutic applications can be explored.

Using flow cytometry and confocal microscopy, here we studied the capacity of these γ-peptides to cross cell membranes in two distinct systems, namely HeLa cells and *Leishmania* as models of mammalian cells and a human protozoal parasite, respectively. The subcellular localization experiments were performed under a range of peptide incubation conditions and with a variety of fluorescent labels that tagged cytoplasm, mitochondria, lysosomes, endoplasmic reticulum and Golgi apparatus in HeLa cells. Furthermore, we addressed the uptake mechanisms of these γ-peptides by means of inhibitors of specific endocytotic pathways.

Our results showed that two specific peptides are taken up in HeLa cells and *Leishmania donovani*, and that they show some remarkable differences with respect to TAT. The TAT peptide is considered a gold standard in the field. In additionally, the γ-peptides showed low cytotoxicity and protease resistance. We propose that the cellular uptake mechanism of γ-peptides involves two or more pathways in the experimental conditions tested.

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COMPARATIVE BIOFABRICATION OF INCLUSION BODIES FOR NANOMEDICAL PURPOSES IN *E. COLI* STRAINS LACKING LIPOPOLYSACCHARIDE

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Although clinical uses of recombinant proteins have exponentially increased during last 30 years, strategies are required to enhance their efficiency as well as biosafety. Being *Escherichia coli* the most commonly used cellular factory, as Gram-negative strain it contains endotoxic lipopolysaccharide (LPS) that is potentially harmful in human therapy [1]. Protein production as inclusion bodies (IBs) as functional protein nanomaterial is becoming an option to increase the efficiency of recombinant protein delivery in cell therapy and regenerative medicine [2-5]. The objective of this study was to comparatively evaluate the production of a reporter GFP-based fluorescent protein soluble and as IBs, in three *E. coli* strains at three different temperatures (37 ºC, 25 ºC and 16 ºC). For that, the *E. coli* wild-type strains MC4100 and BW30270, as well as the LPS-free strain KPM335 were transformed with a plasmid encoding VP1GFP under control of an IPTG-inducible promoter. Protein quality was measured by combining western blot analysis and fluorescence emission, and IBs were isolated and purified to be used as scaffolds in a cellular proliferation test. Results showed a major IBs amount with respect to soluble protein at 37 ºC in all strains, whereas the ratio between IB and soluble protein expression shifted in favor of the soluble fraction at lower temperatures. On the other hand, fluorescence activity was higher in IBs isolated from the LPS-free strain KPM335 when grown at 37 ºC ($p<0.05$). Major specific activities were shown in soluble protein than in IBs at all temperatures ($p<0.001$), while higher specific activities in IBs were shown in KPM335 at 37 ºC and 25 ºC. Finally, 1BRG.3 cells proliferation showed an important increase with respect to control when IBs from KPM335 were used. These data suggest that a higher conformational quality in IBs from this strain may be a result of metabolic differences generated by elimination of LPS-related genes, and IBs from LPS-free KPM335 could be used as scaffolds to stimulate cellular proliferation processes in regenerative medicine.

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TARGET TISSUES OF LIPOSOMES ENCAPSULATING AN LPS/dsRNA-COCKTAIL AFTER ADMINISTRATION BY INTRAPERITONEAL INJECTION AND BATH IMMERSION IN ZEBRAFISH

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A liposomal based nanocarrier that co-encapsulates the bacterial lipopolysaccharide (LPS) and the synthetic analogue of dsRNA viruses, poly (I:C), was developed and its biodistribution *in vivo* was addressed using two different administration routes (intraperitoneal injection and immersion bath) in zebrafish (*D. rerio*). The surface charge of this liposomal carrier was properly designed to show high encapsulation efficiencies for both immunostimulants, and previous results demonstrated that the delivery system had low toxicity and an specific pro-inflammatory and antiviral response both *in vitro* and *in vivo* using zebrafish larvae. The present work compares the biodistribution of these positively charged liposomes by fluorescence imaging using liposomes containing DOPE conjugated to AlexaFluor750. Our results showed that intraperitoneally injected liposomes can be detected even at 72 h post-injection and *ex vivo* organ quantification showed liposome preference mainly for spleen, which is one of the main organs of phagocytic filtration and sites for protective immunity [1], and also for liver. However, when bath immersion was performed liposomes were able to be attached to the gill surface probably on the epithelial cells and underlying phagocytes [2]. Analysis of tissues *ex vivo* (at 12 h post-immersion) showed a different biodistribution pattern with liposomes being located on intestine and liver besides of the gills. This is consistent with previous results where has been observed that immersion induces mainly a mucosal immune response in skin, gills and intestine [3].

This study provides evidence of the *in vivo* distribution pattern of nanoliposomes which will allow for a much more accurate understanding of the behaviour of nanocarriers as a delivery systems for disease control in aquaculture.

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CONFORMATIONAL QUALITY MODULATION BY DNAK CHAPERONE ON JCV VP1 VIRUS-LIKE PARTICLES PRODUCED IN E.COLI

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Promising and fully compatible nano-vehicles for drug delivery can be obtained producing recombinant viral capsid protein in cell factories. These spontaneous self-assembling structures, named Virus Like Particles (VLPs), retain the key viral properties like cellular tropism and the capacity to deliver internalized molecules, while lacking viral infectivity and immunogenicity.

VP1 major capsid protein of Human JC virus (hJCV) can be produced in *E.coli* system and is described to spontaneously form VLPs [1]. These structures elicit the optimal red blood cell hemagglutination level at specific salt and pH conditions.

The conformational quality of VLPs structures and the solubility of VP1 protein are also influenced by the presence or the absence of chaperone DnaK in *E.coli* expression system. Analysis of VP1 hJCV production in different *E.coli* genetic backgrounds indicate a proteolysis targeting role of DnaK: meanwhile the production of the recombinant protein is favored by the absence of DnaK, solubility is highly compromised.

It has been also observed that VLPs hemagglutination efficiency is directly related to the presence of DnaK chaperone in the expression system showing that solubility not necessarily favors biological activity.

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Dissection of nanopill – mammalian cell interaction in drug delivery

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Bacterial inclusion bodies (IBs) are protein clusters occurring during recombinant protein overexpression in microbial hosts. These nanoscale particles historically regarded as waste by -products have been proved able to retain a certain grade of biological activity of the forming protein. In addition, their high purity and mechanical stability coupled with their cost effective production and isolation prompted the appearance of appealing applications in biotechnology, such as their use as a source to extract soluble protein or as immobilized biocatalysts[1]. Very recently, our group has also introduced IBs as a potential biomaterial in bioengineering and nanomedicine. These IBs can modify cell culture topographies, capable to affect cell response in terms of adhesion, proliferation and differentiation[2] or can also work as nanopills[3] with the capacity of rescuing challenged cultured cells by delivering significant amounts of the forming protein with a potential therapeutic biological activity.

Nevertheless, the mechanism by which nanopills can deliver their forming protein to cells remains still unsolved. In this regard, a complete structural and ultrastrucural analysis of eukaryotic cells (HeLa) after the addition of IBs was carried out at different time points, 0h 0.5h, 1h, 3h, 8h and 24h. IBs contacted cells at early incubation times being closely related with cell filopodia. In addition, these particles were progressively engulfed and internalized via endocytosis. Finally immunolocalization of the IB forming protein allowed the visualization of internalized cargo and protein release from the protein particle to the cell cytosol. In summary the present work provides a fine picture of the IB action pathway when added as nanopills in mammalian cell cultures.

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TWO-DIMENSIONAL MICROSCALE ENGINEERING OF PROTEIN BASED NANOPARTICLES FOR CELL GUIDANCE

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Cell responses, such as positioning, morphological changes, proliferation and apoptosis, are the result of complex chemical, topographical, and biological stimuli. We have studied the macroscopic responses of cells when nanoscale profiles made with inclusion bodies (IBs) are used. Fibroblasts cultivated on supports patterned with stripes and dots of green fluorescent protein and human basic fibroblast growth factor-derived IBs were studied. A deep statistical data treatment of fluorescence microscopic images was performed. Obtained data demonstrates that these cells preferentially adhere to the IB areas and align and elongate according to specific patterns. These findings prove the potential of surface patterning with functional IBs as protein-based nanomaterials for the 2D engineering of biological interfaces at the microscale.

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T22-EMPOWERED SELF-ASSEMBLING PROTEIN NANOPARTICLES FOR CXCR4+ CELL-SPECIFIC TARGETING IN METASTATIC COLORECTAL CANCER.

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Targeted delivery of therapeutic molecules has become a promising tool in nanomedicine since it allows not only a local increase of the drug or nucleic acids concentration, but also allows reducing the systemic toxicity.

Many different nanoscale vectors are currently available, but protein-only nanoparticles appear as the most promising ones due to their high biocompatibility, biodegradability and high plasticity of design. Moreover, being the nanoparticle size an important factor for their correct biodistribution *in vivo,* recently described architectonic tags, able to induce the regulatable protein self-assembling, allow to control the formation of optimal size protein nanoparticles [1,2,3].

CXCR4 chemoquine receptor has been described to be a key element during metastasis formation in different types of tumors, including colorectal cancer (CRC), for which metastatic intracellular targeting vehicles are still missing and early metastasis appears in 50% of CRC cases. In this context, four different CXCR4 specific peptide ligands were tested for their ability to specifically internalize green fluorescent protein reporter self-assembling nanoparticles in CXCR4 expressing cells. Eventhough all of them succeeded in their internalization ability, only the 18 mer peptide T22, an engineered segment derivative of the polyphemusin II protein from the horseshoe crab, efficiently penetrated target cells via a rapid, receptor-specific endosomal route. Internalized fully fluorescent nanoparticles, stably accumulated in the perinuclear region of the cultured cells in absence of any detectable toxicity. Performed *in-vivo* biodistribution assays in orthotopic colorectal cancer model mice after i.v. injection of T22-empowered self-assembling protein nanoparticles, showed a stable accumulation of those particles in the primary tumor and all the macro and micro-mestatatic foci for more than 24hours, being internalized in CXCR4 positive cells as it was described *in vitro* showing again no signs of toxicity [4]. Moreover, recently developed T22-empowered multifunctional protein nanoparticles, have proved to be suitable for expressible nucleic acid delivery in CXCR4+ cultured cells after the appropriate downstream processing [5].

Given the big impact that CXCR4 linked pathologies such as colorectal cancer or human immunodeficiency virus infections among others have in the society, there is an urgent demand of finding new effective targeting agents for CXCR4 expressing cells. In this context T22-empowered self-assembling protein nanoparticles appear to be very promising tools not only for the metastatic intracellular targeting of therapeutic molecules, but also as an imaging agent for diagnoses.

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Protein Corona on Microwave Synthesized Magnetic Iron Oxide Nanoparticles: Characterization by Dynamic Light Scattering

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In recent years, magnetic nanoparticles (MNP) have gained a lot of attention in biomedical applications due to their unique magnetic property. Protein corona on NPs surface is one main issue researchers are now faced to when NPs are in contact with biological media. In the present investigation, we studied the protein corona on two MNP with different surface coating by using dynamic light scattering (DLS). In particular, we used microwave synthetic method to synthesize two MNP with an iron core of 6 nm, coated by tetramethylammonium hydroxide (TMAOH) or citrate, respectively. Synthesized MNP were incubated in three biologically relevant media: Phosphate Buffered Saline (PBS), RPMI with and without proteins of serum (RPMI is a high ionic media used in cell culture). Then, we performed DLS to study the behavior of NPs in the tested media in terms of size distribution. DLS results have shown citrate MNP are relatively more stable than TMAOH MNP in the tested media. Big aggregates of MNP-protein are observed for TMAOH MNP, in contrast, for citrate MNP, mainly one size population of MNP-protein complexes is found. These findings highlight the effect of surface coating on the protein corona on MNP surface when they are exposed to biological environments.

BACTERIAL CELLULOSE FILMS AS A NEW SCAFFOLD FOR CELL CULTURE

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Bacterial cellulose (BC) films are an attractive biomaterial as a scaffold for cell culture growing. Their porosity, flexibility and transparency, as well as its biocompatibility, make this material a new target for the generation of three-dimensional cell cultures [1] and for tissue regeneration studies [2, 3].

In this study, we have obtained BC films from *Gluconacetobacter xylinum* cultures. After different drying methods (room temperature, freeze drying and supercritical drying), obtained films were transparent, light with up to 89% of porosity. BC films produced are mechanically robust, flexible with a water absorption capacity up aprox 7000% of its weight and can recover its initial shape after mechanical pressure. Cell cultures (MDA MB 231, HeLa and HepG2 cells) were grown in the surface of the BC films. Viability assays (MTT, calcein AM) showed that BC was not toxic for cells and it did not modify their growth. Cell morphology was also analyzed by fluorescence and confocal microscopy. We have observed no differences in cell morphology when cells were seeded in the films and in classic cell culture flasks (monolayered cultures).

The results indicate that BC films generated by *G.xylinum* can be used as a new scaffold in the cell culture field. Cellulose properties, such as flexibility and transparency, can facilitate cell manipulation and monitoring instead of two-dimensional cultures. Moreover, due to the lack of cytotoxicity showed in our studies, BC should be considered as a new support in further regeneration studies.

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