CIC biomaGUNE
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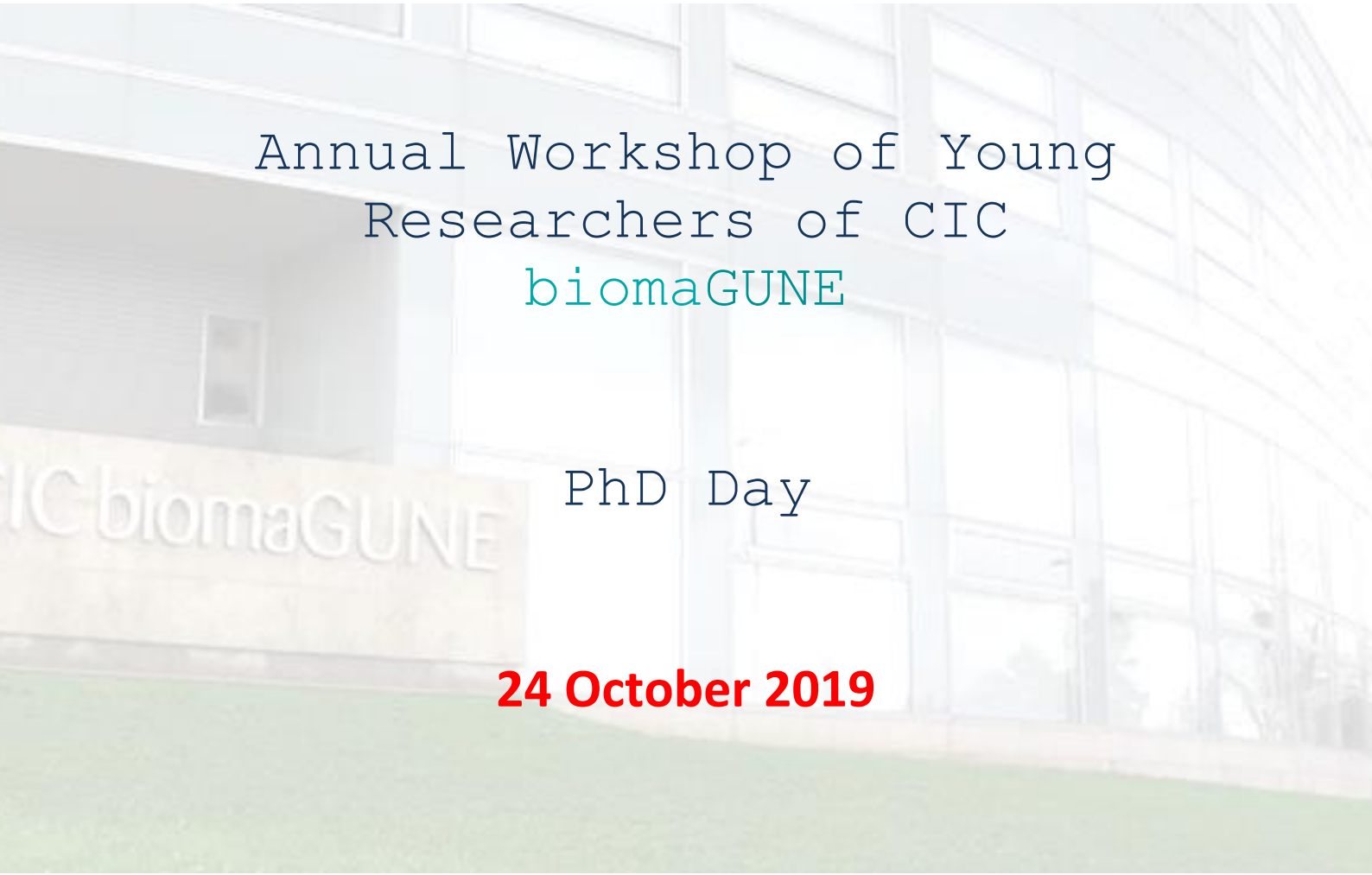
Center for Cooperative Research in Biomaterials



Annual Workshop of Young Researchers of CIC biomaGUNE

PhD Day

24 October 2019

About

The "**Annual Workshop of Young Researchers of CIC biomaGUNE**" is a yearly meeting opened to all researchers, but with a special emphasis on the participation of biomaGUNE's PhD students. The event is envisaged as a 1-day symposium, where PhD students of second and third year have the opportunity to present their latest results within a relaxed environment, and participate in lively scientific debates. All contributions will be in the form of short 5 minutes oral communications, encouraging the students to focus on presenting the most important results of their research in a clear and concise way. As such, PhD Day will tackle all research activities of CIC biomaGUNE, and will provide postdocs and PhD students with an invaluable insight into the latest achievements performed at the institute, all within a reduced timeframe.

Ultimately, PhD Day pretends to be a platform for young researchers within the institute to engage in scientific discussion, share ideas and start new collaborations. Furthermore, the event should help to improve the social and working environment at CIC biomaGUNE, which is of vital importance for the scientific development and wellbeing of researchers at the center.

Program

Thursday 24 October			
9:00-9:10	Welcome: Niels Reichardt		
9:10-9:40	**Invited Talk:** Ana Beloki, CIC nanoGUNE *Polymer-enzyme conjugates for improved biocatalysts*	Inv-1	**Chair:** Antonio Aires Trapote
9:40-9:45	Elena Lopez-Martinez *Novel applications for metal nanoclusters-protein hybrids*	O-1	
9:45-9:50	Idoia Mikelez-Alonso *Enhanced NK cell activity by IL15 functionalized iron oxide nanoparticles*	O-2	
9:50-9:55	Guillermo García-Marquina *Enzymatic engineering of acyltransferase LovD*	O-3	
9:55-10:00	Daniel Sánchez-deAlcázar *Functional nanostructured materials based on designed proteins*	O-4	
10:00-10:05	Cristina Risueño Fernández *Modulating the conformational plasticity of tetraspanin CD81 by ligand binders and its implication in cellular and viral processes*	O-5	
10:05-10:10	Lucia Maio *Designed metallo-tags for probing natural systems*	O-6	
10:10-10:55	Global Discussion		
10:55-11:30	**Coffee Break**		
11:30-12:00	**Invited Talk:** F. Xabier Contreras, BIOFISIKA *Deciphering the busy social network of neurons*	Inv-2	**Chair:** Alejandro Criado Fernández
12:00-12:05	Antonio Dominguez-Alfaro *Innovatives methodologies to develop porous 3D scaffolds based on PEDOT and CNT for tissue engineering*	O-7	
12:05-12:10	Vished Kumar *High yield synthesis of monodisperse gold colloids via micellar modification*	O-8	
12:10-12:15	Donato Mancino *New generation of 3D polymeric scaffolds based on carbon nanotubes doped with biomolecules*	O-9	
12:15-12:20	Mathias Charconnet *Optical properties of gold nanoparticle superlattices*	O-10	
12:20-12:25	Javier Plou Izquierdo *Analysis of the tumor microenvironment by surface-enhanced Raman scattering*	O-11	
12:25-12:30	Verónica Mora-Sanz *Development of bioanalytical assays based on antibodies modified with nanoclusters*	O-12	
12:30-12:35	Juan Pedro Merino *Efficient MALDI-MS array based on functionalized CVD graphene with carbohydrates as potential*	O-13	

	sensing platform		
12:35-13:20	Global Discussion		
13:20-14:30	**Lunch**		
14:30-15:00	**Invited Talk:** María Muñoz Caffarel, Biodonostia *Oncostatin M released by immune cells in the tumour microenvironment activates cancer cells and fibroblasts to promote breast cancer progression*	Inv-3	**Chair:** Malou Henriksen-Lacey
15:00-15:05	Cristian Salvador *Supramolecular polyamine nanocarriers for siRNA delivery in breast cancer therapy*	O-14	
15:05-15:10	Cristina Simó Costa *Biological fate on nanomaterials with nuclear imaging*	O-15	
15:10-15:15	Charles Wiliams *Glycoengineering of Exosomes*	O-16	
15:15-15:20	Marta Martínez-Moro *Translocation, biological fate, stability and effective dose of engineered nanomaterials for nanosafety studies*	O-17	
15:20-15:25	Marcos Navascuez Lominchar *Nanoemulsions as drug carriers: preclinical evaluation of pulmonary delivered hydrophobic drug-loaded nanocapsules*	O-18	
15:25-15:30	Irene Veronika Judith Feiner *A pretargeting strategy for multifunctionalized gold nanoparticles (GNPs) as boron drug delivery agents*	O-19	
15:30-16:00	**Coffee Break**		
16:00-16:05	María Gomez-Higuero *Towards the synthesis of stable isotope labeled N-glycopeptides as internal standards for mass spectrometry based glycan analysis*	O-20	**Chair:** Sonia Serna
16:05-16:10	Pilar Castellnou *In vivo PET study of [11C]L-Alanine and [11C]D-Alanine in a mouse model of prostate cancer*	O-21	
16:10-16:15	Rossana Passannante *Pharmacokinetics evaluation of new drugs with potential application in Duchenne muscular dystrophy*	O-22	
16:15-16:20	Ander Egimendia Tolaretxipi *Functional network characterization by resting-state fMRI in the cuprizone mouse model of multiple sclerosis*	O-23	
16:20-16:25	Ana Joya Villanua *Magnetic Resonance Imaging evaluation of the role of hyperglycemia in experimental subarachnoid haemorrhage*	O-24	
16:25-17:10	Global Discussion		
From 19:30	**Team Building: Evening Snack at Convent Garden (rooftop terrace)**		

Polymer-Enzyme conjugates for improved biocatalysts

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In this talk, I will revise different approaches to the synthesis of polymer-enzyme hybrids for the fabrication of new catalytic materials. From homogeneous to heterogenous hybrid biocatalysts that showcase improved features compared to free enzyme system. Hence, the use of polymeric networks as flexible and porous scaffolds improves significantly the stability and robustness of the biocatalyst under conditions in which free enzyme systems are inactive, i.e. high temperatures or presence of organic solvents. Particularly, I will present the possibilities of Single Enzyme Nanogels (SENs) for the fabrication of organic-inorganic heterogeneous hybrids. SENs are polymeric hydrogels filled with single enzymes. The engineering of the polymeric shell, i.e. introduction of coordination ligands, allows the controlled supramolecular assembly of SENs into catalytic nanoparticles or films through metal-ligand coordination. Finally, I will summarize the technological applications SENs are used for in the field of catalysis, sensing, and energy production.

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Deciphering the busy social network of neurons

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Understanding biological processes at the mechanistic level require a systematic charting of the physical and functional links between all cellular components. While considerable efforts have been made to elucidating the cellular interactome, with the majority of studies focusing on mapping protein-protein, protein-DNA, and protein-metabolite interaction networks, other critical cellular components such as membrane lipids have rarely been studied in large-scale interaction screens. These activities are challenging to characterize on account of the complex scenario where lipids and membrane proteins interact. This lack of information makes protein-lipid interaction one of the fundamental unsolved paradigms in membrane biology research. Unlocking this mystery is the key to a vast number of human diseases. Here, I will discuss strategies to unveil and characterize protein-lipid interactome in living neurons. Characterizing these lipid-protein networks will provide new mechanistic insights into how living cells work depending on protein-lipid cross-talks. Finally, in the long-term, these results will lay the basis for the development of novel, selective, and improved therapies for different diseases and malignancies where lipids have been intimately related.

Oncostatin M released by immune cells in the tumour microenvironment activates cancer cells and fibroblasts to promote breast cancer progression

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Breast cancer metastasis is the main cause of death in patients and finding new therapeutic targets for the advanced disease is an unmet need with high clinical impact. Cytokines are important players in inflammation, a process highly associated with tumour progression. In this work, we investigated the role of the pro-inflammatory cytokine Oncostatin M (OSM) in breast cancer. To address this issue we used a wide array of tools including analysis of clinical samples, in vivo and in vitro models.

Analysis of human breast cancer samples revealed that OSM and its receptor OSMR are upregulated in breast cancer stroma compared to normal stroma. OSMR is mainly expressed by breast cancer cells and fibroblasts while the ligand OSM is produced by macrophages and neutrophils. In vitro, OSM treatment of breast cancer cell lines promotes colony formation, migration and invasion, processes likely mediated by ITGA5. In addition, OSMR activation in cancer cells induces the expression of pro-malignant genes such as VEGFA, Snail and IL6 and induces tumourigenesis and lung metastasis in xenografts of human cancer cells. In vitro 3D cultures of stromal cells show that OSM promotes cancer associated fibroblasts (CAFs) proliferation and activation by inducing α-SMA, FAP expression and fibroblast contractility. Genetic depletion of OSMR in a genetic mouse model delays tumour onset, reduces tumour growth and lung metastasis. Injection of murine cancer cells in OSMR deficient mice shows a decrease in tumour growth compared to wt mice, suggesting that OSMR signalling is important in the stroma.

Our results show that OSM signalling plays a key role in breast cancer progression by inducing the crosstalk between cancer cells and the tumour microenvironment. OSM and its receptor could be blocked by antibody based inhibition, strategy that has had a major impact on breast cancer, which makes them promising candidates for therapeutic targeting.

Novel applications for metal nanoclusters-protein hybrids

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Nanotechnology is an important tool to develop systems in order to lead future changes in industry, environment, information technologies, and medicine. In this context, the use of protein-based materials is optimal because it is inspired by nature's colossal catalogue of protein structures and functions. Specifically, we use repeat proteins, such as consensus tetratricopeptide repeat (CTPR) proteins,[1] that allow modular design approaches while taking advantage of proteins' functional variability. In this thesis work, I focus on the design of metal nanoclusters templated by CTPR proteins[2] and their application as sensors[3] and as labels for subcellular structures for imaging techniques.

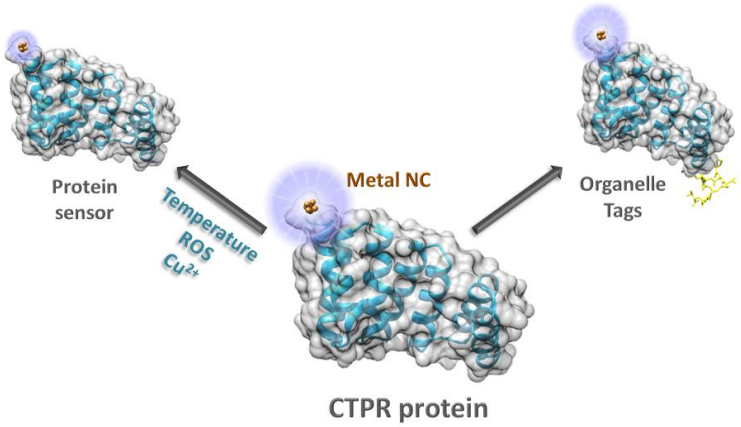

Figure 1. Scheme of proposed applications of fluorescent metal nanocluster-CTPR hybrids.

Keywords: protein design, fluorescence, sensor, tagging, imaging.

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Enhanced NK cell activity by IL15 functionalized Iron Oxide nanoparticles

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In the field of therapy important challenges are targeting to specific locations[1] and the induction of specific cell mediated cytotoxic activity against cancer cells; one example with very promising results are Natural killer (NK) cells. The reason of the success is that they are antigen independent and does not cause graft versus host disease (GvHD) while they exhibit a potent graft versus tumor effect [2]. It is known that the activation of NK cells with cytokines such us interleukin (IL)15 enhances their effector function [3], which is very important for their anticancer effect. In this study we show a nanoformulation, based on a biocompatible, biodegradable and traceable nanomaterial, which selectively enhance NK cell function. This nanoformulation mimics the trans-presentation [4] of IL15 to NK cells by monocytes or Dendritic Cells (DCs) *in vivo* (Fig. 1).

We synthesized two elements to fabricate this novel nanoformulation: 1) recombinant human his-tagged IL15 (hIL15HIS) expression was optimized to obtain larger amounts of protein and in the active monomeric form; 2) spherical hydrophobic Iron Oxide NPs (IONPs) were used; IONPs were synthesized by thermal decomposition and then hydrophilized with PEG phospholipids. Activation of human NK cells from Pheripheral Blood Mononuclear Cells (PBMCs) was explored by incubating the cells with the nanoparticle formulation (IONP@hIL15HIS) compared with corresponding controls. We demonstrated *in vitro* that IONP@hIL15HIS is able to enhance NK cell mediated-activity in a similar way, or even slightly stronger, than standard activation protocols.

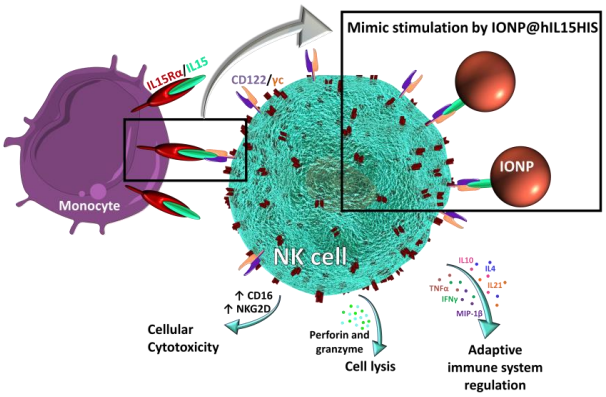

Figure1. Scheme of IL15 transpresentation. On the left it is shown the transpresentation of IL15 by a monocyte. In the box on the right, it is shown how we try to mimic the *in vivo* transpresentation by IONP@hIL15HIS.

Keywords: nanotherapy, immunology, cancer, protein expression

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Enzymatic Engineering of Acyltransferase LovD

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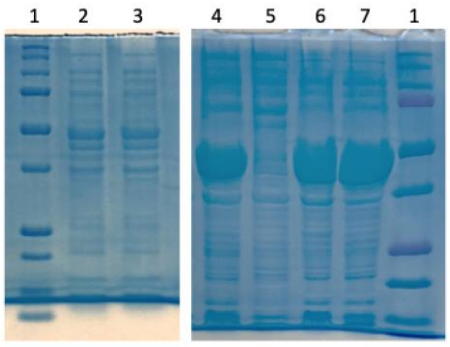
The natural acyltransferase LovD is involved in lovastatin synthesis [1]. The 29-mutant variant recently obtained by directed evolution by Tang and Codexis, Inc (LovD9) accepts a small free acyl thioester eliminating the need for its natural acyl carrier protein partner LovF [2]. This enzyme is 1000-fold more efficient in the synthesis of simvastatin than the wild-type LovD. Computational studies revealed that evolution fixes the active site in the proper arrangement for catalysis, overriding the dependency on protein-protein interactions occurring in the native variant.

A new pool of computationally designed variants was generated in order to probe the mechanisms of laboratory evolution and test the ability to achieve divergent but catalytically efficient engineering pathways. These designs were screened through different computational filters and the most promising candidates were selected for experimental validation.



PAGE 12% of protein extracts. 1: Molecular weight marker. 2, 4, 6: Insoluble fractions of LovD9, LovD9-3 and LovD9-4, respectively. 3, 5, 7: Soluble fractions of LovD9, LovD9-3 and LovD9-4, respectively.

Ten selected mutants were expressed in *E. coli* (together with LovD and LovD9 as controls) and their activity was screened using HPLC. One of them was not expressed, another one was expressed in an insoluble form, two were expressed in a partially soluble form and the other six were expressed in a completely soluble form. Unfortunately, none of them showed enzymatic activity yet, whereas LovD and LovD9 showed a reduced and high activity in simvastatin production, respectively.

Keywords: LovD, Simvastatin, cholesterol, catalysis, evolution.

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Functional nanostructured materials based on designed proteins

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Nowadays state-of-the-art in biotechnology is capable of manipulating the physicochemical properties at molecular scale. Bottom-up design of complex functional nanostructures is of great interest because it allows to synthesize organized structures using the intrinsic self-assembly properties of simple components. In this sense, repeat proteins are useful tools for this task due to their modular and hierarchical structure that can be the basis to construct complex supramolecular assemblies.

Previously, we have reported the formation of self-assembled protein films with proteins organized due to head-to-tail and side-to-side interaction, providing protein directionality[1,2]. Here, we take a step beyond generating functional films based on repeated proteins with applicability in different fields such as optics and heterogeneous catalysis.

In particular, we show the formation of nanopatterned protein films labeled with fluorescent dyes to produce Amplified Spontaneous Emission (ASE) and Distributed Feedback (DFB) lasing relevant for sensing. In parallel, protein films are used to entrap active enzymes, without apparent loss of activity. This film is used to generate energy through the catalase enzymatic activity by coupling the film to a piezoelectric device sensitive to the pressure changes of the produced oxygen.

Keywords: Designed proteins, functional protein materials, biooptical devices, biocatalytic materials, nanopatterning.

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Modulating the Conformational Plasticity of Tetraspanin CD81 by Ligand Binders and its Implication in Cellular and Viral Processes.

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CD81 is a member of the Tetraspanin family, membrane receptors that cluster into microdomains (TEMs) to mediate an enormous variety of physiological processes. However, despite its importance in the normal function of cellular lines as the immune system, CD81 is key in pathological processes such as viral entry and budding. In particular, CD81 is an active player in the internalization of Hepatitis C Virus (HCV) via clathrin-mediated endocytosis (1,2).

Previous structural studies on CD81 head subdomain ($CD81_{LEL}$) show that it can adopt a closed compact fold or open extended conformation depending on the environmental conditions (3-5). Here, in the context of HCV infection we have investigated at molecular level how different endosomal pH (from 7.4 to 4.6) influence the conformational switching of CD81LEL. We hypothesize this flexibility could be exploited by HCV to trigger conformational changes in its glycoproteins to set the time for fusion in the cell (6).

Our structural studies and molecular dynamics simulations i) suggest a new model of binding between E2 and CD81; and ii) point that the structural tuning of $CD81_{LEL}$ could be driven by protein-solvent interactions. Detailed energy analysis at different endosomal pH conditions highlight residues E188 and D139 to guide the $CD81_{LEL}$ switching, as their energy contribution to the protein-solvent interactions is highly destabilizing. *In silico* mutations, E188Q and D139N, recover the stability of the system within the solvent.

Furthermore, ongoing ligand screening studies will help also to shed light about the importance of the plasticity of $CD81_{LEL}$ in HCV infection.

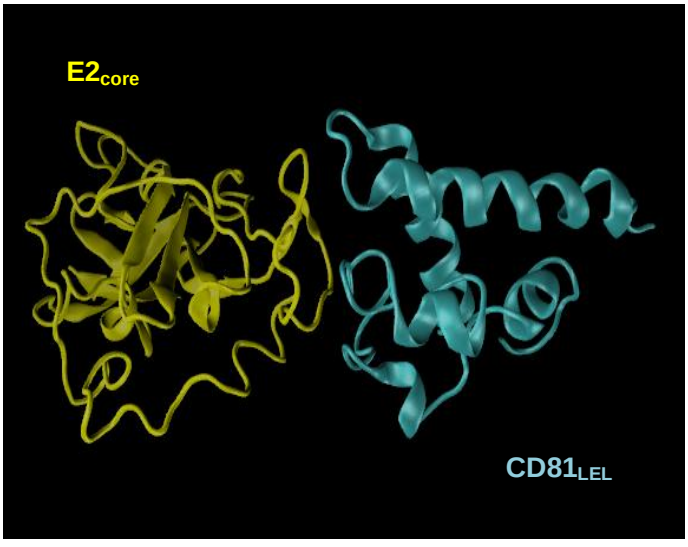

Figure 1. Molecular dynamics simulation of $CD81_{LEL}$ (5) in complex with $E2_{core}$ (7) glycoprotein.

Keywords: tetraspanin, CD81, molecular dynamics, E2, ligand screening

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Designed metallo-tags for probing natural systems

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Protein trafficking plays a pivotal role in maintaining a healthy proteostasis in cells, and its disfunction is inextricably linked to disease states. A growing body of studies has now defined multiple recycling pathways from endosomes to the trans-Golgi network (TGN) and the plasma membrane (PM). The retromer complex is a key component of the endosomal protein sorting machinery and operates by recognising specific membrane proteins termed as cargoes. These are concentrated into discrete regions of the endosomal membrane where coated tubule-vesicles are formed to transport cargo proteins to the appropriate destination.[1]

We work on the design of peptide tags as a toolkit to study distinct mechanisms for cargo sorting into tubule-vesicles *in vitro* reconstituted system. More specifically, taking advantage of natural diversity and specificity of natural effectors, we developed novel fusion tags as fluorescence and electron microscopy tracking tools. In particular, we focus on the design of protein scaffolds to stabilise metal nanoclusters and nanoparticles as tags for fluorescence microscopy and electron microscopy. Indeed, metal nanoclusters (NCs) exhibit very interesting optical, electronical and chemical properties, including strong photoluminescence, electron dense metal core, excellent photostability and good biocompatibility, while having nanometric size. We have previously demonstrated that designed consensus tetratricopeptide repeat (CTPR) proteins are suitable candidates as templates for the stabilization of metal nanoclusters.[2]

Here we design specific protein-NC hybrids for tracking the retromer-dependent retrograde cargo trafficking pathway.

Keywords: membrane trafficking, retromer, nanoclusters, repeat protein, tagging and imaging tools

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Innovatives Methodologies to Develop Porous 3D Scaffolds Based on PEDOT and CNT for Tissue Engineering

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Carbon Nanotubes (CNT) are one of the most promising nanomaterials to be used in tissue engineering, especially, as previously reported by our group, to interface with electrically active tissue, such as neuronal and cardiac tissues. Their inherent electrical properties and cylindrical shape are the key features to improve and boost the neuronal growth and functionality.[1]

Tissue engineering requires the manufacture of tridimensional scaffolds to be implanted, mimicking the tissue of interest to develop the functionality of the region replaced. Nevertheless, CNTs are not suitable to create three-dimensional structures by themselves; therefore polymers can be used as matrix to hold the CNTs in a 3D structure. As first proof of concept, our group developed CNTs-based 3D scaffolds using PDMS as the matrix, which was useful to evaluate the effect of the CNTs in the third dimension in neurological tissue.[2]

Poly (3,4-ethylene dioxythiophene), or commonly known PEDOT, is one of the most used conductive polymers due to his high conductivity, stability and biocompatibility. The design of implants based on this kind of conductive materials offers the opportunity to reduce gliosis, improve the adaptability and increase the charge-transfer energy. However, very little is reported about the combination of conductive polymers and CNTs, and to the best of our knowledge, only 2D films have been synthesized and tested in vitro. [3]

In this work, we have adapted two known methodologies to polymerize conductive polymers to our 3D systems: chemical oxidation, which includes the Vapor Phase Polymerization (VPP)[4] and polymer dispersion in solution, and electrochemical deposition. We present the use of derivatives of these methodologies in order to create 3D structures taking into account considerable characteristics as the controlled composition and porosity, conductivity, mechanical toughness and biocompatibility to be applied in electroactive cells as neurons or cardiomyocytes.

Keywords: PEDOT, CNT, Tissue engineering, Neurons, VPP

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High Yield Synthesis of Monodisperse Gold Colloids via Micellar Modification

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The objective of the study was to exploit the use of additives with Hexadecyltrimethylammonium Bromide (CTAB) to improve the existing synthesis protocols for obtaining highly monodiserse gold colloids for their application in biomedical studies.

In our primary study, we prepared single crystalline small gold nanorods (AuNR) using n-Decanol as a co-surfactant. The Decanol altered the rigidity of the CTAB micelle and promoted facet selective growth of Au seeds at optimum decanol/CTAB ratio. Tuning the conditions for the symmetry breaking step of Au seeds separately from overgrowth step yielded highly monodisperse small AuNR of dimensions 21nm in length and ~7.5nm in width.[1] These as-synthesized small AuNR due to their smaller dimensions have higher absorbance to scattering cross section ratio which make them an ideal candidate for application in photo thermal therapy.[2] Moreover, AuNR of different aspect ratios with narrow plasmon bands could be prepared using these small NR as seeds.
In our second study, we modified the well known Stöber method for preparation of Au@SiO_2 core shell NP by using Binol in presence of CTAB during silica growth. Here, we were able to finely tune the silica shell thickness over a range of 2nm to 6nm around AuNP of different sizes and shapes. This optically transparent and chemically inert silica shell provides thermal and chemical stability to the Au core from agglomerating and losing the optical properties, due to the direct interaction with analyte molecule.[3] Moreover, the highly homogeneous ultra thin silica shell will prevent interference from the SERS active core during sensing while providing ample amplification in the raman signal from the produced electromagnetic field enhancement.[4]

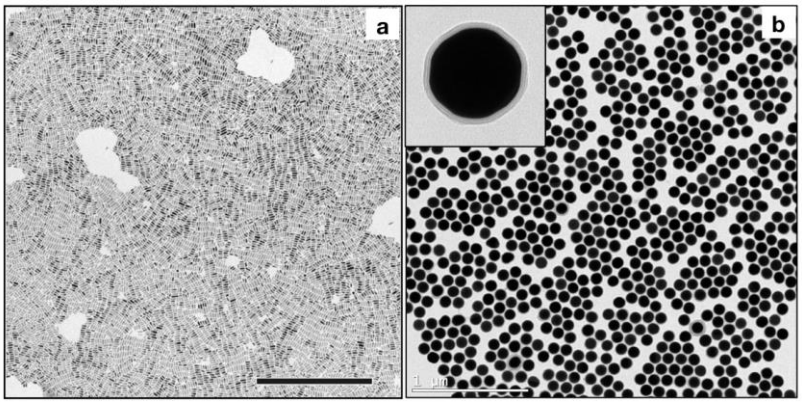

Figure: a) TEM image of small AuNR of 21nm length & 7.5 nm width (scale bar: 500nm); and b) Au@SiO_2 core shell NP with ultra-thin silica shell of 6nm over 65nm AuNP (scale bar: 1µm).

Keywords: Gold Nanorods, Symmetry Breaking, Core Shell Nanoparticles, SERS.

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New generation of 3D polymeric scaffolds based on carbon nanotubes doped with biomolecules.

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In the last decade, carbon nanotubes (CNTs) have become one of the most used and leading edge materials in many engineering fields. Their popularity arises from their unique mechanical, electrical and thermal properties and, in biomedical field, they have been proven to be a valuable tool with application ranging from drug delivery to tissue engineering.[1][2] Promising results arise especially when these materials are applied on the interface with electrically active cells as cardiomyocytes and neurons.[3] Moreover embedding CNTs in a 3D polymeric matrix that can be shaped at will, makes these materials more feasible for *in-vivo* applications.[4] Conductive polymers have been use in this way to help the functional reconnection of damaged tissue.

On the other hand the functionalization of nanomaterial can open the way to a wider range of application. The introduction on different moieties on the CNTs outer wall leads to a change in their physicochemical properties that can have a strong influence on the interaction of living tissue with the nanomaterial.

With this in mind carbon nanotubes have been functionalized with different small molecules and biomolecules of interest. The functionalized CNTs have been used in 2D beds and enclosed in 3D polymeric scaffold for *in-vitro* cell cultures.

Keywords: Carbon nanotubes; conductive polymers; tissue engineering.

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Optical properties of gold nanoparticle superlattices

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Metallic nanoparticles (NPs) display plasmonic properties, allowing them to confine electromagnetic fields to the nanoscale, which can be applied to a wide variety of technologies. The size, shape and composition of the NPs specifically define the wavelength, at which light is absorbed or scattered due to interaction with the metal electrons, and hence, the properties of electromagnetic field enhancement. One strategy that leads to an increase in the performance of plasmonic NPs comprises their arrangement into periodic structures. Such periodic structures feature so-called Rayleigh anomalies, which occur when the incident light is diffracted in-plane. Light diffraction follows the well-known grating equation, which depends on the wavelength of light and the period of the lattice. If the plasmonic resonance of the NPs in the lattice matches the Rayleigh anomaly wavelength, their absorption increases remarkably, giving rise to so-called lattice plasmons [1].

Generally, periodic nanostructures featuring lattice plasmons are fabricated by electron beam lithography. We studied an alternative strategy, which consists of a bottom-up approach for creating periodic structures of pre-formed gold NPs. This thesis revolves around a process for the capillary-assisted self-assembly of differently shaped NPs into superlattices (Figure 1). This presentation will focus on the ability of tuning lattice plasmons by means of changes in the lattice period, as well as on the effect of NP shape on the optical properties of the formed superlattices.

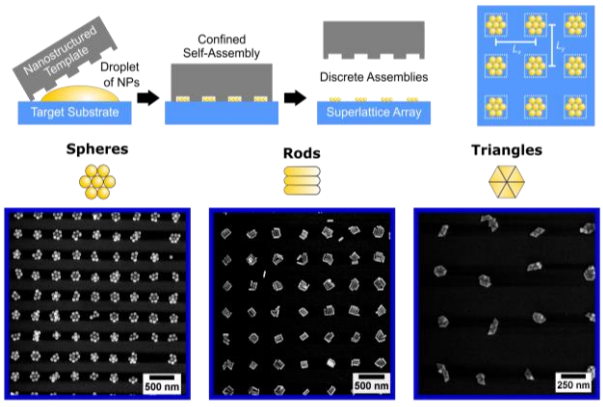

Figure 1. Schematic representation the capillary-assisted self-assembly process and SEM images of superlattices

Keywords: Gold Nanoparticles, Self-Assembly, Lattice Plasmons, Superlattices.

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Analysis of the tumor microenvironment by surface-enhanced Raman scattering

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Cancer cells and the stroma create dynamic pseudo-organs that contain a unique niche with distinct biochemical and physiological properties. The metabolic rewiring that sustains cancer cell growth and proliferation has a profound impact on the properties of this microenvironment, to an extent that monitoring such perturbations could harbor diagnostic and therapeutic relevance. This growing interest has inspired the development and application of novel technologies with sensitivity and resolution to monitor metabolic alterations in the tumor microenvironment. In this context, surface enhanced Raman scattering (SERS) appears as a useful tool for label-free detection and imaging of diverse molecules of interest among the extracellular components.

This PhD thesis focuses on the application of nanostructured plasmonic substrates comprising micropatterned Au nanoparticle superlattices to the precise detection by SERS, of selected tumor metabolites which shape the cancer landscape, such as kynurenine, tryptophan and purine derivatives. SERS allowed the identification of the specific spectral fingerprint of these probe molecules in contact with the plasmonic nanostructure, offering unambiguous identification of analytes with multiplexing capability. Moreover, we employed this plasmonic substrate to monitor *in situ* the tumor response to different stress conditions in 3D models with no need for sample preparation.

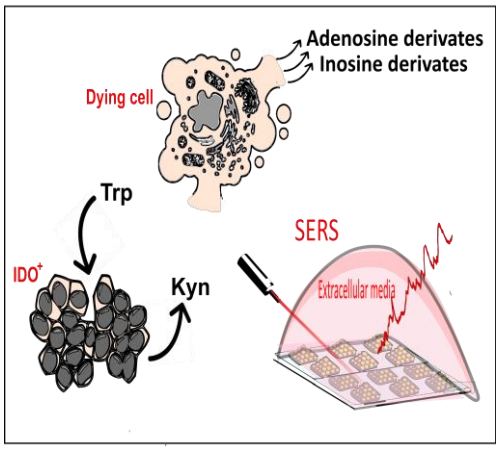

Figure 1. Detection of tumor metabolites by Surface enhanced Raman scattering

Keywords: *SERS, plasmonic substrates, tumor microenvironment, tumor metabolism, 3D cell culture.*

Development of bioanalytical assays based on antibodies modified with nanoclusters

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Nanoclusters have attained great attention in the last few years due to their impressive size dependent optical and chemical properties. Nowadays a large number of methods using proteins as scaffold have been developed for NCs synthesis. However, up to now no synthetic method has been described using an antibody as scaffold. Antibodies are recognition elements used in immunoassays (ELISA) or immunoblots. Its structure highly like that of proteins, makes antibodies suitable biomolecules to act as templates for the incorporation of NCs. Usually the synthetic conditions for the synthesis of NCs stabilized with proteins require extreme conditions of pH or temperature. These conditions cause the denaturalization of the proteins and end up in the loss of their biological functions

In this work we present the first method for the synthesis of photocatalytic or catalytic NCs using antibodies as scaffold and the first immunoassay carried out using antibodies modified with NCs. The synthesis of antibodies modified with NCs is performed under physiological conditions, which do not affect the antibody structure. The resulting antibodies still maintain the affinity for target antigens.

For this issue, semiconductor compounds were chosen as a suitable material for this issue because they exhibit photocatalytic activity. They can generate reactive oxygen species (ROS), as hydrogen peroxide (H_2O_2), triggered by the exposure to ultraviolet light and to oxidize typical peroxidase substrates [1]. Bimetallic nanoclusters are also potentials candidates for the modification of an antibody due to its peroxidase-like activity with peroxidase substrates in the presence of H_2O_2 [2].

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Efficient MALDI-MS array based on Functionalized CVD Graphene with Carbohydrates as Potential Sensing Platform

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Mass spectrometry (MS) is a valuable tool for functional genomic, proteomic, and glycomic studies. Particularly, the combination of MS with microarrays is a powerful technique for analyzing the activity of carbohydrates and identification of carbohydrate-binding proteins (lectins) on cell surface from complex matrices. On the other hand; graphene exhibits high desorption/ionization efficiency, good conductivity and optical transparency, thus standing as a promising candidate as component for matrix-assisted laser desorption/ionization (MALDI) platforms. Besides, the chemical functionalization of graphene increases the adsorption capability of functional biomolecules (e.g. receptors), resulting in very stable interfaces. Taking advantage of the graphene properties, herein, several modified chemical vapor deposited graphene (CVDG)-based arrays with specific carbohydrates were developed in different substrates, as potential sensing platforms. The resulting arrays were fully characterized by MALDI-MS analysis and, in some cases, optical microscopy.

Keywords: graphene, LDI, mass spectrometry, carbohydrate microarrays, lectin.

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Supramolecular polyamine Nanocarriers for siRNA delivery in Breast Cancer Therapy

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In recent years, RNA interference (RNAi) technologies have emerged as a promising therapeutic tool for the treatment of different diseases[1]. For example, siRNAs can be applied to many cancer types as they can inhibit the expression of any gene of interest, being highly specific. However, a major hurdle for clinical translation is to find a suitable and safe delivery vehicle for the siRNA. Unprotected siRNAs are unstable in bloodstream due to nuclease degradation, and are not able to cross cell membranes[2]. Thus, the proposal of this Thesis is to develop and produce a novel nanocarrier for delivering siRNA systemically. This goal is going to be achieved following two strategies. On one hand supramolecular polyamine nanocarriers (PANs), based on the complexation of the phosphate groups of siRNAs with primary amines from PolyAllylamine Hydrochloride (PAH), a polyelectrolyte with MW15KDa. As second strategy, siRNAs will be complexed to Single-chain polymer nanoparticles (SCPNs), based on intramolecular cross-linking of dextran positively charged, driving also to an electrostatic interaction. To improve silencing efficacy and reduce toxicity carriers will be chemically modified. Carriers and polyplexes are characterized by Dynamic Light Scattering and Electron microscopy. The delivery efficiency of these polyplexes is evaluated *in vitro* by Flow Cytometer. The decrease in fluorescence of GFP protein in A549-GFP cell line is quantified after treatment with anti-GFP siRNA. Cytotoxicity of polymers nanoparticles and complexes is evaluated as well by MTS assay. Further work will focus on the use of the polyplexes in the treatment of breast cancer cells by silencing the gen CD47. Gen responsible of the growth, proliferation and aggressiveness of metastasis[3].

Keywords: siRNA, nanocarrier, polyplex, PAH, single-chain

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Biological fate on nanomaterials with nuclear imaging

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After the emergence of nanotechnology, many different types of nanoparticles (NPs) are being used in the biomedical field to encapsulate drugs that are not suitable for direct delivery. Several strategies have been used for the encapsulation of drugs using NPs, where the drugs can be linked to the core or surface of the NP by covalently, electrostatics or hydrogen bonding interactions. In recent years, small interfering RNA (siRNA) has emerged as a promising treatment strategy for various genetic diseases. However, the degradation of siRNA after administration into blood stream of human and animals readily occurs. Hence, a suitable and safe delivery vehicle is required for the pharmacokinetic study of siRNA. One possible strategy is the use of NPs based on the self-assembly of polyamines and phosphate ions as a potential system for siRNA distribution. Here, we describe the use of poly(allylamine hydrochloride) (PAH) as a carrier of siRNA and the application of radiollabelling followed by Positron Emission Tomography (PET) imaging to study its biodistribution *in vivo*. The ^{18}F-labelled SiRNA was prepared by the reacting the amino group-modification of siRNA with 6-[^{18}F]fluoronicotinyl-2,3,5,6-tetrafluorophenyl ester ([^{18}F]FPy-TFP), which was prepared as previously reported [1]. *In vivo* PET Imaging studies in rodents after intravenous administration clearly showed a different biodistribution between free [^{18}F]-siRNA, which was rapidly eliminated via urine, and PAH/[^{18}F]-siRNA nanoformulations, which showed significant accumulation in the liver, the spleen and the lungs (Fig. 1).

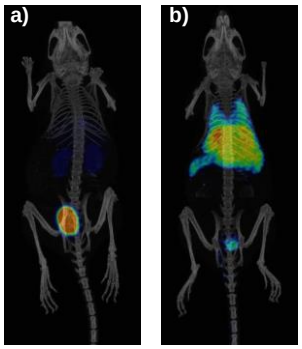

Figure 1. In vivo biodistribution studies of a) [^{18}F]siRNA, and b) PAH/[^{18}F]SiRNA in healthy mice using PET-CT. Images show the biodistribution after intravenous administration at time 30 min.

Keywords: siRNA, nanoparticles, genetic therapy, medical imaging, PET imaging

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Glycoengineering of Exosomes

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Exosomes are extracellular vesicles that are secreted by cells and contain biological material that reflects their cell of origin.[1] There is great interest in using exosomes in regenerative medicine and targeted drug delivery but elements of their biology remain unclear, particularly those elements relating to the role of exosome glycosylation during uptake by recipient cells.[2] To investigate this phenomenon, we have used glycosidase enzymes, glycodendron click chemistry and small molecule inhibition to produce glycoengineered exosomes from a range of cell lines. The glycosylation profiles of these models were characterised using lectin microarray technology to verify glycoengineering efficacy and further vesicle characterisation performed with electron microscopy, Western blot, nanoparticle tracking analysis and dynamic light scattering. Functional effects of glycoengineering on exosome uptake was assayed using flow cytometry, immunofluorescence microscopy, high-content screening and a novel plate-based assay using a genetically engineered luciferase tag.[3] We found that desialylation of exosomes via neuraminidase and de-N-glycosylation through PNGaseF caused changes to uptake, with exact responses varying with cell type.[4] Vesicle characteristics were preserved upon glycosidase treatment, implicating glycans in this process. Elsewise, engineered hypermannosylation of exosomes by kifunensine or click-conjugation of mannose dendrons appeared to increase uptake *in vitro*. Desialylation with a sialyltransferase inhibitor produced uptake incompetent vesicles. Our findings contribute to a body of work that implicates vesicle glycans as important determinants in their uptake by cells. Such work is impactful considering efforts to translate exosomes to the clinic as process scale-up may impact glycosylation and therefore product efficacy and specificity.

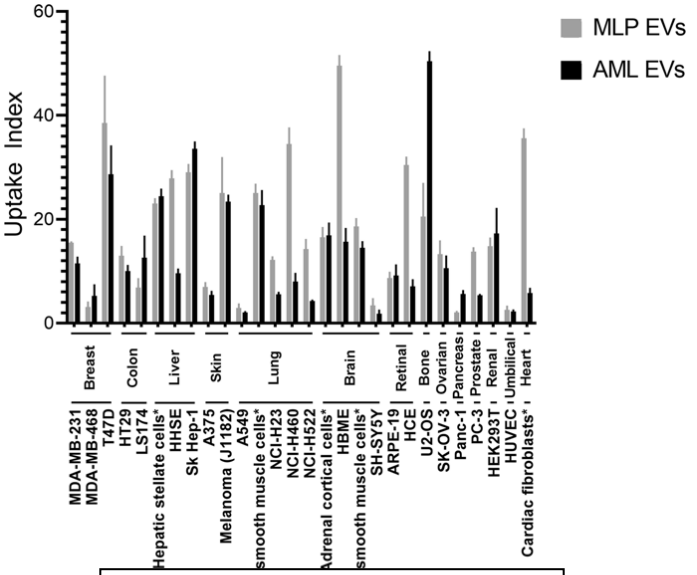

Figure 1. High-content uptake analysis of native EVs incubated with a panel of 28 different human cell lines.[3]

Keywords: exosomes, extracellular vesicles, glycoengineering

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Translocation, biological fate, stability and effective dose of engineered nanomaterials for nanosafety studies

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Nanomedicine aims to develop nanoscale carriers capable of transporting and releasing therapeutic agents to targeted organs or cells in a controlled way. The biological fate of nanocarriers, their aggregation, degradation and their interaction with biomolecules in biological matrixes are issues that must be taken into account when designing nanocarriers for biomedical applications[1]. These issues play a fundamental role in determining if the nanocarrier will fulfill the goal for which it has been designed, such as reaching a specific organ or delivering a drug with certain kinetics.

The main objective of this thesis is to study the biological fate, degradation and formation of protein corona for two soft matter nanocarriers: polyamine/siRNA complexes and poly(NIPAM-co-MAA) hydrogels; and two model nanoparticles: gold nanoparticles and polymer brush engineered ZnO nanoparticles (Fig 1). Flow cytometry, fluorescence correlation spectroscopy (FCS), fluorescence life-time imaging microscopy and confocal Raman microscopy have been used to trace nanomaterial fate and protein corona formation intracellularly[2,3]. By FCS we are able to trace how siRNA/polyamine complexes degrade inside cells. In addition, FCS allows us to study the interaction of lectins with protein corona around gold nanoparticles. Using confocal Raman microscopy, we could assess the impact of brush coatings on ZnO degradation inside cells. Overall, the results obtained from this work provide novel insight into the intracellular behavior of 4 distinct nanocarrier systems, which can be applied to improve their potential use and safety in biomedicine.

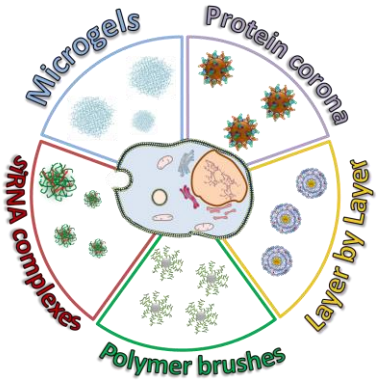

Fig 1: Scheme of the main systems investigated in the thesis

Keywords: Soft Matter Nanotechnology, Biological Fate, Fluorescence Correlation Spectroscopy, Flow Cytometry, Fluorescence Life-Time Imaging

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Nanoemulsions as drug carriers: preclinical evaluation of pulmonary delivered hydrophobic drug-loaded nanocapsules.

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NPs have emerged as ideal drug nanocarriers because they can prolong the residence time of poorly soluble/rapidly metabolized drugs in the lungs after inhalation [1]. In this work, we test cobalt bis(dicarbollide) (COSAN)-stabilized oil-in-water (o/w) nanoemulsions as potential nanocarriers using [^{18}F]Fluoroestradiol (FES) as model drug. To prepare the nanoemulsions, a dissolution containing FES in triglyceride oil was poured into an aqueous COSAN solution under sonication. For Imaging experiments, a solution of FES (4.44±0.74 MBq) either as free drug (solution in physiologic saline) or encapsulated in COSAN-stabilized nanoemulsions was administered intratracheally (IT) to female SD rats, and dynamic PET images were acquired for 40 min. Free FES showed fast lung clearance, with only 50% of the activity present in this organ at t=2 min post administration. This value decreased to 10% at t=10 min. Significant accumulation in the liver and progressive translocation to the gastrointestinal was also observed (Figure 1). The residence time of FES in the lungs could be prolonged by encapsulation with COSAN-stabilized nanoemulsions, with more than 70% of the radioactivity still remaining in the lungs at t=10 min after administration. These results confirm the suitability of our nanoemulsions to alter the biodistribution pattern of hydrophobic drugs. The prolonged residence in the lungs may find application in the treatment of lung diseases.

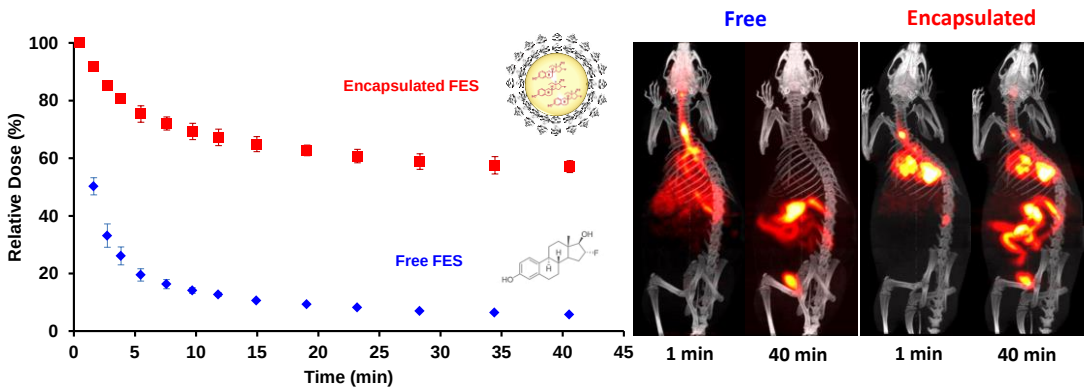

Figure 1. left: Percentage of administered dose in lungs at different time points after intratracheal administration of free (blue) and encapsulated (red) FES; **right:** representative PET images at 1 and 40 min after intratracheal administration.

Keywords: Nanoemulsions, lungs, intratracheal administration, nanocarriers, PET

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A pretargeting strategy for multifunctionalized gold nanoparticles (GNPs) as boron drug delivery agents

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BNCT is a promising cancer treatment exploiting the neutron capture capacity and subsequent fission reaction of boron-10. However drugs need to deposit a sufficient number of ^{10}B specifically in tumor cells.[1] We present a pretargeting strategy[2] using *trans*-cyclooctene conjugated antibodies (TCO-mAbs) capable to undergo a bioorthogonal 'click reaction' *in vivo* with GNPs containing tetrazine derivatives and cobalt *bis*(dicarbollide) (COSAN) boron cluster. Radiolabelling of TCO-mAb and functionalized GNPs was carried out to enable *in vivo* visualization using Positron Emission Tomography (PET).

The mAb (Trastuzumab) was functionalized with TCO-NHS, the chelator DFO-bz-NCS and finally radiolabeled with ^{89}Zr. Copper-64 doped GNPs were prepared by mixing $HAuCl_4$, $^{64}CuCl_2$ and Amino-PEG_{5000}-SH in water in the presence of $NaBH_4$. COSAN-SH and Tetrazine-PEG_4-NHS were subsequently attached to the surface. Characterization followed after purification using UV-Vis, DLS, Z-potential, ICP-MS and TEM. *In vivo* studies were performed on BT474 breast cancer xenograft NOD SCID mice models and PET imaging at different time points.

Uniform, 4nm core sized and stable GNPs functionalized with COSAN, PEG and tetrazine were obtained. Biodistribution studies of mAb and GNPs showed tumor uptake values of 24.5 %ID/cm^3 (46 h p.i.) and 7.8 %ID/cm^3 (16 h p.i.) respectively. The pretargeting strategy resulted in no significant increase in tumor uptake and tumor-to-blood ratios when compared to animals injected with only the GNPs.

Keywords: pre-targeting, gold nanoparticles, antibody, BNCT, PET**.**

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Towards the synthesis of stable isotope labeled N-glycopeptides as internal standards for mass spectrometry based glycan analysis

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Aberrant glycosylation of tumor surface glycans and serum glycoproteins is hallmark of many cancers. The clinical appreciation of this promising class of biomarkers has however lagged behind due to a lack of robust and rapid methods the analysis of serum glycans and glycopeptides. We have recently developed methodology for the absolute quantification of glycans and glycopeptides by MALDI-TOF MS using internal stable isotope labeled standards[1]. As the chemical synthesis of N-glycans remains a time-consuming and elaborate task we have looked into natural sources as starting material for obtaining N- glycans in quantities required for the preparation of glycan libraries and glycoconjugates. Towards this end we have isolated a sialylglycopeptide (SGP) from hen´s egg yolk in gram scale[2,3] (Figure 1). Here we describe our results of our studies on the high level purification of SGP by HPLC-LCMS and its further enzymatic and chemical transformation into free reducing glycans. (Figure 2) Finally, a first account of our studies on the synthesis of defined glycoforms of stable isotope labeled glycopeptides is given.

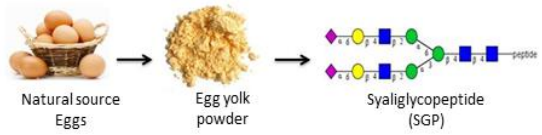

Figure 1. SPG isolation from hen's egg

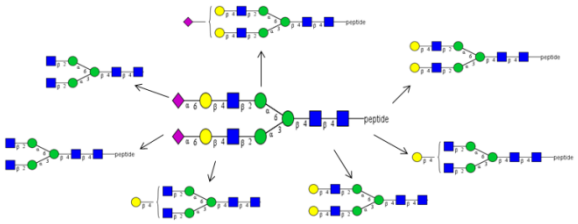

Figure 2. Chemical and enzymatic transformations on SGP

Keywords: N-glycans, isolation, HPLC, labelling.

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In vivo PET study of [^{11}C]*L*-Alanine and [^{11}C]*D*-Alanine in a mouse model of prostate cancer.

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Besides accelerated glucose metabolism, cancer cells present increased uptake of amino acids and incorporation into protein synthesis, enhancing the rapid proliferation and growth of tumor cells. Because of this, radiolabeled amino acids are excellent alternatives to 2-[^{18}F]Fluoro-2-deoxy-d-glucose (FDG) as PET tracers for oncology imaging. Here, we report the development of a methodology that enables the fully automated, enantioselective radiosynthesis of [^{11}C]*L*-Alanine and [^{11}C]*D*-Alanine using phase transfer catalysts of Schiff bases [1]. The preparation of these amino acids was carried out using a GE FX C_{pro} synthesis module. Both isomeric forms could be produced with non-decay-corrected yield above 10% in less than 40 min from the end of bombardment (including purification and reformulation). The radiochemical purity and enantiomeric excess values were >99% and >90% respectively. That radiochemical yields were sufficient to approach *in vivo* PET studies in a mouse model of prostate cancer, driven by the conditional deletion of the tumour suppressor Pten in the prostate epithelia. In vivo biodistribution studies in these rodents after intravenous administration, showed a clearly different biodistribution. Whereas *L*-Alanine presents an elevated uptake in prostate, liver and brown fat (Figure 1a), *D*-Alanine accumulates rapidly to the kidneys due to the elevated amount of DAO (D-Amino acid Oxidase) protein which is present in this organ (Figure 1b).

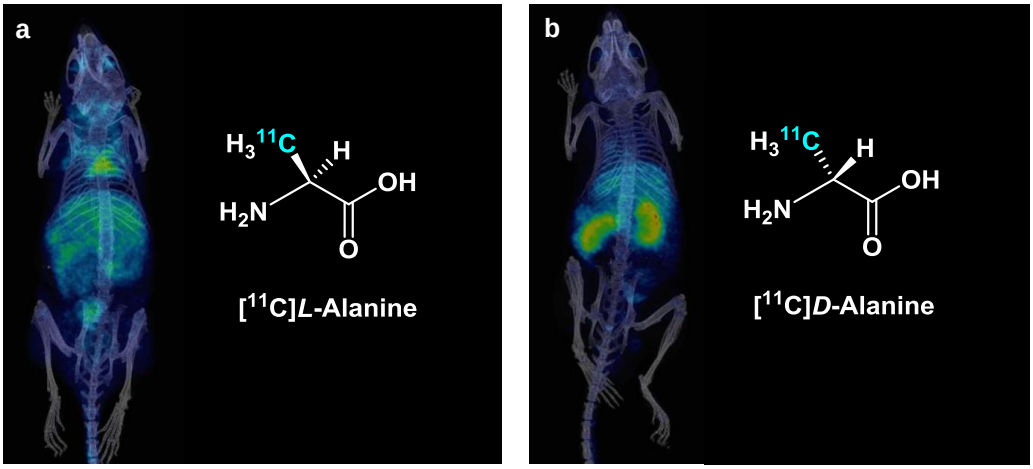

Figure 1. a) [^{11}C]*L*-Alanine and its biodistribution b) [^{11}C]*D*-Alanine and its biodistribution.

Keywords: Amino acids, PET Imaging, Asymmetric Radiosynthesis, Prostate Cancer

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Pharmacokinetics evaluation of new drugs with potential application in Duchenne muscular dystrophy

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Positron emission tomography (PET) is an ideal technique to investigate the pharmacokinetic properties of new chemical entities. In this work, we have tackled the pharmacokinetic evaluation of new triazole-based RyR calcium release channel stabilizers (AHK-1 and AHK-2, Figure 1a) with potential application in Duchenne muscular dystrophy using PET in combination with other *in vivo*/*ex vivo* studies. The drug candidates were labelled either with Carbon-11 via ^{11}C-methylation using [^{11}C]CH_3I or by reduction using tritium gas. Microsomes for *in vitro* metabolism prediction and healthy female Sprague-Dawley rats for *in vivo* studies were used. PET-CT dynamic imaging studies after intravenous and oral administration were combined with determination of arterial blood time-activity curves (TACs) and analysis of plasma samples by HPLC, the latter to investigate the presence of radiolabelled metabolites. Arterial TACs were obtained in continuous mode by using an in-house developed system. The ^{11}C-labelled compounds were obtained in average production time of 30 min with radiochemical yields between 5-15% and molar activity values between 40-140 GBq/μmol. The compounds showed fast blood clearance and fast metabolism in blood under no carrier added conditions, although metabolism was slower at higher administered doses. PET images (Figure 1b) showed accumulation of radioactivity in the liver and kidneys at early time points (0-9 min), and progressive accumulation in the gastrointestinal tract at longer times.

Keywords: RyR calcium release channel stabilizers, radiolabelling, arterial TACs, metabolism.

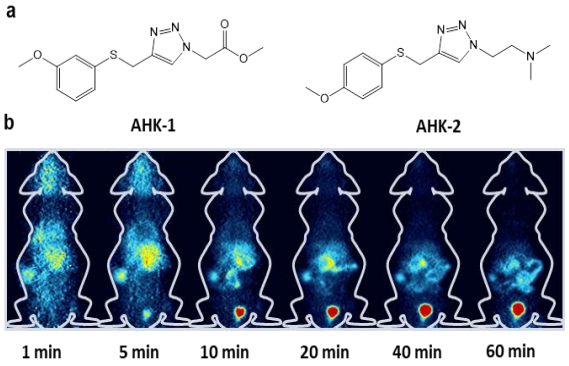

Figure 1. (a) Chemical structure of drug candidates AHK-1 and AHK-2; (b) PET images (coronal projections) at different time points after intravenous administration of AHK-2 at 1 μg/Kg.

Functional network characterization by resting-state fMRI in the cuprizone mouse model of multiple sclerosis

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Resting-state functional Magnetic resonance imaging (rsfMRI) enables de characterization of brain function and connectivity non-invasively [1]. Even though clinical applications of resting state fMRI are at an early stage of development, abnormal brain activity has been detected throught rsfMRI in several pathologies, such us multiple sclerosis. This autoimmune disease is characterized by the attack of the immune system to myelin sheaths, resulting in axon degeneration and neuronal loss. Nevertheless, myelin membrane can be spontaneously remyelinated by oligodendrocytes before the axon degenerates [2][3]. The unsuccessful and uncomplete remyelination observed in post-mortem human studies has triggered a high interest in developing remyelination therapies during the last decade.The aim our study has been to decipher the impact of myelin loss (demyelination) and regeneration (remyelination) in neuronal networks. Moreover, the effect of the potential remyelination capacity of clemastine has been assessed both at an anatomical and functional level in mice. To address this goal, we have exposed C57BL6/J mice to cuprizone containing diet (0.2%) for 5 weeks, leading to an extensive demyelination in white matter tracts. At this point, diet has been switched to an standard one enabling spontaneous remyelination. RsfMRI experiments have been longitudinally conducted during 10 weeks. We have observed how brain activity is increased (hyperconnectivity) at the first stage of demyelination, followed by hypoactivity at the peak of maximum demyelination. Interestingly, remyelination progressively enabled the recovery of damaged regions of the brain. The administration of clemastine for 2 weeks accelerated the remyelination process and enabled a prompt functional recovery of the mice.

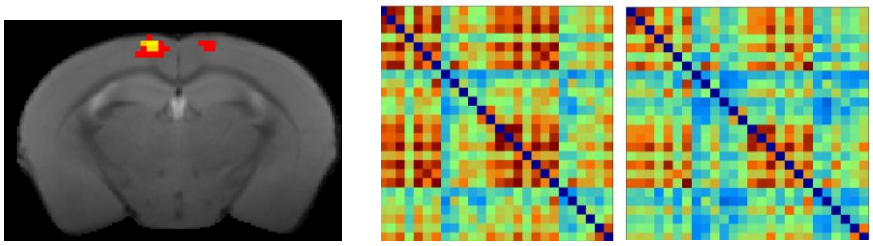

Figure 1: Administration of cuprizone for 5 weeks leads to decreased functional connectivity in the mouse brain.

Keywords: cuprizone, myelin, clemastine, remyelination, rs-fMRI.

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Magnetic Resonance Imaging evaluation of the role of hyperglycemia in experimental subarachnoid haemorrhage

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Spontaneous subarachnoid hemorrhage (SAH) is a devastating cerebrovascular disorder with high mortality and morbidity. It is mostly due to the rupture of an intracranial aneurysm[1]. The occurrence of hyperglycemia is a well-known phenomenon in various types of acute cerebral injury that have been linked with the worsen outcome after SAH[2]. Despite this, the mechanisms involved with this phenomenon are still not well understood. For this reason, the aim of this study is to evaluate the effect of hyperglycemia in a rat model of SAH using Magnetic resonance imaging and neurological evaluation.

In vivo MRI imaging studies using different sequences were carried out to evaluate the effect of hyperglycemia in bleeding, brain damage, BBB disruption and neurofunctional progression at days 1 and 3 after SAH. Hyperglycemia was induced by intraperitoneal injection of dextrose 30 minutes before SAH. Normoglycemic rats received vehicle. The neurological outcome after SAH was measured by a neurological test.

Hyperglycemic rats showed a lower survival rate at different days after SAH in comparison with normoglycemic animals (Figure 1). MRI studies showed a higher bleeding, edema, brain damage and brain barrier disruption together with a worsen in outcome in hyperglycemic rats in relation to normoglycemic ones. In addition, hyperglycemia induced a major impairment of neurofunctional evolution after SAH. These results suggest that high glucose levels in blood during SAH worsen bleeding, edema, brain damage and brain barrier disruption together with the neurofunctional outcome.

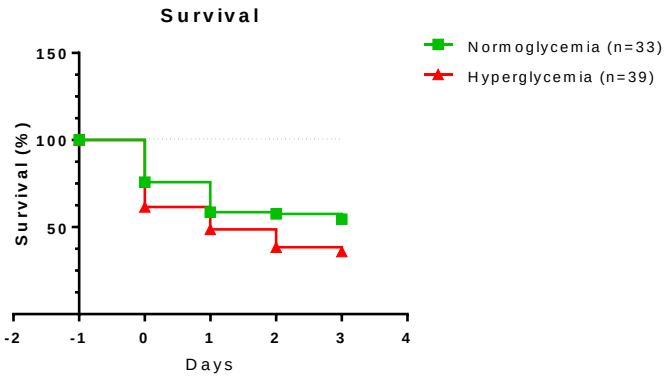

Figure 1. Survival rates in normoglycemic and hyperglycemic conditions

Keywords: Subarachnoid haemorrhage, hyperglycemia, MRI

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