# ABSTRACT BOOK

CRS Spanish Portuguese Local Chapter

## XII Spanish-Portuguese Conference on Controlled Drug Delivery

**Tailoring drug delivery systems to the patients' needs**

**COIMBRA, JANUARY 14-16TH 2018**

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# Welcome message and venue

## Dear Participants,

On behalf of the Board of the Spanish-Portuguese Local Chapter of the Controlled Release Society (SPLC-CRS), we are delighted to welcome you to the *XII Spanish-Portuguese Conference on Controlled Drug Delivery*, taking place at the Auditório da Reitoria of the University of Coimbra, on January 14-16, 2018.

The major theme of the conference will be on *Tailoring drug delivery systems to the patients' needs* – the translational potential of drug delivery systems and how they can tackle existing unmet medical demands.

You will have access to Plenary Lectures presented by ***Nicholas A Peppas,*** and ***Patrick Couvreur***, two well known leading researchers in the field of drug delivery and controlled release; forteen invited speakers from Portugal, Spain and Italy who will address topics in the following areas: Neurological disorders, Cancer, Diagnosis/Medical devices, Regenerative Medicine; Immunotherapy; Design of Monoclonal Antibodies, Vaccines and Viral Systems for Therapeutic Application; a Session fully dedicated to poster discussion, with the scientific committee; a Session with short talks selected from abstracts and a round table - *Bridging the Translational Gap Between Academia and the Patient* with the participation of portuguese pharmaceutical companies (Bluepharma and Blueclinical) and members of the Technology Transfer Office, University of Coimbra, Portugal and from an investment company, FOSUN.

We sincerely hope that you enjoy this scientific event, exchange new ideas and promote future collaborations.

We look forward to welcoming you at this event. Join us in Coimbra!

On behalf of the Board of SPLC-CRS,

*João Nuno Moreira*

*President of the SPLC-CRS*

# SCIENTIFIC PROGRAM

**14/01/2018**

**14.00-16.00** **Registration and setting up the posters**

**16.00** ***Welcome Session***

**João Nuno Moreira** (CNC/UC, FFUC), **Luís Menezes** (Vice-Dean of the University of Coimbra) and **María José Alonso** ("President elect" of the Controlled Release Society)

**16.15** ***Plenary Lecture***

**Nicholas A Peppas –** Protein, Antibody and siRNA Delivery Using Intelligent Polymers with Molecular Recognition Capabilities

Sc.D. Cockrell Family Regents Chair in Engineering, Department of Biomedical Engineering, McKetta Department of Chemical Engineering, Department of Pediatrics, Department of Surgery and Perioperative Care, Dell Medical School, Division of Pharmaceutics, College of Pharmacy Director, Institute for Biomaterials, Drug Delivery and Regenerative Medicine The University of Texas at Austin, USA

**Session Chair** - **María José Alonso** (CIMUS Research Institute, University Santiago de Compostela)

**SESSION 1 – Neurological Disorders**

**Session Chair - Alicia Rodriguez Gascón** (University of the Basque Country, Vitoria)

**17.00** **Ana Paula Pêgo –** Engineering Nanoparticles to Cargo Nucleic Acids to the Nervous System: Breaking Barriers with Nanotools

Instituto de Engenharia Biomédica and Instituto de Investigação e Inovação em Saúde, University of Porto

**17.30** **Elisa Garbayo Atienza –** Brain Regeneration in Parkinson's Disease using Micro/Nanoparticles

Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, Spain

**18.00** **Luís Pereira de Almeida –** Non-Invasive Gene Therapy of the Brain

Center for Neurosciences and Cell Biology; Faculty of Pharmacy, University of Coimbra, Portugal

**SESSION 2 – PhD Thesis Award**

**Session Chair – Dolores Torres** (Vice-President SPLC-CRS)

**18.30** **Award Announcement and Thesis Presentation**

**19.00** **Welcome Cocktail**

| 15/01/2018 | |
|---|---|
| 09.00 | ***Plenary Lecture*** |
| | **Patrick Couvreur –** Nanomedicines for the Treatment of Cancer and Neurological Disorders |
| | Member of the Académie des Sciences, Institut Galien Paris-Sud, UMR CNRS 8612, Université Paris-Sud, Université Paris Saclay, Châtenay-Malabry Cedex, France |
| | **SESSION 3 – Cancer** |
| | **Session Chair – António Almeida** (Faculty of Pharmacy, University of Lisbon) |
| 09.45 | **Davide Prosperi –** Impact of Ligand Moiety on Targeting and Anti-Tumor Effect of Antibody Nanoconjugates |
| | Department of Biotechnology and Biosciences, University of Milano Bicocca, Italy |
| 10.15 | **Maria Jesus Vicent –** Towards the Design of Personalised Polymer-based Combination Conjugates for Advanced Stage Breast Cancer Patients<br>Centro de Investigación Príncipe Felipe, Valencia, Spain |
| 10.45 | **António Duarte –** Targeting Notch Signaling in Mouse Tumor Models<br>Faculdade de Medicina Veterinária, Universidade de Lisboa |
| 11.15-11.45 | **Coffee-break and Speed Dating with Bluepharma** |
| | **SESSION 4 – SPLC-CRS Young Section** |
| | **Session Chair –Simón Pascual-Gil** (University of Navarra) and **Pedro Fonte** (Lusófona University, Lisbon) |
| 11.45-13.10 | **Short Talks Selected from Abstracts** |
| 11.45-11.52 | Eugénia Nogueira, ***Folate-targeted liposomes for rheumatoid arthritis therapy.*** Centre of Biological Engineering, University of Minho. |
| 11.52-11.59 | Jacinta Pinho, ***Targeting AQP3 using a nanotechnological approach for melanoma treatment.*** Research Institute for Medicines, Faculty of Pharmacy, Universidade de Lisboa. |
| 11.59-12.06 | Ainhoa Gonzalez-Pujana, ***Matrix-mediated gene expression regulation in mesenchymal stem cells immobilized in alginate-poly-L-lysine-alginate microcapsules.*** School of Pharmacy, University of the Basque Country. |
| 12.06-12.13 | Edorta Santos-Vizcaino, ***Use of genipin as a quantitative imaging*** |

***biomarker in implanted immunoisolation devices.*** School of Pharmacy, University of the Basque Country.

**12.13-12.20** João Conniot, ***Combination of Dendritic Cell-targeted Nano-vaccines with Immune Checkpoint Therapy for Melanoma.*** Research Institute for Medicines, Faculty of Pharmacy, Universidade de Lisboa.

**12.20-12.27** Laura Saludas, ***Enhanced biological functions of adipose derived stem cells in the injured myocardium of a rat myocardial infarction model when combined with neuregulin-loaded microparticles.*** Faculty of Pharmacy, University of Navarra.

**12.27-12.34** Iago Fernández-Mariño, ***Interaction of small and medium size chitosan nanocapsules with different subsets of immune cells.*** Center for Research in Molecular Medicine and Chronic Diseases, University of Santiago de Compostela.

**12.34-12.41** Duy-Khiet Ho, ***Squalenyl Hydrogen Sulfate Nanocarrier for Dual-Delivery of Anti-infective Quorum Sensing Inhibitor and Aminoglycoside Antibiotic Tobramycin.*** Helmholtz Institute for Pharmaceutical Research Saarland, Saarbrücken.

**12.41-12.48** Josephine Blersch, ***HT-Screening identifies light triggerable NP formulation for efficient in vivo non-coding RNA delivery in wound healing.*** Center for Neuroscience and Cell Biology, University of Coimbra.

**12.48-12.55** Branca Silva, ***Orodispersible films to treat a neurodegenerative disorder: clinical proof of concept.*** Bluepharma Indústria Farmacêutica and Faculty of Pharmacy, University of Coimbra.

**12.55-13.02** Nuno Fonseca, ***Tumor intracellular bioavailability of doxorubicin determines therapeutic efficacy of GLP grade nanoparticle targeted to nucleolin independently of systemic exposure.*** Center for Neuroscience and Cell Biology, University of Coimbra.

**13.02-13.09** Patrícia Albuquerque, ***Exosomes: tiny vesicles with great potential for MJD treatment.*** Center for Neuroscience and Cell Biology, University of Coimbra.

**13.10-14.00** **Lunch and Speed Dating with Bluepharma**

## 14.00-15.00 SESSION 5 – Poster Discussion

| | |
|---|---|
| | **SESSION 6 – Regenerative Medicine**<br><br>**Session Chair – Gorka Orive Arroyo** (University of the Basque Country, Vitoria) |
| **15.00** | **Lino Ferreira –** Drug Delivery in Regenerative Medicine<br><br>Faculty of Medicine, Center for Neuroscience and Cell Biology, University of Coimbra. |
| **15.30** | **Marcos Garcia Fuentes –** mRNA-Activated Reprogramming Matrices<br><br>Center for Research in Molecular Medicine and Chronic Diseases, University of Santiago de Compostela. |
| **16.00** | **Nuno Neves –** Highly Functional Nanostructured Surfaces and Devices for the Development of Effective Tissue Engineering Strategies<br><br>3B's Research Group - Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, Taipas, Guimarães. ICVS/3B's, PT Government Associate Laboratory, Braga/Guimarães. |
| **16.30-17.00** | **Coffee-break and Speed Dating with Bluepharma** |
| | **SESSION 7 – Diagnosis / Medical Devices**<br><br>**Session Chair – Ana Grenha** (Faculdade de Ciências e Tecnologia, Universidade do Algarve) |
| **17.00** | **Pedro Viana Baptista –** Versatile Gold Nanoparticles for Molecular Diagnostics in Leukemia<br><br>Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade NOVA de Lisboa, Campus Caparica, Portugal |
| **17.30** | **Manuel Arruebo –** Near Infrared Dye-labeled Polymeric Micro- and Nanomaterials: *in vivo* imaging and evaluation of their local persistence<br><br>Aragon Nanoscience Institute, University of Zaragoza, Spain |
| **18.00-19.00** | **SPLC-CRS GENERAL ASSEMBLY** |
| **21.00** | **CONFERENCE DINNER** |

## 16/01/2018

| | |
|---|---|
| | **SESSION 8 – Immunotherapy**<br>**Session Chair – Olga Borges** (CNC & Faculty of Pharmacy, University of Coimbra) |
| 09.00 | **João Gonçalves –** Antibody Engineering Strategies for Antiviral Neutralization<br>Research Institute for Medicines, iMed.ULisboa, Faculty of Pharmacy, University of Lisbon, Portugal |
| 09.30 | **Miguel Prudêncio –** Towards Clinical Evaluation of a New Malaria Vaccine<br>Institute of Molecular Medicine, Faculty of Medicine, University of Lisbon |
| 10.00 | **Helena Florindo –** Enhancing Cancer Immunotherapy via Nanotechnology-based Strategies to Engage Innate and Adaptive Immune Responses<br>Research Institute for Medicines, iMed.ULisboa, Faculty of Pharmacy, University of Lisbon, Portugal |
| 10.30-11.00 | **Coffee-break and Speed Dating with Blueclinical** |
| | **SESSION 9 – *ROUND TABLE* – Bridging the Translational Gap Between Academia and the Patient**<br>**Session Chair – Rogério Gaspar** (Faculty of Pharmacy & Institute for Bioengineering and Biosciences, University of Lisbon, Portugal) |
| 11.00-13.00 | **Laura Alho –** Technology Transfer Office, University of Coimbra, Portugal<br>**Cláudia Silva –** Bluepharma Indústria Farmacêutica SA and Luzitin, SA, Portugal<br>**Ricardo Cunha –** Blueclinical, Ltd, Portugal<br>**David Cristina –** Senior Investment Director, FOSUN |
| 13.00-13.15 | **Oral and Poster Communication Prizes**<br>**Concluding Remarks** |

# PLENARY LECTURES

**PL-1**

# Protein, Antibody and siRNA Delivery Using Intelligent Polymers with Molecular Recognition Capabilities

Nicholas A. Peppas

*Sc.D. Cockrell Family Regents Chair in Engineering, Department of Biomedical Engineering, McKetta Department of Chemical Engineering, Department of Pediatrics, Department of Surgery and Perioperative Care, Dell Medical School, Division of Pharmaceutics, College of Pharmacy Director, Institute for Biomaterials, Drug Delivery and Regenerative Medicine The University of Texas at Austin, USA*

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Engineering the molecular design of intelligent biomaterials by controlling structure, recognition and specificity is the first step in coordinating and duplicating complex biological and physiological processes. Recent developments in siRNA and protein delivery have been directed towards the preparation of targeted formulations for protein delivery to specific sites, use of environmentally-responsive polymers to achieve pH- or temperature-triggered delivery, usually in modulated mode, and improvement of the behavior of their mucoadhesive behavior and cell recognition. We address design and synthesis characteristics of novel crosslinked networks capable of protein release as well as artificial molecular structures capable of specific molecular recognition of biological molecules.

**Short Biography**:
Nicholas A. Peppas is the Cockrell Family Distinguished Chair in Biomedical, Chemical Engineering, Pediatrics, Surgery and Pharmacy at UT Austin. Works in biomaterials, polymer physics, drug delivery and bionanotechnology blending modern molecular and cellular biology with engineering and materials. He is a member of NAE, NAM, American Academy of Arts Sciences, NAI, Academies in France, Spain, Greece. NAE Founders Award 2012. Past president of IUSBSE, SFB and CRS. Impact of work at 100,000 citations (H=150). Peppas holds a Dipl. Eng. from the NTU of Athens (1971), a Sc.D. from MIT (1973), honorary doctorates from the Universities of Ghent, Parma, Athens, Patras and Ljubljana and honorary professorships at Sichuan Univ. and Tokyo Union Medical College.

**PL-2**

# Nanomedicines for the treatment of severe diseases

Patrick Couvreur

*University of Paris-Sud, Université Paris-Saclay Institut Galien, UMR CNRS 8612, 5 rue J-B Clément F-92296 Chatenay-Malabry (France)*

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Even if new molecules are discovered to treat severe diseases like cancers, the clinical use and efficacy of conventional chemotherapeutics is hampered by the following limitations: (i) drug resistance at the tissue level due to physiological barriers (non-cellular based mechanisms), (ii) drug resistance at the cellular level (cellular mechanisms), and (iii) non-specific distribution, biotransformation and rapid clearance of the drugs in the body. It is therefore of importance to develop nanodevices able to overcome these limitations.
This will be illustrated by various nanomedicine platforms developed in the laboratory:
- The design of biodegradable doxorubicin-loaded polyalkylcyanoacrylate nanoparticles for the treatment of the multidrug resistant hepatocarcinoma (a nanomedicine with phase III clinical trials ended) [1].
- The construction of nanoparticles made of metal oxide frameworks (NanoMOFs) [2,10], a highly hyperporous material obtained by the complexation of iron oxide clusters with diacids. The nanopores of this material may be designed according to the molecular dimension of the drug molecule to be encapsulated.
- The "squalenoylation" [3,4], a technology that takes advantage of the squalene's dynamically folded molecular conformation, to link this natural and biocompatible lipid to anticancer drug molecules [5] to achieve the spontaneous formation of nanoassemblies (100–300 nm) in water, without the aid of surfactants. Surprisingly, these squalene-based nanoparticles are using the circulating LDL as "indirect" carriers for targeting cancer cells with high expression of LDL receptors [6]. The application of the "squalenoylation" concept for the treatment of brain ischemia and spinal cord injury will be discussed too (Figure 1). The possibility to use other terpenes (natural or synthetic) than squalene to design nanoparticles for the treatment of cancer will be discussed, too [9].

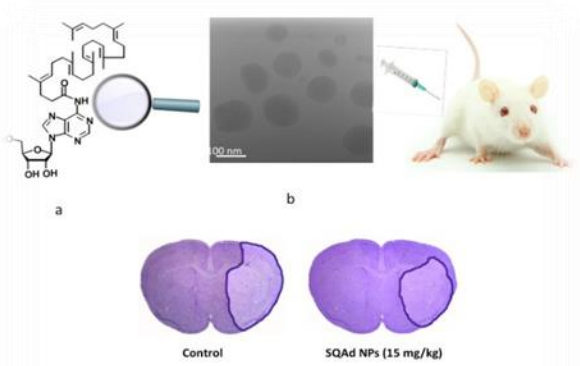


*Figure 1.* Adenosine-Squalene bioconjugate (a) spontaneously self-assemble in water as nanoparticles (SQAd NPs) of ca. 100 nm (b). When injected into mice subject to brain ischemia, nanoparticles induce reduction of ischemic zone (c)

The design of "multidrug" nanoparticles combining in the same nanodevice chemotherapy and imaging (ie., "nanotheranostics") or various drugs with complementary biological targets will be also discussed [7]. Finally, it will be shown that the construction of nanodevices sensitive to endogenous (ie. pH, ionic strenght, enzymes etc.) or exogenous (ie., magnetic or electric field, light, ultrasounds etc.) stimuli may allow the spatio-temporal controlled delivery of drugs and overcome resistance to current treatments [8].

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[10] T. Simon-Yarza et al., *Angewandte Chemie Int. Edition*, DOI: 10.1002/anie.201707346 (2017)

# INVITED COMMUNICATIONS

**IC-1**

# Engineering Nanoparticles to Cargo Nucleic Acids to the Nervous System: Breaking Barriers with Nanotools

A.P. Pêgo[1;2,3;4]*

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[2]*i3S – Instituto de Investigação e Inovação em Saúde*
[3]*Instituto de Ciências Biomédica Abel Salazar, Universidade do Porto, Portugal*
[4]*Faculdade de Engenharia, Universidade do Porto, Portugal*

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Peripheral neuropathies are common and still lack an effective treatment option. Neuron-targeted gene delivery is a promising strategy to treat peripheral neuropathies. We have recently proposed the use of polymeric nanoparticles based on thiolated trimethyl chitosan (TMCSH) to mediate targeted gene delivery to peripheral neurons upon a peripheral and minimally invasive intramuscular administration.

Nanoparticles were grafted with the non-toxic carboxylic fragment of the tetanus neurotoxin (HC) to allow neuron targeting and retrograde transport. Firstly, molecular recognition force spectroscopy (MRFS) was explored to evaluate the specificity of the designed TMCSH-HC nanoparticles to neuronal cell populations in biological samples of different complexity[1]. Subsequently, the retrograde transport of the targeted nanoparticles after a peripheral administration was confirmed using compartmentalized primary neuron cultures and taking advantage of (quantitative) bioimaging tools[2].

Finally, we explored the delivery of a plasmid DNA encoding for the brain-derived neurotrophic factor (BDNF) in a peripheral nerve injury model. The TMCSH-HC/BDNF nanoparticle treatment promoted the release and significant expression of BDNF in neural tissues, which resulted in an enhanced functional recovery after injury as compared to control treatments (vehicle and non-targeted nanoparticles), associated with an improvement in key pro-regenerative events, namely, the increased expression of neurofilament and growth-associated protein GAP-43 in the injured nerves. Moreover, the targeted nanoparticle treatment was correlated with a significantly higher density of myelinated axons in the distal stump of injured nerves, as well as with preservation of unmyelinated axon density as compared with controls and a protective role in injury-denervated muscles, preventing them from denervation[3].

These results highlight the potential of TMCSH-HC nanoparticles as non-viral gene carriers to deliver therapeutic genes into peripheral neurons and thus, pave the way for their use as an effective therapeutic intervention for peripheral neuropathies.

**Acknowledgments**: The financial support of Fundação para a Ciência e a Tecnologia (FCT) (Grants: PTDC/CTM-NAN/115124/2009 and PTDC/CTM-NAN/3547/2014) and INFARMED is gratefully acknowledged.

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[3] Lopes CDF et al. Biomaterials; 121:83-96 (2017)

**IC-2**

# Brain regeneration in Parkinson's disease using micro/nanoparticles

E. Garbayo[1;2]*, E. Ansorena[3], C. Rodriguez-Nogales[1], H. Lana[1;2], M.M. Carmona[4], I. Marcilla[4], J.L. Lanciego[2;5], M. R. Luquin[2;4] and M.J. Blanco-Prieto[1;2]

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Parkinson's disease (PD) is an aged-related neurodegenerative disorder affecting approximately 7 to 10 million people worldwide. Many advances have occurred regarding symptomatic treatment since the first description of the disorder 200 years ago. However, the need for a therapy able to modify the progression of the disease is undoubtely a priority, together with the development of better animal models that recapitulate all the PD features. In this regard, two proteins, glial cell line-derived neurotrophic factor (GDNF) and polo like kinase 2 (PLK2), have been the focus of great interest for many years as therapeutics targets for PD. In the first case, GDNF, a neurotrophic factor capable of promoting the survival of dopaminergic neurons has great potential as a neuroprotective and neurorestorative treatment for PD. Although preclinical studies in multiple animal models were very promising showing a strong neuroprotection, clinical trials examining the efficacy of the free protein failed to confirm the previously observed benefits highlighting the neccesity for better drug delivery vehicles [1]. Regarding PLK2, this serine/threonine serum inducible kinase has been shown to phosphorylate alpha-synuclein at Serine-129, this small peptide being closely linked to PD pathogenesis. Controversial publications have generated debate regarding the putative toxic or protective attributes of PLK2 [2,3]. Moreover, the use of kinase inhitors and gene therapy to elucidate whether PLK2 is a friend or foe has been inconclusive. A suitable strategy to gain insights into PLK2 biological effects might be to increase its intracellular levels with the aim of reproducing the slow progressive neuronal changes that occur in PD. However, a major challenge in enzyme overexpression is the difficulty of administering functional proteins in an active conformation. The use of micro-and nanocarriers, represents a key strategy able to overcome therapeutic protein hurdles such as short *in vivo* half-life and instability [4]. In this talk, recent progress in the use of microparticles (MPs) and nanoparticles (NPs) for effective protein delivery to the brain will be discussed with a focus on the development of a curative therapy for PD based on microencapsulated GDNF [5-7]. The development of a nanotechnology-based approach that could help to clarify the role of PLK2 in PD will also be discussed [8].

**Acknowledgments**: This work was funded by grants provided by the Instituto de Salud Carlos III (PI12/01730) and by Fundación Universidad de Navarra (FUN) (University of Navarra).

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**IC-3**

# Non-invasive gene therapy of the brain

Luís Pereira de Almeida[1;2]

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Gene therapy aims to cure or modify the progression of acquired or hereditary diseases by adding, silencing, replacing or repairing missing, mutated or pathogenic genes.
In the last decades, different viral and non-viral vectors for gene therapy, as well as administration methodologies have been developed. Nevertheless, delivery of vectors through the blood brain barrier to treat brain disorders has been an issue preventing gene therapy from reaching results in line with initial high expectations.
However, recent advances in vector design and groundbreaking successes showing widespread central nervous system gene expression upon intravenous administration and dramatic alleviation of phenotype in animal models and symptoms in clinical trials, suggest that we have reached a new stage that will allow widespread successful implementation of gene therapy to treat brain disorders in clinical practice.
In this talk the main strategies of gene therapy to the CNS will be reviewed, with examples from the literature and our own work on gene therapy of Machado-Joseph disease.

**IC-4**

# Impact of ligand moiety on targeting and anti-tumor effect of antibody nanoconjugates

D. Prosperi[1]*, M. Colombo[1], S. Mazzucchelli[2] and F. Corsi[2]

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**Introduction**. Multifunctional colloidal nanoparticles (MFNs), which combine unique optical and/or magnetic properties and efficient tissue penetration, have been envisaged as promising tracers for noninvasive imaging of cancer cells and as target-directed drug delivery systems. The design of ideal targeted MFNs needs careful optimization of fundamental features including uniform size and shape, surface charge and efficient functionalization with suitable homing ligands to improve the signal amplification and target selectivity toward malignant cells. The selectivity in targeting cancer cells is of primary importance and is usually achieved exploiting the modification of MFNs with biomolecules endowed with high affinity for specific cell membrane receptors, such as antibodies or antibody fragments [1]. One of the greatest challenges in designing MFNs functionalized with homing ligands to optimize molecular recognition resides in the possibility to finely control the ligand density and orientation on the nanoparticle surface.

**Materials and Methods**. Colloidal nanoparticles used within the studies included in this report were gold (AuNPs), iron oxide (IONPs) or nonporous silica (SNPs). AuNPs and IONPs were synthesized in organic solvents and transferred to aqueous phase by surface coating with a pro-functional amphiphilic polymer, termed PMA [2]. SNPs were fabricated by conventional Stöber synthesis [3]. All nanoparticles were characterized by TEM, DLS, and zeta potential. Surface functionalization of MFNs with antibody fragments or full antibodies was accomplished by either chemical or biotech ligation strategies as described in the following section. Antibody-modified MFNs were subjected to in vitro assays with various breast cancer cell cultures using different techniques – including flow cytometry, confocal microscopy and MTT assay – and to *in vivo* assessment in mice models of breast cancer by fluorescence imaging and/or magnetic resonance imaging.

**Results and Discussion.** An elegant strategy to control ligand orientation on the nanoparticle surface involves the use of fusion proteins containing a) a small sequence capable of irreversibly cross-reacting with a suitable functionality, previously introduced on the MFN surface, via a bioorthogonal ligation [4], or b) a low-molecular weight enzyme that recognizes a suicide inhibitor anchored to the solid surface resulting in a covalent immobilization [5,6]. These approaches, which were exploited for the immobilization of scFv variants of human IgGs, present several advantages: 1) the ligand to be immobilized on the surface is a small molecule, 2) binding to the protein occurs under physiological conditions, 3) it is highly specific and irreversible, and 4) all these binding systems involve monovalent recognition partners, which overcomes the crosslinking effects that usually occur with other conventional ligand partners. The application of the above-mentioned strategy to an scFv antibody against HER2 tumor marker led to bioengineered MFNs, which exhibited targeting selectivity toward HER2 receptor in living cells. To illustrate the potential of controlling the surface organization of antibodies on MFNs, the nanoparticles were conjugated to a newly designed recombinant single-domain protein A fragment, which was bioengineered to present a cysteine tripod at the C-terminal end. As protein A recognizes the Fc portion of IgGs, it mediated an orderly Fc site-specific antibody immobilization on MFNs resulting in a target-directed Fab presentation [7]. This novel targeted nanoparticle model was assessed by fluorescence imaging, MRI and ultrastuctural investigation in vivo [8] and compared to a targeting efficiency of the nanoparticle functionalized with different fragments of the same antibody [9]. Finally, to determine the importance of the number of antibodies immobilized on MFNs, a novel method for the synthesis of nanoparticles functionalized with exactly one or exactly two antibodies was developed. These nanoparticles were assessed *in vivo* in tumor bearing mice, revealing that the best targeting efficiency and therapeutic effect was achieved with the less number of attached antibodies per nanoparticle [10].

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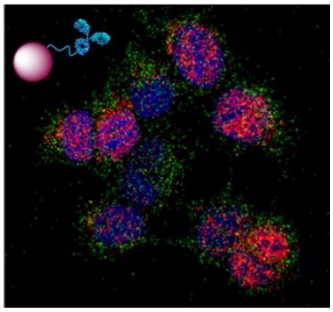

*Figure 1*. Targeting of cancer cells by mono-antibody gold nanoconjugates.

**IC-5**

# Towards the Design of Personalised Polymer-based Combination Conjugates for Advanced Stage Breast Cancer Patients

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Breast cancer (BC) accounts for 21% of all female cancer deaths in Europe, with a mortality rate of ~31 per 100,000. It is considered the main cause of death for women aged 35-64 years [1]. Overall survival rate has vastly improved over recent decades due to early detection and improved therapies, but metastatic disease is largely incurable [2]. Anti-cancer therapeutics research has provided little progress towards improved survival rates for patients with metastatic disease due in part to the complex and heterogeneous nature of the disease. However, the intrinsic advantages of polymer conjugates can be optimised to rationally design targeted combination therapies that would permit enhanced therapeutic efficiency [3,4]. Early clinical trials involving anti-cancer polymer conjugates have shown activity in chemotherapy refractory patients and significantly reduced drug-related toxicity [5].

Our objective is to engineer tumour-targeted polymer-based combination therapies specifically designed to treat metastatic breast cancer in a personalised manner. To achieve this goal, we plan to developed novel multicomponent polymer conjugates with precise control over size, shape, solution conformation, multifunctionality, and bioresponsiveness and assess structure activity relationships clinically relevant models to understand mechanisms of action. In particular, our recent studies using NCA polymerization techniques have allowed us to precisely control the synthesis of well-defined star-based and linear polypeptidic architectures [6,7] that are capable to undergo a self-assembly process according to a structure/conformation-concentration dependency. Based on this behaviour, we described for the first time, a bottom-up methodology for the stabilization of soft-assembled star-shaped polyglutamates by crosslinking. Covalent capture of these labile assemblies provides access to unprecedented architectures as potential nanocarriers [8]

In parallel, we have performed a High Throughput Screening (HTS) to select synergistic drug combinations to be used in polymer-based combination approaches through rationally designed linkers that confer adequate drug release kinetics. To perform this approach, we selected four metastatic human BC cell lines representing the four clinical BC subtypes. Prior to HTS, all cell models have been fully characterized regarding their Cathepsin B activity, intracellular pH, as well as oestrogen, progesterone, Her2 receptors, GSH and exosomes levels; all representing patient-specific biomarkers. Cell viability and exosomes release modulation have been studied following treatments and several drug combinations have been selected for each specific BC subtype.

With selected drug combinations different linking chemistry has been explored (carbamates, hydrazones, disulphides, etc.) yielding different drug(s) release kinetics. This provided different therapeutic outputs in cells and in a orthotopic breast cancer model, not only for the primary tumor but also for metastasis progression in lungs as well as lymph nodes.

The strategy proposed and the results obtained so far open up a wide range of opportunities for the currently unsuccessful clinical approaches to target lymph node metastasis and cancer immunotherapy.

**Acknowledgments**: The authors acknowledge MINECO (grants SAF2013-44848-R, SAF2016-80427-R) and the European Research Council (grant ERC-CoG-2014- 648831 MyNano) for financial support.

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**IC-6**

# Targeting Notch Signaling in Mouse Tumour Models

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**Introduction**,
Delta like 4 (Dll4)/Notch signaling is a key regulator of tumor angiogenesis. Dll4 inhibitors were found to reduce tumor growth due to abnormal vessel formation and reduced perfusion. This raised concerns that targeting Dll4 may diminish efficacy of cytotoxic agents given concurrently. In addition, hypoxic conditions caused by reduced perfusion have been shown to promote the formation of metastasis. To address these two potential caveats of Dll4 inhibition cancer therapy we used the TRAMP prostate adenocarcinoma and the Lewis Lung Carcinoma mouse tumour models.

**Materials and Methods**,
Therapeutical trials assessed the efficacy of a systemically administered Dll4/Notch-inhibitor, soluble Dll4 extracellular domain fused to Fc (Dll4Fc), both alone and concurrently with doxorubicin. Treatments were started when TRAMP mice (C57BL6 background) reached the age of 12 weeks or 18 weeks and continued until mice were 18 or 24 weeks-old, respectively. Doxorubicin concentration in the tumour was determined at endpoint using fluorometry.
Lewis Lung Carcinoma (LLC) cells were used to model tumor metastasis *in vivo* by subcutaneous transplantation into wild-type and endothelial-specific *Dll4* loss-of-function mice.

**Results and Discussion**
As expected, both Dll4Fc and doxorubicin alone were effective in preventing tumour growth. Surprisingly, concurrent therapy produced a much stronger effect than either agent alone. This increased efficacy is likely a consequence of a nearly 4-fold increase observed in the tumour doxorubicin concentration when it is administered together with Dll4Fc.
Endothelial specific *Dll4* deletion also reduced the growth of LLC transplants, inducing an increase in density of blood vessels, that were poorly perfused, with increased leakage and reduced perivascular maturation. Unexpectedly, although hypoxia was increased in the tumour, the number and burden of lung macro-metastases was significantly reduced. Both Epithelial to Mesenchymal Transition (EMT) markers and Cancer Stem Cell (CSC) frequency were decreased along with a concomitant reduction of circulating LLC tumor cells.

**Conclusions**
The present findings suggest tumour vasculature defects induced by Dll4/Notch signaling blockade not only do not adversely interfere with concomitant chemotherapeutic agent delivery to the tumour as may contribute to significantly increase its tumour concentration, with beneficial effects in both efficacy and specificity. Most importantly, despite the hypoxia resulting from reduced blood perfusion, endothelial specific *Dll4* deletion was observed to impair the metastatic process, implicating tumour endothelial Notch signaling in EMT induction and CSC expansion.

**Acknowledgments**:
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**IC-7**

# Drug Delivery in Regenerative Medicine

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New therapies based on the use of biomolecules (e.g. proteins, peptides, non-coding RNAs) as well as vesicles (e.g. exosomes) have emerged during the last years. Due to their instability, side effects, and limitations to cross cell membrane, delivery systems are required to fully show their biological potential. Sophisticated formulations responsive to light offer an excellent opportunity for the controlled release of these biomolecules, enabling the control of timing, duration, location and their dosage. In the context of my presentation, I will discuss the use of light-triggerable systems to enhance the activity of biomolecules in the setting of skin and brain regenerative applications.

**Acknowledgments**: This work was supported by funding of FEDER through COMPETE program and Fundação para a Ciência e a Tecnologia (FCT) (MITP-TB/ECE/0013/2013), European project ERAatUC (ref. 669088) and European project ERC (ref. 307384, “Nanotrigger”).

**IC-8**

# mRNA-activated reprogramming matrices

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**Introduction:** Current tissue engineering and regenerative medicine technologies are unable to prime cell differentiation efficiently, an evident limitation when they are applied to challenging differentiation pathways [1]. Here we present a new technology based on scaffolds that are activated with mRNA complexes coding for transcription factors (TFs) that act as master regulators of cell differentiation. We argue that the transfection efficacy and expression kinetics that can be achieved with mRNA is particularly suited for cell reprogramming technologies [2], and that such complexes can be integrated on in situ gelling scaffold preserving their functionality.

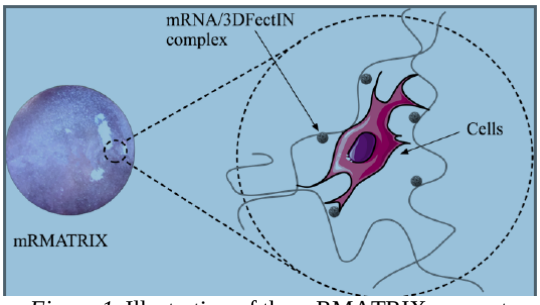


*Figure 1*. Illustration of the mRMATRIX concept.

**Methods:** MRNA encoding master regulator TFs MYOD (muscle) and SOX9 (cartilage) was condensed with 3DfectIN®, and used to activate *in situ* gelling fibrin hydrogels. The activated scaffolds (henceforth "mRMATRIXes") were characterized morphologically by scanning electron microscopy. MRMATRIX toxicity and transfection was tested both in U87M and in mesenchymal stem cell (MSC) cultures. MRMATRIX capacity for inducing cell differentiation and tissue formation was tested in 3D cultures using a pDNA-activated scaffold as a benchmark.

**Results:** MRMATRIXes showed porous morphology and a homogeneus distribution of mRNA/3DFectIN complexes (around 200 nm) in their structure. Under the experimental conditions, mRMATRIXes were not cytotoxic and allowed MSC proliferation. MRMATRIX were able to induce a effient forced expression of TFs in the first 5 days in culture, higher than that achieved with pDNA-activated scaffolds. MRMATRIX scaffolds were able to induce gene expression signatures consistent with successful cell differentiation to a myogenic and a chondrogenic phenotype. Histological and immunohistochemistry analysis of MSCs cultures after 28 days confirmed the formation of cartilage in mRMATRIX grops activated with Sox9.

**Conclusion:** This the first report of a scaffold activated with mRNA encoding TFs for cell differentiation. The high expression of tissue specific markers induced by the mRMATRIXes suggest their potential for regenerative medicine.

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**IC-9**

# Functional Nanofibrous Scaffolds Combined with Stem Cells for Advanced Biomedical Devices and Therapies

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Among the various possible embodiements of Advanced Therapies and in particular of Tissue Engineering the use of temporary scaffolds to regenerate tissue defects is one of the key issues. The scaffolds should be specifically designed to create environments that promote tissue development and not merely to support the maintenance of communities of cells. To achieve that goal, highly functional scaffolds may combine specific morphologies and surface chemistry with the local release of bioactive agents.

Many biomaterials have been proposed to produce scaffolds aiming the regeneration of a wealth of human tissues. We have a particular interest in developing systems based in nanofibrous biodegradable polymers[1,2]. Those demanding applications require a combination of mechanical properties, processability, cell-friendly surfaces and tunable biodegradability that need to be tailored for the specific application envisioned. Those biomaterials are usually processed by different routes into devices with wide range of morphologies such as biodegradable fibers and meshes, films or particles and adaptable to different biomedical applications.

In our approach, we combine the temporary scaffolds populated with therapeutically relevant communities of cells to generate a hybrid implant. For that we have explored different sources of adult and also embryonic stem cells. We are exploring the use of adult MSCs[3], namely obtained from the bone marrow for the development autologous-based therapies. We also develop strategies based in extra-embryonic tissues, such as amniotic fluid (AF) and the perivascular region of the umbilical cord[4] (Wharton´s Jelly, WJ). Those tissues offer many advantages over both embryonic and other adult stem cell sources.

The comparatively large volume of tissue and ease of physical manipulation facilitates the isolation of larger numbers of stem cells. The fetal stem cells appear to have more pronounced immunomodulatory properties than adult MSCs. This allogeneic escape mechanism may be of therapeutic value, because the transplantation of readily available allogeneic human MSCs would be preferable as opposed to the required expansion stage (involving both time and logistic effort) of autologous cells.

**Topics to be covered**

This talk will review our latest developments of nanostructured-based biomaterials and scaffolds in combination with stem cells for bone and cartilage tissue engineering.

**Acknowledgments:**

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**IC-10**

# Versatile gold nanoparticles for molecular diagnostics in leukemia

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Nanotechnology has become a powerful approach to improve the way we diagnose and treat cancer [1]. In particular, gold (AuNPs) possess unique features for enhanced sensitivity and selectivity for earlier detection of circulating cancer biomarkers. *In vivo*, nanoparticles enhance the therapeutic efficacy of anticancer agents when compared to conventional chemotherapy, improving vectorization and delivery, and helping to overcome drug resistance. Nanomedicine has been mostly focused on solid cancers due to take advantage from the enhanced permeability and retention (EPR) effect experienced by tissues in the close vicinity of tumors, which enhance nanomedicine's accumulation and, consequently, improve efficacy. Nanomedicines for leukemia and lymphoma, where EPR effect is not a factor, are addressed differently from solid tumors. Nevertheless, nanoparticles have provided innovative approaches to simple and non-invasive methodologies for diagnosis and treatment in liquid tumors.

We have developed several biosensors towards detection of RNA transcripts from label free amplification strategies relying on cDNA to more complex FRET based spectral codification that allow to question samples directly without prior retro-transcription – BioCode. Such BioCode assay has been effectively applied to for the detection of the BCR-ABL1 fusion transcript, which is the molecular hallmark of chronic myeloid leukaemia (CML). Using the gold nanobeacon configuration - where a gold nanoparticle acts as anchor for recognition and as a quencher - the FRET signature of the beacon allows to assess the molecular profile of the sample. This beacon was designed to detect the most common fusion sequences causing CML, e13a2 and e14a2. The emission spectra indicate that the self-assembly of the several components of the biosensor occurs sequentially, being triggered by the presence of a fully complementary target [2].

This system has further applied in combined therapy with imatinib, where the Au-nanobeacon was capable of specific BCR-ABL1 gene silencing. This nananotheranostics system efficiently silenced the target aberrant gene resulting in a significant increase in cell death. Moreover, combination of the silencing Au-nanobeacon was also capable of inducing the loss of viability of imatinib-resistant K562 cells [3].

We believe that such system might be a starting point for the development of further concepts in nanotheranostics in liquid tumours.

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**IC-11**

# Near infrared dye-labeled polymeric micro- and nanomaterials: *in vivo* imaging and evaluation of their local persistence

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Over the last decades, significant progress has been made in the development of nanoparticulated systems to diagnose, screen, treat, and prevent disease overall improving human health. As a result of those efforts, nowadays several nanomedical products are approved for use in clinical practice mainly for cancer treatment and for antimicrobial therapy [1].

As carriers of drugs, genes or other bioactive molecules nanoparticulated systems have shown an enhanced solubility and improved pharmacokinetics compared to the administration of the free therapeutic molecule. Those carriers also have the ability to prevent therapeutic molecules from metabolizing and to provide them with site-specificity.

To evaluate the *in vivo* biodistribution of those carriers several affordable preclinical imaging technologies are now available. Of all of them, optical imaging allows the non-invasive real-time imaging of those carriers in living organisms using non-ionizing radiation.

Herein, we show how polymeric micro- and nanomaterials of different sizes can be dye-labeled with near infrared dyes to track their *in vivo* biodistribution and local persistence after intramuscular (IM) and subcutaneous (SC) administration in nude mice. We have labeled PLGA (poly lactic-co-glycolic acid) and PNIPAm (Poly(N-isopropylacrylamide)) micro- and nanoparticles with the NIR dye IR-820 and monitored their biodistribution and persistence *in vivo*. Our results show the suitability of the labeled materials for sustained or on-demand local drug delivery regarding the thermoresponsive ability of PNIPAm, avoiding systemic distribution and tissue damage after IM and SC administration.

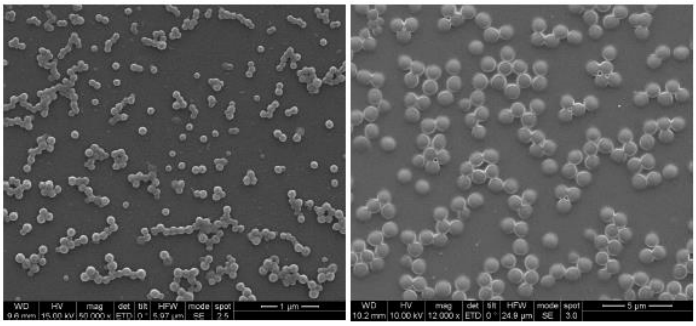

*Figure 1*. Dye-doped PLGA nanoparticles (left) and PNIPAm microparticles (right)

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**IC-12**

# Antibody engineering strategies for antiviral neutralization

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**Introduction:** Antibodies have already proven their importance as therapeutic agents. Despite this fact there is still much space for improvement concerning antibody engineering for antiviral strategies. Here we will present new concepts that improve the development of new antiviral strategies.

**Results:**

A) A new cell based strategy that takes advantage of eukaryotic cell biology to integrate the creation of antibody libraries and the selection of antibody leads will be presented. Using an engineered human T-cell line and the knowledge on DNA specific recognition sites, we created random intracellular diversity on antibodies, mimicking B-cell diversification mechanisms. A cellular platform was developed to integrate this new variability strategy with a selection mechanism where antigen and antibodies interact in their native conditions. No current technologies integrate all the discovery steps in a unique system. This is a completely new approach for antibody generation and selection and it may have profound implications in future technologies for antibody development, contributing for the progress of new immunotherapies, and consequently, reducing the time-to-market. These technologies were used to select antibodies that specifically were used to reprogramme or deliver antiviral strategies. A major goal of lentiviral gene delivery systems is the targeted stable transduction of specific cell types. Here, we pseudotyped lentiviral vectors with a novel Sindbis envelope, which directly encodes a single chain antibody fragment against fluorescein. The resulting pseudotyped viral particles showed a significant enhancement in specific transduction of target cells in combination with fluorescein-labelled anti-CD4, anti-CD7 and anti-VEGFR antibodies.

B) Small interfering RNAs (siRNA) application in therapy still faces a major challenge with the lack of an efficient and specific delivery system. A novel strategy for sucessfully deliver of an anti-Human Immunodeficiency Virus (HIV) siRNA was developed by new small-domain antibodies. Membrane translocation of RNAi effector was addressed by an engineered nanobody towards CXCR4 receptor, a major target expressed on HIV-susceptible T-lymphocytes. A validated siRNA molecule against HIV-1 gene tat was conjugated to fluorescein (FITC) and coupled to the CXCR4-specific nanobody through an anti-FITC single-chain variable fragment. We selected a high-affinity binder of FITC to minimize siRNA loss. The siRNA-fusion protein targeted CXCR4 positive cells, being detected at the surface and in the cytoplasm of a human T-lymphocytic cell line. Additionally, our construct silenced expression of luciferase reporter gene under control of Tat-driven HIV promoter and inhibited HIV-1 infectivity. Similarly to RNA interference technology, zinc-finger transcription factors are potent modulators of gene expression. Accordingly, we further demonstrate the suitability of this nanobody as a vehicle for CXCR4 entry-dependent functionality of a delivered anti-HIV zinc-finger repressor. The present study demonstrates the potential of a specific delivery system for therapeutic HIV molecules based on nanobody chimeras towards CXCR4 receptor.

A) Over the last years, the most used therapy against HIV has been combinational antiretroviral therapy (cART) although, it is not able to eradicate HIV from the host due to HIV latency. Cell receptors fused to antibodies have the ability to generate a response after the binding of an antigen. These receptors can be customized allowing to choose the sensing and response behaviors. With this new concept, we developed a strategy that comprehends the reactivation of latent memmory T-cell reservoirs upon receptor recognition and the induction of T cell activation which leads to the death of the infected cell. We engineered T lymphocytes genetically mod-ified to express a chimeric antigen receptor (CAR-T) that recognizes CD44 bt a new single-domain antibody. However, CAR-based therapies may involve ontarget toxicity against normal tissues expressing low amounts of the targeted CD44 which are not çatently infected. To specify T cells for robust effector function that is selec-tive for latent HIV-infected cells but not normal tissue, we developed a transsignaling CAR strategy, whereby T-cell activation signal 1 (CD3z) is physically dissociated from costimulatory signal 2 (CD28) in two CARs of differing antigen specificity.Trans-signaling CAR-T cells showed weak cytokine secretion against target cells expressing CD44 alone, but showed enhanced cytokine secretion upon encountering natural or engineered T-cells coexpressing CD44 and gp120 upon reactivation in situ. Thus, a dual specificity, trans-signaling CAR ap-proach can potentiate the therapeutic efficacy of CAR-T cells against HIV-latent infected cells while minimiz-ing parallel reactivity against normal tissues bearing single antigen.

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**IC-13**

# Towards clinical evaluation of a new malaria vaccine

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**Introduction.** Whole-sporozoite (WSp) immunization is currently the most effective strategy to induce sterile protection against malaria. However, current WSp malaria vaccines rely on the effective attenuation of deadly *Plasmodium falciparum* (*Pf*) sporozoites [1].
**Materials and Methods.** Here, we describe a novel approach to WSp immunization using rodent *Plasmodium* parasites as vaccination platforms, which can be genetically modified to express antigens of their human-infective counterparts (Fig. 1).
**Results and Discussion.** In a series of pre-clinical studies, we employed sporozoites of the rodent parasite *P. berghei* (*Pb*) to demonstrate that this strategy enables safe and optimal antigen presentation. We show that *Pb* sporozoites unabatedly infect and develop in human hepatocytes but are unable to establish a potentially fatal blood-stage infection, a safety requisite for an attenuated vaccine. We generated *Pb*Vac, a *Pb* parasite genetically engineered to express the major *Pf* immunogen, the circumsporozoite (CS) protein [1], at the surface of sporozoites and hepatic parasite stages. Using an innovative rabbit model, susceptible to hepatic infection but not to blood infection by *Pb*, we show that *Pb*Vac elicits substantial cross-species cellular immune responses and functional *Pf*CS-dependent antibody responses, which can efficiently inhibit *Pf* sporozoite liver invasion and development. An array of additional safety studies completed the pre-clinical package that put *Pb*Vac on the path to clinical assessment.
**Conclusions.** Our findings warrant clinical evaluation of *Pb*Vac in controlled human malaria infection asssays [2] and establish this immunization platform as a new paradigm in malaria vaccination.

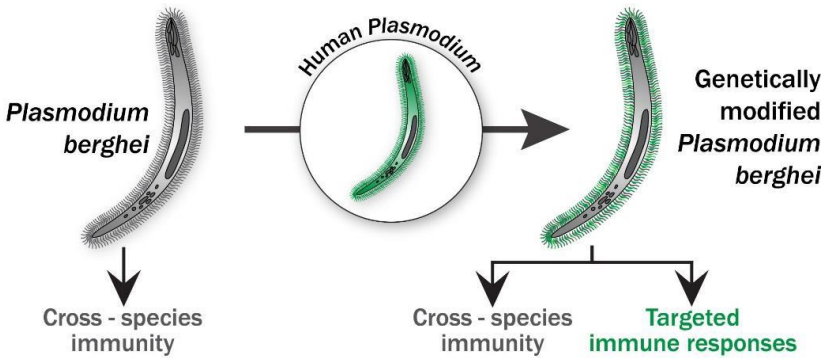


*Figure 1*. Generation of genetically modified *P. berghei* parasites as platforms for immunization against human malaria.

**Acknowledgments**: This work was supported by the Bill & Melinda Gates Foundation, the Malaria Vaccine Initiative, and Fundação para a Ciência e Tecnologia, Portugal.

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**IC-14**

# Enhancing cancer immunotherapy via nanotechnology-based strategies to engage innate and adaptive immune responses

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Cancer vaccines present the unique ability to trigger a memory immune response towards cells presenting specific tumor associated antigens (TAA). Several studies have shown that aliphatic polyester-based biodegradable nanoparticles (NP) are potential vaccine delivery systems for cancer and infectious diseases and suitable platforms for immune modulation [2, 3]. By modulating the properties of those carriers, it will be possible to foster the accumulation of TAA at specific sites within the cytoplasm, potentiating a systemic and/or cellular based immune response.

Our research has been focused on the development of different formulations of biodegradable NP to deliver different combinations of TAA, immune adjuvants, such as the Toll-like receptor ligands (TLRl), and/or gene regulators, such as small interfering RNA (siRNA) to target multiple and complementary tumor progression-related pathways. Mannose-grafted hybrid lipid/polymeric NP and hyaluronic acid-coated NP have been some of the systems prepared to specifically target antigen-presenting cells (APC), such as DC, and tumor cells, respectively. Of particular interest is the elucidation of the effect of particle composition, surface properties and targeting ligands on immune cell activation and functionality, both in vitro and in vivo using wild-type and solid cancer animal models (e.g. melanoma and breast carcinoma).

Our nanovaccines induced a highly prominent long-lasting effector memory Cytotoxic T Lymphocytes (CTL), even 8 weeks after a single immunization with the nanoparticulate vaccine entrapping the combination of antigen and the TLRl, suggesting an efficient cross-presentation and cross-priming that led to a broad and effective immune response pivotal for tumor rejections. In melanoma and breast murine models, our nanovaccines demonstrated to significantly decrease tumor growth rate, especially when a combination of tumor-associated antigens and TLRl, with siRNA regulators or α-GalactosylCeramide (Natural killer T (iNKT) cell agonist), were delivered by a single NP.

Our data reveals the impact of NP surface properties/composition on the type of immune response and overall anti-tumor effect. This deeper understanding on the NP-immune cell crosstalk can guide the rational development of nano-immunotherapeutic systems with improved and specific anti-tumor efficacy and safety, while avoiding off-target effects.

**Acknowledgments**: This work was supported by Fundação para a Ciência e a Tecnologia-Ministério da Ciência, Tecnologia e Ensino Superior (FCT-MCTES), Portugal for PhD Grants SFRH/BD/87869/2012, SFRH/BPD/94111/2013, SFRH/BD/87591/2012 and PD/BD/113959/2015; research projects (UTAP-ICDT/DTP-FTO/0016/2014; ENMed/0051/2016) and iMed.ULisboa grant UID/DTP/04138/2013. This project has received funding from European Structural & Investment Funds through the COMPETE Programme and from National Funds through FCT under the Programme grant SAICTPAC/0019/2015.

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# ORAL COMMUNICATIONS

**OC-1**

# Folate-targeted liposomes for rheumatoid arthritis therapy

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Rheumatoid arthritis is the most common inflammatory rheumatic disease, affecting almost 1% of the world population [1]. Although the cause of rheumatoid arthritis remains unknown, the complex interaction between immune mediators (cytokines and effector cells) is responsible for the joint damage that begins at the synovial membrane [2]. Activated macrophages are critical in the pathogenesis of rheumatoid arthritis [3] and showed specifically express a receptor for the vitamin folic acid, folate receptor β [4]. This particular receptor allows internalization of folate-coupled cargo [5].
Here we propose the encapsulation of methotrexate in a new liposomal formulation using a hydrophobic fragment of surfactant protein conjugated to a linker and folic acid to enhance their tolerance and efficacy [6]. In this study we aim to evaluate the efficiency of this system to treat rheumatoid arthritis, by targeting folate receptor β present at the surface of activated macrophages. The specificity of our liposomal formulation was investigated both *in vitro* as *in vivo* using a mouse model of arthritis (collagen-induced arthritis in DBA/1J mice strain).
In both systems, the liposomal constructs were shown to be highly specific and efficient in targeting folate receptor β. These liposomal formulations also significantly increase the clinical benefit of the encapsulated methotrexate *in vivo* in arthritic mice (Figure 1) [7]. In conclusion, our formulation might be a promising cost-effective way to treat rheumatoid arthritis and delay or reduce methotrexate intolerance.

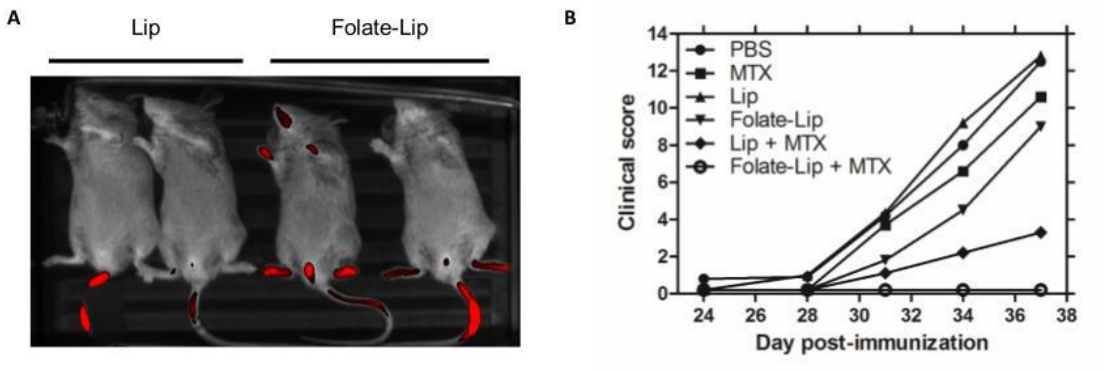


*Figure 1*. *In vivo* specific targeting and prophylactic efficiency of folate receptor-targeted liposomes in arthritic mice. (A) *In vivo* uptake specificity of fluorescently labeled liposomes (30 min). (B) Clinical effects of liposomes encapsulating methotrexate on arthritis. Treatment started 14 days after immunization. The mean clinical score in each group over time is shown [8].

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**OC-2**

# Targeting AQP3 using a nanotechnological approach for melanoma treatment

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**Introduction:** Melanoma is the most aggressive type of skin cancer, presenting a poor prognosis. The high rate of deaths caused by melanoma has encouraged researchers to pursue new and innovative therapeutic approaches. Aquaporins (AQPs) are a family of small transmembrane proteins that facilitate the bidirectional movement of water and small solutes across cell membranes. AQP3 is known to be aberrantly expressed in several cancers, namely melanoma [1, 2]. In this context, the design of selective AQPs inhibitors constitutes a new alternative therapeutic strategy for melanoma. Among these inhibitors, in particular cystein-reactive heavy metal-based compounds, the ones based on copper (Cuphen) and gold (Auphen) have emerged as promising selective inhibitors of AQPs, showing cytotoxic effects towards melanoma cell lines [3, 4]. Moreover, the cytotoxic properties of Auphen towards human epidermoid carcinoma cells (A431) were correlated with AQP3 overexpression [5]. In the present work, we assessed AQP3 expression levels in murine and human melanoma cell lines, in comparison to human keratinocytes as control, enabling the elucidation of the mechanism of action of Cuphen and Auphen. Aiming towards a further preferential targeting to tumor sites *in vivo*, these selective AQPs inhibitors were nanoformulated in lipidic systems – liposomes [4]. The biological evaluation of the so developed nanoformulations was accomplished in a murine melanoma model.

**Materials and Methods:** AQP3 expression, in murine and human melanoma cell lines (B16F10 and MNT-1, respectively), and in healthy human keratinocytes (HaCat) was determined by RNA analysis as previously described [5]. Cuphen and Auphen were incorporated in long circulating liposomes with and without pH-sensitive properties, by the dehydration-rehydration method, followed by an extrusion step to reduce and homogeneize the liposomes [4]. All liposomal nanoformulations were characterized in terms of incorporation parameters, mean size, polydispersity index and zeta potential. Proliferation inhibitory properties of Cuphen and Auphen in B16F10 and MNT-1 cell lines were evaluated by MTS assay. The safety of these metallodrugs was evaluated by determining their hemolytic activity, as described in [6]. The antitumor potential of developed metallodrugs nanoformulations was assessed in a xenograft murine melanoma model.

**Results and Discussion:** Cuphen and Auphen presented inhibitory effects towards murine and human melanoma cell lines, with $IC_{50}$ values in the micromolar range (bellow 7 μM). The designed Cuphen and Auphen nanoformulations were highly homogeneous, presenting a mean size around 0.160 μm, with a polydispersity index <0.1, and the incorporation parameters were lipid composition dependent. Although long circulating liposomes displayed a higher I.E. than pH-sensitive liposomes, the latest ones are more suitable for *in vivo* studies taking into account that tumor's microenvironment is acidic [7], and thus promoting a release of the incorporated material at tumor sites. Cuphen nanoformulations showed an antitumor effect, in comparison to induced and non-treated animals. This effect was also associated to a higher caspase 3/7 activity for groups treated with Cuphen, being this correlated with lower tumor volumes.

**Conclusions:** Cuphen and Auphen are both AQP3 inhibitors, exerting cytotoxic activity against human and murine melanoma cell lines. The *in vivo* model data demonstrated the high potential of liposomes for metallodrugs delivery, constituting a very attractive strategy for melanoma treatment due to their preferential extravasation and accumulation in metastatic sites. The development of lipid nanoformulations combining long-circulation times, pH-sensitive properties with active targeting for receptors overexpressed in melanoma cells should also be considered in following studies.

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**OC-3**

# Matrix-mediated gene expression regulation in mesenchymal stem cells immobilized in alginate-poly-L-lysine-alginate microcapsules

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**Introduction**: Cell encapsulation constitutes a valuable strategy for sustained drug release. However, uncontrolled cell responses importantly limit the safety and efficacy of the therapy. Here, we focused on the properties of the matrix as a tool to regulate cell behavior, since they have a direct impact on cell biology [1]. In particular, we designed a novel strategy to tune the properties of alginate-poly-L-lysine-alginate microcapsules without altering the biomaterial type or proportion. We elaborated two groups of microcapsules, both entrapping D1 Mesenchymal Stem Cells (MSCs), and only differing from the vehicle in which biomaterials were embedded: for the Biological group it contained electrolytes, whereas for the Technological group it contained the innert agent mannitol. Previously, we demonstrated that Biological capsules presented a matrix with suitable mechanical properties that controlled cell proliferation. On the contrary, Technological matrices were more permissive, allowing uncontrolled cell division and leading to enormous aggregates that collapsed the capsule. In this work, our aim was to study if these matrices caused differences at a genic level.

**Materials and Methods**: Cells were encapsulated as previously described [2]. Total RNA was extracted and purified. The Agilent SurePrint G3 Mouse GE v2 8x60K Microarray (Design: ID 074809) was selected for whole mouse gene expression analysis. 100 ng of nucleic acid from each sample were labeled and image was converted into expression data by Agilent Feature Extraction Software vs 10.7.3.1. For data analysis, Agilent GeneSpring GX V 13.0 software was employed. Data was normalized by the Quantile method and filtered based on Coefficient of Variation (CV < 100%), obtaining the log2 of the average value of signal intensity. For differential gene expression analysis, LIMMA statistical package (Smyth, 2004) was employed. Conventional statistical criteria (adjusted p-value < 0.05) were followed, selecting genes with Fold Change (FC) > 3 or < -3.

**Results and Discussion**: Gene expression in Technological cells suggests an excessive division, since proliferative pathways were significantly activated. Such was the case of the PI3K/Akt/mTOR route, which was promoted due to the up-regulation of Gnb3 (FC 3.51), Pik3cg (FC 3.65) and Igbp1b (FC 6.01) and the down-regulation of Inpp5d (FC -3.48). Likewise, the Ras/Raf/MAPK pathway was importantly activated, because of the up-regulation of Ros1 (FC 6.05) or Cyp2c44 (FC 5.34) genes. The proliferative effect was additionally enhanced due the promotion of cell cycle progression caused by the up-regulation of Usp2 (FC 3.03), Usp17le (FC 6.06), and the down-regulation of Basp1 (FC -3.7). Moreover, the down-regulation of the tumor suppressor genes Ptprd (FC -3.18) and Ptprr (FC -3.25), together with the more active cytoskeleton reorganization suggested by up-regulation of Rhoj (FC 4.46) and Eps8l1 (FC 3.72), pointed out to a tumor-like behavior. Regarding cell-fate, results suggested that depending on the properties of the matrix, cells tended to differing lineages: the Technological group might have presented a chondrogenic differentiation (Adamts16, FC 6.77), while the Biological group may have tended to osteogenic differentiation (Col12a1, FC -11.96).

**Conclusion**: Here, we demonstrated that the differing properties of the matrix, in which cells were encapsulated, importantly influenced gene expression. Therefore, our method represents an effective approach to control cell behavior without altering the formulation of the system, and thus achieve safer and more efficient therapies.

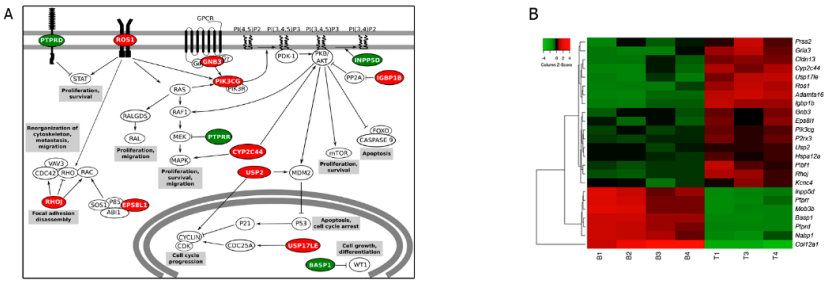


*Figure 1*. (A) Schematic representation of signaling pathways, (B) heatmap representation of differentially expressed genes.

**Acknowledgments**: A. Gonzalez-Pujana thanks the Basque Government for the PhD grant (PRE_2017_2_0295).

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**OC-4**

# Use of genipin as a quantitative imaging biomarker in implanted immunoisolation devices

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**Introduction:** Immunoisolation devices represent one of the leading strategies to deliver bioactive molecules from immobilized allo- or xenogenic cells. To date, despite remarkable outcomes obtained in clinical trials, the inability to visualize the location of microcapsules once implanted in the body leads to biosafety hurdles that must be overcome before making the definitive leap to the clinic [1]. Imaging of implanted hydrogel-based biosystems usually requires indirect labelling of the vehicle or cargo, adding complexity and potential risk of altering functionality. Here, we demonstrate for the first time that incorporating genipin into the design of immunoisolation devices can be harnessed to produce bright, quantitative and stable labelling for *in vivo* imaging.

**Materials and Methods:** We produced alginate microspheres by using an electrostatic droplet generator. Briefly, 1.5% alginate suspension was extruded through a 0,35mm needle and collected in a gelling solution. Beads were then washed and coated with genipin-cross-linked double poly-L-Lysine (GDP) membranes. We implanted 50, 100 or 200 µL of GDP microcapsules (Fig. 1a) subcutaneously into the dorsal region of 15-week-old female NSG mice (n = 4). The University of Michigan IACUC approved all animal procedures. We imaged fluorescence from implanted microcapsules using Ex: 570 nm / Em: 620/20 filter on an IVIS Spectrum (Perkin Elmer) as described previously [2]. To quantify imaging data, we measured radiant efficiency in defined regions-of-interest and then calculated mean values ± SD.

**Results and Discussion:** Obtained images exhibited a strong signal with excellent signal to noise ratio for all injections (Fig. 1b). Importantly, we also achieved a good linearity of fluorescence response to microcapsule dose ($R_2$ = 0.9971) (Fig. 1c). In addition, we estimated the lower limit of detection (LLD) and the lower limit of quantification (LLQ), obtaining doses equivalent to 18.6 µL and 59.6 µL for each of them respectively. We also calculated the instrument error (repeatability). The lowest dose of 50 µL produced a significantly higher coefficient of variation (CV) (46.5%), whereas 100 and 200 µL doses presented 25.7% and 20.8% respectively. By isolating this variability, we determined the human error in the injections of the microcapsules (i.e. dose preparing and administration of microcapsules). Thus, in the present study we estimated a CV of 39.8%, 50.8% and 22.2% for 50, 100 and 200 µL doses respectively. On the other hand, the accuracy of the results with all doses was close to the 100%.

**Conclusions:** We validated genipin as a quantitative imaging biomarker obtaining strong, stable fluorescence with good linearity of signal to microcapsule dose. This enabled us to immediately assess the actual injected dose and monitor its position over time, thereby significantly enhancing the biosafety of the therapy.

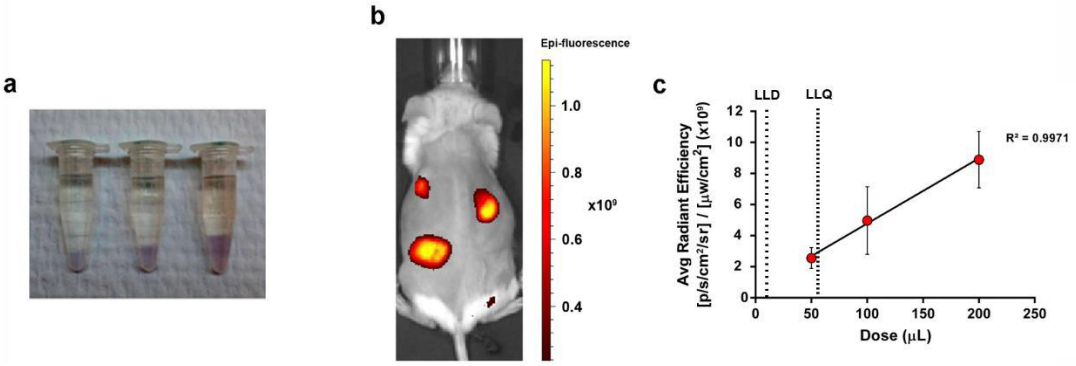


*Figure 1*. Dose-dependent (a) response of average radiant efficiency for GDP microcapsules (b) and its linear correlation (c).

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**OC-5**

# Combination of Dendritic Cell-targeted Nano-vaccines with Immune Checkpoint Therapy for Melanoma

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**Introduction:** Immune checkpoint therapy significantly improved the clinical outcome of melanoma treatment compared to standard therapy. However, results are far from the initially expected. In fact, Programmed cell death protein-1 (PD-1) antibody monotherapy induced effective and durable responses in 30-40% of advanced melanoma patients.[1]
Monoclonal anti-OX40, an immune checkpoint stimulator, member of the tumor necrosis factor (TNF) receptor family, has shown modest monotherapy outcomes in clinical trials.[2] Poor clinical results have been associated with complex mechanisms behind anti-tumor immunity. Currently, it is widely accepted that melanoma therapy will benefit from integrated complementary approaches, which can inhibit tumor immunosuppressive pathways and enhance immunity in an orchestrated manner. We hypothesized that combinatorial therapy with anti-PD-1/anti-OX-40 – to inhibit tumor immunosuppression and to boost T-cell activity, respectively – could be improved by cancer vaccination, which will increase tumor associated antigen recognition, internalization, processing and presentation to those T cells.

**Materials and Methods:** Mannose-poly(lactic-co-glycolic acid)/poly(lactic acid) (PLGA/PLA) were produced as dendritic cell (DC)-targeted nanoparticle vaccine, containing major histocompatibility complex (MHC) class I and MHC class II melanoma MART-1 peptide antigens. Animals were immunized by subcutaneous injection, while anti-mouse anti-PD-1 and anti-OX40 monoclonal antibodies were administered via intraperitoneal injection.

**Results and Discussion:** PLGA/PLA nanoparticles were designed, synthesized and characterized, demonstrating spherical shape, with an average diameter of 170 nm, narrow polydispersity index and near-neutral surface charge. *In vivo* immunization with our nanoparticle vaccine triggered secretion of inflammatory cytokines, splenocyte activation and cytotoxic T-cell activity against melanoma cells. Compared to anti-PD-1/anti-OX-40 alone, treatment with their combination of animals previously immunized with our DC-targeted vaccine induced maximal tumor inhibition with minimal systemic toxicity, leading to 100% of survival 42 days after of tumor inoculation, against 20% obtained for anti-PD-1/anti-OX-40 treatment. In this group, 50% of the animals were still alive two months after tumor inoculation, presenting infiltrating lymphocytes within tumors.

**Acknowledgements:**
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**OC-6**

# Enhanced biological functions of adipose derived stem cells in the injured myocardium of a rat myocardial infarction model when combined with neuregulin-loaded microparticles

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**Introduction**. Recent studies have demonstrated the beneficial effects of adipose-derived stem cells (ADSCs) for treating myocardial infarction (MI) even though low cell survival and engraftment have been observed [1]. ADSCs repair the damaged myocardium by inducing angiogenesis, mainly due to a paracrine effect in the infarcted area mediated by the secretion of proteins, micro-RNA packed exosomes and microvesicles. The possibility of combining ADSCs with cardioprotective growth factors different from those secreted by those cells, such as neuregulin-1 (NRG), may induce a better regenerative response. In this regard, our group has shown that microparticles (MPs) allow NRG controlled delivery in the MI region, accompanied by a significant improvement in cardiac function in rat and pig MI models [2-3]. Therefore, the objective of this study was to enhance the biological functions of ADSCs in the injured myocardium of a rat MI model and likewise to improve tissue repair by the adhesion of the cells to NRG-MPs [4-5]. The potential reparative activity of ADSCs and NRG-MPs, alone or in combination was first investigated. Then, cell survival and cardiac differentiation were analyzed. Finally, the interactions between MPs and ADSCs with the macrophages of the innate immune system were examined.

**Material and Methods.** NRG was encapsulated in poly(lactic-co-glycolic acid) MPs by double-emulsion solvent evaporation technique using Total Recirculation One Machine System (TROMS) [4]. Then MPs were coated with collagen type I and poly-D-lysine (PDL) to favor cell adhesion [4]. ADSCs were obtained from transgenic rats that expressed GFP. The adhesion of ADSCs to NRG-MPs was performed in ultra low attachment plates. The efficacy of the system was then investigated in a chronic rat MI model. MI was induced by permanent occlusion of the left anterior descending coronary artery. A total of 35 animals with left ventricular ejection fraction between 40 and 50% at day 2 post-MI were included in the study. One week post-MI, treatments were locally administered in 2 regions of the peri-infarcted myocardium. At one week and three months post-injection animals were sacrified to perform the histological analysis (infarct size, vasculogenesis, fibrosis, cell survival and differentiation, macrophage polarization…).

**Results and Discusion.** Complete adhesion of ADSCs to collagen-PDL coated particles was observed after 60 min of incubation in ultra low attachment plates. One week after the *in vivo* administration of the treatments, the system promoted macrophage polarization towards a M2 anti-inflammatory phenotype, demonstrating active induction of the immune response. This important finding elucidates the role of macrophages in tissue healing following the delivery of both ADSCs and NRG-MPs in the myocardium. Remarkably, the adhesion of ADSCs to MPs resulted in an increased cell survival, with cells being detectable in the cardiac tissue up to three months. In consonance, better tissue repair was observed in the animals treated with cells attached to MPs, which presented thicker left ventricles than the animals treated with ADSCs alone. Moreover, the presence of NRG in the system promoted a more complete regeneration, reducing infarct expansion, promoting the proliferation of cardiomyocytes and stimulating endogeneous cardiac regeneration. Regarding vasculogenesis, the presence of ADSCs and NRG-MPs alone stimulated vessel formation when compared to the control group, but the combination of both induced the largest vasculogenic effect, promoting the formation of both arterioles and capillaries. Only when ADSCs were administered adhered to MPs, they were incorporated into newly formed vessels.

**Conclusion.** The combination of ADSCs, MPs and NRG resulted in a synergy for inducing a greater and more complete improvement in heart regeneration and positive interactions with the immune system. The approach taken in this study provides strong evidence to move forward with preclinical studies with this strategy.

**Acknowledgments**: This work was supported by the Spanish Ministry of Economy and Competitiveness (SAF2013-42528-R), Spanish Ministry of Education (programa FPI (BES-2014-067838)), Ibercaja, the Spanish Ministry of Science and Innovation (JCI-2011-10737), the "Asociación de Amigos de la Universidad de Navarra" and the Spanish Ministry of Health with the "Instituto Carlos III" (ISCIII-RETIC RD06/0014).

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**OC-7**

# Interaction of small and medium size chitosan nanocapsules with different subsets of immune cells

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**Introduction:** Chitosan (CS) nanocapsules (NCs), nanostructures composed of an oily core stabilized with surfactants and surrounded by a chitosan layer, have shown to be interesting in the vaccination field, especially for mucosal administration [1]. These nanocapsules usually have a particle size between 150 and 300 nm, that has been shown to be adequate for *in vitro* uptake by antigen presenting cells (APCs) [2]. However, it has been reported that for an efficient drainage to the lymph nodes, where most of the APCs are located, a particle size <100 nm is usually required [3]. To elucidate if this threshold is also important for the interaction with immune cells, we study the influence of the particle size of CS NCs on the interaction with different immune cells subsets: macrophages, dendritic cells and hPBMCs.

**Materials and Methods:** Chitosan (hydrosoluble salt, deacetylation degree 88%) was acquired from Heppe Medical Chitosan (Germany). Poloxamer 407 was kindly donated by BASF (Germany). DL-α-tocopherol (vitamin E) was bought to Merck Millipore (Spain). Sodium glycocholate was pursached from Dextra (United Kingdom).
Small and medium nanocapsules were prepared by the solvent displacement technique. In order to obtain the small size nanocapsules the organic phase was injected through a needle into the aqueous phase.
For *in vitro* assays we used mouse macrophage cell line RAW 264.7, human peripheral blood mononuclear cells (hPBMCs) obtained from healthy voluntary donors and from which monocytes were isolated and maturated to dendritic cells. The possible cytotoxic effect of chitosan nanocapsules on macrophages was evaluated using *MTS* assays and *xCELLigence RTCA Systems*. Nanocapsules uptake by macrophage and dendritic cells viability were evaluated by flow cytometry.

**Results and Discussion:** New chitosan nanocapsules composed of vitamin E in the oily core and poloxamer 407 and sodium glycocholate as surfactants were developed. With a slight modification of the solvent displacement technique two differentiated particles sizes were obtained: small size (64 ± 9 nm) and medium size (173 ± 13 nm) with similar surface charge (≈ +40 mV) and the same theoretical composition (9 mg/mL).
*In vitro* toxicity of both formulations was evaluated in macrophages and dendritic cells. None of the systems showed significant cell death after 6 h of incubation (400 μg/mL) with macrophages. Results with dendritic cells after 24 h of incubation indicated greater toxicity of medium size nanocapsules compared with the small ones (LC50: 107 μg/mL *vs* 248 μg/mL, respectively). Nanocapsules were labeled with Nile Red to evaluate the uptake in macrophages by flow cytometry. Independently on the particle size, both formulations were efficiently captured by the cells.
Regarding the activation of the immune cells, none of the NCs produce hPBMCs activation, with undetectable levels of cytokines. Being a heterogenous mixture of immune cells, the activation of some subset of cells can be compensated with inhibition of others. With dendritic cells, the scenario is different, only NCs of 173 nm generated important levels of cytokines, including IL12-p70, IFN-γ, IL-1β, TNF-α and IL-10.
Finally, in allogenic cultures 173 nm NCs have significantly more capacity to activate CD4+ T lymphocytes, as show by the increase of activated CD25+ cells. Being the composition of the NCs the same, probably the higher toxicity of the 173 nm NCs is related with a higher uptake or different internalization routes, causing a higher activation and production of cytokines.

**Conclusions:** Particle size of chitosan NCs influences the *in vitro* activation of immune cells depending of the studied subset of cells. Meanwhile no differences were found regarding activation of hPBMCs, dendritic cells treated with 173 nm NCs produced substantially higher levels of cytokines compared with 64 nm NCs.

**Acknowledgments:** This work was supported by the Spanish Ministry of Economy, Industry and Competitiveness - Project NanoTAID (Refs. BIO2014-53091-C3-1/2/3-R). Iago Fernández Mariño acknowledges a predoctoral FPU grant from the Spanish Ministry of Education, Culture and Sports (grant number FPU16/03509).

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**OC-8**

# Squalenyl Hydrogen Sulfate Nanocarrier for Dual-Delivery of Anti-infective Quorum Sensing Inhibitor and Aminoglycoside Antibiotic Tobramycin

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**Introduction.** Completely eliminating *Pseudomonas aeruginosa* infections, especially in cystic fibrosis lungs, remains challenging due to the fast resistance developement of this pathogen. Complementary to the established antibiotics, so-called quorum sensing inhibitors (QSI) are currently being developed capable to interfere with the formation of bacterial biofilms [1]. One might hypothesize that dual-delivery of such complementary anti-infective agents will further improve the efficacy against biofilm forming bacteria. Nanocarriers for delivery of either antibiotic or QSI have been already desbribed [2,3]. A liposomal dual-delivery system for QSI Farnesol and Ciprofloxacin has also been described, but in this case both drugs were rather lipophilic [4]. Taking advantage of the self-assembling properties of the anionic amphiphilic lipid Squlenic Hydrogen Sulfate, we here describe a dual-delivery nanocarrier with high loading capacity for both a lipophilic QSI and the hydrophilic aminoglycoside antibiotic Tobramycin.

**Materials and Methods**. A core-shell nanocarrier system was prepared by self-assembly of Squalenyl Hydrogen Sulfate. A novel alkylquinolone-derived QSI discovered by Kamal *et al.*[1], was encapsulated in the core of the carrier, while its surface was decorated with the aminoglycoside antibiotic Tobramycin by electrostatic interaction. Drug encapsulation rate and efficacy, as well as release characteristics for both drugs were evaluated by LC-MS. The efficacy against *P. aeruginosa* (PA14) was evaluated *in vitro* by measuring minimum inhibitory concentration (MIC). Quorum sensing inhibition was measured by reduction of virulence factor pyocyanin. Drug-free particles, as well as the two drugs in free form served as controls.

**Results and Discussion**. The spherical shape of the particles was confirmed by Cryo-TEM, the hydrodynamic average size ranging from 150 nm to 450 nm depended from the initial lipid concentration. Dual-loading rates up to 10% for the QSI and 20% for Tobramycin, resp., could be achieved at encapsulation efficacy >85%. While the drug-free carrier system did not show any effect either in MIC assay or pyocyanin assay, the drug-loaded carriers achieved similar IC90 values as free Tobramycin in MIC assay. Interestingly enough, however, the QSI loaded nanocarrier reduced the virulence factor pyocyanin up to 2*x* more than the free drug.

**Conclusions**. (i) Stable core-shell nanoparticles could be obtained by self-assembly of the new amphiphilic lipid Squalenyl Hydrogen Sulfate; (ii) the size of the nanoparticle could be tuned by varying the lipid concentration; (iii) a high dual-loading capacity of 10% and 20%, resp., were achieved for the hydrophobic QSI, and the hydrophilic aminoglycosic antibiotic; (iv) the nanoparticles did not compromise the activity of the antibiotic, while the activity of the QSI was markedly improved compared to the free drug. We might conclude that this carrier platform has good prospects for improving the treatment of infections by biofilm forming bacteria.

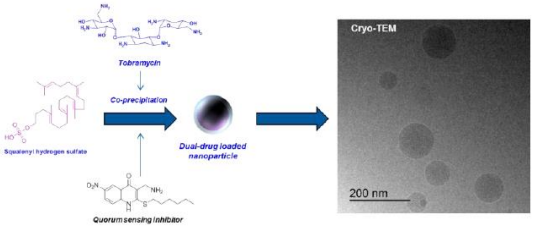


*Figure 1*. Dual-drug loaded nanoparticles preparation and Cryo-TEM image

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**OC-9**

# HT-Screening identifies light triggerable NP formulation for efficient *in vivo* non-coding RNA delivery in wound healing

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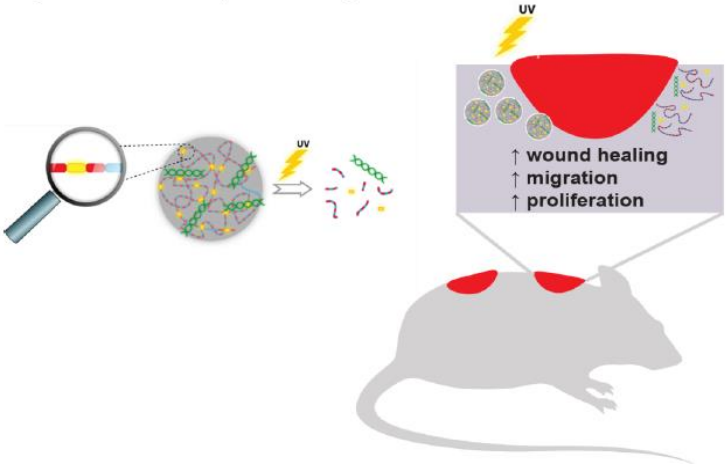
Impaired wound healing and its medical complications remain one of the most prevalent and economically burdensome healthcare issues in the world. RNA-based therapies have emerged recently as promising drugs for skin regeneration [1,2], with distinct advantages over conventional drug therapies such as small molecules or other biomolecules, such as specificity, potency, number of accessible targets, species crossreactivity, manufacturing, etc… [3]. However, several obstacles need to be addressed before the clinical translation of RNA-based therapeutics. In particular, the design of formulations that enable their delivery to a target cell in the skin reducing potential off-target effects and simultaneously increase their efficacy in the intracellular delivery. The hypothesis of the current work was that biocompatible light-activatable nanoparticles (NPs) allowing precise control of the timing and spatial release of the RNA molecules could accelerate the translation of these therapies.

Our experimental results indicate that we can produce a library of light-activatable NPs (more than 300 NPs) that dissociate at different rates once activated by UV or a blue laser. We have performed high-throughput screenings in reporter cells to identify formulations that were rapidly taken up by cells and deliver efficiently siRNA (more effectively than the commercial transfection agent lipofectamine RNAiMAX). We have identified candidates that were further characterized in secondary tests regarding their specificity to skin cells (some NPs were more internalized by a specific type of cell than other), endolysosomal escape and functional studies before and after light activation. Moreover, we have confirmed the advantages of one of the candidate formulations in a wound healing animal model, for the delivery of a skin regenerative miRNA identified recently by us. In conclusion, we have developed a powerful platform for the delivery of RNA-based therapeutics delivery both *in vitro* and *in vivo*.

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**OC-10**

# Orodispersible films to treat a neurodegenerative disorder: clinical proof of concept

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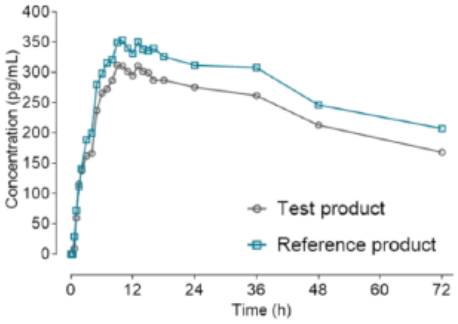
**Introduction:** Orodispersible films (ODFs) along with other orodispersible dosage forms (tablets, minitablets, granules, powders) offer several advantages such as ease of administration, no need for water intake and dose accuracy. Orodispersible films are preferred over other orodispersible formulations due to their faster disintegration, ease of handling, decreased risk of particles aspiration and improved patient compliance [1-3]. These characteristics are particularly relevant for patients with neurodegenerative disorders frequently affected by dysphagia where the treatment is administered in the form of injectables, tablets or capsules. Therefore, ODFs containing the same strength of drug substance (DS) as in the commercially available capsules were developed and their pharmacokinetics, safety and tolerability were assessed in healthy subjects.

**Materials and Methods:** Twenty-four healthy subjects were enrolled in an open-label, randomized, parallel-group study. Subjects were given a single dose of the reference product (RP) commercially available (capsules) or a single dose in the form of ODFs. Blood samples were collected at specified time points for drug substance and its phosphate metabolite for pharmacokinetics evaluation. The disintegration time and taste acceptability of ODFs was also evaluated.

**Results and Discussion:** The pharmacokinetic parameters of the drug substance determined in this clinical trial were in accordance with the results found in literature. Pharmacokinetic parameters and statistical analysis of the results indicated that the rate (as assessed by $C_{max}$) and extend (as assessed by $AUC_{0-72}$) of systemic exposure from ODFs were not significantly different from the RP (Figure 1 A and B). Based on the acceptance criteria for bioequivalence, this exploratory study provides evidence that the test product is bioequivalent to the RP.

Similarly to the observed in ODFs formulations developed by others [4, 5], the test product included in its composition flavors and sweeteners for taste masking. ODFs' taste was well accepted by the volunteers of the study.

**A**




**B**

| Parameter | ANOVA p-value | Geometric LSmeans | | Test/Reference GMR(%) | 90% CI |
|---|---|---|---|---|---|
| | | Test | Reference | | |
| $C_{max}$ | 0.210 | 324.0 | 365.8 | 88.58 | 75.38-104.08 |
| $C_{max}$ /NF | 0.766 | 45395.0 | 44284.5 | 102.51 | 89.02-118.04 |
| $AUC_{0-72}$ | 0.158 | 16434.9 | 18925.7 | 86.84 | 73.57-102.50 |
| $AUC_{0-72}$ /NF | 0.955 | 2302485.6 | 2291149.2 | 100.49 | 86.59-116.63 |

$C_{max}$ maximum observed blood concentration post dose, $AUC_{0-72}$ area under the blood concentration versus time curve from time zero to 72 h, *NF* normalization factor , *LSmeans* least square means, *GMR* geometric means ration, *CI* confidence interval

*Figure 1.* A- Drug substance mean blood concentration versus time profile of ODFs (test product) and capsules (reference product). B- Drug substance least square means, test-to-reference geometric means ratio and 90% confidence intervals for $C_{max}$, AUC0-72, $C_{max}$ /NF, and $AUC_{0-72}$ /NF.

**Conclusions:** The ODFs were well-tolerated in this population of healthy subjects either in terms of adverse events or in terms of taste acceptability. The systemic exposure to the drug substance was similar after the administration of the reference product and ODFs demonstrating that both formulations are bioequivalent. This proof of concept clinical trial demonstrates the potential to improve patient's compliance using ODFs, because it addresses an unmet medical need of having an easy to swallow formulation able to facilitate the administration of DS to dysphagic patients.

**Acknowledgments**: This work was supported by FCT grant, reference SFRH/BDE/52098/2013 and QREN SI-I&DT Project No.38480

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**OC-11**

# Tumor intracellular bioavailability of doxorubicin determines therapeutic efficacy of GLP grade nanoparticle targeted to nucleolin independently of systemic exposure

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**Introduction:** Caelyx® (non-targeted liposomal doxorubicin) has had a major impact on improving the safety profile of conventional (free) doxorubicin in patients [1]. However, those with breast or ovarian cancer have not benefited from improved efficacy relative to free doxorubicin, contrasting with preclinical data. This may arise from the limited extent of the enhanced permeability and retention (EPR) effect in those patients, thus limiting drug bioavailability [2]. Drug bioavailability at the tumor and/or at the tumor cell level is therefore of utmost importance for overall efficacy [3, 4]. Accordingly, there is a need for novel mechanisms of drug delivery, different from the one based on the EPR effect. Targeting readily available overexpressed markers that promote cell internalization, combined with efficient intracellular drug release, may offer increased efficacy and safety [3]. Accordingly, the objective of this work was to assess *in vivo*, the mechanism of action of a novel doxorubicin-containing pH-sensitive liposome functionalized with the nucleolinbinding F3 peptide (codenamed PEGASEMP®). It was hypothesized that nucleolin deregulated overexpression in the tumor microenvironment (endothelial cells from tumor blood vessels), besides cancer cells, will enable improved tumor bioavailability of doxorubicin delivered by PEGASEMP® relative to the non-targeted non-pH-sensitive counterpart [5].

**Materials and Methods:** Doxorubicin (DXR) blood profile was determined in female BALB/c mice, upon intravenous administration of PEGASEMP® (at 5, 6 or 7 mg DXR/Kg; Good Laboratory Practices [GLP] grade). The corresponding tumor accumulation was assessed in female BALB/cnu/nu mice, bearing nucleolin-overexpressing MDAMB-435S-derived mammary tumors (100-150 mm3). Lip-DXR, non-targeted non-pH-sensitive liposomal DXR (at 5 mg DXR/Kg) was used as control. Tumors were collected 24 h after administration. Plasma and tumor samples were analyzed by LC-MS/MS. Furthermore, a group of tumors were sectioned and blindly swept for DXR fluorescence image acquisition using confocal microscopy. Antitumor effect against MDA-MB-435S-derived mammary tumors was assessed upon i.v. administration of PEGASEMP® at 6 or 7 mg DXR/Kg (q7dx5w). Lip-DXR (5 mg DXR/Kg, same schedule) and saline solution were used as controls. Tumor volume and body condition were assessed at least twice-a-week.

**Results and Discussion:** The systemic exposure (AUC5 min-48 h) of DXR delivered by PEGASEMP® was 8.7-fold lower than the one from Lip-DXR, at same dose (5 mg/Kg). Notwithstanding DXR delivered by PEGASEMP® presented a dose-dependent systemic exposure (4.5-fold higher AUC5 min-48 h at 7 mg/kg than at 5 mg/Kg), the corresponding bulk tumor accumulation at 24 h was not dependent on the administered dose, and, at least, 4.3-fold lower than the one delivered by Lip-DXR. Strinkingly, intracellular delivery of DXR at 6 and 7 mg/Kg by PEGASEMP®, at the tumor level, (assessed by the number of DXR+ cells per area), was equal or 2.3-fold higher, respectively, than the levels provided by Lip-DXR, which is consistent with the enhanced intracellular delivery and proposed release mechanism. This translated into a relevant improvement of overall survival (80% for PEGASEMP® at 7 mg/kg compared to 43% of Lip-DXR). The antitumor effect was accompanied by a reduction of the area of dividing cells (Ki-67+) and nucleolin-positive tumor blood vessels.

**Conclusions:** Delivery of a small molecular weight drug as DXR by pH-sensitive liposomes, targeted towards a marker as nucleolin, overexpressed in the tumor blood vessels (and cancer cells), enabled a significant improvement of drug bioavailability at the intracellular tumor level, relative to the non-targeted non-pH-sensitive counterpart (with a higher systemic exposure and higher bulk tumor accumulation) [4]. Accordingly, the antitumor efficacy of PEGASEMP® relies on efficient, systemic exposure-independent, intracellular bioavailability of DXR rather than overall tumor accumulation.

**Acknowledgments:** The work was supported by the grants QREN/FEDER MultiNanoMed (Ref: 23240), by the European Regional Development Fund (ERDF) through COMPETE 2020 - Operational Programme for Competitiveness and Internationalisation and Portuguese national funds via FCT – Fundação para a Ciência e a Tecnologia, under the project POCI-01-0145-FEDER-007440.

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**OC-12**

# Exosomes: tiny vesicles with great potential for MJD treatment

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**Introduction:** Machado-Joseph disease (MJD) is a neurodegenerative disorder that associates with an unstable expansion of a CAG tract in the coding region of *ATXN3* gene, which translates into a polyglutamine repeat expansion in the resultant ataxin- 3 protein [1]. This abnormally expanded tract confers a toxic gain of function to the protein, leading to neuronal dysfunction and death in several regions of the central nervous system (CNS). Consequently, MJD patients present diverse clinical manifestations, losing voluntary movement control, which culminates in premature death [2]. Despite the effort of the scientific community towards the development of a therapy for this condition, MJD still remains incurable and patients only have available symptomatic care [3]. Exosomes have recently emerged as promising tools for efficient delivery of therapeutic strategies aimed at treating CNS diseases, due to their intrinsic features, such as the stability and stealth capacity in bloodstream, the ability to overcome natural barriers and targeting properties [4].

Based on that, the aim of this work was to develop an exosome-based gene delivery system for the treatment of MJD.

**Materials and Methods:** We developed exosome-based vector systems that express on their surface a fusion protein constituted by a transmembrane exosomal domain, and brain targeting peptides that assure brain targeting and allow blood-brain barrier (BBB) crossing. These exosomes were characterized concerning to their size (Nanoparticle Tracking Analysis), morphology (Transmission Electron Microscope) and typical protein markers (Western Blotting). In parallel, to evaluate brain-targeting specificity, they were loaded with luciferase to evaluate exosomal biodistribution along time, through bioluminescence imaging using IVIS Lumina II XR. After this evaluation, exosomes were incorporated with silencing sequences for mutant ataxin-3 mRNA and intravenously injected in the tail vein of a transgenic mouse model of MJD. Control transgenic mice were injected with modified exosomes containing control sequences. In an attempt to assess transgenic mice behavior performance, we carried out behavioral tests each 3 weeks during 3 months. To evaluate neuropathological features, mice brain sections were analyzed for mutant ataxin-3 protein aggregates (immunohistochemistry) and granular and molecular layers thickness of cerebellum (cresyl violet staining). Finally, to access mutant ataxin-3 protein and mRNA expression levels in cerebellum, we performed Western Blotting and qRT-PCR, respectively.

**Results and Discussion:** Engineered-exosomes specifically and efficiently delivered genetic material to mice brains, as confirmed by bioluminescence imaging analysis. Importantly, transgenic mice IV-administered with therapeutic exosomes displayed better performance in behavioral assessment when in comparison with the control ones. Treated mice displayed also reduced mutant ataxin-3 mRNA and protein levels, promoted attenuation in cerebellar-associated neuropathology, particularly regarding number of cells with protein aggregates, and amelioration in cerebellar layers thickness.

**Conclusions:** In conclusion, we have developed an original exosome-based gene delivery system for the treatment of MJD which involved the use of brain-targeted exosomes including silencing sequences targeting the causative mRNA. These modified exosomes were able to cross the BBB, silence the mutant ataxin-3 mRNA, and ameliorate the neuropathology and mitigate motor deficits in a mouse model of MJD, through minimally invasive routes of administration. This is the first exosome-based gene delivery system for the treatment of MJD and constitutes a promising delivery tool for the treatment of other brain-related diseases.

**Acknowledgments**: This work was funded by the ERDF through the Regional Operational Program Center 2020, Competitiveness Factors Operational Program (COMPETE 2020) and National Funds through FCT (Foundation for Science and Technology) - BrainHealth2020 projects (CENTRO-01-0145-FEDER-000008), ViraVector (CENTRO-01-0145-FEDER-022095), CortaCAGs (POCI-01-0145-FEDER-016719) and POCI-01-0145-FEDER-007440, as well as the EXPL/NMC/0331/2012 (FCT) and the SynSpread, ESMI and ModelPolyQ under the EU Joint Program - Neurodegenerative Disease Research (JPND), the last two co-funded by the European Union H2020 program, GA No.643417; by National Ataxia Foundation (USA), the American Portuguese Biomedical Research Fund (APBRF), AFM-Telethon no. 21163 and the Richard Chin and Lily Lock Machado-Joseph Disease Research Fund.

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# POSTER COMMUNICATIONS

**PC-1**

# Development of nanocarriers for intracellular delivery of protein drugs

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**Introduction:** Cancer is one of the main causes of death in the developed countries. To treat this kind of diseases chemotherapy continues to be the main therapeutic option, although the side effects (e.g. non specificity) of these drugs represent a big problem that needs to be solved. The use of proteins as therapeutics coupled with nanotechnology can overcome the drawbacks of the classical therapy, enabling more selectivity and specificity. Based on this, the main objective of this work was to develop a nanocarrier with the capacity to associate protein drugs and to deliver them in the intracellular environment of cancer cells. The carrier is based on natural compounds (i.e. hyaluronic acid, HA) and surfactants, well tolerated by the human's body.

**Methods:** an HA-based nanocarrier based on both hydrophilic and hydrophobic interactions of the biomaterials and the protein was developed. The nanocarrier's properties (average size and zeta potential, encapsulation efficiency, loading capacity and protein release) were evaluated and the effect of both different formulation media (milliQ water and phosphate buffer 10 mM pH 7.2) and the presence or not of two model proteins (BSA and an IgG) was studied. Finally, the stability of the particles in simulated biological fluids (PBS and RPMI) and also upon storage was monitored.

**Results and discussion:** particles with a size below 300 nm and a round shape were obtained in both milliQ water and phosphate buffer 10 mM pH 7.2. Selected particles showed a negative zeta potential (around -25 mV and -30 mV) and were able to encapsulate BSA with high efficiencies (more than 90%). The IgG was just loaded into the nanoparticles formed in milliQ water, since big particles with a low reproducibility were obtained when loaded into the ones formed in phosphate buffer. The BSA was found to be fundamental to guarantee the stability of the particles in simulated biological media for at least 24h and, for this reason, particles containing both BSA and IgG were developed. The selected particles showed a 40% (if formed in phosphate buffer 10 mM pH 7.2) or 60% (if formed in milliQ water) burst release profile. The nanoparticles were found to be stable upon freeze drying.

**Conclusions:** the developed delivery system is able to efficiently encapsulate both BSA and IgG. The type of media in which the particles are formed influenced the nanoparticles properties. It was found that BSA is fundamental for stabilizing the IgG encapsulating nanoparticles in simulated biological fluids and good preliminary results were obtained regarding the storage stability. These results suggest that the developed nanocarrier could be promising for protein delivery to cancer cells.

**Acknowledgments**: This work was supported by the European Union's Horizon 2020 - Research and Innovation Framework Programme under the Marie Sklodowska - Curie Grant agreement No. 642028 (NABBA).

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**PC-2**

# First use of polyurethane Pearlbond™ 523 in prolonged drug release matrices by direct compression. Estimation of critical points by Percolation theory

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## Introduction

Nowadays, polyurethanes are one of the most versatile polymers because of their wide variety of applications as well as outstanding aspects like adhesiveness, strength, elasticity, non-toxicity and low production price. Thermoplastic polyurethanes (TPU) have been successfully used for biomedical purposes but the use of these polymers for drug delivery systems is more limited. Recently, TPUs have been used in sustained release matrices prepared by hot melt extrusion or injection molding [1]. Nevertheless, the ability of these polymers as excipients in direct compression has not been already developed. The objective of the present work is to evaluate the ability of a thermoplastic polymer to form controlled release matrices by direct compression and to estimate the percolation threshold of the polyurethane.

## Materials and Methods

Thermoplastic polyurethane (TPU) was kindly supplied by Merquinsa (Pearlbond-523, Lubrizol, USA) and anhydrous theophylline (Acofarma, Spain) has been used as model drug.

The powdered TPU was rheologically characterized using the SeDeM method [2] to determine whether the polymer is suitable for direct compression. Blends of theophylline and TPU were prepared with varying concentrations: 10/90, 20/80, 30/70, 40/60, 50/50 w/w. Matrix tablets were obtained by direct compression with an eccentric tableting machine and technologically characterized according to European Pharmacopoeia. Drug release studies were carried out in a dissolution apparatus 2 (USP 23) using 900 ml of purified water (37± 0.5 ºC) during 12 h. The percentage of drug released was measured by UV–Vis spectrophotometry (272 nm).

According to the percolation theory, critical points represent discontinuities of the system properties, due to geometrical phase transitions of the components. Kinetic parameters obtained from dissolution profiles were represented as a function of the polymer weight fraction (%) in order to estimate the percolation threshold.

## Results and Discussion

Rheological results of powdered TPU indicate that the polymer has suitable properties to be processed through direct compression, with values comparable to commercial controlled release excipients, as HPMC or ethylcellulose. Moreover TPU matrix tablets have a suitable crushing strength and disintegration times higher than 30 min. Dissolution profiles (Figure 1) from TPU tablets indicate the ability of the polymer to prolong drug release. With respect to the excipient percolation threshold, kinetic parameters as Higuchi's and Peppas-Sahlin constants were represented as a function of the TPU weight fraction (%) (see for example the relaxation constant in Figure 2). These results indicate that TPU percolation threshold falls between 23.03-25.45% w/w.

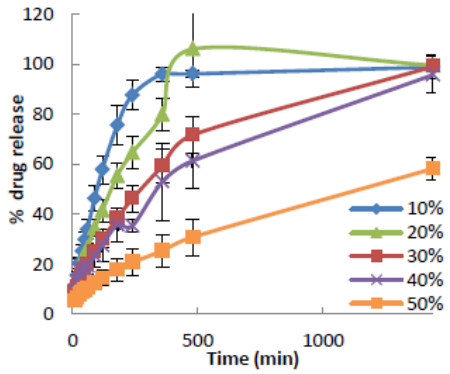


*Figure 1*. Dissolution profiles from TPU matrix tablets

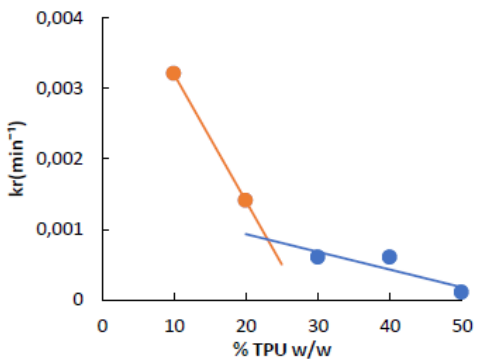


*Figure 2*. Relaxation constant *vs* % thermoplastic polyurethane

## Conclusions

Biodegradable polyurethane Pearlbond™ 523 shows suitable rheological characteristics to be processed by direct compression, according to the SeDeM methodology. Matrix tablets prepared with this polymer confirm its ability to prolong drug release, showing a percolation threshold between 23.03-25.45% w/w.

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**PC-3**

# BSA Nanoparticles with Parvifloron D for Pancreatic Cancer Treatment

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**Introduction:**
Pancreatic cancer is the thirteenth most common cancer and the eighth leading cause of cancer death worldwide. The reason for this high mortality rate is that most patients are diagnosed with advanced disease. The prognosis of this type of cancer remains very poor with only a 5-year survival in 5% of most reports. An improvement in the outcome of patients with pancreatic cancer is strongly dependent on the development of more effective therapies [1].
Nanotechnology can play a crucial role by targeting the drugs to the malignant cells [2]. In another hand, medicinal plants studies have led to the discovery of new attractive bioactive compounds. As an example, Plectranthus species can really be used as a chemotherapy option because they have cytotoxic and antiproliferative activities against human tumor cells like the present abietane diterpenoid, Parvifloron D [3]. However, Parvifloron D has solubility problems and lacks some specificity to tumor cells. The aims of this work were to extract and isolate Parvifloron D from *P. ecklonii*; to prepare and characterize albumin nanoparticles using different processes.

**Material & Methods:**
Parvifloron D was extracted by an acetone ultrasound-assisted method and isolated by a chromatography column over silica gel using eluents mixtures of increasing polarity.
Albumin nanoparticles were produced through desolvation method [4]. The formulation was optimized using five different cross-linking processes (glutaraldehyde, glucose, glucose with UV light and UV light), albumin concentration (30mg, 50mg and 150mg), different cross-linking times (30min and 24h) and organic solvents (DMSO, acetone, ethanol and hexane). Resultant particles were then characterized in terms of stability, particle size by photon correlation spectroscopy, zeta potential by laser Doppler anemometry, cross-linking efficacy by Bradford method and shape by atomic force microscopy (AFM). Parvifloron D was encapsulated in the optimized formulation and then characterized in using the same techniques. Encapsulation Efficacy (%) was determined by measuring the non-encapsulated drug lost in the supernantant (i.e., indirect quantification) by HPLC analysis.

**Results & Discussion:**
For the extraction and isolation of Parvifloron D from *P. ecklonii*, it was used a total mass of 197.55g of dry plant. Extraction was carried out as previously described [5]. Whole plant was macerated with acetone as solvent and it was subjected to ultrasound-assisted at room temperature. The extract was obtained by filtration and evaporation of the solvent under vacuum at low temperature (50˚C) to yield 28.54 g of extract (14.44% (w/w) of the dry plant). The extract was subjected to successive chromatographic processes and a mass of 0.882 g of Parvifloron D was isolated (0.45% (w/w) of the dry plant).
The majority of nanoparticle population showed a particle size range between 90 and 520nm. Some agglomerates were visible in some of the methods. All nanoparticles showed a negative zeta potential, independently of the different production conditions. In terms of morphology, nanoparticles were spherical and AFM confirmed the particle size. Differents cross-linking efficacies in the methods were observed and ranged between 43.8% and 99.6%. Then, Parvifloron D was encapsulated in the chosen formulation. Particle size was around 95nm and nanoparticles maintained a negative zeta potential. The cross-linking efficacy was up to 85% and the encapsulation efficacy was 91.2%.

**Conclusions:**
This study confirms the feasibility of producing uniform and well-defined albumin nanoparticles, as well as it shows to be a good method to encapsulate the Parvifloron D. Further ligand-attachment onto the nanoparticle surface and efficacy studies activity against tumor cells will be performed.

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**PC-4**

# Highly concentrated emulsions based on cubic liquid crystals for the release of diclofenac sodium

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## Introduction

Highly concentrated emulsions or high internal phase ratio emulsions (HIPREs) have a volume fraction of droplets higher than 0.74, value corresponding to the highest packing density of monodisperse spherical droplets. HIPREs exhibit gel-like properties and have been used as controlled drug delivery systems [1]. The continuous phase of HIPREs surrounding the droplets can be constituted by a liquid crystal [2], with a key role on active solubilization, drug release properties and emulsion stability. The present work describes the preparation and characterization of O/W HIPREs containing 1% diclofenac sodium (DS) and the influence of liquid crystal structure in the continuous phase on emulsion stability and drug release.

## Materials and Methods

Miglyol 812® (medium chain triglycerides, Fagron Ibérica S.A.V.) was used as oil component. Cremophor RH 40 (Polyoxyl 40 hydrogenated castor oil) from BASF and Tween 20 (Polyoxyethylene (20) sorbitan monolaurate) from Sigma-Aldrich were used as surfactants. MilliQ® water was the aqueous component. Diclofenac sodium (DS) was purchased from Sigma-Aldrich. Phosphate buffer solution pH 7.4 (PBS) was used as receptor solution in release studies. Commercial gel, Solaraze® (Almirall) was used as a reference in release studies.
O/W HIPREs were prepared by stepwise addition of the oil phase to surfactant and water mixtures under continuous stirring (2500 rpm) at 25ºC. The stability of the emulsions was assessed by visual observation, optical microscopy and rheology. Liquid crystalline structures were characterized by Small-Angle X-Ray scattering (SAXS). DS release profiles were studied at 32ºC by Franz diffusion cells with cellulose membranes (Cellu SepT3®, Orange Scientific). Quantification of DS was carried out by HPLC (Shimadzu, Nexera X2) and with UV detection.

## Results and Discussion

HIPREs were prepared at a constant surfactant: water weight ratio of 40:60. SAXS patterns at 25ºC at various oil concentrations and the optical isotropy of the samples point the presence of cubic phases in the continuous phase of HIPREs prepared from Cremophor RH40, which results in a higher colloidal stability. The liquid crystalline structure was preserved after addition of DS. HIPREs presented sustained release of DS during the experiment (24 hours) comparing to commercial gel in which DS was released during the first hours of assay. Molecular diffusion through the solvent seems to be the main controlling step in the release process of DS from the HIPREs studied.

## Conclusions

Cubic liquid crystalline structures were identified by SAXS in O/W emulsions and containing 1%DS. The higher stability of HIPREs prepared with Cremophor RH40 could be attributed to the formation of liquid crystalline structures. HIPREs are suitable vehicles for sustained release of DS.

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**PC-5**

# Microgels, obtained in water-in-water (W/W) emulsions, as carriers for the encapsulation of lactase

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Water-in-water (W/W) emulsions are liquid/liquid dispersions of two immiscible aqueous phases, in absence of both oil and surfactant. They consist in droplets of an aqueous solution, dispersed into another aqueous solution. These colloidal dispersions can be prepared in aqueous mixtures of two hydrophilic polymers, in which segregative phase separation occurs because of mutual incompatibility between the two polymers [1,2]. In the present work, W/W emulsions were prepared in the $H_2O$/gelatin/maltodextrin system ternary system. Gelatin microgels were obtained in this system by cross-linking the gelatin, located mainly into the disperse phase of the emulsion, with genipin, which is a natural reagent. Fig. 1 shows an example of cross-linked gelatin microgel particles.
These microgels have been used as carriers for the encapsulation of lactase (also known as β-galactosidase), and preliminary experiments of release of this enzyme have been performed. Different methods of encapsulation have been tested, and the best results were obtained when adding the enzyme into the gelatin solution, before emulsifying and crosslinking. The results clearly showed that crosslinking improved the efficiency of encapsulation. Moreover, crosslinking was able to retard and control the release of the enzyme, as expected. It is interesting to note that the enzyme-loaded microgels could be freeze-dried, and resuspended again in water, but nevertheless the activity of the enzyme was preserved. Further studies of enzyme release and activity are currently being performed.

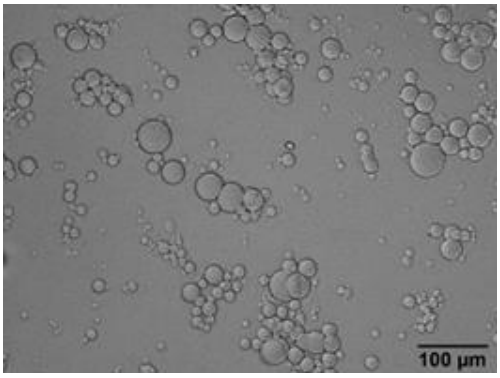


*Figure 1*. Example of a microgel, obtained by cross-linking in the disperse phase of water-in-water emulsions.

**Acknowledgments**: This work was supported mainly by a Marie Curie Action of the European Union's Seventh Framework Programme, under REA grant agreement n°606713 (BIBAFOODS project).

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**PC-6**

# Study of nanostructured fibroin/dextran biomatrices for controlled protein release

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## Introduction

Micro- and nanoporous films are structures with interesting features for drug delivery due to their high porosity and intrinsic surface area [1]. In drug delivery applications, the films need to be formed of biocompatible materials that are also preferably biodegradable. Unfortunately, most biocompatible materials useful for micro- and nanoporous film formation are suboptimal for drug delivery because they cannot control drug release or/and they require organic solvents for film preparation. Silk-based materials have attracted attention in this area due to their versatility, excellent safety record, and capacity for sustained drug release. Fibroin (SF) is a high molecular weight protein that is the main component of silk. SF can be combined with other polymers [2] and processed in several forms and by different methods, including some performed entirely in aqueous envioronments [3]; thus, SF is ideal material for the development of green medical technologies. SF blends can change drastically the properties of the final material, affecting characteristics such as water absorption or the secondary structure of SF. In the current work, we have evaluated the effect the anionic polymer dextran sulfate (DSS) on the physical properties of SF/DSS blend films, and how this blending affects the controlled release of a model protein, horseradish peroxidase II (HRP).

## Materials and Methods

SF was purified from silkworm coccons as previously described [2,3], and several blend SF/DSS ratios were employed for film formation. The changes on the structure of SF/DSS films were studied by FTIR and scanning electron microscopy (SEM). We also tested the physical and chemical properties of the different films, both as prepared and after incubation in simulated physilogical medium. HRP was labelled with fluorescein isothiocyanate and HRP release from the SF blend films was investigated and fitted to different mathematical kinetic models.

## Results and Discussion

As a result of the blending, it was possible to obtain micro- and nanostructured films (Fig. 1) with different physical and structural properties. DSS acted as a functional porogen, where the size of nano and microdomains varied with the blend ratio. High blend ratios showed a higher swelling ratio, porosity, and crystallinity. They also resulted in lower degradation rate and modified protein release kinetics as compared to pure SF films.

## Conclusions

SF/DDS films can be prepared though a water-only process that leads to a microporous structure. Their physical and pharmaceutical properties make these devices ideal for the sustained release of proteins upon implantation.

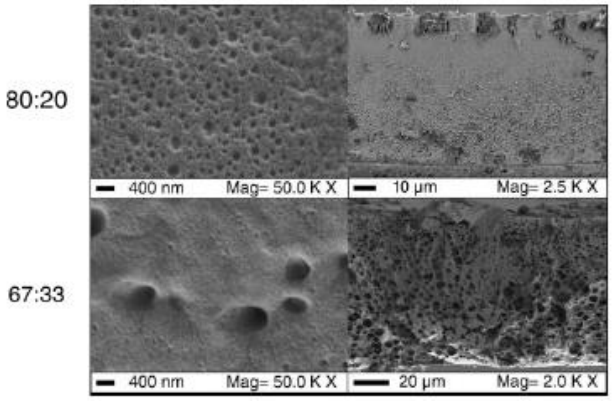


*Figure 1*. Surface and section areas of fibroin/dextran films

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**PC-7**

# Near-infrared light triggered release of bioactive molecules from supramolecular modified gold nanorods

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Nanotechnologies are an emerging platform to control the activity of endogenous (stem) cells [1,2]. In the last years, we have demonstrated that small molecules such as retinoic acid released by nanoparticles may control the biological activity of neural stem cells [3,4], leukemia cells [5], and endothelial progenitor cells [6]. We have also demonstrated the possibility to control *in vivo* the release profile of nanoparticles containing retinoic acid by a blue laser, giving us an unprecedent spatio-temporal control in the biological effect of retinoic acid. Unfortunately, the tissue penetration of the blue laser is relatively low [5]. Here, we have developed a near infrared-triggerable release system of retinoic acid that allow us high tissue penetration. The formulation is based in gold nanorods (AuNRs) conjugated with cucubit[6]uril (CB6) host which was complexed with retinoic acid modified with polyamines (Figure1). After exposure to near infrared laser, the AuNRs generate plasmonic heat which is enough to disrupt the host-guest interaction between CB6 and the modified retinoic acid. We have demonstrated the extracellular and intracellular release of retinoic acid in a NB4-RARE reporter cell line.

The functionalized AuNR were synthesized using seed-mediated method with CTAB as a stabilizer, followed by ligand exchange with CB6 modified hyaluronic acid (CB6HA). The ligand exchange was evaluated by UV-Vis and Zeta potential analysis. The three retinoic acid analogs were synthesized by adapting a reported procedure [7].

To demonstrate whether molecules may be released from the CB6HA@AuNR system upon NIR irradiation, a fluorescent dye was selected as a proof-of-concept. 4-(1H-Imidazol-1-yl)aniline (IA) is a fluorescent compound that form a stable 1:1 host-guest complex with CB6 ($K_a = 1\times10^5\ M^{-1}$). Since this complexation is an exothermic process, the binding strength of the host-guest complex between IA and CB6 will remarkably decrease with increasing the temperature [8]. Therefore, the increase in temperature near the gold nanorod surface due to photothermal conversion after irradiation will lead to the displacement of the IA from the CB6 cavity. The fluorescence of IA increases upon complexation with the macrocycle and decreases when the molecule is free in the bulk. When an aqueous solution with IA@CB6HA@AuNR system was exposed for 120 s to a red laser at 780 nm at 2 $Wcm^{-2}$ power, the fluorescence intensity of IA decreased revealing the release of 25% of IA molecules from the CB6HA@AuNR system.

To assess the photothermal efficacy for the delivery of retinoic acid into leukaemia cells, the RA analogs was complexed with gold nanorods. The three RA analogs synthesized have different binding affinities with CB6, which allow us to control the number of RA molecules released from the nanoparticles after irradiation. Furthermore, we confirmed the high drug loading capacity of the CB6HA@AuNR system, since 0.1 nM of AuNR can complex up to 20 μM of RA. The delivery of RA was monitored using the firefly luciferase assay in a NB4-RARE reporter cell line. The results show that after NIR light exposure, the nanoparticles with the RA analog with lower binding affinity released up to 50% of RA molecules, while the RA analog with higher affinity approximately 10 % of RA is released.

In summary, we have developed a system that has the potential for spatiotemporal release of therapeutics, with the advantage to fine-tune the number of molecules to be released.

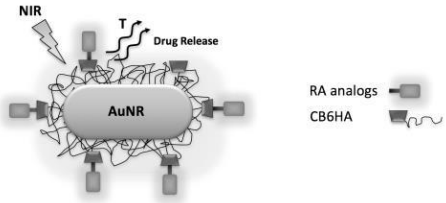


*Figure 1*. Schematic representation of the macrocycle conjugated gold nanorods for NIR light photothermal drug delivery.

**Acknowledgments**: Authors would like to acknowledge to FCT (Portugal, SFRH/BPD/102103/2014) and ERC funding (ERC project nº 307384, 'Nanotrigger') and Compete 2020- Operational Programme for Competitiveness and Internationalisation, POCI-01-0145-FEDER-016390:CANCEL STEM project for supporting this work.

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**PC-8**

# Ionic liquids and nanoparticles hybrid systems as new tools to deliver poorly soluble drugs

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**Introduction:** Poor drug solubility and low permeation are two major problems to consider in the development of drug delivery systems. The encapsulation of drugs into nanoparticles is a useful strategy to avoid such problems and deliver it in a targeted or controlled manner [1]. Ionic liquids (ILs) are salts, with an organic cation and an organic or inorganic anion, that are liquid below 100ºC, and may be used as functional excipients in pharmaceutical formulations, since they may be added to water, oils or hydroalcoholic solutions to increase drug solubility [2]. The combination of nanosystems and ILs, may represent an interesting improvement in the delivery of poorly soluble drugs. Therefore, the aim of this study was to develop a IL-nanoparticle hybrid system as a new strategy to deliver a poorly soluble drug model, rutin.

**Materials and Methods:** Poly(lactic-co-glycolic acid), (PLGA), nanoparticles are widely used because of its biocompatible, biodegradable and good sustained release properties [3]. PLGA 50:50 or PLGA 75:25 were used to produce nanoparticles by a modified solvent evaporation, w/o/w double emulsion technique described by Fonte et al [4]. The inner phase was an aqueous solution of 0.2% (v/v) IL dissolving rutin [2,5]. The ILs used were the prepared choline-based ILs [2], namely (2-hydroxyethyl)-trimethylammonium-L-phenylalaninate [Cho][Phe] and (2-hydroxyethyl)-trimethylammonium-L-glutamate [Cho][Glu]. After nanoparticles production, it was evaluated the diameter, polydispersity index (PDI), zeta potential (ZP) and association efficiency (AE) of the system. The stability and activity of rutin was also evaluated after production and an *in vitro* release study.

**Results and Discussion:** For PLGA 50:50, both ILs had similars diameter and PDI. Regarding the ZP, the formulation with [Cho][Glu] showed a higher colloidal stability (Table 1). For PLGA 75:25, all analyzed parameters were similar for both ILs (Table 1). The overall results showed that the hybrid systems had a diameter in the range of 250-280 nm, with good PDI and colloidal stability. The AE of rutin was higher than 70%, demonstrating that the combination of polymeric nanoparticles with ILs, not only, obtained stable nanoparticles, but also allowed the incorporation of a higher amount of rutin in the nanosystems, comparatively to nanoparticles without IL.

*Table 1*. Diameter (nm), PDI and ZP (mV) of Rutin-loaded PLGA nanoparticles-choline-based ILs hybrid systems. n=3, mean ± SD.

| Polymer | IL | Diameter (nm) | PDI | ZP (mv) |
|---|---|---|---|---|
| PLGA 50:50 | [Cho][Phe] | 251 ± 6.0 | 0.189 ± 0.022 | -29.75 ± 1.17 |
| | [Cho][Glu] | 254 ± 5.1 | 0.178 ± 0.013 | -43.34 ± 2.45 |
| PLGA 75:25 | [Cho][Phe] | 279 ± 5.2 | 0.181 ± 0.013 | -31.26 ± 0.30 |
| | [Cho][Glu] | 275 ± 4.0 | 0.224 ± 0.014 | -32.10 ± 2.22 |

**Conclusions:** This work demonstrated the potential of nanoparticle-IL hybrid systems to deliver poorly soluble drugs. The developed system allowed the loading of a higher amount of rutin in presence of IL, comparatively to nanoparticles without the IL, obtaining simultaneously a robust delivery system.

**Acknowledgments:** The authors would like to thank to Fundação para a Ciência e a Tecnologia, Portugal (FCT/MCTES (PIDDAC), UID/DTP/04567/2016).

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**PC-9**

# Advanced production of vectors for drug delivery applications using continuous flow

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## Introduction

Despite the current advances in the production of biomedical vectors for drug delivery applications, their translation into the clinic has been slow because it remains challenging to produce vectors that are consistent "batch-to-batch" and in sufficient quantities for clinical production scale [1]. Consequently, the development of new technologies tackling some of these challenges could significantly accelerate the clinical translation of nanomedicines.

## Materials and Methods

A plethora of biomedical vectors were produced by the fine manipulation of reagent streams using microfluidic micromixers and membrane-assisted systems (See Figure 1).

## Results and Discussion

Biodegardable polymeric poly(d,l-lactic acid/glycolic acid)-PLGA and PLGA-PEG nanoparticles, as well as niosomes were produced in continuous flow using the shear stress produced at the interphase of immiscible liquid microflows. This shear stress is energetic enough to promote the emulsion formation without the need of external mechanical or ultrasonic forces. The nanoparticle size was tuned by the control of the residence time and temperature of the inlet streams [2]. Membrane-assisted nanoprecipitacion was successfully developed to produce PLGA-PEG nanoparticles by controlling the solvent phase volume/non-solvent phase flow ratio [3]. Multi-lamelar niosomes were produced by microfluidic hydrodynamic flow focusing without the need of a size reduction step, as is required for the conventional methods. The production of these vectors were carried out using commercial systems, which is of outstanding importance in order to reproduce the resulting vectors and ease the translation of nanomedicines.

## Conclusions

The methodology based on microflow manipulation by micromixers and micrometric membranes allowed to produce drug loaded polymeric nanoparticles for delivery applications with a high productivity, reproducibility and easy scalability.

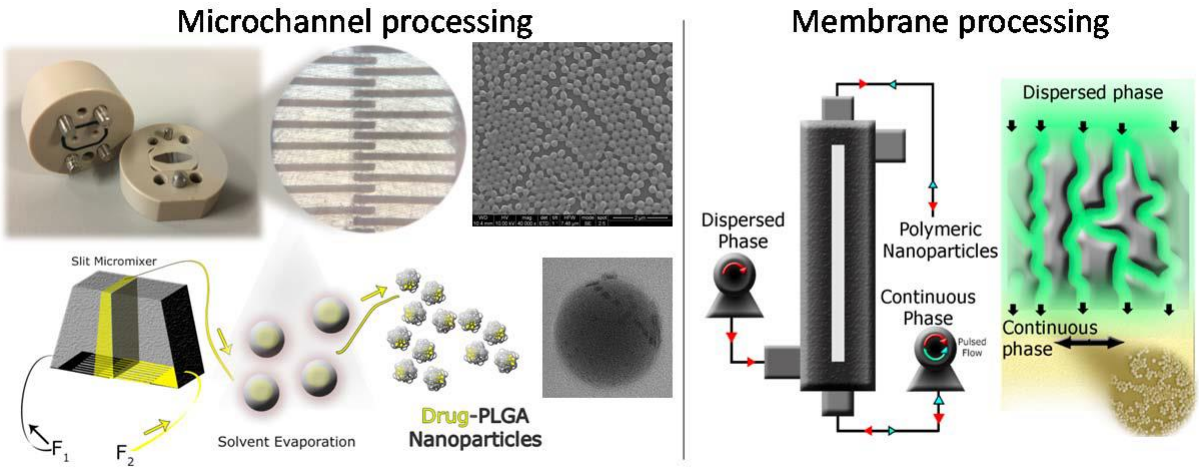


*Figure 1*. Microchannel and membranes processing of reagents to produce biomedical vectors for drug delivery applications

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**PC-10**

# Optimization of orodispersible films manufacture process using retrospective quality by design (rQbD)

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**Introduction**: The present work shows the application of quality by design (QbD) [1] based on data generated during the development of an orodispersible film (ODF) - retrospective QbD (rQbD). More precisely, the study explored the bases for the slower drug release observed during storage of the product. Through risk assessment tools, the critical process parameters (CPPs) (room temperature, room relative humidity, drying temperature and mixing equipment) were identified as potentially affecting ODFs critical quality attributes (CQAs), such as residual water content and percent drug release. Retrospective data of CPPs were used for statistical modeling and the estimated models generated were then applied to define the feasible working region (design space) [2].

**Materials and Methods:** The CPPs identified through the risk estimation matrix (REM) were analyzed, in order to identify the parameters that significantly influenced the drug release, as well as, to define the design space region. This analysis consisted in the construction of predictive multiple regression models for the responses of interest and in the evaluation of which effect was statistically significant for the process. The design space was then defined through the simultaneous combination of all the individual acceptance regions for each CPP.

**Results and discussion**: Statistical modeling indicated that the initial residual water content of the ODFs was mainly affected by second order interactions of room relative humidity vs. drying temperature, room temperature vs. drying temperature, while the stability of drug release profile was mostly influenced by the room temperature and drying temperature. Depending on the drying temperature employed, the effect of room temperature and room relative humidity change significantly. For the definition of the design space, although 40ºC would be the preferred drying temperature, it was not possible to determine the feasible working region at this condition (Figure 1). The results further indicated that for drying temperatures of 50ºC and 60ºC, a narrow design space could be established (Figure 1). It is important to point out that the quality data showed that the impurities content did not increase at these conditions.

**Conclusion**: This work exemplifies the application of QbD principles using retrospective data (rQbD) and illustrates its added value for increasing knowledge of investigational medicinal products being developed in general (e. g. tablets and capsules) and ODFs in particular.

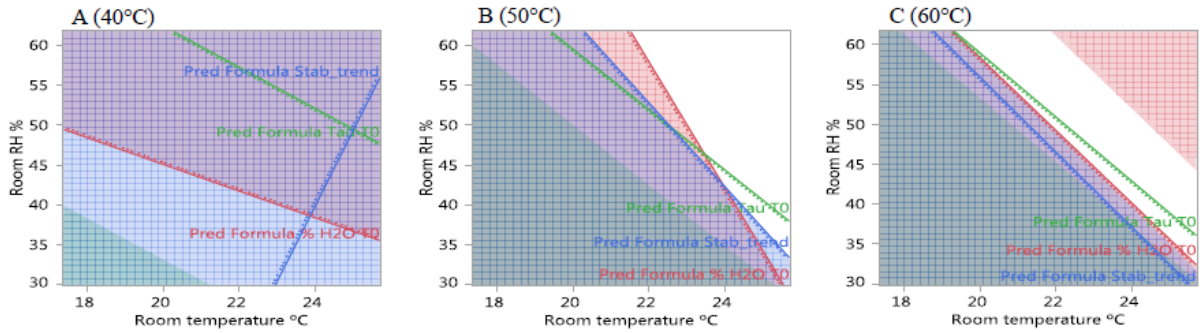


*Figure 1.*Design space for different drying temperature when Mixer is used: 40□C (A), 50□C (B) and 60□C (C). The unshaded white area represents the feasible working region and the dotted lines show the direction of increasing response values. Each response is represented by a color, initial residual water content is red, initial drug release rate is green and stability trend is blue.

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**PC-11**

# Fast Forward Screening for Hot Melt Extrusion: an effective tool for formulation development of poorly soluble drugs

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**Introduction:** In the last three decades, the use of high-throughput screening methodology generated a large number of new drug candidates with poor aqueous solubility. The poor drug release rate is the rate limiting step for absorption, which generally leads to low bioavailability (BA). To overcome solubility issues, Hot-Melt Extrusion (HME) have been adopted as an effective tool to produce amorphous solid dispersions (ASDs) [1, 2] with promising performances [3, 4]. HME formulations are complex mixtures of functional excipients and drug substance [5]. The first stage of formulation development includes the design of drug-polymer/excipients solubility and stability studies, aiming to drive more effectively the development of extrudates with improved manufacturability, BA and stability. At a later stage, these HME-based ASDs are formulated to yield the desired final dosage form.

**Materials and Methods:** A BCS class IV drug was used as model drug in this study [6]. The screening of polymers and solubility enhancers was performed by high-throughput screening using a solvent evaporation technique [7]. The experiments were evaluated in what concerns solubilisation capacity, measured by HPLC, as well as physical stability over 2 months, assessed by polarized light microscopy (PLM). The first stage of screening included binary systems composed by 7 different polymers and drug in 7 charge levels, ranging from 1% to 50%. At the target loading of drug (20%), a third component was added, where 5 polymers were combined with 10 solubilizers in two charge levels (1 and 4%). The most promising systems proceeded to the next stage, where they were tested by HME to assess manufacturability and solubility. Extrudates were characterized by assay (UPLC) and purity testing (HPLC), X-Ray Powder Diffraction (XRPD), thermal analysis (DSC) and dissolution testing (Apparatus II, quantification by HPLC). Two HME-based compositions were selected for further formulation development (extragranular phase) and processability into an adequate pharmaceutical form (tablets).

**Results and Discussion:** Through the solvent evaporation technique, 156 formulations were screened for solubilisation capacity and physical stability. Based on the results, 6 binary/trinary systems were selected for HME tests. In general, good correlation was found between results of solvent evaporation and HME. PVP-K12-based formulations were shown to be easily extrudable, with clear and transparent appearance. Moreover, their amorphicity was confirmed by XRPD and DSC analysis. Drug release rate was around twofold higher as compared to crystalline drug substance (Figure 1). Two of these systems were shown to have adequate compressibility.

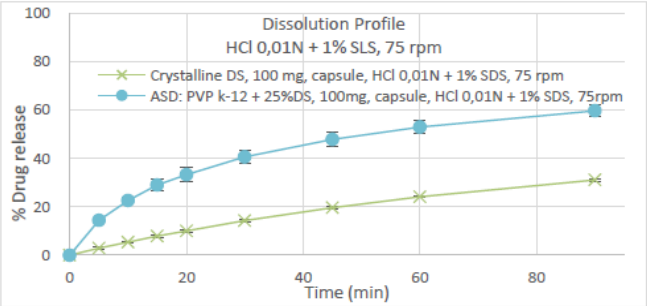


*Figure 1.* Dissolution profile in HCl 0.01N + 1% SLS at 75rpm of capsules contanining crystalline BCS class IV drug (100mg) or the corresponding ASD (100 mg of drug): PVP k-12 + 25% drug. Error bars represent standard deviation.

**Conclusions:** A fast and effective screening technique to develop stable ASDs for a poorly soluble drug was successfully developed and implemented. The given method is easy to use, requires low amount of drug and is fairly accurate in predicting the amorphization of the drug in when formulated. The success of HME formulation development was undoubtedly enhanced with this high-throughput tool, which led to the identification of extrudates with improved biopharmaceutical properties, such as drug release and compressibility.

**Acknowledgments**: This work was supported by FCT grant, reference PD/BDE/135149/2017.

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**PC-12**

# High gene delivery efficiency of a novel nanosystem based on a synergistic polymeric combination

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**Introduction:** Gene therapy is a very promising therapeutic strategy that allows not only to treat but also to prevent or eliminate the causes of a disease, changing the current medicine's paradigm. However, there are several obstacles that limit the clinical application of this technique, such as the number of barriers the genetic material has to surpass to reach the final target and the complexity of the procedure. The development of new and improved nanocarriers has been a vital field in medicine and health care, including for gene therapy application. Cationic polymer-based vectors have been considered a promising strategy in gene therapy area due to their relative ease fine tuning of physicochemical properties and inexpensive production. In this regard, the main goal of this work was to develop a new gene delivery nanosystem, based on the combination of PDMAEMA and PβAE homopolymers, in order to obtain polyplexes with reduced cytotoxicity and high biological activity, even in the presence of serum.

**Materials and Method**s: Polymers were synthesized according to our previously reported procedures [1,2]. Cell viability studies were conducted using the Alamar Blue assay. Cell uptake and the transfection efficiency were evaluated by confocal microscopy, flow cytometry analysis, and fluorescence microscopy [3]. The endocytic pathway involved in the internalization of polyplexes was determined by evaluating the cell uptake and biological activity of polyplexes in the presence of different endocytosis inhibitors. The biological activity of the different polyplex formulations was evaluated on different cell lines by luminescence assay. Size and surface charge of the polyplexes were measured by dynamic light scattering and zeta potential analyses.

**Results and discussion:** The biological activity of the combined polymeric-based formulations is approximately 200-fold and 35-fold higher than that observed with the best PDMAEMA-based and bPEI-based polyplexes, respectively. Among the diverse mixtures between PDMAEMA and PβAE homopolymers, the best formulation, prepared with the combination PDMAEMA/4PβAE at the 25/1 N/P ratio, presented a 700-fold and 220-fold higher transfection activity, in the presence of serum, than that obtained with bPEI-based and block copolymer-based polyplexes, respectively. The treatment of COS-7 cells with this new nanosystems, resulted in much higher percentage of transfected cells (~75%) than that achieved with the standard bPEI-based polyplexes (~10%), as observed both by flow cytometry and by fluorescence microscopy assays. Moreover, the obtained results showed that this physical mixture-based polyplexes is an efficient transfection agent in different human cells, including hard-to-transfect normal human astrocytes. Additionally, our results demonstrated that this novel formulation is mainly internalized by the clathrin-mediated endocytic pathway, showing, at the same time, that it presents the ability to escape from endosomes, avoiding degradation in the lysosomes and, consequently, allowing the above-presented high levels of biological activity. Regarding the physicochemical properties, the developed nanosystems presented high protection of genetic material and reduced sizes, which are suitable features for *in vivo* applications.

**Conclusions:** The non-covalent combination of PDMAEMA and PβAE homopolymers resulted in a noticeable and synergistic effect in terms of transfection activity, without causing substantial toxicity, constituting a new platform for the development of gene delivery nanosystems.

**Acknowledgments**: This work was financed by the grants PTDC/QUI-BIQ/116080/2009, IF/01007/2015, and PEst-C/SAU/LA0001/2013−2014 from the Portuguese Foundation for Science and Technology (FCT) and the European Regional Development Fund (FEDER) through the COMPETE program (Operational Program for Competitiveness).

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**PC-13**

# Development of polymer-based combination therapeutics for the treatment of Castration-Resistant Prostate Cancer (CRPC)

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**Introduction:** Prostate cancer (PCa) is the first most common cancer in men and leads to 10% of cancer deaths in Europe [1]. Therefore, identification of biomarkers for aggressive PCa would facilitate therapeutic targets. A potential biomarker is the presence of a fusion gene between TMPRSS2 (androgen-dependent serine protease) and ERG (transcription factor belonging to the ETS family) causing overexpression of TMPRSS2-ERG (T2E) transcript, which is present in 50% of diagnosed PCa always indicating the presence of cancer [2,3]. Androgen Receptor (AR) and Insuline Growth Factor 1 Receptor (IGF1R) are involved in PCa [4]. Preliminary results showed a reciprocal feedback loop regulation between both pathways [5], but the combination of an IGF1R inhibitor (monoclonal antibody mAb) with an anti-androgen drug (Abiraterone) results in synergistic effects in T2E positive cells [6]. Therefore, our work has focused on the construction of a polymer-based combination therapy composed of a polymer-antibody conjugate (PGA-mAb) with Abiraterone to produce a T2E-targeted treatment for aggressive PCa.

**Materials and Methods:** To evaluate the mAb and PGA-mAb response in PCa cell models the cells were seeded in 96 well plate, after 48h the cells were treated with the compounds and 72h later the cell viability was analysed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. To determine the selectivity against T2E fusion gen the cells were seeded in 24 well plate 2 days prior transfection. ERG siRNA and non-silencing siRNA were added to cells for 24 h. Following this time the medium was replaced and after another 24h cells were incubated with mAb, PGA-mAb alone and in combination with Abiraterone along 72 h, in this point the activity was measured by MTS assay. To check the mAb and PGA-mAb localization cell trafficking studies were performed, both compounds were labeled with a fluorophore Cyan 5.5. The compounds were incubated in VCaP cells for 1h and then was studied the cellular fate by immunofluorescence using confocal and STORM microscopy. To optimized *in vivo* model, VCap cell line was virally infected with Lentis pRNA tin Luc 2 vector to express luciferase, 24 h later the medium was replaced and the cells were selected by G-418 antibiotic. Cells were implanted into C.B-17/IcrHanHsd-Prkdc-scid male prostate gland and tumor growth was measured twice a week by IVIS spectrum along 7 weeks.

**Results and Discussion:** An antibody drug conjugate (poly-L-glutamic acid (PGA)-mAb) has been synthesized and fully characterized. This immunoconjugate has been evaluated in a panel of PCa cell lines (VCap, LNCap, PC3, RWPE1, DU145 and 22RV1) and compared with the parent drug. Only VCap cells showed response to both compounds due to the presence of T2E fusion gene. In order to design the above-mentioned combination approach, selectivity against T2E was a major goal apart from synergism. Therefore, to achieve this proof, ERG gene was silenced in VCap cell line and the mAb, Abiraterone as free single drugs and its combination, PGA-mAb conjugate and its combination with free Abiraterone were evaluated both in the silenced and non silenced cell lines. This study clearly demonstrated that, antitumor activity was directly correlated with the presence of T2E fusion gene since the silencing of ERG gene was always accompanied by an increase of survival. Important to note, the performance of PGA-mAb conjugate in combination with free Abiraterone showed an enhanced selectivity towards the presence of T2E fusion gene in comparison with the combination of free antibody. In order to explain this selectivity, differential cellular trafficking of the mAb upon polymer conjugation and testing the activity in an optimized orthotopic PCa model employing luciferase-expressing VCaP cells are being explored.

**Conclusions:** Our results suggest that the combination of PGA-mAb conjugate with Abiraterone could be a promising therapy in T2E PCa patient subtype as an enhancement in activity as well as selectivity has been observed in T2E positive models when compared with the parent free antibody combination.

**Acknowledgements:** This study is funded by PROMETEO/2016/103, SOGUG, Plan Nacional I+D; SAF2013-44848-R and FPI grant BES-2014-068439.

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**PC-14**

# Design of Polymer Therapeutics as combination therapy for the treatment of Triple Negative Breast Cancer

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**Introduction** Breast Cancer (BC) is the leading cause of death from cancer in women. Survival rate has improved over the last decade thanks to early diagnosis and therapeutic improvements that come from the development of combination therapy together with endocrine- and HER2-targeting therapies. Nevertheless, Triple Negative Breast Cancer (TNBC) patients cannot benefit from these improvements due to the lack of expression of progesterone and estrogen receptors and HER2 [1]. Moreover, this is the most aggressive subtype, with high risk of metastasis and the worst prognosis [2]. Thus, there is an urgent need to develop new systemic therapies against TNBC. In our lab, nanocarriers based on self-assembled star-shaped polyglutamates (St-PGA) have been developed and tested *in vivo* showing accumulation in lymph nodes [3]. Moreover, by the incorporation of suitable targeting vectors, they have also demonstrated its ability to cross the Blood Brain Barrier [4]. Thus, the versatility of these systems makes them ideal candidates for the treatment of primary BC tumors and their metastasis. These preliminar data have encouraged us to develop combination therapies for the treatment of metastatic TNBC using these nanocarriers. **Materials and Methods** St-PGAs have been synthesized by controlled polymerization techniques via ring opening polymerization of Ncarboxyanhydrides and subsequent protecting group removal, following a protocol previously reported by our group [5]. This star-shaped polymer undergoes a spontaneous self-assembly process in non-salty aqueous solution via charge-like interactions leading to the formation of spherical nanosystems [3]. To covalently entrap the self-assembled structure, the polymer has been modified with different % of pyridyl dithiol (PD) by DMTMM chemistry in organic solvents aiming for a posterior stimuli-responsive cross-linking strategy. A chemotherapeutic agent (Dox) and a Tyrosine Kinase inhibitor (TKi) have been conjugated separately to these PD-modified polymers. Dox has been conjugated through an amide bond, as an example of a non-stimuli-responsive linker, or a pH-labile hydrazone linker. TKi has been conjugated by a pH-labile ester bond. Cross-linking reactions have been performed in aqueous solutions using DTT as reductive agent. The kinetics of the reaction have been monitored by UV-Vis and 1H-NMR. Exhaustive physico-chemical characterization has been performed for the whole family of crosslinked nanosystems including 1H-NMR, size-exclusion chromatography, size and z-potential by Dynamic Light Scattering, Circular Dichroism and UV-Vis for drug loading. Finally, the cross-linked single and combination conjugates have been tested *in vitro* in TNBC cell lines (MDA-MB-231, MDA-MB-453, ZR-75, MCF-7 and 4T1). MTS assay was used for cell viability studies.

**Results and Discussion** We have successfully synthesized St-PGAs and modified them with PD moieties for reversible cross-linking strategies. These systems showed a concentration-dependent size in pure water that could be additionally modulated by modifying the ionic strength of the aqueous media, as observed by DLS measurements. Therefore, a wide range of sizes going from 10-1000 nm can be precisely obtained, offering a myriad of possible architectures to be used in drug delivery. By using stimuli-responsive reversible chemistry (disulfide bonds) to cross-link the structures, we have obtained nanosystems that will be stable in blood circulation (low GSH) but will disassemble at the tumor site (high GSH). Reaction kinetics showed that complete cross-linking was achieved within 5 minutes under super-mild conditions, what expands the use of this strategy to drugs susceptible to degradation. Furthermore, this system provides high versatility for the design of combination therapies by the co-assembly of different drug-linked St-PGAs. Following this strategy, we have conjugated the anticancer drugs Dox and TKi in different St-PGA molecules. Then, by self- and co-assembly, we obtained cross-linked nanosystems with one drug or combinations at the desired synergistic ratios. These cross-linked nanosystems showed promising results in cell viability studies, with $IC_{50}$ values ranging from 0.1 to 1.7 µM. These studies also revealed that stimuli-responsive linkers between the drug and the polymer (hydrazone-Dox) had a better performance compared to those nonbioresponsive linkers (amide-Dox).

**Conclusions** In this study, we have proven the high versatility of St-PGAs in the synthesis of polymer-drug combination conjugates. The self-assembly behavior of this polymer allows us to reach stable nanosystems bearing synergistic combinations of drugs with precise control of size and drug ratio, which has been translated into a good performance *in vitro*, thus, expanding the horizons for combination therapy.

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**PC-15**

# A Hierarchical Transfer of Structural Information from Dendrimers to Polyion Complexes for the Control of Size and Biodistribution

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Dendrimers are well-defined, monodisperse, and globular macromolecules [1]. Herein we describe that the information stored within dendrimer G can be efficiently transferred to micellar and vesicular nanoassembles and determine in a controlled fashion their size and biological response (Figure 1). So, we selected polyion complex (PIC) [2] PIC prepared by electrostatic interaction from oppositely charged polymers in stoichiometric charge ratios. Their electrical neutrality, PEG corona, nanometric size and narrow size distribution are well suited properties for drug delivery (DD) applications.[3] The intrinsic rigidity and globular nature of a GATG (gallic acid-triethylene glycol)[4] dendritic block ensures the formation of PIC of ca. 30 nm with unprecedented stability in serum and up to ionic strengths higher than 3 M, the highest attained for PIC micelles.[5] we propose the use of charged dendrimers, where information stored within G might be transferred to PIC with oppositely charged PEGylated linear copolymers (Figure 1). However, considering the large differences in local dynamics between linear polymers [dependent on segment, not MW] and dendrimers (dependent on G and hence, MW),[6] we decided to explore in detail the "dendrimer to PIC" transfer of structural information (size control) by screening a large library of eight GATG amino-dendrimers (6-243 terminal amines) and five different PEG-PGA blocks. To this end, GATG dendrimers rather than wedged dendrons previously described by our group [7] were envisaged to enhance the solubility of the dendritic partner at high G and so, facilitate PIC formation in a broad range of sizes. Accordingly, two new families of GATG dendrimers, namely 2[Gn] and 3[Gn], were designed starting from symmetrical di- and trifunctionalized cores. Following a divergent growth sequence involving an amide coupling with the GATG repeating unit (EDC, HOBt, 93-96%) followed by azide reduction ($Ph_3P$, 90-100%).
Larger dendrimers afforded smaller PIC, an effect associated to their higher rigidity and multivalency. These dendrit-ic PIC display greater stability in saline at 37ºC than PIC form linear polymers. The easy formation, high stability, low toxicity, ability to internalize cells and favorable *in vivo* profile of these dendritic PIC encourage us to continue exploring the potential of this hierarchical transfer of structural information in DD and diagnosis.

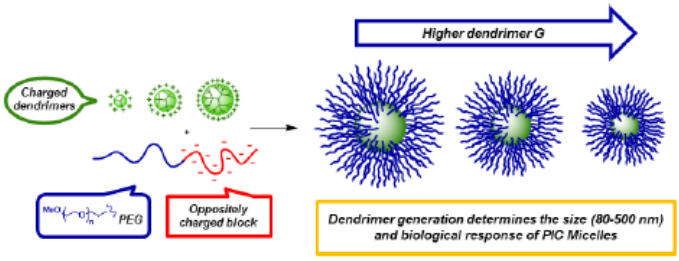


*Figure 1*. Preparation of PIC micelles from oppositely charged dendrimers and PEG-linear block copolymers

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[3] *The European Union (European Regional Development Fund - ERDF).*

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**PC-16**

# Synthesis and characterization of polyphosphazane based nanosystems and their application in gene therapy

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Important advances in drug delivery have been possible thanks to the application of concepts and materials from polymer science. Polymeric systems can control the release of different therapeutic agents, both in time and space, which, in turn enhances drug efficacy and safety. Polymers have been either, obtained naturally, modified from the natural source or synthetized [1]. Polymer synthesis allows the manufacturer the possibility to obtain the polymers with well-defined structures and tailored properties.

In this work, we have developed and characterized new polymeric nanoparticles for gene therapy based on a polyphosphazenes (PPZ). Polyphosphazenes are characterized by an inorganic phosphorous/nitrogen backbone, and by two organic side chains that can be modulated. Some polyphosphazenes have been investigated previously for gene therapy, and they have showed remarkable activity/toxicity ratio [2]. First, we synthesized a random copolymer polyphosphazene, Poly[(Cysteamine)(1-Mercapto-2-Propanol)] Phosphazene (PPZ-CMP) and the homopolymer Poly(Cysteamine) Phosphazene (PPZ-Cys). These polymers were characterized for their chemical structure (NMR, FTIR) and for their physicochemical properties (pKa, solubility). Next, PPZ-CMP was used to form complexes with pDNA, by selecting appropriate N:P ratios (Figure 1A) . The resulting nanoparticles were characterized for their size, stability, pDNA association/dissociation capacity and toxicity.

The results showed that PPZ-CMP exhibits aqueous solubility and is thermally stable. PPZ-CMP showed also considerable buffering capacity, which might aid in promoting endosomal release and ultimately, enhanced transfection. The nanosystems formed showed a small particle size (85-95 nm) and presented high stability at different polymer ratios. PPZ-CMP was able to condense the genetic material and to release it the presence of a polyanionic competitor. Finally, the results obtained from the toxicity test showed enhanced cell viability for the PPZ-CMP copolymer nanoparticles as compared to the cationic homopolymer PPZ-Cys (Figure 1B).
The physicochemical and cell toxicity data obtained herein indicate the potential of the PPZ-CMP nanoparticles as new formulations for gene delivery.

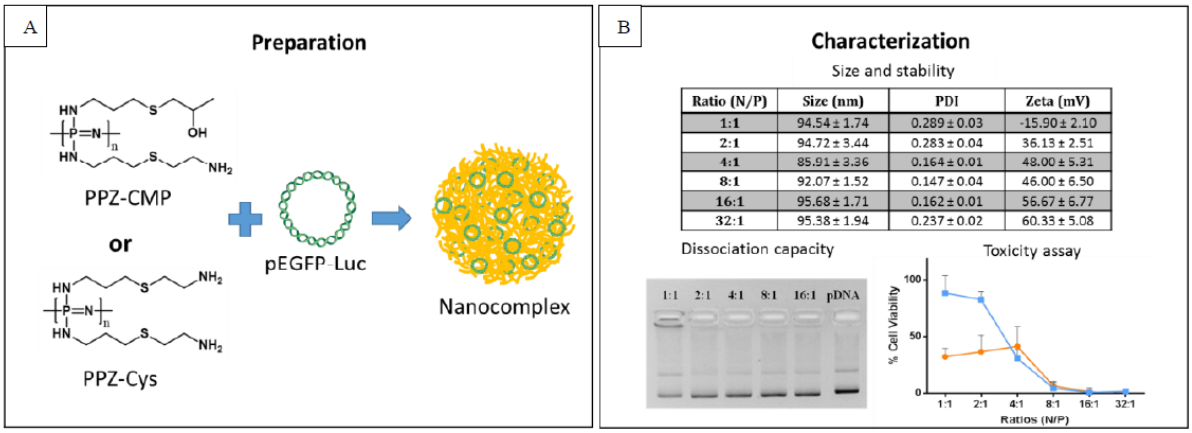


*Figure 1*. Preparation (A) and characterization (B) of the DNA-polymer nanocomplexes.

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**PC-17**

# Co-assembly of alkyl-lysophospholipid edelfosine and squalenoyl-gemcitabine form new nanosystems applicable to cancer therapy

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**Introduction.** Nanoparticulate drug delivery systems for antitumor agents represent an alternative strategy in the search of novel therapeutic approaches in cancer [1,2]. Several therapeutic modalities of squalenoyl nanocomposites have obtained encouraging outcomes in a great variety of tumors [3–5]. Among them, the prodrug squalenoyl-gemcitabine has been chosen to construct a novel nanosystem in combination with edelfosine, an alkyl-lysophopholipid with proven anticancer activity [6–8]. Given their amphiphilic nature as well as their complementary cellular targets, our main goal is to study if the physical mixture of both compounds could lead to the formation of a new antitumor nanomedicine.

**Material and Methods.** Chemical synthesis of squalenoyl-gemcitabine was performed according to previous works [9,10] and nanoparticles were formulated by nanoprecipitation method. Particle size and zeta potential were measured by dynamic light scattering, particle morphology by transmission electron microscopy, chemical analysis by X-ray photoelectron spectroscopy and nanoparticle drug content was quantified by UPLC-MS-MS.

**Results and Discussion.** Our results determined that these molecules spontaneously self-assembled as stable and monodisperse nanoparticles of 51 ± 1 nm in a surfactant/polymer free-aqueous solution. Particle morphology analysis of edelfosine-squalenoyl gemcitabine nanoparticles (Fig. 1A) showed a particle size reduction and different particle morphology in comparison with squalenoyl gemcitabine nanoassemblies (Fig. 1B). In addition, cell culture viability tests in the pediatric osteosarcoma cell line U2-OS showed that the novel formulation displayed comparable antiproliferative and cytotoxic effects.

**Conclusion.** The combination of squalenoyl gemcitabine with edelfosine have resulted in a smaller particle size with higher stability and drug content whereas its antitumoral potential has remained intact. Further studies in cells and *in vivo* will allow to validate the therapeutic applicability of this novel nanomedicine.

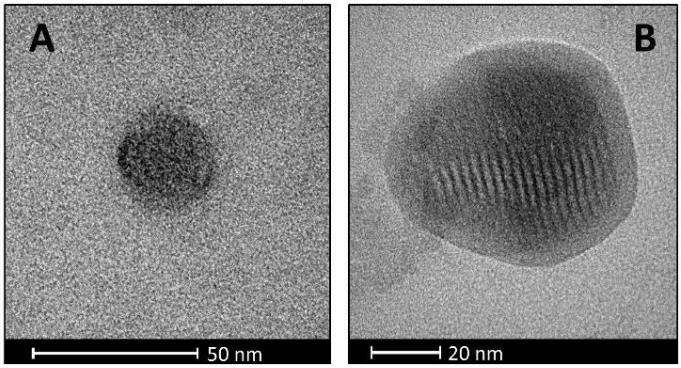


*Figure 1*. Transmission electron micrographs of (A) edelfosine-squalenoyl gemcitabine nanoparticles and (B) squalenoyl gemcitabine nanoassemblies

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**PC-18**

# A Dendrimer-Hydrophobic Interaction Synergy Improves the Stability of Polyion Complex Micelles

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Polyion complex (PIC) micelles are prepared by electrostatic interaction from oppositely charged polymers or biopolymers in stoichiometric charge ratios, with at least one of the components carrying a neutral hydrophilic block, usually poly(ethylene glycol) (PEG). Their electrical neutrality, nanometric scale, narrow size distribution, and core-corona structure are well suited properties for drug delivery (DD) to ensure an improved biocompatibility, long circulation times in the bloodstream, and passive accumulation into solid tumors [1]. In PIC micelles: a program to unveil the relative influence of the linear *vs* dendritic architecture as well as other structural elements on the stability of these systems (see Figure 1).

In brief, three generation (G) of PEG GATG block copolymers carrying terminal azides (PEG-[Gn]-$N_3$; n = 2, 3, 4) and PEG chains of different lengths [2],[3] were peripherally decorated at the dendritic block with alkynated anionic residues *via* triazol linkers generated by (CuAAC).[4]

Preliminary experiments on the formation of PIC micelles were performed in 10 mM PB, pH 7.4 with $PLL_{108}$ and $PEG_{5000}$-[G3]-$CO_2^-$, a dendritic block copolymer displaying 27 carboxylate groups. In conclusions: First, there is a minimum chain length for the hydrophilic PEG block necessary to stabilize the PIC micelles. Second, variations at the dendritic block reveal G3 as the optimal generation, with G2 (not enough multivalency) and G4 (reduced solubility) copolymers affording less stable micelles. Third, regarding the terminal anionic group, sulfate and sulfonate render more stable micelles than carboxylate groups in agreement with the reduced net charge of the latter at pH 7.4. Fourth, increasing the hydrophobicity of the linker (phenyl-triazol) results in micelles with higher stability due to additional hydrophobic contributions. Advantage of this stabilizing dendritic effect has been taken for the design of a robust, pH-sensitive micelle for the controlled intracellular release of the anticancer drug doxorubicin. This micelle displays a slightly higher toxicity, and distinctive mechanisms of cell uptake and intracellular trafficking relative to the free drug.

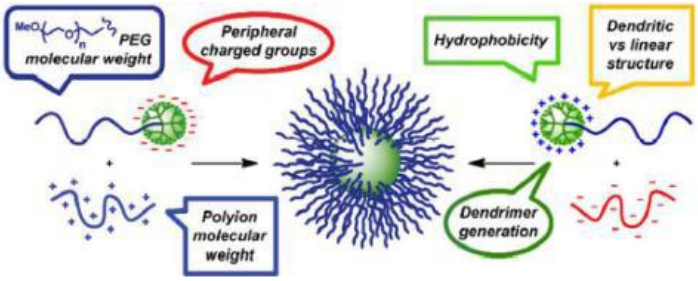


*Figure 1*. Structural elements controlling the stability of PIC micelles.

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**PC-19**

# Conjugation of squalene to gemcitabine as unique approach exploiting endogenous lipoproteins for drug delivery

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**Introduction**. An amphiphilic prodrug of gemcitabine (Gem) has been synthesized by its covalent conjugation to the squalene (SQ), a natural lipid, precursor in the cholesterol biosynthesis. Nanoparticles made of this bioconjugate (SQGem NPs) revealed higher anticancer activity in various animal tumor models compared to the free drug. [1] However, the mechanism behind the tumor cell recognition by these prodrug nanoparticles remained unclear. After intravenous (iv) administration, NPs interact with a complex biological environment and acquire a complex signature, which can significantly affect the *in vivo* fate. In this context, the lipid nature of SQ and the capacity of circulating lipoproteins (LP) to transport hydrophobic molecules led us to believe that the interaction between the SQGem NPS and lipoproteins deserved to be deeply explored.

**Materials and Methods**. *Formulation*. A radiolabeled bioconjugate ($^3$H-SQGem) has been synthesized and tritiated NPs were prepared by nanoprecipitation. Size and polydispersity index were determined by DLS. *Interaction with lipoproteins:* Radiolabeled NPs and free $^3$H-Gem were incubated *in vitro* with human blood or (ii) administered intravenously to healthy Sprague Dawley rats. 5 minutes after, blood was collected, centrifuged and obtained plasma was separated into lipoprotein and LP deficient fraction (LPDF). The radioactivity found in each fraction was measured using a β-scintillation counter.

**Results and Discussion.** We clearly showed that SQGem bioconjugates spontaneously interact with the plasma lipoproteins in particular with the cholesterol-rich ones, both *in vitro* in human blood and *in vivo* in rodents, whereas the free drug does not interact with LPs. [2] (Figure 1) To be noted that in rodents, due to their specific lipid metabolism, the cholesterol transport is mediated by the HDL, which play the same role as LDL in Humans. Thanks to this interaction, the cholesterol rich particles behaved as endogenous carriers of the bioconjugates and allowed an "indirect" transport of the gemcitabine to high LDL accumulating Human cancer cells. This led to an improved efficacy of the drug both *in vitro* as well as *in vivo* in an experimental tumor model. [3]

**Conclusions.** We have demonstrated that is possible to exploit the lipoproteins as indirect carriers by simply taking advantage of the spontaneous intravascular events that occurs in the circulation post administration. To the best of our knowledge, using squalene to drive a drug insertion into LDL and subsequent targeting represents a novel concept in drug delivery.

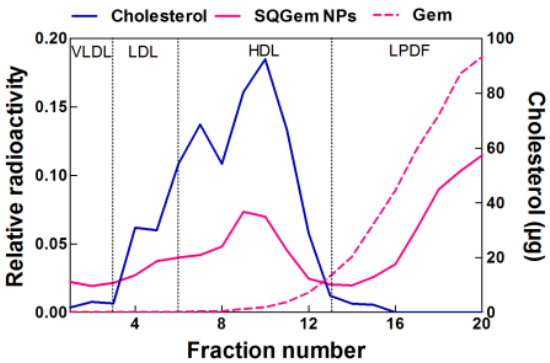


*Figure 1.* Radioactivity (magenta lines) and cholesterol (blue line) distribution among the collected fractions of plasma obtained from rats treated with 3H-SQGem or free 3H-Gem, 5 min post administration.

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**PC-20**

# Engineering novel anti-nucleolin antibody fragments with antibody-dependent cell-mediated cytotoxicity

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**Introduction.** Nucleolin is a protein overexpressed at the surface of cancer and angiogenic endothelial cells from tumor blood vessels [1], thus allowing a dual tumor targeting. This has been explored for the targeted delivery of cytotoxic drugs, using internalizing targeting ligands such as the nucleolin-binding F3 peptide [2]. Additionally, some nucleolin-binding ligands (including an aptamer and pseudopeptides) presented cytotoxic activity against nucleolin-overexpressing cells [3]. Despite several nucleolin-based targeting approaches that have been developed, strategies focused on triggering anti-tumor immune responses remain largely unexplored. The mechanism of antibody-dependent cell-mediated cytotoxicity (ADCC), of proven relevance for the therapeutic outcome of antibodies, has not been described for nucleolin-targeting approaches. Therefore, the objective of this work was to develop novel anti-nucleolin antibody fragments with ADCC activity.

**Materials and Methods.** A grafting strategy was used for the development of anti-nucleolin nanobodies/VHHs (Figure 1), using F3 peptide-derived sequences grafted onto the complementarity determining region (CDR) 1 or 3 of a parental VHH. Binding of the nanobodies to MDA-MB-435S and 4T1 cancer cells was evaluated by flow cytometry. To confirm that the binding was nucleolin-mediated, competitive inhibition assays with the F3 peptide were performed. One of the nanobodies was further fused to the Fc region of a human IgG1, resulting in a nanobody-Fc antibody fragment (Figure 1). Cytotoxicity of the nanobodies and nanobody-Fc were evaluated against MDA-MB-435S and 4T1 cancer cells. ADCC activity of the anti-nucleolin nanobody-Fc was assessed using the xCELLigence system, upon assessing MDA-MB-435S cell death following incubation with nanobody-Fc and human peripheral blood mononuclear cells (PBMCs).

**Results and Discussion.** The four generated nanobodies, with the F3 peptide-derived sequence, presented increased binding to the cancer cells, as well as increased cytotoxicity, relative to the parental VHH. CDR3-grafted nanobodies revealed increased binding and cytotoxicity relative to the CDR1-grafted nanobodies. Pre-incubation with the F3 peptide resulted in decreased binding of all the generated nanobodies, confirming that the observed binding was nucleolin-mediated. Based on these results, a CDR3-grafted nanobody was fused to the Fc region of a human IgG1, generating a nanobody-Fc antibody fragment that displayed improved cytotoxicity within the nanomolar range, relative to the parental nanobody-Fc and the nanobodies (devoided of the Fc domain). Upon incubation with human PBMCs, the anti-nucleolin nanobody-Fc led to increased MDA-MB-435S cell death, when compared to its nanobody counterpart and the parental nanobody-Fc. These results supported an ADCC activity of the anti-nucleolin nanobody-Fc antibody fragment.

**Conclusions.** The grafting strategy used for the development of anti-nucleolin nanobodies resulted in new antibody fragments with cytotoxic activity. Subsequent fusion to a Fc region resulted in an anti-nucleolin antibody fragment with ADCC activity, which could pave the way for nucleolin-targeting immunotherapeutic approaches.

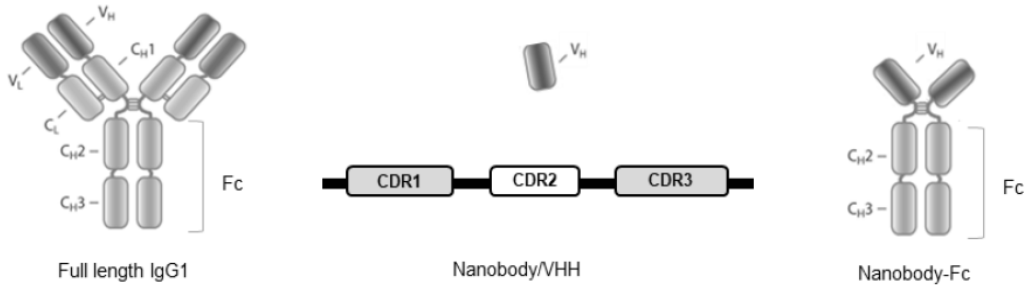


*Figure 1.* Schematic representation of antibody fragments, depicting the complementarity determining regions (CDRs) of a VHH.

**Acknowledgments**: Sofia Romano was the recipient of a fellowship from FCT (SFRH/BD/51680/2011). This work was supported by the European Regional Development Fund (ERDF) through COMPETE 2020 - Operational Programme for Competitiveness and Internationalisation and Portuguese national funds via FCT – Fundação para a Ciência e a Tecnologia, under the project POCI-01-0145-FEDER-007440.

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**PC-21**

# Synthesis and evaluation of heterotelechelic polymer prodrugs for anticancer therapy

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**Introduction.** Drug-loaded polymer nanoparticles are meant to increase the therapeutic efficacy of drugs and to decrease toxicity to healthy tissues [1]. However, they also have some disadvantages, including the "burst release" and poor drug loadings. Establishing a covalent linkage between the polymer scaffold and the drug (the so-called "prodrug" approach) [2], aims to circumvent these issues.
The "drug-initiated" method [3], where the drug is linked to an initiator prior polymerization, was combined with controlled/living radical polymerization (CLRP) to obtain different drug-bearing polymers, such as Gemcitabine-polyisoprene (Gem-PI) [4]. Interestingly, the polymer ω-chain end, end-capped by a nitroxide, is still available for further post-functionalization. Rhodamine B was chosen as first moiety to synthesize dual-functionalized polymer prodrugs, as a simple solution towards theranostic application.

**Materials and Methods.** A small library of Gemcitabine-polyisoprene (Gem-PI) with different molecular weight was prepared following a previously published procedure [4]. The nitroxide exchange reaction was selected to post-functionalize the polymers [5] that were then characterized by GPC (equipped with a fluorescent detector) and NMR.

**Results and Discussion.** A library of Gemcitabine-Polyisoprene-Rhodamine (Gem-PI-Rho) of different molecular weight (1000-5000 $M_n$) was synthesized using, for the first time, the nitroxide exchange as an efficient and nearly quantitative post-functionalization reaction. The reaction was followed by GPC and the optimal conditions were determined. Moreover, the drug-initiated method allows to obtain polymers with a high drug loading (17%) and narrow dispersity (1.2-1.3).

**Conclusions.** Heterotelechelic polymers were successfully prepared by the "drug-initiated" method followed by the nitroxide exchange reaction. The presence of a fluorophore could permit biological studies, for example cells internalization or *in vivo* imaging, providing a NIR emitting dye is used. The nitroxide exchange reaction proved to be a valid post-functionalization strategy and can be used to attach a variety of different moieties to the polymer ω-chain end.

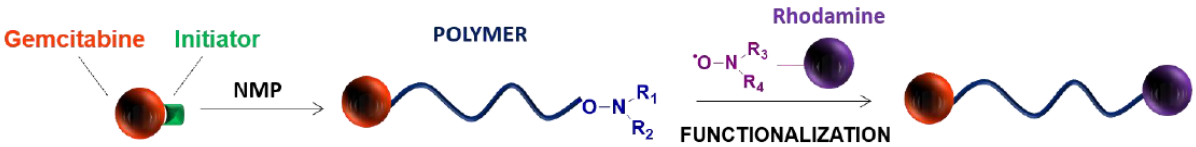


*Figure 1*. Synthesis and post-functionalization strategy to obtain heterotelechelic polymer.

**Acknowledgments**: This work was supported by the European Union's Horizon 2020 research and innovation programme under Marie Skłodowska Curie grant agreement no. 642028 (NABBA), the CNRS and the French Ministry of Research.

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**PC-22**

# Optimization of linalool-loaded solid lipid nanoparticles (SLNs) by experimental factorial design

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Linalool ($C_{10}H_{18}O$), also known as 3,7-dimethyl-1,6-octadien-3-ol, is a monoterpene found in essential oils and teas that has anti-oxidative properties [1]. According to recent studies oral administration of linalool has a hypocholesterolemic effect since it reduces body weight in mice and decreases cholesterol levels through the reduction of mRNA and protein expression of sterol regulatory element binding protein-2 (SREBP-2) and the suppression of the transcription of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) [2]. Recently, the encapsulation of linalool in solid lipid nanoparticles (SLN) has demonstrated to overcome its physicochemical limitations improving therapeutic efficacy [3,4]. The present study aims to development and optimize linalool-loaded SLN (Lin-SLN) by $2^2$ factorial design.

SLN dispersions were produced by the hot high-pressure homogenization (HPH) technique (EmulsiFlex-C3, Avestin, Portugal). The dependent variables were the mean particle size, polydispersity index (PI) analysed by dynamic light scattering (DLS) and zeta potential (ZP) by electrophoretic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK, respectively). The influence of the independent variables, surfactant (kolliphor® P 188) and lipid concentrations (imwitor® 900 K) on Lin-SLN was evaluated by a $2^2$ factorial design composed of 2 variables which were set at 2-levels each (-1 and +1). The data was analysed by STATISTICA 7.0 (Stafsoft, Inc.) software.

The mean particle size varied from 94.8 ± 0.8 nm to 459.3 ± 231.8 nm, whereas the PI ranged from 0.1 ± 0.01 to 0.5 ± 0.08. Zeta potential was approximately 0 mV in all formulations since the surfactant used has a non-ionic nature. For each of the three dependent variables, analysis of the variance (ANOVA) was performed using a confidence level of 95% ($\alpha$= 0.05). The concentration of surfactant as well as the interaction between the different concentrations of lipid and surfactant has a statistically significant effect ($p$-value < 0.05) on the particle size and PI. Experimental factorial design has been successfully employed to develop an optimal SLN dispersion composed of 1% (w/v) linalool 2% (w/v) of imwitor® 900 K and 5% (w/v) of kolliphor® P 188 that is currently being studied by *in vitro* assays.

**Acknowledgments**: The authors wish to acknowledge the financial support received from Portuguese Science and Technology Foundation (FCT/MCT) and from European Funds (PRODER/COMPETE) under the projects M-ERA-NET-0004/2015-PAIRED, co-financed by FEDER, under the Partnership Agreement PT2020.

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**PC-23**

# *In vitro* permeability and dissolution of nimesulide from HA-coated PLGA nanoparticles

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## Introduction

Although intratumoral (IT) administration is a promising approach for the treatment of various solid tumors by achieving high local drug concentrations with minimal systemic toxicity, its efficacy is strongly dependent on frequency of the drug injections because of its rapid clearance from the tumor site [1]. To achieve this goal we have formulated poly lactide-co-glycolide (PLGA) nanoparticles (NPs) loaded with Nimesulide (NS) as a model drug (BCS class II). In recent studies, NPs coating with hydrophilic polymers such as hyaluronic acid (HA) has been also evaluated [2]. This study focused on the characterization of the *in vitro* release and permeation of NS as free drug and loaded in PLGA NPs.

## Materials and methods

NPs were obtained by the emulsion-solvent evaporation method and coated by electrostatic interactions with two counter-ion polymer solutions, 0.1% chitosan (CS) and 0.05% HA [3]. The coated NPs (NPCSHA) were evaluated for drug permeability using the PAMPA method at different NS and NPs concentrations. After 6 h of incubation at room temperature the amounts of NS in the receptor compartment were determined. In order to estimate the combined effect of drug release and permeability from NPs an *in vitro* system based on apical and basal chambers separated by a polyvinylidene fluoride membrane was used. The experiment was performed at 37ºC under constant magnetic stirring and solvent recirculation to keep sink conditions. This study was also conducted with an equivalent amount of free NS as control.

## Results and discussion

PAMPA studies confirmed the high permeability of NS across lipid barriers (Pe=1-1.5x$10^{-5}$ cm/s) and revealed a slight restrictive effect of the polymeric carrier probably related to the affinity between the drug and PLGA (Pe=6.5-8.2x$10^{-6}$; 0.58-1.44x$10^{-6}$ and 4.04-5.63x$10^{-6}$ cm/s for NP 1% PVA, NP 0.25% PVA and NPCSHA 0.25% PVA, respectively). In the solution/permeability study, free NS permeability follows first-order kinetic whereas the Higuchi model is the best to fit the results obtained with NPs formulations. In addition, lag times between 30 and 65 min were obtained.

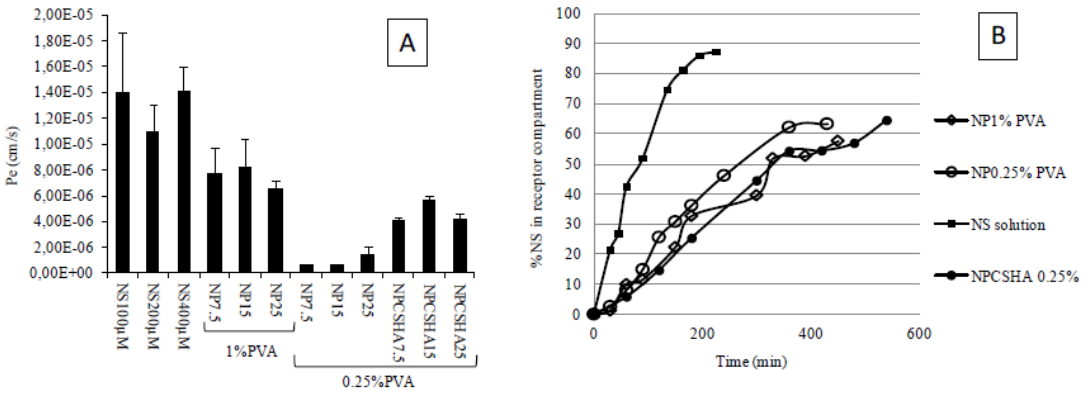


*Figure 1.* Permeability coefficients (Pe) estimated with the PAMPA method (A) and %NS in receptor compartment of dissolution/permeability system (B)

## Conclusions

In both experiments, NPs show a clearly restriction effect on the NS Pe. The amount of residual PVA and the presence of HA have an important role in PAMPA results but, in the dissolution/permeability study all the NPs regardless of their composition show the same behaviour in contrast with free drug.

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**PC-24**

# Effect of sucrose in freeze-dried liposomes encapsulating drugs

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The use of liposomes as drug delivery system is very promising due to their ability to encapsulate hydrophilic and hydrophobic drugs [1]. However, the long-term storage of liposomes reveals physical and chemical instabilities which limits the use in therapeutic applications [2]. The development of a dry powder formulation can be a solution to improve these problems. The production of a freeze-dried liposomes encapsulating drugs is considered a key challenge, since the drugs can leak out from the liposomes during the freeze-drying process, occurring drug leakage [3]. The stress caused by the main steps of the process may affect the structure of liposomes. Therefore, cryoprotectants can be used to prevent damage in the integrity of the liposome bilayer [2].

The aim of this study was to optimize a liposomal formulation that after freeze-drying continues to be stable and able to maintain drugs with few leakages. The protective effect of five sugars at different concentration was tested in terms of size distribution, morphology and concentration.

Results showed that sucrose, in a concentration dependent manner, effectively prevents liposomal fusion or aggregation and protects the integrity of freeze-dried liposomes (Figure 1). This liposomal formulation encapsulating a hydrophobic drug Tamoxifen can be freeze-dried and stored without significant drug leakage. The biological activity of drug after freeze-drying was also evaluated. In sum, the results indicated that this optimized liposomal formulation can be a good approach to long-term storage.

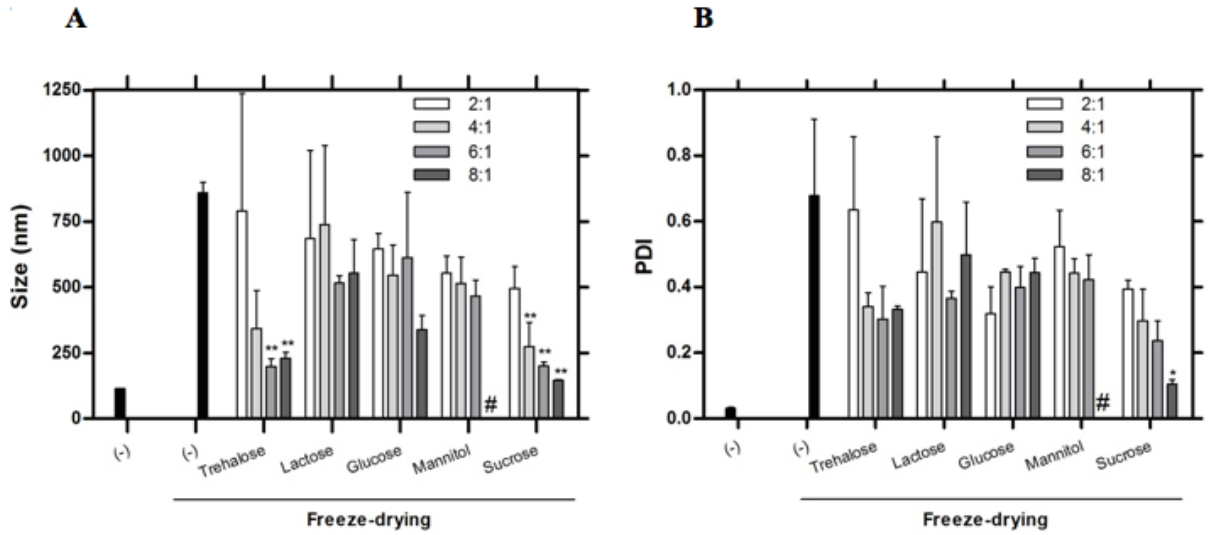


*Figure 1.* Influence of several sugars in size distribution after freeze-drying of liposomes. Determination of (A) size and (B) PdI of liposomes with and without (-) cryoprotectants, before and after freeze-drying, by DLS. # Values not determined due to non-homogenous dispersion obtained. Values represent the mean +SD of 2 independent experiments. Significant differences were detected as shown by an * ($P<0.05$) and ** ($P<0.005$).

**Acknowledgments**: This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684) and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 – Programa Operacional Regional do Norte. This work has also received funding from the European Union Horizon 2020 research and innovation programme under grant agreement NMP-06-2015- 683356 FOLSMART.

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**PC-25**

# Nanosized glucan particles as a delivery system for curcumin

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**Introduction:** Curcumin is a natural chemical yellow compound isolated from the rhizome of the plant *Curcuma longa*. It is a hydrophobic diphenol known for its diverse pharmacological activities including antioxidant, anti-inflammatory, antimicrobial and anticarcinogenic properties [1]. However, despite its advantages, curcumin does not have a good clinical efficacy due to its low aqueous solubility, its poor bioavailability and rapid systemic elimination [1]. A promising approach to solve this problem and enhance curcumin's clinical relevance is to encapsulate it using a drug delivery system that is dispersible in aqueous media. In this regard, nanoparticles (NPs) have gained importance as delivery systems since they improve the therapeutic impact of the drug by enhancing its retention time and allowing a controlled release or even providing better targeting and tissue penetration [2]. More recently, biodegradable polymeric nanoparticles have grown interest since they enable a greater stability in body fluids, they present low toxicity and they can act as immunostimulants or adjuvants [3]. Curdlan is a neutral polymer consisting of β-1,3-linked glucose residues [4]. It is extracted from *Alcaligenes faecalis* and it is insoluble in water, alcohols and the majority of organic solvents [4]. Curdlan has many applications based on its physico-chemical properties and in its relevance in the medical field [4]. In the literature glucan NPs have been addressed in different medical fields like for cancer treatment or as a vaccine or gene delivery system. Taking this into account, this work aims to describe the production of curcumin encapsulated glucan NPs to overcome this drug poor efficacy and characterize the delivery system and evaluate its toxicity *in vitro*.

**Materials and Methods:** Curcumin encapsulated glucan NPs were produced using a nanoprecipitation technique. Curdlan 0.025% (w/v) was dissolved in sodium hydroxide 2% (w/v) containing Tween 80® under magnetic stirring for three hours. To produce the NPs, curcumin dissolved in acetic acid 8% (w/v) was added to the polymer solution. For comparison, a control of curcumin free NPs (blank NPs) was produced using the same technique without dissolving the drug in the acetic acid solution. The suspensions of NPs were characterized regarding its size and zeta potential using Dynamic Light Scattering and Electrophoretic Light Scattering, respectively. Its morphology was visualized by Transmission Electron Microscopy (TEM). Both dispersion of NPs were washed and concentrated using Vivaspin 20 centrifugal concentrator (MWCO 300KD). Concentrated curcumin encapsulated glucan NPs and blank NPs were evaluated *in vitro* regarding its cytotoxic effects on a murine monocyte-macrophage cell line (RAW 264.7) using MTT cell viability assay.

**Results and Discussion:** Immediately after production, blank NPs and curcumin encapsulated glucan NPs presented an average size of 132 ± 19 nm and 106 ± 18 nm, respectively, both with minor variations between each batch. The zeta potential for both formulations was similar and was about 0 mV, which was expected because curdlan is a neutral polymer. Nonetheless, the NPs suspensions showed no aggregation or changes in size during short term storages (≤ 1 week) or after the washing with purified water and concentration. TEM analysis showed that the particles have a spherical shape and confirmed that they have an average size of 50-110 nm, which is suitable for a drug delivery system. The cell viability studies performed showed that although curcumin encapsulated NPs appear slightly more toxic than blank NPs, for concentrations below 32.5 µg of glucan per mL in the well there are no cytotoxic effects.

**Conclusions:** In this work we reported the successful production of curcumin encapsulated glucan NPs as a strategy to improve curcumin low bioavailability. Further studies are now required to access eventual immunological effects of this delivery system in order to safely use it as a vector for curcumin.

**Acknowledgments**: This work was funded by FEDER funds through the Operational Programme Competitiveness Factors - COMPETE 2020 and national funds by FCT - Foundation for Science and Technology under the project PROSAFE/0001/2016 and strategic project POCI-01-0145-FEDER-007440.

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**PC-26**

# Near-infrared triggered release of miRNAs for modulation of cell activity

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**Introduction.** Ischemic diseases are a cause of morbidity in the contemporary world. Several pre-clinical and clinical trials are exploring the therapeutic effect of cell-based therapies, including endothelial progenitor cells [1]. Outgrowth endothelial cells (OECs), a sub-population of endothelial progenitor cells [2], may be beneficial for the treatment of ischemic diseases [3] and for tissue engineering applications [4]. However, the rates of survival and vascular engraftment of transplanted cells are very poor, forcing cells to work mainly via time-limited paracrine actions. Therefore, the development of strategies to modulate the activity of these cells (e.g. proliferation, survival, etc…) is highly desirable to enhance their therapeutic effect. Previous studies have demonstrated that miRNAs are powerful modulators of angiogenesis [5], although the delivery of multiple miRNAs with spatio-temporal control has not been demonstrated yet.

**Material and Methods.** We developed a plasmonic nanocarrier to control the release of two microRNAs that regulate cell proliferation and survival. The nanocarrier is formed by gold nanorods (AuNRs) modified with single stranded DNA (ssDNA) for hybridization of complementary DNA-conjugated microRNAs. DNA strands with distinct melting temperatures allow independent release of each microRNA with a near-infrared laser (780 nm) using different powers. In addition, we have used an antimicrobial peptide (AMP) with membrane-perturbing ability to enhance the uptake of the nanocarriers and destabilize the endosomal membrane during intracellular uptake. Light-triggered sequential release of each miRNA was tested in a reporter cell line expressing two fluorescent proteins and in OECs derived from $CD34^+$ cells isolated from umbilical cord blood. Finally, we tested the system in an *in vivo* wound model. OECs transfected with AuNRs conjugated with miRNAs were transplanted subcutaneously in nude mice and irradiated at 780 nm.

**Results and Discussion.** AuNRs were conjugated with controllable amounts of ssDNAs and ssDNA-conjugated miRNAs. Incubation with AMP enhanced 63-fold the uptake of AuNRs and increased endosomal escape. The sequential release of two miRNAs (miR-155-5p and miR-302a-3p) was demonstrated in a dual-reporter cell line expressing mCherry and EGFP. A stimulus of 1.25 $W/cm^2$ caused the release of miR-155, decreasing mCherry fluorescence and a second stimulus of 2 $W/cm_2$ induced the release of miR-302a, decreasing EGFP fluorescence. Tests in human OECs indicate that this system can be used to silence different targets sequentially and to modulate cell activity with spatio-temporal resolution, increasing cell survival (6.5 fold) and proliferation (1.8 fold). Two sequences of delivery were tested *in vivo*. Particularly, delivery of miR-302a followed by delivery of miR-155 had a positive impact in wound healing kinetics. Moreover, increased amount of human DNA was detected in this condition 5 days after treatment.

**Conclusion.** In this work, we have developed a light-responsive system that, by the addition of an antimicrobial peptide, can be internalized in higher amounts and escape the endosomes to deliver more than one miRNA, working as an optical switch of biological circuits with spatial and temporal control. Our results show that the order of the sequential release of 2 miRNAs has significant impact on the modulation of cell activity.

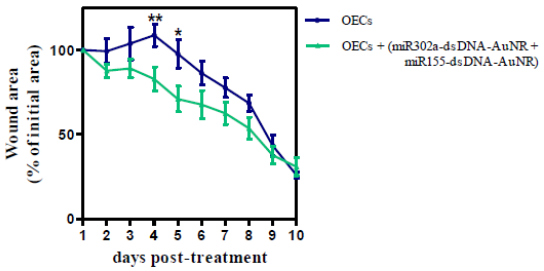


*Figure 1*. Wound closure in nude mice treated with cells previously incubated or not with miR-ssDNA-AuNR. (Unpaired t-test, * $p < 0.05$, ** $p < 0.01$)

**Acknowledgments**: The authors would like to thank the financial support of FCT (SFRH/BD/81705/2011 to ML and SFRH/BPD/105327/2014 to SS) and EC (ERC project nº 307384, "Nanotrigger").

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**PC-27**

# GoNanoBioMat Project - Polymeric NanoBioMaterials for drug delivery: developing and implementation of safe-by-design concept enabling safe healthcare solutions

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**Introduction:** Polymeric nanobiomaterials applied for medical use in diagnosis, drug delivery, or nanodevices were pioneered in Europe and have already led to innovative products. Nonetheless, the number of health applications based on nanobiomaterials on the market remains small due to the unclear situation of the future regulatory assessment of efficacy and safety. Pre-clinical data on the polymer chemistry, sourcing, Nanoparticles (NPs) preparation methods at the lab scale, physicochemical characterization, *in vitro* characterization, cytotoxicity, immunotoxicity, animal and human toxicology, and environmental risks are to some extent available in the literature. However, data are not well structured, characterization assays not harmonized and/or validated, and scale-up to manufacturing at larger volumes with necessary in-process controls not established. Furthermore, the correlations between NPs physicochemical properties and clinical outcome are the subject of current research efforts by regulatory authorities and industry. It was observed that in the nanoscale, changes in properties such as size and surface charge may lead to dramatic changes in efficacy, safety and ecological impact.
The GoNanoBioMat project aims to help Biotech, Nanotech, Medtech and Pharma SMEs (small and medium size enterprises), their suppliers, and Research Institutes to work on the development and production of polymeric nanobiomaterials focussing on drug delivery.

**Methods:** The project contemplates different tasks performed by a multidisciplinary and transnational consortium. After consulting the industry and stakeholders to understand theirs needs regarding polymeric nanobiomaterials for drug delivery, a verified knowledge base will be built on peer-reviewed scientific publications on polymeric nanobiomaterials, their environmental and human health risks and the regulatory aspects. The consortium will then produce guidelines to implement a safe-by-design (SbD) approach for polymeric nanobiomaterial drug delivery systems. Simultaneously, 3 case studies (Chitosan, Polylactic acid and Polyhydroxyalkanoates) will be evaluated regarding the immunotoxicological effects of the bulk material and the nanoparticulate drug delivery systems.

**Results and Discussion:** With this project, the GoNanoBioMat consortium expects to generate an added value regarding the use of polymeric nanobiomaterials for drug delivery. The knowledge base and the guidelines may serve as a basis for discussion among the different stakeholders and to increase and harmonize the knowledge and the terminology in this interdisciplinary field. Also, we intend to provide a systematic proceeding to develop safe polymeric nanobiomaterials, facilitate the understanding of the regulatory framework and provide guidance for how to characterize and test these materials for the toxicological point of view.

**Conclusion:**
By combining the GoNanoBioMat consortium expertise in the area of nanobiomaterial science and technology, life science, pharmaceutical science, as well as nanosafety and life cycle thinking, the main goal of this project is to reduce uncertainties and commercial risks for polymeric nanobiomaterials drug delivery applications, accelerating innovation outcome and market access for safe nanobiomaterials.

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**PC-28**

# Effect of different sterilization methods on drug loaded silicone-based hydrogels for soft contact lenses

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**Introduction:** Contact lenses (CLs) have raised particular interest as potential drug delivery vehicles for the treatment and prophylaxis of several ocular diseases, since they are capable of increasing drugs bioavailability in at least 50% when compared to eyedrops (1-5%) [1]. Because of the close contact with the cornea surface these devices are required to be sterile [2]. However, there is a lack of knowledge concerning the possible effects of terminal sterilization methods on the drug release profiles. The main goal of this study was to investigate the effects of an ozone-based sterilization method on different systems of drug loaded hydrogels intended for therapeutic CLs, and compare them with the effects produced by two conventional sterilization methods (steam heat and gamma irradiation).

**Materials and Methods:** A silicon hydrogel containing hydroxylethyl methacrylate (HEMA) and [tris(trimethylsiloxy)silyl]propyl methacrylate (TRIS) was produced and soaked in different drug solutions, commonly used for treatment of ocular diseases (levofloxacin, chlorhexidine, diclofenac and timolol maleate). The drug release profiles and several properties relevant to CLs materials were evaluated before and after the sterilization. Namely, swelling capacity was determined by water uptake studies, transparency was accessed by UV-Vis spectroscopy, surface topography/morphology by scanning electron microscopy (SEM) and mechanical properties by performing tensile tests. The drug released was quantified by high performance liquid chromatography (HPLC). The effectiveness of the sterilization procedures was assured by performing sterility tests.

**Results and Discussion:** Ozone gas method led to a significant reduction of drug released and to the formation of degradation products, especially for diclofenac and levofloxacin. Gamma irradiation led to darkening of the loaded hydrogels and to the complete degradation of levofloxacin, although significant degradation of the other drugs was have been also observed. Steam heat led to smoother surfaces and to a decrease of the amount of drug released, however, with no formation of degradation products. It was observed that steam heat is the sterilization method with less impact on the devices. The results of the swelling and mechanical properties tests strongly indicate the occurrence of specific drug-polymer interactions promoted by the sterilization. In general, these interactions led to a decrease on the amount of drug released.

**Conclusions:** The present work shows that the development of efficient and functional drug delivery devices for ophthalmic purposes cannot be done independently of a careful analysis of the influence of the sterilization procedures and methods on the degradation of these polymeric systems as a whole. The outcome of the each sterilization method is highly dependent on the drug/matrix system involved. There is no possible generalization, therefore validation must be carried out case by case.

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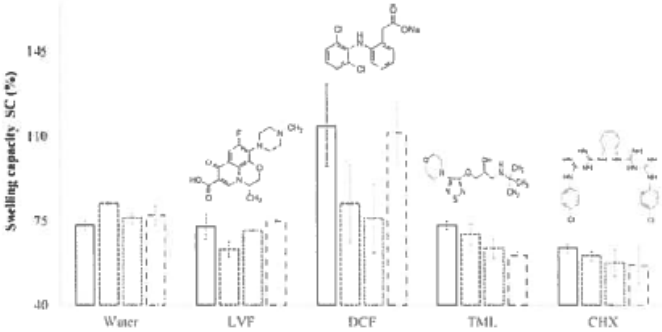


*Figure 1.* Swelling capacity of the hydrogel samples in water and in drug solution (levofloxacin, diclofenac, timolol and chlorhexidinee), before and after different sterilization processes (SII - steam heat, GI - gamma irradiation, OZ -ozonation).

**PC-29**

# Alginate-Hyaluronic acid hybrid microcapsules mimic extracellular matrix stimuli for mesenchymal stem cells

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**Introduction.** Alginate cell microencapsulation is used in research as a delivery system for therapeutic drugs. Microcapsule structure allows nutrients, oxygen and carbon dioxide diffusion flow between outer and inner side, allowing also, the release of the therapeutic molecules produced by encapsulated cells [1,2]. Our research group has previously developed sustainable release systems based on cell microencapsulation [3,4]. Despite the great promise of the cell encapsulation technology, cell death within microcapsules remains unsolved. Hyaluronic Acid (HA), one of major components in the extracelular matrix, improves cell viability [5]. The aim of our study is to combine HA with cell microencapsulation to recreate the extracelular matrix environment and enhace cell viability while maintaining the mesenchymal stem cells phenotype and differentiation potential.

**Materials and Methods.** Several mixtures of alginate and hyaluronic acid were charactarized in terms of viscosity behavior, selecting those that were similar to 1.5% alginate. Next, HA content inside microcapsules, microcapsules surface analysis by SEM and swelling of selected microcapsules containing HA were evaluated. Mesenchymal stem cells were next immobilized into the selected alginate-hyaluronic acid hybrid beads with similar properties than 1.5% alginate using an electrostatic atomization generator (Nisco®), and covered with poly-L-lysine-alginate. Then, cell viability, apoptosis, cell membrane integrity, metabolic activity and erythropoietin and vascular endothelial growth factor release in hybrid alginate-HA microcapsules were quantified. Finally, the differentiation potential of mesenchymal stem cells after encapsulation was tested.

**Results and Discussion.** 1% alginate 0.25% HA and 0.5% alginate 0.5% HA mixtures showed similar viscosity profile to 1.5%. However, our results showed that only 1% alginate 0.25% hyaluronic acid microcapsules retain 1.5% alginate physicochemical properties, with similar microcapsule surface and swelling properties, thanks to the similarities between the macromolecular structures of alginate and hyaluronate. Moreover, mesenchymal stem cells encapsulated in these hybrid beads showed enhanced viability therapeutic protein release and mesenchymal stem cells potential to differentiate into chondrogenic lineage, maybe due to an induction of aggrecan and proteoglycan accumulation, nodule formation, and inhibition of TNF-alpha induced chondrogenic differentiation.

**Conclusions.** HA protects mesenchymal stem cells when encapsulated within alginate, providing a niche-like environment and improving the beneficial effects of alginate microcapsules after encapsulated mesenchymal stem cells implantation. Encapsulated mesenchymal stem cells into such bio-artificial niches are protected and remain competent in terms of cell delivery or sustained drug release systems.

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**PC-30**

# An inhalable micro/nano-structured system for protein drug delivery

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**Introduction**: There is an increasing interest in the lung for the delivery of proteins. Besides its physiological advantages, this interest stems from the need to develop needle-free delivery procedures. Hybrid nanoparticles (NPs) based on chitosan (CS) and hyluronic acid (HA) were prepared via ionotropic gelation, showing potential for treating asthma, when loaded with heparin [1]. However, direct inhalation of NPs is not plausible because of having a strong tendency to agglomerate, resulting in difficult physical handling. We have thereby proposed the microencapsulation of NPs in a carrier matrix by spray drying. This method results in micro-scale powders, which can be tuned to bear aerodynamic diameters of less than 5 µm, being suitable for lung deposition. Herein, we have incorporated insulin (INS) in the hybrid CS/HA NPs that were subsequently microencapsulated in microspheres of mannitol. The microencapsulated NPs were characterized and optimized, in order to obtain reproducible particles with appropriate lung deposition properties and high INS content. Particularly, this work focused on the assessment of structural integrity of the protein nanoencapsulated in polymeric matrices, following spray drying, with the thermoprotectant, mannitol.

**Materials and methods:** Chitosan hydrochloride salt (CS) [Protasan® UP Cl 113, FMC Biopolymers, Norway]; hyaluronic acid (HA) [~166 kDa, Bioiberica, Spain]; pentasodium tripolyphosphate (TPP), D-mannitol and bovine insulin (INS) [Sigma-Aldrich]. ***Preparation of the hybrid NPs:*** The INS-loaded CS/HA NPs were produced, in the presence of the crosslinker TPP, by the ionotropic gelation method [2]. ***Spray drying of NPs:*** NPs were scaled up, isolated, dispersed in mannitol solution and, then spray-dried, using a laboratory scale drier [Büchi® Mini Spray Dryer, B-290]. ***Assessment of protein structural integrity by circular dichroism (CD):*** Far-and near-ultraviolet CD spectra were acquired using a JASCO-715 automatic recording spectropolarimeter [Jasco, Japan]. The mean residue ellipticity, $\theta$ (deg cm$^2$ dmol$^{-1)}$ was calculated from the formula: $\theta = (\theta_{o\beta\sigma}/10).(MRM/lc)$ ($\theta obs$ = observed ellipticity in deg; $MRM$ = mean residue molecular mass; $l$ = optical path length, in cm; $c$ = protein concentration, in g.mL$^{-1}$). To calculate the composition of the secondary structure of the protein, SELCON3, CONTIN, and DSST algorithms (from the DichroWeb on-line server) were used to analyze far-UV CD spectra. INS concentration for spectra acquisition amounted to 0.5 mg/mL. CD spectra obtained from buffer solutions, NPs without INS and mannitol, at the concentration employed for microspheres formation, were subtracted as needed from INS spectra prior to data analyses.

**Results and discussion:** The NPs exhibited a size and positive zeta potential of 218 nm and +33 mV, respectively, with a high protein association efficiency. The resulting micro-scale powder showed a spherical morphology and adequate aerodynamic properties. Most importantly, the CD data elucidated that the secondary and tertiary structure of the protein, incorporated into the hybrid NPs prior to microencapsulation with mannitol by spray drying, was preserved, as compared with its native form. This indicates the non-invasiveness of the spray drying-based microencapsulation technique to proteins.

**Conclusions:** Protein encapsulation in nanoparticulate matrices, besides the use of a thermoprotectant, could hinder its diffusion to the air/liquid interface during spray drying and, consequently, evading its degradation.

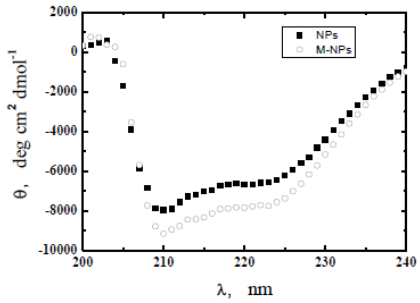


*Figure 1:* FUV-CD spectra of insulin-loaded inside nanoparticles (NPs) and mannitol microspheres (M-NPs).

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**PC-31**

# Budesonide Loaded Policraprolactone Microparticles for Pulmonary Administration: Preparation and Characterization

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**Introduction.-**

Respiratory systemic pathologies, such as ASMA or Chronic Obstructive Pulmonary Disease (COPD), affect a high percentage of the world's population. According to WHO [1], in this year 2017, asthma will affect to 235 million people worldwide and predicts that COPD will have become the fourth leading cause of death by 2030. Glucocorticoids such as budesonide (BDS), administered by nebulizers or metered dose inhaler (MDI), are one therapeutic option for their treatment. But these diseases being chronic require frequent administration of of high repeated doses [2]. Recent therapeutic strategies suggest that drug targeting to macrophages could to improve corticosteroid response in COPD patients [3]. On these bases we set as goal developing a biodegradable pharmaceutical system of polyΣcaprolactone (PCL) microspheres (MSs) loaded with budesonide and with adequate size (<5µm) to reach alveoli [4].

**Materials and Methods.-**

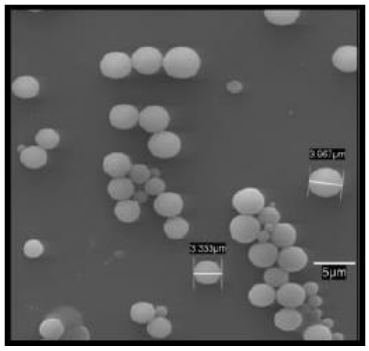
MSs were obtained from a solid in oil in water (S/O/W) emulsion, according to the procedure described previously [5]. Influence of the polymer concentration [PCL 50, 100 mg/ml], dispersant concentration [PVA 1, 1.5 mg/ml] and homogenization rate [RPM 8000, 13500] on particle size were evaluated by an experimental design $3_2$. Next, budesonide (5, 10 and 20 mg) loaded microspheres were prepared according to the optimization performed in order to obtain part i-cles suitables for deposition in the alveolar region.

MSs were tested for particle size (DLS), zeta potential (DLE), surface morphology (SEM), encapsulation efficiency (EE), thermal behavior (DSC). Also, preliminary cytotoxicity essays on rat macrophages J774 and HELA cells was carry on (MTT and flow cytometry).

**Results and Discussion.-**

✓ Experimental design data were fitted to a multiple linear regression model getting the following equation, which allows predicting MSs's size as function of the three setting factors, with a high statistical significance and high predictive value R2 (adjusted to freedom degrees) = 99,97 %; p = 0.0121

$$Size\ (\mu m) = 14{,}783 - 0{,}846*RPM\cdot 10^{-3} - 1.25*PVA + 0{,}635*PCL - 0{,}049*RPM*PCL - 0{,}443*PCL*PVA + 0{,}035*PCL*PVA*RPM$$




✓ Blank and BDS-loaded MSs does not present statistically significant differences by each other, being their hydrodynamic particle size 2,84±0,65 µm. These values were corroborated by SEM microphotographies (Fig.1).

✓ EE was calculated by removing the external medium from MSs after centrifugation, and the pellet analyzed by UV/Vis spectroscopy. Maximum encapsulation was achieved with 20 mg BDS vs 100 mg PCL, being EE 97.86 ± 1.67%. No BDS melting or crystalline peaks were observed by DSC, suggesting that the drug was integrated into the matrix

✓ Cell culture assays during 24h have demonstrated high viability of J774 macrophages and Hela cells, cultured with blank and BDS-loaded MSs.

*Figure 1.* SEM BDS-MSs

**Conclusions.-**

Preparation method optimized in this work, allows obtaining MSs with high drug encapsulation degree and uniform and suitable average size to be administered by the pulmonary route for treatment of chronic lung diseases, with the aim that they might be able to transport the drug to the pulmonary alveoli, releasing it in a controlled way and being able to be captured by the alveolar macrophages.

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**PC-32**

# Reproducible preparation of 5-FU-loaded poly(butylcyanoacrylate) nanoparticles

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**Introduction.** Colon cancer has an excellent prognosis if it is diagnosed at an early stage. However, 25% of colon cancer patients develop metastasis, thus requiring chemotherapy which is frequently based on 5-fluorouracil (5-FU).
The clinical use of 5-fluorouracil, one of the drugs of choice in colon cancer therapy, is limited by a nonuniform oral absorption, a short plasma half-life, and by the development of drug resistances by malignant cells. We hypothesized that the formulation of biodegradable nanocarriers for the efficient delivery of this antitumor drug may improve its therapeutic effect against advanced or recurrent colon cancer.

**Materials and Methods.** Butylcyanoacrylate is a generous gift from Henkel Loctite (Ireland). All chemicals were of analytical quality from Sigma-Aldrich Chemical Co. (Spain). We have engineered a 5-FU-loaded nanosystems based on the biodegradable polymer poly(butylcyanoacrylate) (PBCA). PBCA NPs were formulated by emulsion polymerization of the monomer in an aqueous solution [1,2]. Mean particle diameter was determined in triplicate at 25.0 ± 0.5°C by photon correlation spectroscopy (PCS; Malvern Autosizer® 4700, Malvern Instruments Ltd., UK).
Drug incorporation to the colloid was accomplished by entrapment (absorption) within the polymeric network during nanoparticle synthesis. Ultraviolet (UV) absorbance measurements (8500 UV–Vis Dinko Spectrophotometer, Dinko, Spain) were carried out to determine the 5-FU concentration in all the nanoformulations. Main factors determining 5-FU incorporation within the polymeric nanomatrices were investigated. Finally, the electrokinetic characteristics of both blank (drug-unloaded) and drug-loaded NPs were determined by electrophoresis measurements (Malvern Zetasizer® 2000 Electrophoresis Device, Malvern Instruments Ltd., UK).

**Results.** PBCA NPs were characterized by a spherical shape and an average diameter under 100 nm. Neither particle size nor the quality of the polymeric suspensions varied considerably when different drug quantities were loaded. Both the 5-FU entrapment efficiency (%) and 5-FU-loading values (%) were significantly greater when the drug was added to the organic phase, whatever the initially fixed 5-FU concentration. It was further observed that the use of dextran-70 (within the concentration range tested) ensured the formation of homogeneous distributions of 5-FU-loaded PBCA NPs, with reduced size and highly uniform, but without significantly influencing 5-FU vehiculization.
Conversely, 5-FU entrapment was clearly lower in the absence of dextran-70. Similarly, monomer concentration did not significantly influence the entrapment of 5-FU within the NPs. Finally, results from the electrophoretic characterization of the NPs clearly highlighted the great similarity between the $\zeta$ values of blank PBCA NPs and 5-FU-loaded PBCA NPs: from an electrokinetic point of view, blank and drug-loaded NPs were indistinguishable, thus it could be assumed that the molecules of chemotherapy agent were very efficiently entrapped within the polymeric nanomatrix.

**Conclusions.** A reproducible formulation methodology has been developed to obtain PBCA nanostructures for the efficient delivery of 5-FU on the basis of an emulsion polymerization procedure.

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**PC-33**

# Effect of drug incorporation methodology on its release from nanochitosan

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**Introduction.** Chitosan nanoparticles (NPs) are under extensive investigation both for parenteral and oral delivery of many active agents to improve the bioavailability of degradable substances such as proteins or to enhance the uptake of hydrophilic substances across epithelial layers. The *in vivo* susceptibility of chitosan to lysozyme makes it biodegradable and an ideal material to provide controlled release of many drugs [1]. This work will focus on the formulation of tegafur-loaded chitosan NPs. The therapeutic efficiency of this antitumor drug could be significantly enhanced by its incorporation into this polymeric colloid, thanks to: *i*) a controlled delivery to the targeted site that will enhance its pharmacokinetic profile; and, *ii*) the minimization of the adverse side effects, a consequence of a minimized biodistribution.

**Materials and Methods.** Chitosan NPs were prepared by a coacervation method [2]. In this procedure, the addition of sodium sulfate to the solution of chitosan in acetic acid resulted in decreased solubility of chitosan, rapidly leading to its precipitation as a poorly soluble derivative. Tegafur loading to the NPs was achieved by following two procedures.
The first method (entrapment procedure) was similar to that followed for the preparation of chitosan NPs, except that the aqueous phase was a 2% (w/v) acetic acid and a 1% (w/v) pluronic® F-68 aqueous solution, with appropriate amounts of the antitumor drug. In this method, the influence on drug entrapment of the concentration of polymer and stabilizing agent was also investigated. The second one (adsorption procedure) involved single drug surface adsorption onto the preformed polymeric NPs. Drug release from NPs was performed *in vitro* following the dialysis bag method, and using phosphate-buffered saline (pH 7.4 ± 0.1) as the release medium.

**Results.** The coacervation method followed for the synthesis of chitosan NPs allowed the formation of wellstabilized spherical nanospheres consisting of a well-defined matrix, with an average diameter ≈ 160 nm and a narrow size distribution. Tegafur incorporation into the polymeric matrix during the coacervation process yielded higher drug loading values and a sustained drug release profile, compared to single surface adsorption. The optimal formulation conditions to obtain tegafur-loaded chitosan NPs suitable for parenteral administration were determined.

**Conclusions.** The contributions of both the surface and the polymer network to the overall drug loading were investigated by means of electrophoretic mobility and optical absorbance determinations. These very interesting results suggest that chitosan NPs are potential carriers for efficient delivery of tegafur to cancer.

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**PC-34**

# Optimization of the formulation conditions to maximize doxorubicin vehiculization in poly(ε-caprolactone) nanoparticles

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**Introduction.** Despite doxorubicin (DOX) having an important antitumor effect, its lack of specificity for the tumor tissue makes its use limited. In fact, one of the major limitations of this drug is the production of cardiotoxicity caused by mechanisms such as reactive oxygen species production [1]. For these reasons, it is important to develop new drug administration pathways to enhance the antitumor effect and improve specificity for tumor tissues. In this context, the aim of our study was to develop a reproducible procedure to prepare poly(ε-caprolactone) (PCL) nanoparticles (NPs) for transporting DOX to potentially optimize cancer treatment.

**Materials and Methods.** All chemicals were of analytical quality from Sigma-Aldrich Chemical Co. (Spain).
Deionized and filtered water was used in all the experiments (Milli-Q Academic, Millipore, Molsheim, France). PCL NPs were prepared by a modified nanoprecipitation solvent evaporation procedure using a probe sonicator [2]. Particle size was determined in quadruplicate at room temperature by photon correlation spectroscopy (Malvern Autosizer® 4700, Malvern Instruments Ltd., UK). Surface electrical charge of both blank (DOX unloaded) and DOX-loaded NPs was investigated by electrokinetic determinations (Malvern Zetasizer® 2000 electrophoresis device, Malvern Instruments Ltd., UK). Finally, Ultraviolet-Visible (UV–Vis) absorbance measurements (8500 UV–Vis Dinko spectrophotometer, Dinko, Spain) were done in quadruplicate at the maximum absorbance wavelength (481 nm) to quantify drug concentration in all the formulations. Drug release experiments were performed in quadruplicate following the dialysis bag method (phosphate buffered saline, PBS, pH 7.4 ± 0.1; 37.0 ± 0.5°C).

**Results.** PCL NPs were characterized by having an average diameter < 90 nm, a spherical shape, and by a negative surface electrical charge in water. No differences were observed in particle size and quality of the colloid when the NPs were loaded with different amounts of DOX. Finally, the colloidal formulations were found to be stable during a threemonth storage period at 4.0 ± 0.5°C: no appreciable change in particle size and surface electrical charge, no existence of aggregates/sediments, nor any DOX precipitation and/or release were detected.
DOX entrapment efficiency (%) and DOX loading values (%) were found to be significantly greater when DOX was added to the organic phase, whatever the initially fixed drug quantity. As an example, when the initial DOX concentration was 0.01 M these parameters rose from ≈59.8% and ≈32.5% (when the drug was in the aqueous phase) to ≈ 90.6% and ≈ 49.2% (when DOX was incorporated to the DCM solution), respectively. In addition, drug concentration positively influenced the efficiency of DOX entrapment into the NPs. Great similarity was found between the electrokinetics ($\zeta$ values) of blank NPs and DOX-loaded NPs. Due to the fact that $\zeta$ values did not change upon drug incorporation, it can be assumed that the DOX molecules were entrapped within the NP structure. Finally, DOX release from the NPs, a biphasic process was observed at pH 7.4, where an initial rapid (burst) release phase (up to ≈ 30% in 12h) was followed by a phase in which the remaining drug molecules were released in a more progressive way.

**Conclusions.** A reproducible methology has been developed to obtain DOX-loaded NPs with appropriate properties for the parenteral route of administration, further exhibiting high drug loading values and sustained release properties.

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**PC-35**

# Physical transfection of DNA encoding green fluorescence protein to mammalian COS-7 cells with photoacoustic waves

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**Introduction:** Approved gene therapies rely on viral vectors, which are the mainstream of gene transfer methods. They offer high transduction efficiencies, but have limited packing potential, raise concerns related to mutagenesis and immunogenicity, and have a high cost. The success of gene therapy depends on the development of safe and effective methods to deliver foreign DNA to target cells. Cellular membranes offer a barrier to foreign molecules that must be overcome. Gene transfection mediated by laser-induced stress waves was proposed as an alternative and shown to be safe and spatially controllable, although the transfection efficiency is lower than 1% [1,2]. Laser-induced stress waves have high peak pressures, high impulses (pressure over time) and high stress gradient (peak pressure divided by wave rise time), but require very laser fluences. We showed that photoacoustic waves generated with much lower laser fluences, compatible with the use of optical fibers, when irradiating piezophotonic materials [3,4], attain similar stress garadients as laser-induced stress. In this work, short laser pulses with laser fluences below 100 mJ/cm2 are used to generate photoacoustic waves using piezophotonic materials. The properties of the photoacoustic waves are presented and are shown to be safe. This work relates the properties of photoacoustic waves and the duration of their exposure to COS-7 mammalian cells, to the transfection efficiency of a plasmid coding for green fluorescent protein (GFP).

**Materials and Methods:** Nd:YAG lasers with FWHM pulses of 30 picoseconds and 8 nanoseconds were used to irradiated thin films of a specially designed dye homogeneously incorporated in a polystyrene matrix, to generate photoacoustic waves. The pressure transients were fully characterized. Cell viability and transfection efficiency were evaluated and compared with gene transfection with lipofectamine using flow cytometry and fluorescence microscopy. The influence on gene trasnfection of laser energy fluence, duration of exposure to photoacoustic waves and plasmid concentration were evaluated.

**Results ans Discussion:** Gene transfection efficiencies were related with the photoacoustic waves properties and the results show that the most distinct feature of the photoacoustic waves is the generation of steep pressure gradients that can be generated repeatedly at low laser fluences (below 100 mJ/cm$^2$). We demonstrate the ability of photoacoustic waves to efficiently and transiently permeabilize the cell membrane with GFP plasmid DNA transfection efficiencies higher than 5% in COS-7 cell line without cytotoxicity.

**Conclusions:** Photoacoustic pressure waves generated by picoseconds laser pulses safely permeabilize mammalian cellular membranes. The reversible permeabilization allows cells to remain viable and enables transfection efficiencies over 5% at room temperature. Maximization of the transfection efficiency may be achieved by varying factors such as temperature, laser fluence, duration of exposure, and plasmid concentration.

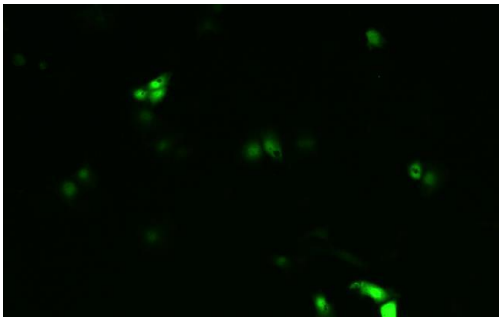

*Figure 1*. Representative image of gene transfection with gWizGFP [500 µg/mL] 24 hours after the application of PA waves for 10 minutes in COS-7 cell line with 100 mJ/cm$_2$ of laser fluence (30 ps pulse duration).

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**PC-36**

# Intracellular delivery of multiple proteins with spatio-temporal control

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**Introduction.** Intracellular delivery of proteins is extremely useful for the manipulation of cellular processes and cell reprogramming [1]. Although some formulations have been described in the last years for the successful intracellular delivery of proteins [2], so far no formulation has the capacity to orchestrate the delivery of multiple proteins. This is an important issue in many biological applications. For example, lineage-switching experiments in the hematopoietic system have shown that the order in which two transcription factors become expressed in a progenitor can decide lineage outcome and thus timing is of utmost importance [3]. Here we describe a formulation that can orchestrate the delivery of multiple proteins by light. The user can control the delivery of a specific protein immobilized on a nanoparticle by controlling the power of a near-infrared light source.

**Materials and Methods**. Our formulation is based on gold nanorods (AuNRs) conjugated with more than one single stranded DNA (ssDNA) having different physico-chemical properties. These ssDNAs were used for the attachment of proteins on the gold surface via DNA directed immobilization. Proof of concept experiments were performed with β-galactosidase (βGal) and with BSA labelled with DyLight fluorophores (DL-BSA). DL-BSA-DNA-AuNR were used to test the sequential release of fluorescent proteins with three laser powers at 780 nm. Intracellular laser-induced release of proteins and colocalization studies using confocal microscopy and transmission electron microscopy were performed in mouse fibroblasts.

**Results and Discussion.** Different laser powers (0.8, 1.25 and 2 W/cm$^2$) were used to modulate the release of fluorescent proteins immobilized on AuNRs. A stimulus of 1.25 W/cm2 induced the release of one of the proteins (86%) and a stimulus of 2 W/cm$^2$ induced the release of the other protein (93%). In fibroblasts incubated with DL-BSA-DNA-AuNR, the protein fluorescence increased, the signal was more diffuse and the colocalization with AuNR decreased significantly in the irradiated samples, indicating a displacement of the protein from the AuNR. Moreover, transmission electron microscopy studies indicated that, upon irradiation, the number of AuNRs in the endosomes decreased, suggesting a laser-induced endosomal escape. In fibroblasts incubated with βGal-DNA-AuNR, the fluorescence from XGal staining increased 1.5 fold when the cells were irradiated at 1.25 W/cm$^2$. The protein was distributed homogeneously in the cytosol and the colocalization with AuNR decreased. The same level of enzymatic activity was achieved when the photo-activation was done up to 24 hours after incubation.

**Conclusion.** We report a light-responsive nanomaterial that efficiently escapes endolysomal compartment and allows precise control over the release of more than one protein from a single nanomaterial using an external near infrared laser.

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**PC-37**

# Development of neuregulin-loaded microparticles combined with cardiomyocytes for cardiac repair

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**Introduction**. The lack of effective therapies to repair the heart and reestablish cardiac function after a myocardial infarction have boosted the search for new therapeutic options [1]. Among them, cell and protein therapies represent promising strategies to repair the heart after an ischemic event [2,3]. However, preclinical and clinical studies using these therapies have generated modest results due to the short half-life of molecules and the low engraftment and survival of cells [4,5]. Therefore, new approaches are needed to overcome these issues. In this context, drug delivery systems have emerged as very advantageous tools. Here, we propose the use of microparticles (MPs) with a dual function: as a reservoir for therapeutic proteins and as a 3D scaffold to support cell functions.

**Material and Methods**. Neuregulin (NRG)-releasing poly(lactic-co-glicolic acid) MPs were formulated by multiple emulsion solvent evaporation method and characterized. In order to favour cell attachment, the surface of MPs was functionalized with biomimetic molecules and zeta potential was measured. Different molecules and concentrations were tested: collagen type I (30 µg/ml, 60 µg/ml) in combination with poly-D-lysine (PDL, 100 µg/ml) or fibronectin (30 µg/ml) with PDL (100 µg/ml). Next, 150.000 cardiomyocytes derived from induced pluripotent stem cells (CM-iPS) were incubated with 0.150 mg of differently coated MPs for 2h at 37ºC in order to enable cell adhesion. Once the most appropiate coating was selected, the ability of the MPs-cells complexes to pass through a 27G needle was assessed. The viability and proliferation of cells after passing through the needle were studied by Live/Dead assay and Click-iT Plus EdU Imaging kit, respectively.

**Results and Discussion**. We obtained MPs of a mean size of 10 µm and a zeta potential of -20 mV. After functionalization, zeta potential of MPs increased to positive values (23 mV for collagen 30 µg/ml and PDL 100 µg/ml, 16 mV for collagen 60 µg/ml and PDL 100 µg/ml and 8 mV for fibronectin 30 µg/ml and PDL 100 µg/ml). Encapsulation efficiency of NRG was 55%. Incubation of CM-iPS with overlaid MPs revealed that better cell adhesion was obtained after coating of MPs with collagen type I (30 µg/ml) and PDL (100 µg/ml) (see Figure 1). Furthermore, complexes passed through a 27G needle, which reveals the suitability of the systems to be injected in the infarcted myocardium in mice.

**Conclusion.** The developed complexes will be tested in a murine myocardial infarction model in order to elucidate their potential for heart repair.

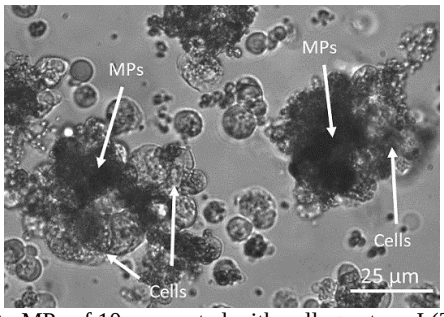


*Figure 1*. Adhesion of CM-iPS to MPs of 10 µm coated with collagen type I (30 µg/ml) and PDL (100 µg/ml).

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**PC-38**

# Shedding lights on cancer cells and their microenvironment: an *in vitro* 3D tumor model of pancreatic cancer for preclinical prediction of *in vivo* behavior of nanomedicines

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**Introduction.** Nanomedicines offer the possibility to improve the efficacy of anticancer drugs, however progresses in pancreatic cancer therapy have remained exceedingly slow mainly as consequence of an inefficient drug delivery to cancer cells [1]. Hallmark of this tumor is an extensive desmoplastic reaction, which acts as a physical barrier sequestrating nanomedicines, blocking their diffusion and therefore limiting the effectiveness of the treatment [2]. Thus, appropriate preclinical evaluation of nanomedicines urgently requires the use of *in vitro* models capable to reliably mimic the tumor microenvironment, such as the 3D multicellular tumor spheroids (MCTS) [3].

**Materials and Methods.** Mono-type (cancer cells) and hetero-type (cancer cells + fibroblasts) MCTS were prepared according to the liquid overlay technique. Cell suspensions were seeded at different densities in poly-HEMAcoated 96 round-bottom well plates (w/v). Spheroid analysis has been performed through image processing algorithms, which allowed reproducible area selection to collect morphometric parameters. Complete characterization included also the evaluation of cell viability and cyto-architectural organization.

**Results and Discussion.** MTCS were prepared using PANC-1 cancer cells and viable 3D mono-cultures have been maintained *in vitro* up to 17 days. Extremely compact aggregates of poorly viable cells were instead obtained using the BxPC3 cells. Co-cultures of PANC-1 cells and fibroblasts were successfully created and we observed a clear growth promoting effect of fibroblasts on cancer cells. Immunostaining revealed that the spheroid core was characterized by the presence of cytokeratin (CK AE1/AE3) negative cells and it was positively stained for fibronectin, thus suggesting the accumulation of the fibroblasts in this area and the secretion of the ECM proteins (Figure 1).
Investigation of a triple 3D co-cultures with endothelial cells is ongoing aiming to reproduce the structural and functional integration of the various cell types constituting the complex pancreatic tumor.

**Conclusions.** Mono-type and hetero-type MCTS were succesfuly prepared and extensively characterized. Once introduced also the endothelial component the model will be used to screen a library of variously engineered nanomedicines in order to identify the strategies which will prompt the penetration of the drug delivery systems through the whole tumor mass.

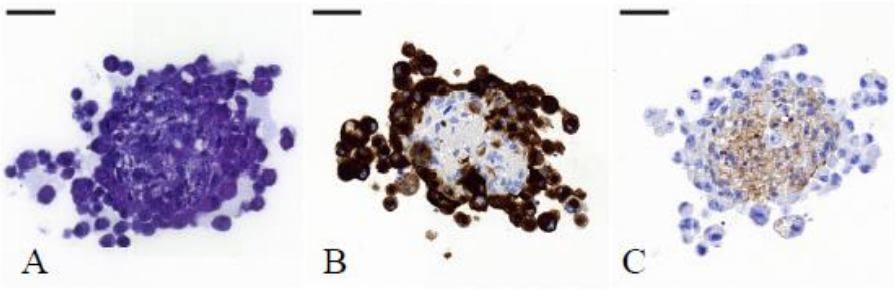


*Figure 1*. Immunohistochemistry on paraffin sections (5 µm thick) of hetero-type spheroids at d4 post seeding. A) Hematoxilin & Eosin staining; B) Cytokeratin staining of cancer cells; C) Fibronectin staining of fibroblast-associated fibronectin. Scale bar: 50µm

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**PC-39**

# Targeting of human CD44v6 with Fab-decorated PLGA-based nanoparticles

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## Introduction

Variant isoforms of CD44 containing exon v6 (CD44v6) have been implicated in many of the malignant processes associated with carcinoma including metastasis [1], sustained Ras activation [2], and pre-metastatic niche formation [3]. As a co-receptor for the tyrosine kinase c-Met, CD44v6 is required for HGF-induced signalling [4]. It is also expressed in colon cancer stem cells [5] and (pre-)malignant gastric lesions [6]. Targeted drug delivery with antibody-decorated nanoparticles (NPs) [7] offers a promising strategy for the treatment and/or diagnosis of (metastatic) carcinomas.

## Materials and Methods

Fluorescent, PEGylated poly (lactic-co-glycolic acid) (PLGA)-based NPs were produced by nanoprecipitation or water-in-oil-in-water double emulsion and engrafted with a human Fab [8-9] that specifically targets human CD44v6 (v6 Fab-PLGA NPs) with ~50 nM affinity. The Fab was engineered to express C-terminal cysteines to allow for site-directed conjugation to the maleimide-functionalized NPs, thus allowing for Fab orientation that maximizes CD44v6 recognition. These and control NPs were characterized for their physical qualities by dynamic light scattering, laser Doppler anemometry, and trasmission electron microscopy. Human CD44v6 specificity was confirmed through binding assays based on a CD44v6-derived peptide (ELISA) as well as gastric cancer cell lines expressing CD44v6 (FACS). Live cell internalization was determined by light microscopy and FACS and cytotoxicity by MTT assays. Lastly, resistance to simulated intestinal fluid (SIF; +/- pancreatin) exposure and freeze-drying were monitored by cell binding (FACS).

## Results and Discussion

The NPs had sizes ranging from 200-400 nm with reasonable polydispersity, overall negative charge, and spherical morphology. The v6 Fab-PLGA NPs specifically recognized human CD44v6 when presented as a peptide as well as on the surface of cells from *in vitro* culture. These NPs were internalized in living cells and are not cytotoxic at 50 µg/mL. Lastly, CD44v6-specific cell binding was maintained when the v6 Fab-PLGA NPs were exposed to SIF, but binding was lost with the addition of pancreatin. Lastly, the NPs also maintained cell binding when subjected to freeze-drying in 10% trehalose and stored for two weeks at various temperatures, expanding our group's previous work on the crypreservation of biotherapeutics within PLGA NPs [10-11].

## Conclusions

The PLGA NPs developed in this study have the capacity to harbor both hydrophobic and hydrophilic payloads and recognize human CD44v6 expressed on the surface of cells. We envision NPs targeting CD44v6, like those developed here, could serve as drug and/or diagnostic delivery agents in patients previously stratified with CD44v6+ carcinomas.

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**PC-40**

# PEGylated cationic liposome – DNA nanoparticle assembly in cell culture media: pathway effects and clues to enhanced control and transfection efficiency optimization

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**Introduction:** Cationic Liposome – DNA nanoparticles constitute a promising approach for safe and efficient delivery of genes for therapeutic applications. In order to be used *in vivo*, these particles can be coated with an inert and hydrophilic polymer, such as polyethylene-glycol (PEG), which improves blood circulation time by providing steric stabilization against removal by the immune system. In this work we study the influence of the initial salt concentration, which controls the electrostatic atraction between cationic liposomes and anionic DNA, on the structure of PEGylated CL–DNA nanoparticles.

**Materials and Methods:** The lipids DOPC, DOTAP, and 18:1 PEG2000 PE were purchased from Avanti Polar Lipids and used as received. Two parameters are systematically changed in the lipid composition: (i) the lipid membrane charge density (σM), which is proportional to the amount of DOTAP in the membrane; and (ii) the amount of PEG2K lipid (0, 5 and 10% molar fraction). Two CL-DNA preparation methods are studied in this work. In the first, the complexes are prepared in water, and transferred later to saline media. In the second, when cationic lipid and DNA are mixed, they are already in saline media. Small-angle X-ray Scattering (SAXS) and cryo-TEM are the main characterization techniques.

**Results and Discussion:** Previous results have shown that if non-PEGylated or PEGylated CL–DNA lamellar complexes are prepared in water, their structure is well defined with a high number of lipid membrane–DNA layers [1,2]. Here we show that if these complexes are transferred to saline media (150 mM NaCl or DMEM, both near physiological conditions), this structure remains nearly unchanged. Contrariwise, if PEGylated complexes are prepared in saline media from the beginning, their lamellar structure is much looser, with a small number of layers [3]. This pathway-dependent behavior of PEGylated complex formation in salty media is controlled by the liposome membrane charge density and the mole fraction of PEG in the membranes, with the average number of layers decreasing with increasing salt concentration and increasing amount of PEG-lipid. Each of these structures (high and low number of layers) is stable with time, suggesting that in spite of particle formation being thermodynamically favored, the assembly process between DNA and cationionc PEGylated membranes is largely controlled by kinetics.

**Conclusion:** One important result of these findings is the realization that subtle differences in the preparation protocol (differences which are common between different laboratories and researchers), can lead to important differences over the structure of PEGylated CL-DNA-NPs, and possibly to the way these particles interact with cells, influencing gene delivery. Ongoing work is aimed at elucidating the influence of these differences in the transfection efficiency, and how they can be exploited towards achieving more efficient therapeutic formulations.

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**PC-41**

# Cell surface/cytoplasmic relative levels of nucleolin: a key feature for successful anticancer nanotherapies targeting nucleolin

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**Introduction:** Although the latest technologies have promoted a high rate of early diagnosis of cancer, breast cancer still accounts for 25% of all cancers and presents a high death rate worldwide [1] associated to chemotherapy resistance and development of metastasis. Therefore, it is mandatory to unravel the molecular mechanisms underlying breast cancer initiation, progression and metastasis, both at the intrinsic and tumour microenvironment levels [2]. As nucleolin is crucial for cell proliferation and signal transduction, and it is overexpressed in cancer- and endothelial-cells [3], [4] mediating significant cytotoxicity of synergistic drug combination encapsulated in nanoparticle functionalized with nucleolin-binding F3 peptide against breast cancer stem cells and non-stem breast cancer cells [5], the goal of this work was to evaluate the subcellular relative levels of nucleolin in different breast cancer cell lines to further exploit its subcellular contribution for the anti-cancer efficacy and the underlying intracellular mechanisms.

**Materials and Methods:** MCF-7 (luminal), MDA-MB-231, MDA-MB-468, Hs578T (triple negative) breast cancer cell lines, and MCF-12A (non-tumorigenic) and MDA-MB-435S (nucleolin-overexpressing) cell lines were used. Two-million cells of each were collected for Western blot analysis of nucleolin protein levels of whole cell and cell fractionated extracts (by centrifugation method).

**Results and Discussion:** Preliminary data pointed out that in all breast cancer cell lines tested, a 0.10 to 0.22-fold increase of nucleolin protein levels was observed, compared to the non-tumorigenic cell line, whereas the nucleolin-overexpressing cell line (MDA-MB-435S) presented 0.52-fold increase (as expected). Most of the nucleolin protein of MDA-MB-231 and MDA-MB-468 cell lines was identified in the nucleus (63% and 67%, respectively), in contrast with the predominant expression in the cell surface/cytoplasm of MDA-MB-435S and Hs578T cell lines (63% and 58%, respectively).

**Conclusion:** Based on these data, it is of major interest to further address the efficacy of the previous developed synergistic drug combination encapsulated in nanoparticle functionalized with nucleolin-binding F3 peptide against the Hs578T cell line. This will further support the exploitation of the higher expression of cell surface/cytoplasmic nucleolin relative protein levels, as a key molecular feature of the underlying pharmacodynamic associated with the synergistic drug combination-based nanomedicine targeting nucleolin.

**Acknowledgments**: This work was supported by the European Regional Development Fund (ERDF), through the Operational Programme for Competitiveness and Internationalisation - COMPETE 2020, and Portuguese national funds via FCT – Fundação para a Ciência e a Tecnologia, under projects POCI-01-0145-FEDER-016390: CANCEL STEM and POCI-01-0145-FEDER-007440.

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**PC-42**

# $S4_{13}$-PV-derived peptides as promising nucleic acid delivery systems in hard-to-transfect cell cultures

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**Introduction:** Gene therapy for treating genetic and acquired diseases holds great promise, but its successful application has been limited due to difficulties related to the delivery efficiency of nucleic acids into target cells, as well as to cytotoxicity issues. In this regard, research on cell penetrating peptides (CPPs) as potential delivery vehicles has contributed to important advances in the field.
The $S4_{13}$-PV CPP, a dermaseptin S4-derived peptide fused to the nuclear localization sequence of the SV40 large T antigen, has been extensively studied in our laboratory and, recently, modulated by systematic structural modifications to increase its efficiency for nucleic acid delivery. Such modifications include the introduction of a five-histidine tail to the N-terminus of $S4_{13}$-PV (H5-$S4_{13}$-PV), acylation at the N-terminus of $S4_{13}$-PV with a lauroyl group (C12-$S4_{13}$-PV), and combination of these two modifications to obtain C12-H5-$S4_{13}$-PV and H5-$S4_{13}$-PV-C12, which differ in the location of the lauroyl group, at the N- and C-terminus, respectively. The doubly modified peptides have shown to mediate efficient gene silencing in human glioblastoma (U87) and cervical carcinoma (HeLa) cells, even in the presence of serum. In the present work, the nucleic acid delivery efficiency of $S4_{13}$-PV derivatives was assessed in human THP-1 monocytic leukemia cells, which grow in suspension and are known to be difficult to transfect.

**Materials and Methods:** THP-1 cells were incubated with complexes formed through electrostacic interactions between nucleic acids and peptide. Peptide-mediated delivery of labeled small interference RNA (siRNA) and labeled Locked Nucleic Acid (LNA™) was evaluated after 4 hours incubation with the cells in the presence of 10 % serum by flow cytometry.

**Results and Discussion:** In contrast to the results obtained in adherent U87 and HeLa cells, C12-$S4_{13}$-PV proved to be the most efficient peptide to mediate the delivery of both FAM-labeled siRNAs and LNAs into the suspension THP-1 cells, allowing to transfect almost 90% of the cells. Monolayer cultured cells have been commonly used in transfection studies to evaluate the *in vitro* efficacy of non-viral gene vectors [1]. However, two-dimensional (2D) cell cultures do not accurately mimic the biology of *in vivo* tumors The ability of these peptides to mediate the delivery of small nucleic acids into both adherent and suspension cell lines prompted us to challenge $S4_{13}$-PV-derived peptides to transfect three-dimensional (3D) cell cultures (see Figure 1). These studies are currently in progress in our laboratory.

**Conclusions:** Altogether, our results might contribute to the establishment of a more rational design of new and efficient peptide-based nucleic acid delivery systems, in conditions mimicking *in vivo* environments.

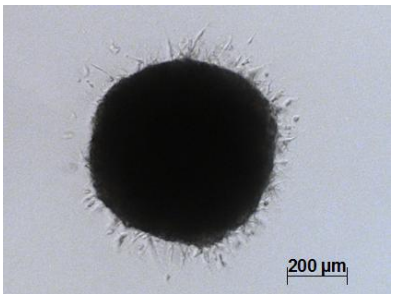


*Figure 1.* Transmission light image of a U87 spheroid generated by the GravityPLUS hanging drop system and embedded in a collagen I matrix.

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**PC-43**

# Aptamer-based targeted delivery of G-quadruplex ligands in HeLa cervical cancer cells

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**Introduction:** Human papillomavirus (HPV) are oncogenic viruses that cause head, neck and angenital cancers [1]. Notably, high-risk HPV types 16 and 18 are responsible for 70% of all cervical cancer cases. The oncogenic potential of the HPV is related to the expression of the oncoproteins E6 and E7 which inactivate p53 and members of pRb family leading to carcinoma development [2]. Despite extensive studies during the past decades, there is still no specific cure against HPV infection.
Recently, it has been reported that the genome of certain HPV types contain G-rich regions capable of forming G-quadruplex (G4) structures [1,3]. Moreover, these were found prevalent in important regulatory regions, thus being potential targets for G4-targeted ligand design, interfering with the regulation of viral gene expression. This strategy could be an promissing method for the control of viral replication and transcription. Moreover, using the well-studied aptamer AS1411 [4], we intend to develop an aptamer-based drug delivery system to deliver therapeutic compounds (*i.e.* HPV G4 ligands) to HPV infected cells in pre-cancerous state. AS1411 targets the cell-surface protein nucleolin which is also involved in HPV18 oncogene transcription in cervical cancer and HPV16 genome stability maintenance [5,6].

**Materials and Methods:** Circular dichroism and FRET-melting were used to assess G4 formation in three HPV genomes, HPV 9, 32 and 57, and to study the ability of different ligands to stabilize these G4 structures. The use of AS1411 as a drug delivery system in cervical cancer HeLa cell line was investigated by confocal microscopy and the MTT cell viability assay.

**Results and Discussion:** Several ligands were found to bind and stabilize G4 structures of HPV 9, 32 and 57. The ligand, [32]$Phen_2N_6$ stabilizes the HPV G4s structure by around 20 °C. Using confocal fluorescence microscopy HeLa cells were shown to overexpress nucleolin at the cell surface, being a cancer cell specific target for the delivery of G4 ligands using AS1411 aptamer. The G4 AS1411 aptamer was conjugated with [32]$Phen_2N_6$ via supramolecular approach, and its transfection in HeLa cells led to an anti-proliferative effect.

**Conclusions:** Three HPV genome derived sequences were used as targets for G4 ligands that could act as transcription inhibitors with antiviral effect. Using HeLa cervical cancer cell line, we showed the feasibility of using nucleolin and AS1411 aptamer as a potential ligand delivery route to HPV infected cells. This study suggests the G4 regions of HPV genomes as promising targets for drug development and the use of AS1411 as a cell-specific drug delivery system.

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**PC-44**

# Stabilization of modified AS1411 G-quadruplex aptamer by acridine orange derivative for cervical cancer therapy

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## Introduction

G-quadruplex (G4) AS1411 aptamer has been recently employed as a drug delivery system with efficient cellular internalization compared to non-G4 DNA sequences, and it exhibits anticancer activity by interfering with nucelolin oncogenic functions [1]. Clinical trials of AS1411 have indicated that it is well tolerated with therapeutic activity but improved pharmacology and potency are required for optimal efficacy [2].
Since G4 AS1411 aptamer has high affinity for nucleolin and it is able to enter more easily into malignant cells that can be used for diagnosis (when associated with imaging agents) and for treatment of cancer (by ligands association) [3].
The synthetized ligands used in this study are derivatives of acridine orange [4]. Acridines are a class of heterocyclic compounds that have a wide range of biological and pharmaceutical properties [5].
In order to have greater entrance in the cell, greater stability and better pharmacological properties, AS1411 has been structurally modified to be used as therapeutic agent.
The aim of this study is to evaluate the AS1411 properties with and without modification and ligands in cancer cell lines.

## Materials and Methods

Circular dichroism is performed to evaluate the structure of the AS1411 aptamer modified and G-quadruplex maintenance/interconversion after acridine orange binding. The binding affinity of G-quadruplex AS1411 derivative aptamer with and without acridine orange ligand to nucleolin is assessed by surface plasmon resonance (SPR) biosensor
Cell viability assays are performed to evaluate the cytotoxic effect of G-quadruplex AS1411 derivative aptamer on cervical cancer cells.

## Results and Discussion

The G-quadruplex AS1411 derivative aptamer showed high stabilization with the acridine orange derivative by circular dichroism. The binding G-quadruplex AS1411 derivative aptamer with and without ligand assessed by SPR biosensor showed high affinity to nucleolin. Cytotoxic studies revealed that the ligand is cytotoxic for cervical cancer cells with IC50 around 2.4-8.9 μM.

## Conclusions

Overall, the G-quadruplex AS1411 derivative showed high stability in the presence of acridine orange ligand. The binding AS1411 aptamer modified with and without ligand presented high affinity to nucleolin and cytotoxic studies revealed that the ligand is cytotoxic for cervical cancer. The G-quadruplex AS1411 derivative is an effective targeting agent that can be used to deliver cargoes to cancer cells.

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**PC-45**

# Nanosystem based on AS1411 G-quadruplex derivative for cancer theraphy

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**Introduction**: AS1411 is a G-rich oligonucleotide that has been found as a potent cancer target agent, due to its ability to folding into a variety of four-stranded structures called G-quadruplexes (G4) in the presence of K+ and Na+ [1],[2],[3],[4]. It was found that G4 AS1411 has cancer-selective antiproliferative activity, mainly because it works as an aptamer to nucleolin, a multifunctional protein which is present at high levels on the surface of cancer cells[2][3]. Previous studies reported that G4 aptamer AS1411 binds NCL and inhibit the growth of cancer cells without affecting normal cells and was reach human clinical trials Phases 1 and 2. However, it shown low potency (μM) and suboptimal pharmacology that may hamper its potential for approved clinical use [1] [5]. Several issues need to be considered to use AS1411 namely, accumulation in cancer cells, AS1411-linked particles or conjugates trafficking, the nature of the cargoes and the ability to reach the nucleus. Furthermore, AS1411 is highly polymorphic, and there are at least 8 different forms of G-quadruplexes with important biological activities [1].
One prominent strategy to explore the therapeutic potential of G4 AS1411 is stabilize it with specific ligands, therefore, the development of novel, selective G4 stabilizing agents is very important since these are suitable candidates in cancer therapeutic treatments [1] [4].

**Materials and Methods:** Pyridostatin and TMPyP4 porphyrin ligands are conjugated to AS1411 derivative via supramolecular approach.
The nucleolin binding affinity to G4 AS1411 derivative (with and without pyridostatin and TMPyP4 porphyrin ligands) is evaluated by surface plasmon resonance (SPR) biosensor.
The evaluation of maintenance/interconversion of the G4 in AS1411 derivative with and without pyridostatin and TMPyP4 porphyrin ligands is evaluated by circular dichroism. Cytotoxicity and co-localization studies are performed using HeLa cells versus non-malignant epithelial cells, namely through MTT assay and confocal microscopy, respectively.

**Results and Discussion**: Pyridostatin and porphyrin TMPyP4 ligands bind efficiently G4 AS1411 derivative and stabilize it, by increasing their melting temperature ($T_m$). SPR results demonstrates an improvement in the binding between the G4 AS1411 derivative-ligand and nucleolin when compared with AS1411. Improvements in cytotoxicity are also being achieved, using the G4 AS1411 derivative-ligand.

**Conclusions**: The results suggest the importance of this kind of ligands in stabilizing modified G4 AS1411 structures; which make them potential anti-cancer drugs. The modifications introduced in the AS1411 increase the chemical and enzymatic stability of aptamer, improving their binding afinity and specificity to nucleolin. In summary, the use of G4 AS1411 derivative-ligands provides a suitable approach to delivery ligands acting as anticancer agents.

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**PC-46**

# G-quadruplex AS1411 derivative-phenanthroline nanosystem for cervical cancer therapy

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**Introduction:** AS1411 is a G-rich oligonucleotide sequence and are highly polymorphic, presenting a wide variety of G-quadruplexes (G4) structures. This aptamer exhibits selectivity for nucleolin, a protein overexpressed in many tumor cells and which binds preferentially to G-quadruplex structures. Due to the binding to nucleolin, AS1411 can be internalized by the cell, presenting itself as a potential therapeutic target [1], [2].
The use of this aptamer for therapeutic purposes requires its stabilization through ligands [3]. In addition, the ligands can be used as cargoes with therapeutic effect against HPV-associated oncogenicity. Phenanthroline macrocycles, such as [32]$phen_2N_4$ and [16]$phenN_2$, are presented as potential stabilizers of G4 AS1411 derivative.
The aim of this study is to produce a nanosystem based on G4 aptamer AS1411-ligands and evaluate it as cancer-selective antiproliferative agent in cervical cancer cells.

**Materials and Methods:** The G4 aptamers sequences AS1411 and AT11 are used in these studies.
Phenantroline macrocycles were synthesised as previously described [4]. Circular dichroism (CD) was performed to evaluate stability of ligands with aptamers AS1411 and AT11. The binding affinity of nucleolin to the G4 aptamers is evaluated by Surface Plasmon Resonance (SPR) biosensor.
Finally, the G4 aptamers AS1411 and AT11 was bounded to the phenanthroline ligands via supramolecular approach. The G4 aptamer AS1411-ligand was transfected into human cervical cancer cell line (HeLa) following by assement of cytotoxicity and co-localization by MTT assay and confocal microscopy, respectively.

**Results and Discussion:**
The results of CD revealed high affinity and G4 thermal stabilization of [32]$phen_2N_4$ and [16]$phenN_2$ to G4 aptamers AS1411 and AT11. The modified G4 AT11 showed better results comparatively to AS1411.
The binding affinity determined by SPR biosensor between nucleolin and G4 aptamers AS1411 and AT11 is high being promising candidates for the *in vitro* studies.
Cells incubated with G4 aptamer AS1411-ligands showed decreased cell viability and proliferation.

**Conclusions:**
The present work reports the importance of the study of G4 aptamers AS1411 and AT11 bound to phenanthroline ligands being promising anticancer agents, inhibiting the proliferation of cancer cells, with minimal damage to healthy tissues.

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**PC-47**

# Nucleolin as a functional driver of breast cancer cells and a target for anti-cancer therapies

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**Introduction:** Breast cancer is a complex disease at both cellular and molecular levels, being mortality associated with the formation of metastasis [1]. Cancer stem cells (CSCs), which are naturally resistant to chemotherapy, have been associated with disease recurrence and metastases formation [2]. For successful therapeutic intervention, the identification of surface proteins overexpressed in both CSCs and non-SCCs is crucial, as well as to deeply understand the underlying biology [3]. In line with this, our group has previously demonstrated nucleolin overexpression in breast cancer cell sub-populations with different stem-like properties, enabling intracellular delivery of liposomal synergistic drug combination [4].
According to the state-of-the-art, the focus of this work was to establish an association between nucleolin expression and different phenotypic features of breast cancer cells that could further support this protein as marker for targeted breast cancer therapy.

**Materials and Methods:** Aiming at studying the impact of the modulation of nucleolin expression in the phenotypic features of breast cancer cells, molecular tools enabling the control of nucleolin expression were developed. MCF-7 (luminal A) and MDA-MB-231 (triple negative) breast cancer cell lines were transduced with nucleolin coding sequence or a specific anti-nucleolin short-hairpin RNA (anti-NCL shRNA) sequence, enabling overexpression or downregulation of the protein, respectively. Nucleolin overexpression-encoding lentiviral plasmids were engineered with a Tetracycline On (TetOn) system, allowing to control the expression of the gene of interest. Upon transduction, nucleolin overexpression (and expression of the reporter gene mCherry) was induced in the presence of doxycycline.

**Results and Discussion:** An increase in the mammospheres formation efficiency on the 1$^{st}$ and 2$^{nd}$ generation of mammospheres formed by nucleolin overexpressing-MDA-MB-231cells was observed, compared with non-transduced cells and transduced cells without activation of nucleolin overexpression. In contrast, no differences were observed in MCF-7 cells upon modulation of nucleolin expression. Wound healing assay data demonstrated that nucleolin knockdown with an anti-NCL shRNA decreased the migration capacity of both MCF-7 and MDA-MB-231 cells by approximately 2- and 3.3-fold, respectively, relative to non-transduced cells, 12 h after wound scratch.

**Conclusions:** The observed phenotypic differences, as a function of the levels of nucleolin expression, suggested that this protein may influence the stem-like phenotype of MDA-MB-231 breast cancer cells and might be a relevant protein for cell migration machinery of both breast cancer cell lines tested. Overall, these results suggested cell surface nucleolin as a common marker between CSCs and non-stem cancer cells (non-SCCs) in MDA-MB-231 cells, further supporting this protein as a functional driver and a target for targeted anti-cancer therapy, in indications with recognized unmet medical need as triple negative breast cancer.

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**PC-48**

# Cytotoxicity of targeted liposomal C6-ceramide and doxorubicin against nucleolin-overexpressing cancer cell lines is supported by the downregulation of the Akt pathway and is more effective than conventional drug combinations

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**Introduction:** Cancer stem cells (CSCs) have been described as a relevant contributor to tumorigenicity, metastasis, tumor recurrence and drug resistance, making this cell population a relevant target in solid tumors. $C_6$-ceramide has been demonstrated to downregulate PI3K/AKT/mTOR pathway that is essential to CSC proliferation and survival [1]. Metformin, a standard drug for treating diabetes, has been described to selectively target CSCs and enabling sensitization to chemotherapy [2]. However, the recognition of the potential of cancer cells to interconvert in response to environmental stimulus, turned both CSCs and non-stem cancer cells into two relevant therapeutic targets [3]. One of the strategies to target these different tumor cell populations, relies on the combination of conventional chemotherapeutic drugs (as tumor debulking agents, targeting non-stem cancer cells) with a drug that has been shown to target CSCs.

Nucleolin overexpression has been demonstrated on the surface of both breast CSC and non-stem breast cancer cells [4] and endothelial cells from tumor blood vessels. We have previously developed a lipid-based nanoparticle containing doxorubicin (DXR) and functionalized with the nucleolin-binding F3 peptide, [F3]L-D. This pH-sensitive lipid-based nanoparticle was recently modified to encapsulate a synergistic combination of a sphingolipid ($C_6$-ceramide) and DXR, [F3]L-$DC_6$. The aim of this work was to assess the therapeutic potential, at the molecular level, of this lipid-based nanoparticle containing a synergistic combination of $C_6$-ceramide and DXR against nucleolin-overexpression cancer cell lines. In addition, we aim at demonstrating the gain in efficacy arising from the intracellular delivery of the developed synergistic combination, relative to the drug combinations commonly used in clinical practice for the treatment of triple-negative breast cancer (doxorubicin+cyclophosphamide and doxorubicin+cisplatin) and [F3]L-D+metformin.

**Materials and Methods**: Cytotoxicity was assessed by the rezasurin assay, upon incubation of triple negative breast cancer cells (MDA-MB-231, MDA-MB-468 and HS-578T), breast cancer MCF-7 (estrogen and progesterone receptors positive) and ovarian cancer SKOV-3 cells with serial dilutions of F3 peptide-targeted liposomes containing DXR:$C_6$-ceramide in 1:1 molar ratio, [F3]L-$DC_6$, and the counterpart containing only DXR, [F3]L-D. In addition, cytotoxicity was assessed upon incubation of breast cancer cell lines with combinations of drugs at their $IC_{50}$ (DXR+cyclophosphamide, DXR+cisplatin, [F3]L-D+metformin). Akt and p-Akt protein levels were evaluated by western blot analysis. AKT1 and S6K1 mRNA levels were evaluated by RT-PCR.

**Results:** The targeted drug combination ([F3]L-$DC_6$) enabled a 90% death of MDA-MB-231 and SKOV-3 cell lines, following 4 h incubation, and 100% death following 24 h incubation, a level of cell killing not achieved by the counterpart containing only DXR. In the case of the breast cancer cell lines, the associations doxorubicin+cyclophosphamide, doxorubicin+cisplatin or [F3]L-D+metformin, incubated at their $IC_{50}$ did not enable a significant increase in cell death compared with single drugs at the $IC_{50}$. At the molecular level, [F3]L-$C_6$ induced a p-Akt downregulation of 5.4 and 3.2-fold in MDA-MB-231 and SKOV-3 cell lines, respectively. Moreover, S6K1 mRNA levels were decreased at least 1.5-fold in all cell lines tested, following incubation with 2 μM [F3]L-$C_6$, for 24 h, thus supporting $C_6$-ceramide mediated inhibition.

**Discussion and Conclusions:** The enhanced efficacy of the lipid-based nanoparticle functionalized with the nucleolin-binding F3 peptide containing a synergistic drug combination ($C_6$-ceramide and DXR) was supported by the downregulation of the Akt pathway. Moreover, the delivery of the targeted liposomal drug combination to cancer cells enabled 100% of cell death, in contrast with the non-liposomal (conventional) drug combinations commonly used for the treatment of triple-negative breast cancer (doxorubicin+cyclophosphamide and doxorubicin+cisplatin) that did not reach 90% of cell death.

**Acknowledgments**: The work was supported by national funds through FCT within the grant ENMed/0005/2015 (Euronanomed/Portuguese Foundation for Science and Technology, FCT), the European Regional Development Fund (ERDF) through the Centro 2020 Regional Operational Programme: project CENTRO-01-0145-FEDER-000012-HealthyAging2020, the COMPETE 2020 - Operational Program for Competitiveness and Internationalization, and the Portuguese national funds via FCT under project POCI-01-0145-FEDER-007440.

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**PC-49**

# Folic acid-tagged protein nanoemulsions with controllable size for cancer therapy

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Cancer is one of the most devastating diseases and the conventional chemotherapeutics agents distribute nonspecifically in the body, inducing a number of drawbacks [1, 2]. Protein-based nanoparticles have gained considerable interest as drug delivery devices due to their exceptional characteristics [3, 4]. Additionally, protein-based nanoparticles can also be easily amenable for surface modification and covalent attachment of drugs and targeting ligands [3, 5].

The aim of this work was the development of albumin nanoemulsions as drug delivery systems for cancer therapy. The production of albumin nanoemulsions was achieved by high pressure homogenization of an aqueous solution with an organic solvent (vegetable oil), subjecting the mixture to varying number of homogenization cycles at high pressure. In order to determine the best formulation for therapeutic applications, physicochemical and biological (*in vitro* and *in vivo*) characterizations were performed.

Albumin nanoemulsions were produced by high pressure homogenization with and without a tri-block copolymer (Poloxamer 407), which presents a central hydrophobic chain of polyoxypropylene (PPO) and two identical lateral hydrophilic chains of polyethylene glycol (PEG). We observed a linear correlation between tri-block copolymer concentration and size – the use of 5 mg/mL of Poloxamer 407 yields nanoemulsions smaller than 100 nm. Molecular dynamics and fluorescent tagging of the tri-block copolymer highlight their mechanistic role on the size of emulsions. Folic acid (FA)-tagged protein nanoemulsions were shown to promote specific folate receptor (FR)-mediated targeting in FR positive cells. Carbon monoxide releasing molecule-2 (CORM-2) was incorporated in the oil phase of the initial formulation. FA-tagged nanoemulsions loaded with CORM-2 exhibited a considerable antitumor effect and an increased survival of BALB/c mice bearing subcutaneous A20 lymphoma tumors (Figure 1). The developed nanoemulsions also demonstrated to be well tolerated by these immunocompetent mice.

The novel strategy presented here enables the construction of highly stable, size controlled, functionalized protein-based nanoemulsions with excellent characteristics for active targeting in cancer therapy.

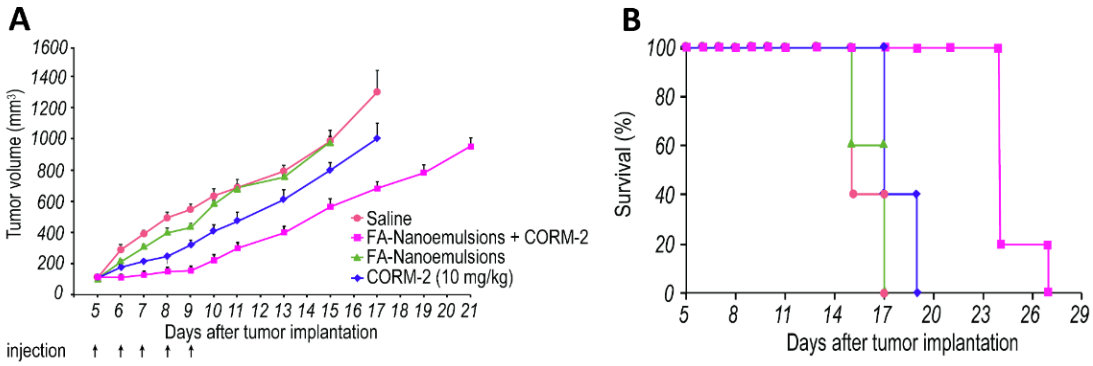


*Figure 1*. (A) Tumor growth curves of immunocompetent BALB/c mice bearing subcutaneous A20 lymphoma tumors treated intravenously with FA-tagged nanoemulsions loaded CORM-2, empty FA-tagged nanoemulsions, CORM-2 or saline. Data represent mean tumor volumes (±SE). (B) Survival curves of mice treated with FA-tagged nanoemulsions loaded CORM-2 and control groups.

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**PC-50**

# Impact of ligand conjugation method and PEG composition in the behavior of new PD-L1 targeted immunoliposomes

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**Introduction:** Immunotherapy has emerged in recent years as a new strategy to treat various kinds of diseases. In oncology, the majority of solid tumors are characterized by the up-regulation of several types of membrane molecules like the PD-L1 (Programmed cell Death Ligand 1), involved in different processes promoting tumor progression. This molecule, together with its receptor PD-1 expressed in T-cells, is involved in self-tolerance maintaining but is also responsible of tumor resistance, promoting tumor escape from immune control [1]. Monoclonal antibodies (mAbs) against PD-1/ PD-L1, block this interaction and provide antitumor effect by the activation of T-cells infiltration into tumour [2]. Despite of their low toxicity, their limited efficacy has led to test combinations. Thus, immunoliposomes conjugating anti-PD-L1 or even its Fab'-fragment, represents a combinatory strategy to promote the immune response and to provide a flexible system for incorporating antitumor agents. However, to attain this, adequate targeted vectors are necessary. Thus, the aim of this work was to develop and characterize PD-L1 targeted liposomes, using different methods for conjugation of ligand: the whole mAb and its monovalent Fab'-fragment, both *in vitro* and *in vivo*.

**Materials and Methods:** Fluorescent liposomes (LP) were prepared by film hydration method [3] using two different PEG concentrations (2 and 5%). PD-L1 targeted liposomes conjugating mAb or Fab' (LPmAb or LPFab') were formulated using two methods: post insertion (PI) and conventional (CV), respectively. For PI method, DSPE-$PEG_{2000}$-Mal micelles were coupled to anti-PD-L1-Fab' fragments or the anti-PD-L1 mAb, and then, incorporated in preformed liposomes; whereas for CV, both ligans, whole mAb and Fab', were directly conjugated to Maleimide contained in preformed fluorescent liposomes. Particle size, PDI and Zeta potential were analyzed by laser diffractometry and coupling efficiency was quantified using the Coomassie Protein assay reagent. Ligand affinity to the rm B7-H1/Fc chimera mAb was studied for all the formulations as well as their uptake, measured by FACS and confocal microscopy after different times of exposure, between 4 and 24h. Finally, *in vivo* biodistribution was evaluated in melanoma tumor bearing mice for all formulations and tumors were also collected and the immunogenic activity visualized by immunohistochemistry.

**Results and Discussion:** Ligand conjugation did not statistically increase particle size, which was around 130 nm associated with a PDI < 0.1. No significant differences were found for mAb efficiency conjugation (EC) using PI or CV, or formulating with 2 or 5% of PEG. However, CV method displayed an EC double than PI for Fab' fragment, without any influence of the PEG percentage, although in all cases EC was lower for Fab' (40%) than for the whole antibody (75%). Binding affinity showed higher fluorescent signal for LPmAb containing 2% PEG while for LPFab', the highest was for 5% PEG. In both cases PI method showed better affinity than the CV. These results were in accordance with the fluorescent signal measured in the studies performed by FACS and confocal microscopy, allowing us to select these formulations as possible candidates for their *in vivo* evaluation, encapsulating several antitumor drugs. Regarding *in vivo* biodistribution, targeted formulations seem to accumulate later than non-targeted; in addition, this accumulation resulted higher for LPFab' than for LPmAb, suggesting that mAb influenced the clearance of the formulation. A fact previously described for other mAbs conjugated to LP. Nevertheless, in this case Fc' fragment plays a relevant role in the immunogenic activity, influencing the balance between elimination and functional activity. For exploring this, immunohistochemistry assay of tumor tissue to evaluate the immune effect was done.

**Conclusion:** LPmAb and LPFab' containing 2 or 5% of PEG have been successfully developed using PI and CV methods, respectively. PD-L1 affinity was higher for LP formulated with PI method and in particular for 2% PEG in the case of whole antibody and 5% PEG for the Fab' fragment. The maximum cell and tumor accumulation was achieved at 8h, being the tumor accumulation more important for Fab'-LP than for mAb-LP.

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**PC-51**

# CBD-loaded-microparticles as therapeutic strategy in ovarian cancer

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## Introduction

Cannabinoids have shown a potential therapeutic utility in numerous pathological disorders such as neurodegenerative diseases, pain, nausea, vomiting and cancer among others. One of the most promising cannabinoids is Cannabidiol (CBD), a non-psychoactive cannabinoid [1]. In cancer disease, it has proved to have a high interest due to its ability to inhibit the proliferation, migration, invasion and angiogenesis of cancer cells in a great number of carcinomas like breast, prostate, lung, and brain tumors [2]. In spite of its potential clinical interest, the high lipophilicity and the stability problems of cannabidiol may difficult the development of effective CBD formulations [3]. Microencapsulation may resolve these questions and also allow to get a controlled release of the active with a single administration. The objectives of this research work are to evaluate the efficacy of cannabidiol in ovarian cancer cells (i) and to design, develop and characterize polymeric microparticles (MPS) loaded with CBD for intraperitoneal administration.

## Materials and methods

The antiproliferative efficacy of CBD has been proved *in vitro* using SKOV-3 and OAW-42 cells as ovarian cancer models. Cells were seeded and treated with CBD in a range of 6-50 μM for 24 and 48 hours.Cell proliferation was determined by MTT assay. Microparticles have been prepared by the solvent evaporation technique, using the poly(lactic-co-glycolic acid) (PLGA) RG® 502 as polymer and loaded with CBD at 10%. Then they were characterized by determining particle size by laser diffraction, shape and surface by scanning electron microscopy (SEM), drug loading and entrapment efficiency by HPLC and drug physical state into microparticles by differential scanning calorimetry (DSC). The antitumor efficacy was also determined in both *in vitro* and *in vivo* models. The *in vivo* activity was determined using the chick embryo chorioallantoic membrane model. SKOV-3 derived tumors were treated with free CBD or CBD-loaded-microparticles (100 μM), and the tumor growth was evaluated after 2 days and a half of administration.

## Results

On the one hand, CBD inhibited the proliferation of SKOV-3 and OAW-42 ovarian cancer cells with $IC_{50}$ values of 21.13 and 31.80 μM, respectively after 48 hours of incubation.

On the other hand, spherical, non-porous and uniform microparticles were obtained with a mean particle size, expressed as volume diameter, of 28.5 ± 1.08 μm, suitable for parenteral administration. The drug loading was high (865.06±17.54 μg CBD/10mg MPS) and the process yield and entrapment efficiency were above 90%. DSC studies showed that CBD is dissolved into the polymeric matrix and that it has a plasticizer effect. The *in vitro* release studies reported a controlled release of the active compound during a month with a slightly burst effect around 3% during the first 2 hours followed by a faster release phase during 2 days and a slower one during 29 days. While blank microparticles were not toxic, CBD formulations reported an antitumor activity. In this way, after a single administration of CBD-MPS, they showed an *in vitro* antiproliferative activity during at least the 10 days tested and an *in vivo* tumor growth inhibition similar to the CBD daily administered.

## Conclusions

Firstly, due to the anticancer activity of CBD in ovarian cancer cells, it may be a good strategy to treat ovarian carcinoma with this drug. Secondly, the developed CBD formulation, that showed a suitable size to be administered intraperitoneally, exhibited an extended antitumor activity in both *in vitro* and *in vivo*, suggesting its utility for ovarian cancer treatment.

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**PC-52**

# Multifunctional gold-nanoparticles: a nanovectorization tool for the targeted delivery of novel chemotherapeutic agents

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**Introduction**: Cancer is one of the main causes of death, being responsible for more than 8 million deaths worldwide every year. Chemotherapy, despite being widely used, is not completely effective due to: i) intrinsic or acquired multidrug resistance by cancer cells, ii) to unselective effect and iii) high toxicity to healthy cells. The development of novel compounds and novel nanoformulations (*e.g.* using gold nanoparticles, AuNPs) arises in demand to more effective and selective cancer therapies. AuNPs can delivery and enhance drug concentration and therapy effect inside cancer cells. **Materials and Methods:** We have developed two novel AuNPs-based systems - AuNP@PEG@BSA@TS265 (NanoTS265) and AuNP@PEG@Anti-EGFR_BSA@TS265 (Target NanoTS265) - for the vectorization and active targeting of a metallic compound with high anti-proliferative potential - $CoCl(H_2O)(Phendione)_2$ $[BF_4]$ (TS265) - in colorectal cancer cell line (HCT116). **Results and Discussion**: The nanoconjugates were characterized by UV-Vis, DLS, Bradford and Ellman's assay, allowing the quantification of PEG, BSA and metallic compound binding to the surface. Compared to the free compound, TargetNanoTS265 and NanoTS265 efficiently delivered the cytotoxic cargo in a controlled selective manner due to the active targeting, boosting tumor cytotoxicity. Treatment of HCT116-derived xenographs tumors with TargetNanoTS265 led to 93% tumor reduction. **Conclusion:** This simple conceptual nanoformulation demonstrates the potential of nanovectorization of chemotherapeutics via simple assembly onto AuNPs of BSA/HAS-drug conjugates that may easily be expanded to suit other cargo of novel compounds that require optimized controlled delivery to cancer target [1].

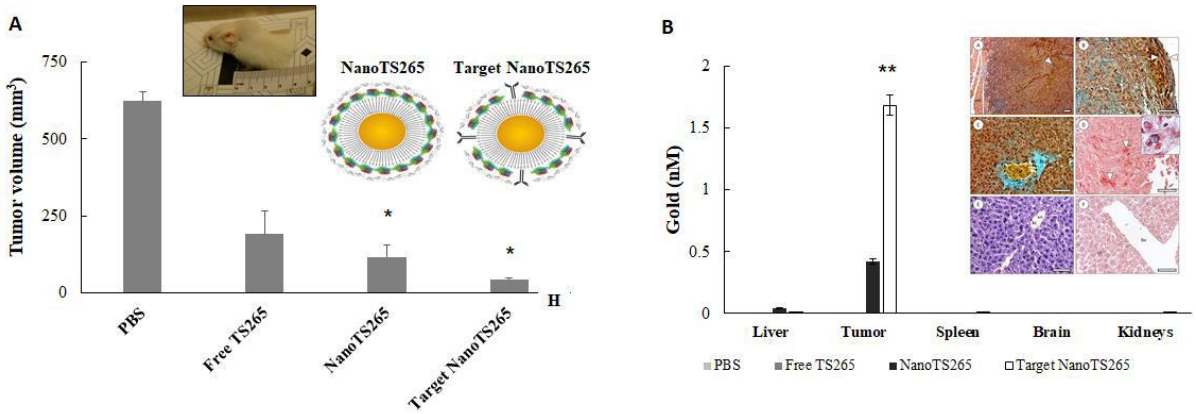


*Figure 1*. A. Inhibition of tumor growth in NOD/scid mice treated with PBS, free TS265 and the NanoTS265 and TargetNanoTS265 nanoconjugates. Tumor burden was determined by the sum of tumor volumes per mouse (n=3), in xenografted mice treated with PBS, the free TS265 or the nanoconjugates. All the results are expressed as the mean ± SEM. B. Concentration of gold (nM) based on ICP-MS in livers, spleens, brains, kidneys and tumors in NOD/scid mice treated with PBS, free TS265 and the NanoTS265 and TargetNanoTS265 nanoconjugates. * $p < 0.01$ relative to PBS; ** $p < 0.005$ relative to PBS. Values represented were normalized to the mass of each organ.

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**PC-53**

# Multi-targeted directed triazene-based hybrid molecules against melanoma and colon cancer

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**Introduction:** Melanoma and colon cancer are two types of tumors responsible for a huge number of deaths. Triazenes are well-known antitumor molecules, being dacarbazine (DTIC) and temozolomide (TMZ), two examples with clinical application in chemotherapy. However, the main problems of these molecules are their lack of specificity, therapeutic resistance and the frequency of side effects leading to disappointing therapeutic benefits [1]. To overcome these disadvantages, the design of series of new compounds involving the conjugation of a triazene with cytotoxic phenols or sulfur analogues of tyrosine has been accomplished [2]. These new compounds, hybrid molecules (HM), present two distinct moieties, covalently joined and acting through two different mechanisms of action, were rationally designed to achieve synergistic effect towards tumor cells. The cytotoxic effect of these HM was evaluated in human and murine melanoma and colon cancer cell lines (MNT-1, HCT-116, B16F10 and CT-26). For the most promising HM in terms of antiproliferative properties towards tumor cells and aiming to further improve its *in vivo* profile, the association to a lipidic system, liposomes, was performed. The stability of compounds in free and liposomal forms, in the presence of human plasma, was assessed. Preliminary *in vivo* studies were performed for one of the selected HM in a melanoma murine model.

**Materials and Methods:** Inhibitory proliferation properties of HM in MNT-1, HCT-116, B16F10 and CT-26 cell lines was evaluated by MTS assay [2,3]. Selected HM were incorporated in long circulating liposomes, by the dehydration-rehydration method, followed by an extrusion step to reduce and homogeneize the liposomes [3]. All liposomal nanoformulations were characterized in terms of incorporation parameters, mean size, polydispersity index and zeta potential. HPLC methodologies using an isocratic solvent system allowed quantifying the incorporated HM in liposomes. The stability of HM formulations, either in free or liposomal forms, in the presence of human plasma, was performed by HPLC [2,3]. The antitumor potential of the selected HM was assessed in a xenograft murine melanoma model [4].

**Results:** In the cell lines used, some of the tested HM showed $IC_{50}$ values ranging from 10 to 45 μM. The lowest $IC_{50}$ values were observed for the murine colon cancer cell line CT-26, that ranged from 10 to 35 μM. Two HM with higher antiproliferative properties towards tumor cell lines were selected for incorporation in liposomes, presenting octanol/water partition coefficients (LogP) values of 6.8 and 4.1. While the HM with lower LogP achieved incorporation efficiencies (I.E.) of around 100%, the other one presented I.E. below 25%.

HM, either in free or in liposomal forms, presented a good stability in plasma medium, ensuring that selected compounds are able to reach tumor sites after parenteral administration.

For one of the selected HM, preliminary *in vivo* studies were performed in a xenograft murine melanoma model, which showed that HM liposomes were able to reduce, in a very high extent, the tumor progression in comparison to mice induced and non-treated or treated with HM in the free form.

**Conclusions:** The developement of new triazene-based hybrid molecules and their incorporation in lipidic systems was successfully accomplished. *In vitro*, these novel compounds showed great promise against human and murine melanoma and colon cancer cell lines and preliminary *in vivo* results demonstrate the therapeutic advantage of using liposomes for HM delivery.

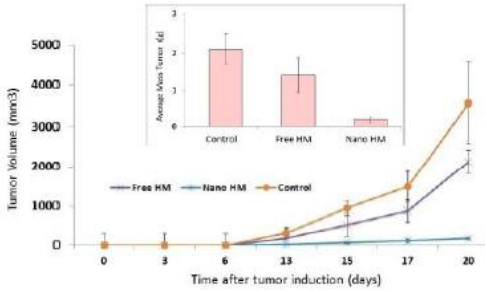


*Figure 1*. Tumor volume evolution: influence of treatment group

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**PC-54**

# Brain drug delivery by intranasal nanosystems: a systematic review of pre-clinical studies

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**Introduction:** The treatment of neurodegenerative and psychiatric disorders remains a challenge for medical research, mainly due to the blood-brain barrier's very low permeability. Several strategies have been developed over the years, either to overcome the blood-brain barrier, or achieve a safer or faster brain drug delivery. One of them is intranasal (IN) administration. The possibility of direct nose-to-brain transport offers enhanced targeting and reduced systemic side effects. Nevertheless, labile, low soluble, low permeant and/or less potent drugs might need a formulation other than the common solutions or suspensions. For that, formulation into carrier nanosystems is considered to be a promising approach, by protecting drugs from chemical and/or metabolic degradation, enhancing their solubility, or offering transport through the biological membranes [1]. However, the understanding of the factors promoting efficient brain targeting, when using nanosystems for delivery through the IN route, is currently patchy and incomplete.
The main purpose of the present work was to evaluate the overall evidence in current literature of an association between brain delivery efficacy (in terms of brain targeting, brain bioavailability and time to reach the brain) and nanosystem type.

**Materials and Methods:** We performed a systematic bibliographic search and analysis. Articles were obtained via search on the "Web of Science™" database with use of specific search terms to include all possible carrier nanosystems, IN drug administration to *in vivo* animal models, and comparison with intravenous drug administration. Statistical analysis was performed using the Prism software, version 6.0, from GraphPad.

**Results and Discussion:** Only 56 of the 243 search results met the inclusion/exclusion criteria. Represented nanosystem classes included: microemulsions (36 cases), nanoemulsions (14 cases), polymeric nanoparticles (21 cases), polymeric micelles (5 cases), solid lipid nanoparticles (3 cases), nanostructured lipid carriers (5 cases), liposomes (4 cases), transfersomes (2 cases) and other liposome related nanosystems (4 cases). Among the groups with at least 5 formulation cases, all mean/median values of drug targeting efficiency (DTE%), direct transport percentage (DTP%) and brain biovailability ($B\%_{brain}$) showed a significant advantage of IN nanosystem drug delivery (both in comparison with the IV route and the plain IN drug solution). Microemulsions, the most used nanosystem type, were among the classes with the lowest benefit in drug targeting and biovailability (as compared to IV administration). The best values belonged to the polymeric micelles group, however their superiority disappeared when in comparison with the respective IN drug solution. Furthermore, there was a strong negative correlation of brain targeting with the ability of a drug to achieve the brain through the IV route, meaning that the drug itself strongly influences brain targeting when administrated through the IN route while using carrier nanosystems. In what concerns the time it takes the drug to reach the brain, brain delivery through IN administration appears to be at least as fast as IV administration, which is in accordance with the conceived idea that the IN route is a fast route to the brain.

**Conclusions:** Some drugs reached the brain so efficiently through the IN route, even as drug solutions, that further benefit from formulation into nanosystems became less evident. That being said, overall it was not possible, from the current global analysis, to clearly discriminate the superiority of a nanosystem class in relation to another with respect to brain targeting and bioavailability, only that, in reported works, nanosystems were better than the respective drug solutions or IV drug administration.

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**PC-55**

# Evolution of the protein corona across the blood brain barrier

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**Introduction**: Nanoparticles (NPs) are a promising tool to deliver drugs across biological barriers, which can hinder effective pharmacological treatment for a variety of diseases. The corona, a layer of proteins attached to the surface of NPs formed upon contact with biological fluids, affects bioavailabilty, toxicity, and clearance of NPs [1]. Though the corona is relatively well defined under various conditions, its evolution following interactions with biological barriers is unknown. We investigated if the protein composition of the corona formed on gold NPs [2] changes upon passage through an *in vitro* model of the blood-brain barrier (BBB) [3].

**Materials and Methods**: Gold NPs coated with 11-mercaptoundecane-1-sulphonate (MUS-NP) were incubated with 5% FBS in cell medium and allowed to pass through an *in vitro* model of the BBB for 3 h at 37ºC. This consisted of hCMEC/D3 (human brain microvascular endothelial cells) seeded on transwell inserts, separating an apical "blood" side and basolateral "brain" side. Samples in both compartments were qualitatively and semi-quantitatively analysed using SDS-PAGE and mass spectrometry.

**Results and Discussion**: The qualitative protein content of the corona changed dramatically following passage through the BBB. Many proteins were excluded, and 15 out of 381 were enriched in the "brain" side compared to the "blood" side. This clearly indicates the dynamic nature of the corona, and the ability or inability of specific proteins bound to NPs to traverse the BBB. Once beyond the barrier, the corona was stable upon incubation with other proteins.

The changes in corona composition following passage through barriers should be considered when formulating NPs, as they could affect targeting or the targeted tissue itself. Theoretically, proteins that are enriched following passage could be used to functionalize NPs and boost their (and any associated drugs) passage through the BBB or other biological barriers.

**Conclusions**: Crossing biological barriers has a significant qualitative and quantitative effect on the protein corona on NPs. The corona loses proteins following passage through the *in vitro* BBB, and is enriched in a limited number of specific proteins. This change is particularly relevant when designing NPs that are required to traverse any biological barrier and its consideration may lead to more successful development of therapeutic and/or diagnostic nanodevices. Moreover, proteins that are enriched upon passage could be theoretically used to functionalize NPs and boost their passage (and of any associated drugs) through the BBB or other biological barriers. This hypothesis is under evaluation.

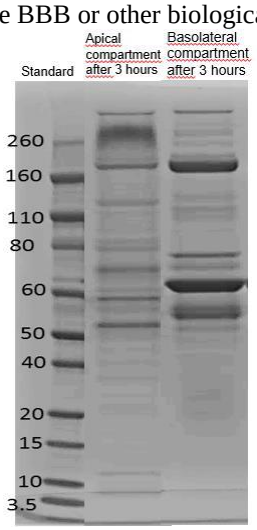


*Figure 1*. Protein corona on MUS-NP changed dramatically following passage through *in vitro* BBB. Enrichment of certain proteins is visible where bands of similar molecular weight are darker in the basolateral "brain" side compared to the apical "blood" side.

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**PC-56**

# Ultrasosound-induced microbubble formation in perfluorohexane nanoemulsions for blood-brain barrier opening

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## Introduction

Recently, it has been shown that microbubbles (MB) can be used to open the blood-brain barrier (BBB) in a noninvasive, reversible and local manner when stimulated by focused ultrasound (FUS) [1]. In this context, perfluorohexane (PFH) nano-emulsions may constitute an advantageous approach. PFH is liquid at body temperature and shows interesting properties such as echogenicity, high oxygen carrying ability and extremely low solubility in aqueous media, including blood [2,3]. The formulation of PFH in O/W nano-emulsions allows intravenous administration and facilitates PFH droplets reaching fine brain capillaries, where they may undergo a phase transition to microbubble formation upon *in vitro* activation with focused ultrasounds (FUS), thus contributing to loosening the tight junctions of brain capillaries endothelial cells. In this study, the incorporation of PFH into polymeric nano-emulsions prepared by a low-energy method has been investigated, as well as its use for the preparation of PFH-loaded nanocapsules as microbubble precursors.

## Materials and Methods

MATERIALS: Perfluorohexane (PFH); PLGA (MW ≈ 10.000 g/mol), ethyl acetate, nonionic surfactant (HLB = 15), Apolar Low Density Oil (ALDO), phosphate buffered saline (pH=7.4; 0.16M and 0.33 M). METHODS: Nano-emulsions were prepared by the phase inversion composition (PIC) method. Nanocapsules were obtained from the nano-emulsions by dialysis against PBS. Nano-emulsions and nanocapsules were characterized by Dynamic Light Scattering (DLS), Enhanced Dark Field Microscopy (EDFM), and Transmission Electron Microscopy (TEM). Nano-emulsion stability was assessed by light transmission and backscattering (Turbiscan Lab Expert) at 25ºC. PFH in the nanocapsules was determined by elemental fluor microanalysis and hyperspectral mapper classification of hyperspectral images of NC. Biocompatiblity of the nanocapsules was assessed by means of MTT assay.

## Results and Discussion

PFH nano-emulsions were obtained at high oil-to-surfactant ratios with hydrodynamic droplet sizes typically below 300 nm. The incorporation of the PFH in the oil component of the polymeric nano-emulsion dramatically decreases its stability against sedimentation, but this can be improved by the addition of apolar low-density oil. The PFH nanoemulsions were used as templates for the preparation of PFH-loaded polymeric nanocapsules (NC) using a dialysis method. The as-obtained NC show globular shape and sizes below 250 nm by DLS, smaller than the corresponding template nano-emulsion. Successful PFH encapsulation in the NC has been evidenced by VNIR spectral angle mapper classification of hyperspectral images of single NC as well as F elemental microanalysis. Cytotoxicity tests suggest that loading of PFH in the NC does not produce a cytotoxic effect, while ALDO has a negative impact on cell viability.
Further, preliminary assays suggest that the as-prepared PFH-loaded NC are promising MB precursors for FUS-assisted BBB opening.

## Conclusions

PFH was successfully incorporated in polymeric nano-emulsions (NE) prepared by a PIC method. PFH-loaded NC have been obtained from NE by dialysis. The addition of anapolar low density oil into the oil component of the nanoemulsion improves the stability of the PFH nano-emulsion but has a negative impact on the biocompatibility of the NC. The as prepared PFH-loaded NC are promising MB precursors for FUS-assisted BBB opening.

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**PC-57**

# Using microfluidic platforms to develop CNS-targeted polymeric nanoparticles for HIV therapy

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**Introduction.** The human immunodeficiency virus (HIV) uses the central nervous system (CNS) as reservoir, especially the brain, which turns it as a promising target to fight this pathology [1]. Nanoparticles (NPs) hold tremendous potential as anti-HIV drugs carriers to the CNS, since most of these antiretrovirals cannot surpass the blood-brain barrier (BBB), as efavirenz (EFV) [2,3]. Forasmuch as the conventional bulk approaches to produce NPs lack a precise control over their final properties [4], microfluidic platforms emerged as a prospective solution to circumvent this drawback, providing much more uniform and tunable formulations [5].

**Materials and Methods.** EFV-loaded PLGA NPs were produced using the nanoprecipitation technique performed through the bulk and microfluidic approaches. After production, the nanosystem was physico-chemically characterized through dynamic light scattering (size and respective polydispersity), laser Doppler anemometry (zeta-potential), and high-performance liquid chromatography (EFV association efficiency and loading), as well as through an *in vitro* drug release assay. A microfluidics-associated scaling experiment was conducted, by decreasing (scale-down) and increasing (scale-up) the final volume of the NPs batch, followed by a new physico-chemical characterizion of the particles. Their safety was tested through a metabolic viability assay on BBB endothelial (hCMEC/D3 cell line) and brain parenchyma neuron (ND7/23 cell line) cells, and a quantitative and qualitative (scanning electorn microscopy) hemolysis study. The microfluidics-associated NPs were further functionalized with a transferrin receptor-binding peptide. Bradford test and proton nuclear magnetic resonance ($_1$H NMR) were used to evaluate the functionalization efficacy, and flow cytometry studies were performed to evaluate the interaction of hCMEC/D3 cells with functionalized NPs. Finally, a permeability study was conducted using a BBB *in vitro* model, consisting on a monolayer of hCMEC/D3 cells.

**Results and Discussion.** In comparison with the conventionally-performed nanoprecipitation, the nanoformulation obtained through microfluidics resulted in reduced NPs average size (133.0 nm for the conventional method; 72.8 nm for microfluidics), comparable NPs polydispersity (0.090 for the conventional method; 0.086 for microfluidics), and less negative zeta-potential (-28.0 mV for the conventional method; -14.1 mV for microfluidics). This decrease in size is an advantageous feature for brain-targeted drug delivery. With microfluidics, higher EFV association efficiency (32.7% for the conventional method; 80.7% for microfluidics) and drug loading (3.2% for the conventional method; 10.8% for microfluidics) were obtained, which are key aspects to achieve the desired drug delivery nanosystem therapeutic effect. The microfluidics-associated EFV-loaded NPs also demonstrated a sustained *in vitro* drug release profile over time from their matrix. The robustness of the microfluidic method was successfully demonstrated by a scaling experiment, which proved that physico-chemical properties of NPs were independent on the volume of the batch. This was a great achievement, since it symbolized a preliminary step towards the application of this technology to produce drug-loaded NPs in industrial dimensions. The EFV-loaded NPs proved to be safe to hCMEC/D3 and ND7/23 cells, providing protection compared to the free drug. Hemolysis studies demonstrated a nonhemolytic behavior of the NPs, which presented only approximately 1-2% of hemolysis and did not cause morphological changes in red blood cells. This was important to support the intravenous administration of the nanosystem. The microfluidics-associated EFV-loaded PLGA NPs were further bioconjugated with a transferrin receptor-binding peptide since this receptor is overexpressed at the BBB level, and the results suggested an effective surface functionalization of NPs. Finally, functionalized NPs were able to interact with hCMEC/D3 cells, and EFV permeated through the BBB *in vitro* model.

**Conclusions.** This work confirmed the advantages of microfluidics over the bulk approach in the loading of EFV in PLGA NPs, which presented suitable properties to be possibly administered by the intravenous route. The additive effect of functional targeting to the BBB was also successfully approached, in order to allow the permeation of the drug through this biological barrier, thus reaching the CNS.

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**PC-58**

# Does cannabinoid decoration enhance the permeability of lipid nanocapsules across the *in vitro* blood-brain barrier model hCMEC/D3?

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## Introduction

A rapidly evolving knowledge about tumor biochemistry would in principle enable various new drug molecules to be designed as treatments. However, malignant brain tumors remain untreatable due to the failure to expose the entire tumor to such therapeutics at pharmacologically meaningful concentrations. In this regard, nanomedicine poses an appealing platform for efficient brain drug delivery, since it may provide optimal drug availability, and, subsequently, fewer side-effects.

## Materials and Methods

In an attempt to develop nanocarriers that can help overcome the blood-brain barrier, we have developed monodisperse lipid nanocapsules (LNC) using the phase inversion technique (PIT) [1], a solvent-free low-energy method based on the formation of nanoemulsions. By varying the percentage of their excipients - namely Labrafac WL 1349 (caprylic-capric acid triglycerides), Solutol HS15 (mixture of free and hydroxystearate polyethylene glycol 660) and Lipoïd S75 (soybean lecithin at 70% of phosphatidylcholine)-, monodisperse LNCs in different sizes were prepared. For particle tracking purposes, the fluorescent dye 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) was encapsulated into the LNCs by dissolving it in the oily core. Furthermore, LNCs have been decorated with cannabidiol (CBD) under the assumption that it might confer brain targeting properties to the nanocarriers, given the enhanced biodistribution across the blood-brain barrier of the cannabinoid.

*In vitro* assays have been conducted on the human brain endothelial cell line hCMEC/D3 [2] to assess the cytotoxicity of these nanocarriers and to screen the potential to cross the blood-brain barrier [3]. The role played by the presence of cannabinoids and the particle size on the uptake pattern has likewise been explored. Cell viability experiments have been conducted by MTT, uptake experiments have been performed both by flow cytometry and confocal microscopy, and permeability experiments have been carried out across 1.0 μm Transwell® plates.

## Results

LNCs show a remarkable size-dependent cytotoxicity on the human brain endothelial cell line hCMEC/D3: LNC80>LNC40>LNC20. Fluorescently-labelled LNCs tested at non-toxic concentrations were internalized by the human brain endothelial cell line hCMEC/D3. The uptake extent as evidenced by flow cytometry revealed a slight size-dependent uptake pattern (LNC20>LNC50), whereas the functionalization with cannabidiol did not show any significant improvement in the *in vitro* brain endothelial-targeting ability of LNCs. Importantly, these results were corroborated by confocal laser scanning microscopy (LCSM).

Nevertheless, when the permeability of the cannabinoid-decorated nanocarriers was tested *in vitro* across the hCMEC/D3 cell line, a statistically significant enhancement was found in comparison with their undecorated counterparts. This enhancement was evidenced regardless of the size of the colloid systems (LNC20 and LNC50). An evaluation of the role of the particle size on the permeability pattern revealed a higher permeability coefficient for the smaller nanocapsules.

## Discussion and Conclusions

1. Cannabinoid-decorated lipid nanocapsules prepared by the phase inversion technique may represent promising platforms to overcome the blood-brain barrier and enable brain delivery of chemotherapy, as evidenced by the uptake and permeability experiments performed on the hCMEC/D3 cell line.
2. According to the *in vitro* screening, the parameters that most influence the permeability rate across the brain endothelium are the particle size (being the smaller the size, the higher the permeability coefficient) and the cannabinoid decoration (likewise, with a positive correlation).

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**PC-59**

# Effect of gamma irradiation on a PLGA multipaticulate drug delivery system developed for rasagiline

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## Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease after Alzheimer´s [1]. Rasagiline mesylate (RM) is a monoamine oxidase inhibitor (IMAO) with selectivity and specificity for MAO type B (MAO-B), which provides symptomatic relief as monotherapy and as adjunctive therapy combined with L-dopa [2]. Rasagiline exhibits an oral bioavailability of only 36% and a very short elimination half-life (0.6-2 h) [3]. These biopharmaceutic and pharmacokinetics characteristics and the fact that rasagiline is used for the treatment of a chronic disease, make it a suitable candidate for the development of controlled release systems.

## Materials and Methods

Microspheres (MPs) were prepared using PLGA as polymer (PLGA 50:50, Resomer-502®) by the solvent evaporation technique from an O/W emulsion. The amounts of (RM) and polymer used were 40 mg and 400 mg, respectively. Blank PLGA MPs were also obtained. Sterilization of blank and RM-loaded PLGA MPs was performed by γ-irradiation (25 kGy). All formulations were characterized before and after sterilization by SEM, laser light diffraction, DSC, XRDP, GPC, drug loading (EE) and *in vitro* RM release at pH 7.4. RM was quantified by HPLC [4].

## Results and Discussion

Sterilization of RM-loaded MPs by γ-irradiation induced modifications on surface morphology with irregular surfaces with small pores which were also present in the polymeric matrix. Particle size distribution of sterilized MPs (mean particle size 105.3±54 μm) practically overlapped that of non-irradiated particles with the sterilization procedure inducing a slight aggregation of the particles, which correlates with the Span value obtained (1.83). Sterilization did not affect ($p<0.05$) the EE of RM into PLGA MPs (51.3±2.6%). XRDP analysis of RM-loaded PLGA microspheres before and after γ-irradiation did not show the maxima of crystalline RM. This loss of crystallinity could be explained by the fact that RM can be partially dissolved in polymer matrix being therefore necessary for the drug to be solubilized in the medium thereby prolonging its release from the particles. DSC thermograms of both non-sterilized and sterilized RM-loaded PLGA MPs showed melting endotherms at 211.2ºC, values slightly lower than those of RM, probably due to an ionic interaction between amino groups of RM and the terminal carboxylic anions of the polymer. GPC analysis was performed at time zero and after five days of *in vitro* release. After 5 days, both weight-average molecular weight (Mw) and number-average molecular weight (Mn) decreased for irradiated MPs, with Mn decreasing faster than Mw, indicating that that once in an aqueous medium the content of monomers and oligomers significantly increases in the irradiated polymer being Mn more sensitive than Mw towards γ-irradiation. It has been hypothesized a cleavage mechanism which primary affects the terminal groups of polymer chains causing a faster decay of Mn [5]. *In vitro* release of RM from MPs was significantly increased after γ-irradiation. For instance, after 7 days 79% and 55% of RM was released from irradiated and non-irradiated microspheres respectively, thereby indicating that the sterilization technique significantly modifies the release characteristics of RM from the particles. After two weeks approximately 90% of RM was released from both, non-irradiated and irradiated particles. The similarity factor (f2) obtained was 37.47 thereby indicating that both release profiles cannot be considered similar.

## Conclusions

Gamma-irradiation of RM-loaded PLGA microspheres significantly modifies the release characteristics of the drug therefore; this sterilization procedure could not be applied for the final sterilization of the formulation due to its detrimental effects on the polymer.

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**PC-60**

# Modulating neural stem cells activity by nanoparticle light-activation

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**Introduction**: Restoration of brain function represents a major challenge in today's society. To date, no current therapeutic strategy is able to completely restore brain function upon demand. As neurogenesis persists throughout life within specific regions of the brain (namely the subventricular zone- SVZ), modulation of endogenous pools of neural stem cells (NSCs) present a straight forward solution for brain disorders. Protein delivery is a powerful tool that can potentially be used to manipulate of NCSs behavior. Intracellular delivery of proteins is extremely useful for the manipulation of cellular processes and cell reprogramming [1]. In the last years, reports of formulation with the ability of intracellular delivery of proteins have imerged [2]. Nevertheless, so far no formulation has the capacity to orchestrate the temporal delivery of proteins. In this framework, nanoparticle-mediated protein delivery to NSCs holds great promise as therapeutic agents.

**Materials and Methods**: We have designed a protein delivery system based on lanthanide-doped upconversion nanocrystals that respond to near infra-red (NIR) light to intracellularly release functional Cre recombinase (CRE-UCNPs). A new photo-cleavable linker was synthesized and linked to the UCNPs through one end, while on the other was used to immobilized Cre recombinase. Cytotoxicity of the nanoformulation, as well as, the laser effect was evaluated. A reporter cell line (mouse fibroblasts) for Cre activity was used to access the delivery and activation of the Cre recombinase *in vitro*. Deep-tissue photoactivation was performed in mice to consolidate the hypothesis of in deep activation using NIR-light. Mice with a transgenic reporter strain for Cre recombinase enzyme activity were used and and recombination efficiency was evaluated by fluorescence microscopy.

**Results and Discussion**: We show the potential of CRE-UCNPs in the internalization of Cre recombinase and its escape from endocytic compartments. We further confirm the specificity of NIR-light-induced cargo release both *in vitro* and *in vivo*, with the key achievement of *in vivo* DNA recombination, within the SVZ region of transgenic mice. This gene editing platform with a spatio-temporal control over intracellular Cre enzyme delivery represents a generalist tool for *in vivo* modulation of SVZ cells.

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**PC-61**

# sdAb-Immunoliposomes for Brain Targeting and Drug Delivery

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**Background:** Neurological disorders are one of the major causes of mortality representing 12% of total global deaths each year. Despite the efforts, effective brain drug delivery has systematically been challenged by the impenetrability of the blood brain barrier (BBB). This is especially true for drugs that address major unmet needs in the Central Nervous System (CNS) area such as Alzheimer's, Parkinson's, meningitis and brain cancers making the development of BBB penetrating drugs and brain targeting drug delivery systems the main goal of neuroscience research [1,2]. One approach is to target specific BBB transport systems and develop CNS drug delivery strategies that exploit these natural portals of entry into the brain. To achieve this there has been a strong interest in the development of monoclonal antibodies toward BBB receptors. Some of these mAbs transmigrate the BBB and have been tested as vectors to deliver drugs into the brain. However, these mAbs rely on broadly expressed receptors such as transferrin and insulin receptors possibly bringing unwanted side effects and resulting in a low fraction of the injected dose actually reaching the brain. Promising alternatives are single domain antibodies (sdAb) [3,4].These small antibody molecules show improved tissue penetration reaching targets not easily accessible by con-ventional antibodies, low immunogenicity and lower manufacturing costs. Within this context, sdAbs are a promising alternative to conventional antibodies as potential transvascular brain delivery vectors. Liposomes are vesicular concentric bilayer structures composed of relatively biocompatible and biodegradable materials. They consist of one or several lipid bilayers, separated by aqueous compartments and they form spontaneously when certain lipids are hydrated in aqueous media.

Liposomes offer several advantages over other delivery systems such as their unique characteristics to incorporate hydrophilic and hydrophobic drugs, biocompatibility, low toxicity, lack of immune system activation and targeted delivery of bioactive compounds to the site of actions [5,6]. Therefore, to develop an improved delivery system to CNS and to successfully contribute to overcome the brain drug delivery bottleneck we are developing novel anti-body-conjugated liposomes (immunoliposomes). For that, highly specific BBB transmigrating sdAbs are being developed and conjugated with liposomes containing antibiotics for drug delivery validation in a meningitis model.

**Materials and Methods:** We developed a rabbit-derived immune sdAb library as an antibody source of highly specific and improved BBB-transmigrating sdAbs. Specific BBB sdAbs were obtained by immunizing two New Zealand White rabbits with brain endothelial cells. Rabbits were then sacrificed and with the spleen and bone marrow we constructed a phage displayed-sdAbs library. Paralleled screens are currently being performed in both *in vitro* and *in vivo* models for selection and identification of sdAbs towards new BBB receptors with potent BBB penetration properties. To select the best liposome formulation different lipid compositions were prepared and tested for vancoymcin encapsulation. The major neutral lipidic constituents included dipalmitoyl phosphatidyl choline (DPPC) or dimiristoyl phosphatidyl choline (DMPC). Sphingomyelin (SM) and cholesterol (Ch) were incorporated to improve the biological properties of the liposome. Liposomes were characterized in terms of lipid and antibiotic contents by spectrophotometry. Mean particle size and surface charge was measured by laser light scattering methodologies.

**Results and Discussion:** A specific immune response towards brain endothelial cells was obtained for both rabbits. Both serums showed BBB crossing properties in *in vitro* and *in vivo* models. sdAbs libraries against brain endothelial cells were efficiently constructed with a diversity with ~$10^7$. Phage display selections and functional screenings are currently being performed to identify potent BBB crossing sdAbs and novel BBB endogenous receptors that can provide a more selectively delivery into the brain. Ten different liposomal formulations have been developed with DPPC: DPPG (dipalmitoyl phosphatidyl glycerol) and SM:Chol:DcP (dicetyl phosphate) being the most promising formulations and reaching up to 42% of encapsulation efficiency.

**Conclusions:** We have selected brain specific sdAb with BBB transposition properties. Two promising vancomycin liposomal formulations have been selected for antibody linkage and drug delivery validation.

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**PC-62**

# Modeling of nanoparticle surface charge for targeting glioblastoma

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## Introduction

Glioblastoma multiforme (GBM) is an aggressive brain tumor with poor prognosis, mainly because standard treatment is not always effective enough in reaching tumor cells. Blood-brain barrier (BBB) is pointed out as one of great challenges in this field [1]. Considering the negative charge of BBB surface and its restricted permeability to small compounds, positively-charged nanoparticles have been developed to facilitate the transport of drugs through the BBB [2]. This work aimed at studying the interaction of different cationic surfactants used in lipid nanoparticle (LN) formulations with BBB, using atomistic simulations. Surfactants incorporating natural structural motifs, specifically serine, were chosen instead of the conventional synthetic surfactants, due to the lower cytotoxicity and higher biodegradability, thus being environmentally friendly [2].

## Methods

Molecular dynamics simulations were performed on 4 systems containing different serine-based surfactants, two of them monomeric (16SerTFA and 12SerTFA) and the other two dimeric ((12ser)2CON12 and (12ser)2N5), in a fully hydrated palmitoyloleoylphosphatidylcholine (POPC) lipid model, intended to mimic cell membranes of both the BBB and tumor. The topology for each compound was generated using the Automated Topology Builder (ATB) platform [4]. All simulations were carried out under GROMACS (version 4.5.6) using simple point charge (SPC) water and the united-atom lipid parameters of Berger *et al*. [5,6].

## Results and discussion

The systems were evaluated in terms of effects induced by the surfactants in this type of membranes and rationalize the interactions at molecular level. It was seen that serine-based monomeric surfactants (12SerTFA and 16SerTFA) are preferentially positioned close to the interface, while gemini molecules are mostly embedded in the hydrophobic region. The bulky heads of the monomeric molecules act at the interface, while that of the gemini disrupt a more internal region. As such, the monomeric surfactants interact more strongly with the polar heads of POPC. The longer chain length of 16SerTFA allows the respective terminal groups to be positioned in the center of the bilayer and the molecule is, overall, leveled with those of POPC. This may indicate that serine monomeric surfactants, especially 12SerTFA, are more available to interact with BBB/GBM cells. Wider distributions of terminal methyl groups are observed for (12Ser)2N5 and (12Ser)2CON12. This indicates that the mobility of the tails within the POPC membrane is more pronounced, with the chains being able to switch between the two leaflets.
The gemini polar heads are less mobile, especially for (12Ser)2CON12, while fully embedded in the inter-leaflet region of the membrane. Further details on the ordering of hydrocarbon tails of POPC, when monomeric or gemini surfactants are introduced, can be provided by estimating the order parameter (SCD). Accordingly, the surfactants induce a general stabilization effect in the following order, (12Ser)2CON12<(12Ser)2N5≈12SerTFA<16SerTFA. A stronger and uniform stabilization effect is observed for the longest monomeric molecule, 16SerTFA. In contrast, the (12Ser)2CON12 molecules induce a significant disordering effect close to the interface, and ordering in the internal region of the bilayer.

## Conclusions

Overall, this computational study suggests the viability of cationic serine-based surfactants as appealing compounds in LN formulations for targeted GBM therapy.

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**PC-63**

# Exploring SCD1 silencing in GBM

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**Introduction**: Glioblastoma multiforme (GBM) is the most aggressive form of brain tumor as it rapidly grows and invades surrounding normal brain tissue, and is highly resistant to cytotoxic treatments, remaining a fatal disease [1]. Aberrant lipid metabolism is recognized as one of the key features of cancer [2]. Lipid metabolism is rewired to meet the highly proliferative phenotype of cancer cells, with some lipid species being increased while others decreased. Fatty acid synthesis is commonly up-regulated to fit the demand for membrane biogenesis, while the generation of apoptotic lipids, such as ceramide, is prevented. Stearoyl-CoA desaturase-1 (SCD1), a key enzyme in the formation of monounsaturated fatty acids, is constitutively active in cancer cells [3].

**Materials and Methods:** In this work, human U87 GBM cells were used to test a strategy to reduce GBM cell survival that combines gene therapy, through SCD1 silencing, with chemotherapeutic agents, namely, etoposide, temozolomide, sunitinib and axitinib. Lipofectamine® RNAiMAX was used to transfect the cells with siRNAs targeting SCD1. SCD1 downregulation was evaluated at the mRNA, protein and fatty acid composition level. Cells were analysed for viability, cycle and caspase-3/7 activation as an indicator of apoptosis induction.

**Results and Discussion:** We found that silencing of SCD1 is detrimental to human U87 GBM cells by inducing apoptosis. In addition, combination of SCD1 silencing with sunitinib treatment, a multi-targeted receptor tyrosine kinase inhibitor, further increased the cytotoxity mediated by the gene silencing *per se* (see Figure 1). These results were observed only when the cells were grown in low-serum medium. It has been suggested that SCD1 has an important role to support the enhanced *de novo* lipid synthesis and desaturation in cancer cells under low-oxygen and lipid-depleted conditions, which are in fact those at which tumor cells are exposed [4]. Therefore, in order to better mimic this metabolically challenging environment experienced by GBM cells and the relevance of SCD1 silencing in this context, multicellular tumor spheroid models are currently being established for human U87 and NCH82 GBM cells using the hanging-drop method.

**Conclusions:** Silencing of SCD1 may represent a promising target for GBM treatment. In addition, since three-dimensional (3D) cell culture systems are currently viewed as an intermediate model between *in vitro* cancer cell line cultures and *in vivo* solid tumors [5], by using GBM cells grown in 3D, we expect to obtain better clues about the therapeutic potential of SCD1 silencing against this deadly tumor towards an *in vivo* application.

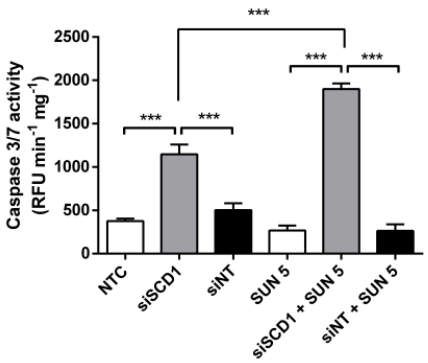


*Figure 1.* Effect of sunitinib on caspase 3/7 activation in U87 cells transfected with siRNAs targeting SCD1 (siSCD1) and control siRNAs (siNT).

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**PC-64**

# Differentiation therapy toward glioblastoma stem-like cells

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**Introduction:** Glioblastoma (GBM) is the most prevalent and malignant primary brain tumor. Since stem-like cells were identified in GBM tumor, their presence was recognized to be the reason for GBM treatment failure and tumor recurrence, due to their survival advantage over the proliferative non-tumorigenic glioma cells [1]. Consistently, high percentages of GBM stem-like cells (GSCs) in resected tumors have been related with poor GBM prognosis [2]. The development of strategies able to overcome GSC pleiotropic characteristics are, therefore, crucial to effectively treat GBM.

**Materials and Methods:** G144 and G26, two GSC lines established by Pollard and coworkers in 2009 [3], were grown in media containing either growth factors or bone morphogenetic protein 4 (BMP4). The expression of stemness (OLIG2 and EdU) and differentiation (O4 and GFAP) markers was evaluated by immunocytochemistry, and cell proliferation rate was assessed by using thesulforhodamine B (SRB) assay. Additionaly, the viability of G144 and G26 cells upon incubation with sunitinib, a tyrosine kinase inhibitor that is currently being tested in phase II/III clinical trials for GBM treatment, was assessed through the Alamar blue assay.

**Results and Discussion:** The evaluation of the cytoxicity of sunitinib in undifferentiated and differentiated G144 and G26 cells showed that the latter are more susceptible to the drug than the former (see Figure 1). Accordingly, the IC50 of cells supplemented with growth factors was ~2-fold higher when compared to that of cells cultured in medium with BMP4. Parallel studies are planned using axitinib (another tyrosine kinase inhibitor) and temozolomide (currently being used as a first-line GBM chemotherapeutic). In order to implement a robust therapeutic approach able to permanently induce cell differentiation, we are currently exploring a gene therapy strategy that will mimic the action of BMP4 on GSCs, such as the silencing of miR-302c [5]. With this purpose, anti-miR oligonucleotides will be encapsulated into chlorotoxin-coupled stable nucleic acid lipid particles (SNALPs; [6]), to mediate efficient therapeutic delivery targeting GSCs.

**Conclusions:** Taken together, our results indicate that a differentiation strategy increases the susceptibility of GSCs to sunitinib, holding promise as a viable therapeuticapproach against GBM.

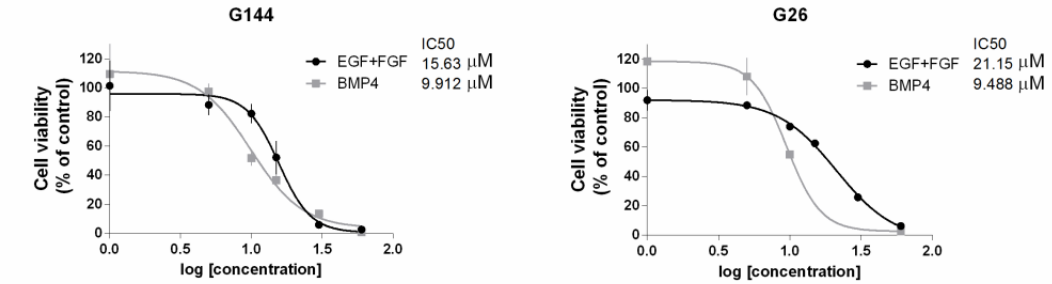


*Figure 1.*Differentiated GSCs are rendered susceptible to sutininib.

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**PC-65**

# Novel promising vesicles carrying artificial miRNAs against Machado-Joseph Disease

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**Introduction**: Extracellular vesicles (EVs) are membrane-contained vesicles that are produced by the majority of cells [1]. These promising vesicles were found to be important modulators of cell-to-cell communication due to the easiness of their membranes to interact and be incorporated by other cells [2]. Furthermore, they are able to deliver specific proteins, lipids and genetic material, especially small nucleic acids [3]. The content carried make them promising delivery systems to be used in therapy.
Among the species of nucleic acids, miRNAs are small non-coding RNAs with around 22 nucleotides in size that are able to regulate gene expression at the post-transcriptional level. In this context, a wide range of artificial miRNAs have been designed to target specific mRNAs [4].
In the present work, our aim was to evaluate EVs as vehicles to deliver artificial nucleic acids to treat neurodegenerative disorders, namely Machado-Joseph Disease – a neurodegenerative disorder which is associated with an abnormal over-repetition of the CAG tract in ataxin 3 (ATXN3) gene, conferring toxic properties to ATXN3 protein [5].

**Materials and Methods**: Firstly, we designed 7 artificial miRNAs (miR-A, miR-B, miR-C, mir-D, mir- E, miR-F and mir-G) targeting mutant ataxin 3 (mutATXN3) mRNA. The silencing efficiency of each miRNA was then tested in a stable line of HEK293T previously infected with lentivirus encoding mutATXN3 (mutATXN3 HEK293T). Transfected mutATXN3 HEK293T cells were collected after 48h and 72h and mutATXN3 protein levels were evaluated by Western Blotting. In the second part, in order to develop and evaluate the efficacy of EVs to carry artificial miRNAs, HEK293T cells were transfected with each artificial miRNA. Six hours after transfection the cellular medium was changed and collected 48h later. A differential ultracentrifugation protocol, already optimized in our lab, was then performed to isolate EVs from each condition. All EVs were characterized by Nanoparticle Tracking Analysis (NTA) to evaluate size distribution and particles concentration. The same amount of EVs was then placed in mutATXN3 HEK293T cells and the levels of mutATXN3 mRNA were subsequently evaluated by RT-PCR.

**Results and Discussion:** Regarding to the first task we verified that mir-D presents the best silencing efficiency in both time points. At 48h mir-D presents a silencing efficiency of 42.2%, followed by mir-C (39.6%) and mir-G (36.3%). Concerning to the 72h time point, mir-D presents an increase of silencing to 51.4%, followed by mir-G (49.9%) and mir-B (38.5%). Finally, we evaluated the capacity of modified EVs to deliver artificial miRNAs and silence mutant ATXN3 mRNA. According to the results of RT-PCR, it has been confirmed that EVs can deliver small nucleic acids, particularly artificial miRNAs.

**Conclusion:** In conclusion, this study supports that EVs are promising natural vehicles to deliver artificial miRNAs.

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**PC-66**

# Development of nanostructured lipid carriers (NLC) for nose-to-brain delivery by simple quality by design (QbD) approach

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## Introduction

Intranasal route has been suggested as a promising alternative for drug targeting to the brain, avoiding the need of bypass the blood brain barrier (BBB) [1]. After intranasal administration, drugs can reach the brain directly, along olfactory and trigeminal nerves, or indirectly, entering in vasculature or lymphatic system, crossing the BBB. Lipid nanoparticles (e.g. nanostructured lipid carriers, NLC) have been showing effectiveness for nose-to-brain drug delivery. These systems are preferred over other nanocarriers, due to their unique properties that increase drug bioavailability [2]. The aim of this work was to develop an intranasal formulation of NLC loaded with carbamazepine, a drug used in the treatment of neuropsychiatric disorders, employing a simple quality by design (QbD) approach.

## Methods

NLC dispersions with different surfactant proportions were prepared from the method previously employed by Silva *et al.* [3]. Afterwards, particle size and encapsulation efficiency (EE) were measured. Finally, the pH and osmolarity of the NLC formulation that showed the best quality attributes for intranasal delivery (lower particle size and higher EE) were adjusted to physiological values, and the impact on particle size was evaluated.

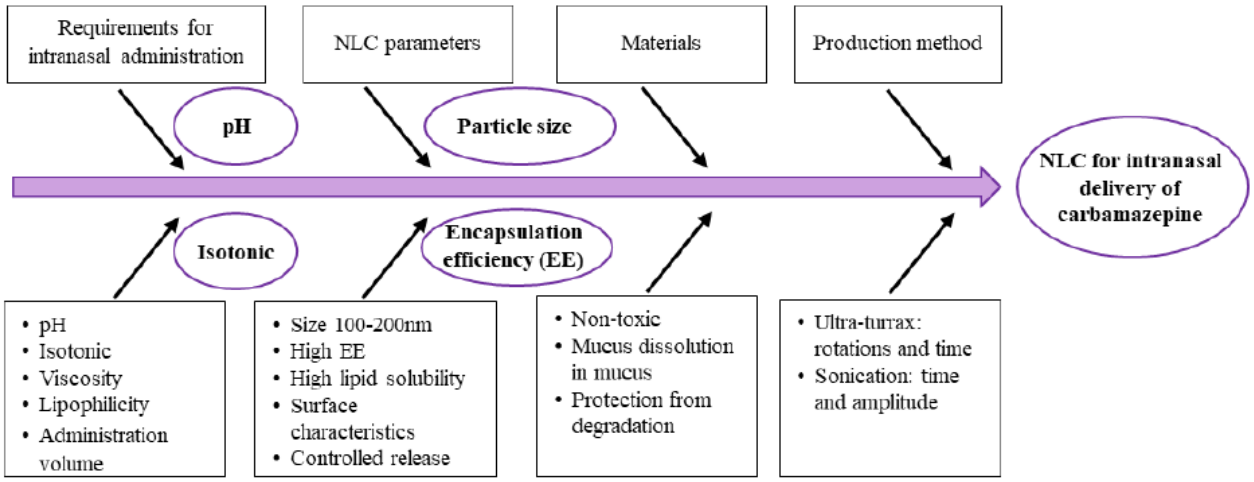


*Figure 1:* Ishikawa diagram showing factors affecting the critical requirements for intranasal administration of NLC formulations [4].

## Results and discussion

The developed NLC formulations revealed the presence of 50% of nanoparticles with sizes ≤ 103-161 nm and 90% with sizes ≤ 321-501 nm, which means that most nanoparticles might direct transport carbamazepine from the nose-to-brain, although some may reach the brain via indirect pathway [5]. High EE values (96.8-98.8%) were obtained for all formulations, which confirm the solubility of the drug in the lipids used for NLC preparation [4]. After pH and osmolarity adjustment, no significant changes of the NLC size were observed.

## Conclusion

From this study, we conclude that the QbD approach is an efficient method for the optimization of intranasal NLC formulations, reducing the development time. Further studies will be performed, with more critical paramenters, using specific statistical software for data analysis.

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**PC-67**

# Electrospun nanofibers for bone regeneration

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## Introduction

The repair of large bone defects is still a major challenge in orthopedic and maxillofacial surgery. Scaffolds play a crucial role in bone tissue engineering acting as a template to facilitate cell growth and differentiation within bone defects. Fibers of mainly submicron sizes produced by electrospinning resemble the extracellular matrix structure and are an excellent framework for cell adhesion, proliferation and differentiation [1]. Polycaprolactone (PCL) is a low cost, biocompatible and appropriate for electrospinning process.In order to improve the osteinduction and osteoconduction the addition of hydroxyapatite nanorods (HA) to PCL/polyvinyl acetate (PVAc) core shell fibers have proven to be a good strategy [2]. The incorporation of osteogenic grown factors, such as morphogenetic protein-2 (BMP-2) is an interesting alternative to increase the osteogenic activity of these materials.

## Materials and Methods

HA/PCL/PVAc fibers decorated with BMP-2 loaded Poly(D,L-lactide-co-glycolide)lactide:glycolide 50:50 (PLGA) particles were obatined by simultaneous electropinning-electrospraying process in a a Yflow 2.2.D-500 electrospinner. Morphology of materials was analyzed using a SEM and TEM microscopy. Fourier transform infrared (FTIR) spectra were used to evaluate the molecular structure. BMP-2 *in vitro* release from loaded scaffolds was carried out at 37ºC in Dulbecco's phosphate-buffered saline for 24 hours. Human osteoblasts seeded scaffolds were cultured up to 21 days.

## Results and Discussion

Size distribution and morphology of the electrosprayed PLGA particles were investigated by SEM and TEM giving mean diameter of 1.0±0.6 µm. The histogram clearly shows the presence of an important population in the range 0.4-1.2 µm. TEM images confirm the spherical shape of the obtained particles. BSA fluorescein conjugate (BSA-FTIC) was added to the PLGA solution. No significant phase separation of polymer and/or drug was observed by confocal laser scanning microscopy. Once the particles synthesis was optimized, the simultaneous electrospray-electrospinning process was carried out to obtain the final material. Figure 1 shows SEM images of the obtained HAn loaded PCL/PVAc fibers decorated with PLGA particles. Due to the simultaneous synthesis of both morphologies, particles are homogeneously distributed in the entire fiber mat. BMP-2 encapsulation efficiency was 40±11% and around 35% of the loaded protein was release in the first 48 h. The effect of the growth factor is clearly seen after 21 days culture, when the viability increases by 18% compare to the scaffolds without BMP-2.

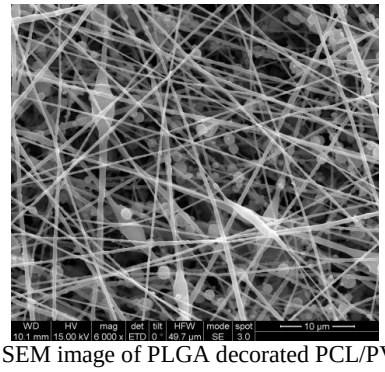


*Figure 1*. SEM image of PLGA decorated PCL/PVAc fibers

## Conclusions

PCL/PVAc fibers loadad with hydroxiapatite were decorated by simultaneous electrospinning-electrospraying process with PLGA particles loaded with BMP-2 grown factor. The presence of BMP-2 clearly increases the cell viability and imporves human osteoblast grown.

**Acknowledgments**: This work was supported by the Ministerio de Economía y Competitividad, SPAIN (grant number CTQ2014-52384-R).

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**PC-68**

# Multiple growth factor delivery from enzymatically crosslinked gelatin-based 3D systems

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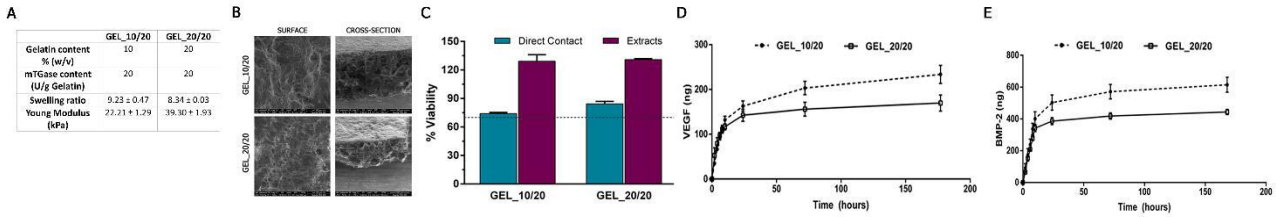
**Introduction:** Great efforts have been made in recent years in order to develop three-dimensional (3D) systems that promote the regeneration of new tissues. Gelatin is a natural origin polymer that has been shown to be biocompatible and biodegradable [1]. Although diverse crosslinking agents have been used so far, the use of enzymes that catalyze the specific reaction between amino acids seems to be an interesting strategy for designing structures that deliver therapeutic growth factors in a controlled manner for tissue engineering [2]. The synthesis and characterization of gelatin-based scaffolds crosslinked with microbial transglutaminase (mTGase) enzyme was the main goal of this experimental work. Furthermore, the swelling, biomechanical behavior, biocompatibility and growth factors *in vitro* release from these new gelatin-based scaffolds is reported.

**Materials and Methods:** 3D scaffolds were prepared by the freeze-drying technique. Gelatin powder was dissolved in distilled water at 40 ºC under constant agitation. Enzymatic crosslinking was produced by the addition of the enzyme mTGase to the homogeneous dispersion of the polymer. This solution was cast into Petri dish before the complete reticulation would have happened. Finally, 8 mm diameter scaffolds were freeze-dried. Morphology of dry scaffolds was assessed by scanning electron microscopy (SEM). Swelling ratio was measured in PBS at room temperature for at 24 hours. Uniaxial unconfined static compression tests were performed to determine scaffolds mechanical properties. The biocompatibility of the scaffolds was evaluated following the ISO 10993-5 guidelines using L-929 fibroblasts. Toxicity of extracts and toxicity by direct contact were evaluated. *In vitro* release experiments were completed with Vascular Endothelial Growth Factor (VEGF) and Bone Morphogenetic Protein-2 (BMP-2) as therapeutic molecules models. The assay was carried out with Protein LoBind tubes containing PBS at 37ºC under orbital shaking (40 rpm). At preestablished times samples were taken and the concentration of the growth factors was measured by ELISA kits.

**Results and Discussion:** Two different prototypes of enzymatically crosslinked gelatin–based scaffolds were developed and lyophilized (GEL_10/20 and GEL_20/20). Both systems showed great ability to raise and retain water, presenting swelling ratio between 8 and 9. However, differences in mechanical properties were identified since GEL_20/20 scaffold presented higher Young Modulus value (Fig. 1A). It is well established that scaffolds designed for tissue engineering must mimic the mechanical characteristics of the tissue to be regenerated or replaced. The images that were taken by SEM demonstrated that the 3D systems showed sponge-like structures with pores between 150-230 µm distributed throughout the entire surface (Fig. 1B). The porosity of the systems determines the capacity of host cells to migrate and invade the scaffold promoting the formation of a new tissue. Based on the results obtained in the cytotoxicity tests, the 3D systems were considered as non-cytotoxic. In fact, both preparations showed viability values higher than 70% respect to the control (Fig. 1C). In the *in vitro* release assay both prototypes demonstrated the ability to retain both bioactive molecules, achieving in all cases encapsulation efficiencies of around 90%. Depending on the method used for collagen pretreatment prior to the extraction process, two types of gelatin are obtained. The isoelectric point (pI) of the gelatin used in this test (4.7-5.2) allows the development of structures that can serve as carriers of basic bioactive molecules such as VEGF (pI 7.65) and BMP-2 (pI 9.15). The GEL_20/20 prototype showed greater ability to retain these growth factors, since only the 61% of absorbed VEGF and the 50% of loaded BMP-2 were released (Fig. 1D-E).

**Conclusions:** The new 3D scaffolds fabricated with enzymatically crosslinked gelatin have shown to be biocompatible and functional as vehicles for the controlled delivery of growth factors. These prototypes could be suitable candidates to evaluate their osteoregenerative capacity in future *in vivo* studies in animals.




| | GEL_10/20 | GEL_20/20 |
|---|---|---|
| Gelatin content % (w/v) | 10 | 20 |
| mTGase content (U/g Gelatin) | 20 | 20 |
| Swelling ratio | 9.23 ± 0.47 | 8.34 ± 0.03 |
| Young Modulus (kPa) | 22.21 ± 1.29 | 39.30 ± 1.93 |

*Figure 1*. SEM images and results of the biomechanical, biocompatibility and *in vitro* release tests

**Acknowledgments**: M.C. Echave thanks the Basque Country Government for the granted fellowship (PRE_2016_2_0185).

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**PC-69**

# Porosity enhancement of drug-loaded PCL scaffolds for bone regeneration processed using supercritical fluid technology

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## 1.-Introduction

Synthetic scaffolds for bone regeneration are designed in regenerative medicine to overcome the problems of availability, morbility and disease transmission of biological grafts [1]. Scaffolds should be biocompatible and possess porosities ranging from 65 to 80%, with pore sizes in the 100-500 µm range and full interconnectivity to allow tissue growth and vascularization [2]. Supercritical foaming is a solvent-free processing approach able to prepare scaffolds at moderate operating conditions [3]. However, this technique has certain limitations regarding the control of the macroporosity and its interconnectivity. This work focuses on enhancing the macroporosity of synthetic scaffolds of poly(ε-caprolactone) (PCL) prepared by supercritical foaming through the addition of ammonium bicarbonate to the polymeric matrix as a sacrificial porogen that can be removed through thermal degradation at mild temperatures. Ketoprofen, a non steroideal anti-inflammatory drug, ws also incorporated in the scaffold formulation.

## 2.-Materials and Methods

*Materials:* PCL (50 kDa, Polysciences); ketoprofen (99.7% purity; Acofarma); ammonium bicarbonate ($NH_4HCO_3$; 250 to 500 mm size; 30% min. content in $NH_3$; Panreac); $CO_2$ (99.8%, Praxair); release medium: phosphate buffered solution (PBS) pH 7.4.

*Preparation of scaffolds:* Materials were weighed, mixed, compacted, and placed in Teflon cylindrical moulds. Three $NH_4HCO_3$ (0, 50 and 75 wt. %) and ketoprofen contents (0, 5 and 10 wt. %) were used. The moulds were inserted in a high-pressure autoclave and soaked in supercritical $CO_2$ at 37ºC and 140 bar for 60 min in the static mode. Then, the autoclave was depressurized to ambient pressure at a venting rate of 3 bar/min. The scaffolds were removed from the moulds and placed in an oven at 37ºC under vacuum to remove the $NH_4HCO_3$ particles.

*Characterization of scaffolds:* Scaffoldss were measured and weighed before and after removing the porogen to determine bulk density. Helium pycnometry was employed to determine skeletal density. Porosity was calculated using both parameters. The morphology of the scaffolds was studied by digital imaging using a CCD microscope and scanning electron microscopy (SEM).

*Release profiles:* Scaffolds were cut in pieces (10 mg) and suspended in 50 mL of PBS pH 7.4 medium, at 37ºC with an agitation of 60 rpm for 21 days. At selected times, alliquots of PBS were sampled and replaced with fresh medium. The concentration of ketoprofen was determined by UV-Vis spectrophotometry at $\lambda$=260 nm.

## 3.-Results and discussion

The addition and later removal of $NH_4HCO_3$ lead to an increase in the porosity of the obtained PCL-based scaffolds from 54% without addition of porogento 72% and 83% when adding 50 wt. % and 75 wt. % of $NH_4HCO_3$, respectively. Moreover, ketoprofen incorporation yields to the scaffolds were close to 100%.

Ketoprofen release assays show faster release profiles when $NH_4HCO_3$ was initially added to the formulation. These results indicate that a high pore interconnectivity was attained when scaffolds were processed with the sacrificial salts, which allows the PBS medium to circulate through the porous structure of the scaffolds and to dissolve the drug.

## 4.-Conclusions

Ammonium bicarbonate was successfully employed as a porogen to increase the overall porosity, particularly macroporosity, of ketoprofen-loaded polymeric scaffolds processed by supercritical foaming. The porogen herein used was degraded under mild temperatures and vacuum leading to full incorporation yields, unlike other commonly used salts (e.g., NaCl) where the drug is washed away upon lixiviation of the salt. The processing strategy herein presented allows a fine control on the macroporosity of the scaffolds to get the optimum morphology for bone regeneration to promote bone tissue formation and growth as well as suitable mechanical properties.

**Acknowledgments:** Work funded by MINECO (SAF2014-52632-R), Xunta de Galicia (ED431F 2016/010) & (ED431C 2016/008), Banco Santander SA (JPI-2017), and FEDER. C.A. G.-G. acknowledges MINECO for a Ramón y Cajal Fellowship (RYC-2014-15239).

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**PC-70**

# *In vivo* protein release in bone and cartilage for tissue regeneration

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**Introduction:** Osteochondral defects in articular cartilage appear after degenerative diseases or traumatic lesions, and bone defects results from fractures, trauma, tumors or infections. The current treatments for this type of defects are, among others, the autologous and allogeneic graft transplantation with some limitations that have limited their use [1-3]. Lack of capacity of cartilage for self-repairing and loss of bone mass that exceeds the bone capacity of self-healing, compromises the regeneration of tissue, being the current treatments not able to fully regenerate the osteochondral and bone defects [2,4]. Direct application of GF in the defects have shown a great pre-clinical potential, but due to their short biological half-lives and the difficulties to retain them in the defect site, high and repeated doses are required, making these therapies costly [2,5]. Controlled delivery systems that combine GF and biomaterial based-scaffolds with porous structures have emerged as effective systems for local regeneration [3]. The aim of this contribution is to compare the *in vivo* release profiles achieved with different systems, containing encapsulated GF, applied in critical calvaria defects in rats or cartilage and intramedullary femur defects in rabbits for tissue regeneration.

**Materials and Methods:** Several osteogenic and angiogenic radiolabeled growth factors, like $^{125}$I-rhBMP-2 or $^{125}$I-TGF-β1, among others, were loaded into PLGA microspheres (PLGAμE) that were prepared by double emulsion method [6], and included in different delivery systems. Regarding to cartilage repair, the following systems, among others, were used, tri-layered scaffolds composed of a tablet of blank PLGAμE, $^{125}$I-rhBMP-2-loaded PLGAμE in Pluronic® F-127 and an external electrospun PLGA layer [1]; and biphasic systems composed of a PLGA cylinder and a layer with a mixture of alginate [4] or segmented polyurethane (SPU) [7] with $^{125}$I-rhBMP-2 or $^{125}$I-TGF-β1 loaded PLGAμE. With regard to bone defects, some systems were used, like hollow PLGA cylinders filled with $^{125}$I-rhBMP-2-loaded PLGAμE [6]; cylindrical rings of β-tricalcium phosphate, including $^{125}$I-rhBMP-2-loaded PLGAμE and SPU [8]; $^{125}$I-rhBMP-2-loaded PLGAμE dispersed in Tetronic® (T908 or T1307) [9], or new mixtures of Pluronic® F-127, T1307 and α-cyclodextrine or T1307, alginate and $CaCl_2$; new tixotropic mixtures of $CaCl_2$ and alginate, including $^{125}$I-rhBMP-2-loaded PLGAμE, and crosslinked with $CaCl_2$; and innovative mixtures of collagen, hydroxylpropyl-γ-cyclodextrin/rivoflavin, chitosan and PEG, including $^{125}$I-rhBMP-2-loaded PLGAμE, and crosslinked by blue light and tripolyphosphate. Release *in vivo* was performed at the defect site by a validated method with an external probe-type gamma counter [10].

**Results and Discussion:** BMP-2 release profiles from most systems reveals a two-phase behavior, a moderate burst release of around 30% and a controlled release period until the total degradation of PLGA microspheres, around 4 weeks, being the total release of more than the 90%. Burst release in the delivery systems may cause high concentrations of GF in the defect site and consequently loss of protein that would be required for a longer release. For that reason, more complex systems were designed, like the cylindrical tri-layered or bi-layered PLGA scaffolds that reduce such burst profile at 17-20% and maintain the total release in values around 90% in a timeframe of 6 weeks, highlighting the use of SPU that contributed to an efficient control over the GF release that decreased their burst release up to the 1.6% [1,4,7]. Promising results are been obtained with new tixotropic and collagen based hydrogels with a reduced burst of 18%, due to a dense polymer network and interactions between components created before crosslinking. In the case of other GF, release profiles were similar when compared with BMP-2, with slight modifications. Since no radioactivity was detected in blood, released GF seem to be located around the defect site.

**Conclusions:** Release profiles of encapsulated GF in different delivery systems for tissue regeneration have been compared, obtaining the commonly two-phase release profiles with a first burst effect, but allowing a controlled release over more than 4 weeks that provide the correct repair concentrations for regeneration. Some promising systems with a reduced burst profile and an increased timeframe of 6 weeks of release have been reported.

**Acknowledgments**: This work was supported by the Ministry of Economy, Industry and Competitiveness (MAT2014-55657-R).

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**PC-71**

# Combined sustained release of BMP2 and MMP10 for bone regeneration

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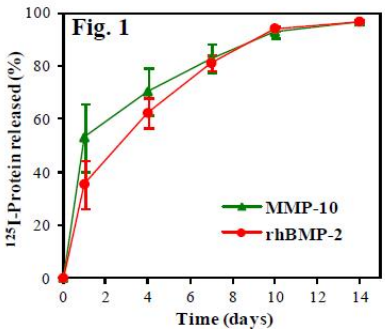
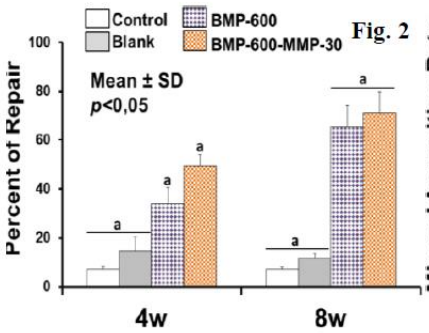
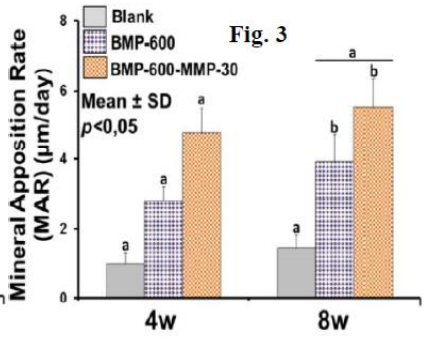
**Introduction:** The repair of critical bone defects remains a major clinical orthopaedic challenge. Bone morphogenetic protein 2 (BMP-2), is involved in all steps of bone repair process [1]. Moreover, metalloproteinase 10 (MMP10) augmented the differentiation of myoblastic cells into osteoblastic cells induced by BMP-2, leading to propose that MMP10 promotes the differentiation of myoblasts into osteoblasts by interacting with the BMP signaling pathway [2]. Therefore, in the present study combination of BMP-2 and MMP-10 (20:1) formulated in a controlled release microspheres suspension was tested to promote bone repair in a murine model of critical-size bone defect.

**Materials and Methods:** Microspheres of polylactide-co-glycolide (PLGA, Resomer® 504, Evonik) containing BMP-2 (GenScript, USA) or MMP10 [3] were prepared by a double emulsion process. Batches for encapsulation yield and protein release assay were prepared with $^{125}$I-BMP-2 and $^{125}$I-MMP labeled by the iodogen method. The implanted system consisted in 2 mg of microspheres suspended in 7 µL of a 15% aqueous solution of Pluronic F-127®. Microspheres were morphologically characterized by SEM and sized by laser diffractometry. 4 mm calvaria critical defect in 30 g mice was carried out under isoflurane and the delivery system was inserted in the defect and the skin was stapled. Release experiments were performed at the defect site using an external probe-type gamma counter [4]. Defects of 4 groups of 8 mice each (4 animals per time point): a control group (C) of mice with an empty defect, a blank group of a implanted suspension of 2 mg of blank microspheres, BMP-600 group of a implanted 600 ng BMP-2 in 2 mg of microspheres suspension and BMP-600-MMP30 group of a implanted 600 ng BMP-2 and 30 ng MMP10 in 2 mg of microspheres suspension, were histological examined to determine the bone-regenerative effect of BMP-2 and MMP10 combination after 4 and 8 weeks postimplantation. To label the mineralization front, the animals were injected oxytetracycline-HCl (40 mg/kg, IM) and calcein blue (15 mg/kg, SC) 12 and 4 days before euthanasia, respectively.

**Results and Discussion:** The mean volume diameter and the encapsulation efficiency of BMP-2 and MMP-10 microspheres were 67 µm and 67% ± 4.9% and, 52 µm and 65.5 % ± 6.8%, respectively. The protein release profiles are reflected in fig. 1 and the percent of defect regenerated in fig. 2. The role of BMP-2 as an inducer of osteogenesis and its effects promoting osteoblastic differentiation during bone repair processes are well known. However, in the present study the combination of BMP-2 and MMP10 not only enhanced the repair response at shorter times but, even more importantly, increased significantly the mineral apposition rate throughout the experimental period (Fig. 3).




**Conclusions:** The sustained release of the combination of MMP10 and BMP-2 in a ratio of 1:20 from the injectable and biodegradable delivery system made in this study enhanced bone healing, and improved the mineralization rate.

**Acknowledgments**: This work was supported by the Ministry of Economy, Industry and Competitiveness (MAT2014-55657-R).

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**PC-72**

# Vascular endothelial factor-bound microparticles modulate human endothelial progenitor cell survival and miRNA expression

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**Introduction**

Several cell-based therapies are under pre-clinical and clinical evaluation for the treatment of ischemic diseases. Poor survival and vascular engraftment rates of transplanted cells force them to work mainly via time-limited paracrine actions. Although several approaches, including the use of soluble $VEGF_{165}$ (sVEGF; vascular endothelial growth factor), have been developed in the last 10 years to enhance cell survival, they showed limited efficacy. Here, we report a pro-survival approach based on VEGF-immobilized microparticles (VEGF-MPs) and propose an action mechanism for the VEGF-MPs on cord blood-derived outgrowth endothelial progenitor cells (OEPCs).

**Results and Discussion**

The conjugated VEGF amount on MPs was between 271.8±44.3 to 425.4±50.2 ng *per* $10^6$ MPs depending on the initial conditions. VEGF-MPs showed prolonged VEGFR-2 phosphorylation, intracellular $Ca^{2+}$ signaling (up to 1 h), and Akt phosphorylation relatively to sVEGF. Under hypoxia conditions (0.5% $O_2$), the survival of OEPCs incubated with VEGF-MPs was 1.6 times higher than sVEGF group after 24 h. In addition, VEGF-MPs decreased caspase 9 activity ~20% compared to sVEGF while the reduction compared to cell control group (cells incubated with blank MPs) was >35%. Both total network length and the number of branch points were higher in the presence of VEGF-MPs. After 60 h, the number of branch points decreased more than 50% for all the conditions, while the decrease was just 30% for VEGF-MP group. After 10 days, the cells transplanted in the presence of VEGF-MPs had 35.5% (±28.4%; n=7) of the initial fluorescent signal while the cells transplanted with blank MPs and soluble VEGF lost the signal after 3 and 4 days, respectively, *in vivo*. Our results show that the VEGF-MP modulates the cell activity by decreasing the expression miRNAs (especially miR-17) related to cell apoptosis and senescence while soluble VEGF does not affect the expression of these miRNAs. In order to understand the effect of miR-17 downregulation on cell survival and angiogenesis, OEPCs were transfected with antagomiR-17. AntagomiR-17 increased OEPC survival at least 1.5 times (n=6) and sprout formation on Matrigel at least 2 times (n=5) compared to all groups. The effect of antagomiR-17 was more pronounced under hypoxia conditions (1% $O_2$). *In vivo*, antagomiR-17 accelerated hemodynamic recovery of the whole limb (n=12) in unilateral limb ischemia obtained by occlusion of the left femoral artery. Blood flow recovery evaluated by Laser Doppler analysis was significantly higher 21 days after surgery in antagomiR-17 group compared to all other groups. Immunohistochemical analyses showed an increase in the capillary density of skeletal muscle in antagomiR-17 condition. In order to determine the gene target and potential pathway involved in the biological effect of antagomiR-17, next generation mRNA sequencing was performed. The results of mRNA sequencing and subsequent siRNA studies indicated that antagomiR-17 increased cell survival by upregulating the gene expression of *CDKN1A* and *ZNF652*.

**Conclusions**

We show that VEGF-MPs improve the survival and angiogenesis of OEPCs both *in vitro* and *in vivo*. Immobilized VEGF prolonged the VEGFR-2 phosphorylation and Akt signaling up to 1 h, which were diminished in 10 min when sVEGF was used. VEGF-MPs promoted OEPC survival up to 10 days in subcutaneous injections. Our work also reveals that miR-17 is an important molecular target of VEGF-MPs in OEPCs. The downregulation of miR-17 both *in vitro* and *in vivo* is associated with an up-regulation of *CDKN1A* and *ZNF652* transcripts. Our study provides insights about the molecular mechanism of immobilized VEGF in terms of OEPC angiogenesis and survival.

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**PC-73**

# Application of micelleplexes of Pluronic® L64-PEI-miR-145 in osteosarcoma therapy

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**Introduction:** Osteosarcoma (OS) is the most common type of bone cancer, being normally found at the end of long bones, frequently around the knee. Moreover, the current treatments available for OS have not increased the survival rate, in the last 30 years [1]. Therefore, gene therapy appears as a possible and suitable alternative to treat OS, overcoming the issues related to the conventional therapies, and taking advantage of the therapeutic genes associated with this disease. I.e. since associated with OS exists a dysregulation of several microRNAs (miRNAs) involved in important cellular processes, the use of therapeutic miRNAs, such as miR-145, which are downregulated, can be a viable option to restore the apoptotic processes in OS cells [2].
Thus, in this work, the authors intended to develop a micellar complex composed of an amphiphilic copolymer, namely Pluronic® L64, linked to a cationic polymer, namely polyethyleneimine (PEI), to form a poly (ester amine) PEA, in order to have a more stable and less toxic system to encapsulate and to deliver efficiently the therapeutic miR-145 into OS cells [3,4].
**Materials and Methods:** PEA nanosystems were synthesized by a two-step chemical reaction and were characterized through the evaluation of their zeta potential, size, chemical structure, and morphology, by ELS, DLS, $^{1}$H-NMR and FT-IR, and TEM, respectively. After this characterization, it was developed micelleplexes based on the conjugation between PEA systems and miR-145, through the electrostatic interactions between the negative charge of miR-145 and the positive charge of PEI, at different N/P ratios. Micelleplexes were evaluated in terms of structural and morphologic properties, by DLS, ELS, agarose gel electrophoresis, and TEM, as well as, in terms of capability to be internalized by OS cells, cytotoxicity and cell death assessment in MG-63 cell line.
**Results and Discussion:** The chemical characterization of PEA has shown that Pluronic® L64 was successful bond to PEI. In this sense, PEA nanosystems presented sizes between 132.1-158.4 nm, polydispersion index (PDI) between 0.281-0.316, and zeta potential values around 30 mV. Besides this, PEA formulations had non-cytotoxicity until concentrations of 40 µg/mL. In this way, it was chosen the PEA formulation with a molar ratio of 0.2/1 (Pluronic® L64/PEI) since presented the better results. Thereby, the characterization of micelleplexes has shown better results at an N/P ratio 10/1 using 50 nM of miR-145, once this micellar complex was able to obtain sizes around 200 nm and zeta potential around 8 mV. The complexation of miR-145 into PEA systems modified the well-defined spherical structure to a more irregulated spherical shape of micelleplexes (see figure 1). Regarding this, these micelleplexes at an N/P ratio of 10/1, with 50 nM of miR-145, was capable to induce cell death by activating apoptosis and necrosis pathways in OS cells.
**Conclusions:** Consequently, the results obtained in this research work shown that it was developed a novel, stable and efficient nanocarrier to deliver a therapeutic miRNA, such as miR-145, in future gene therapy strategies for OS.

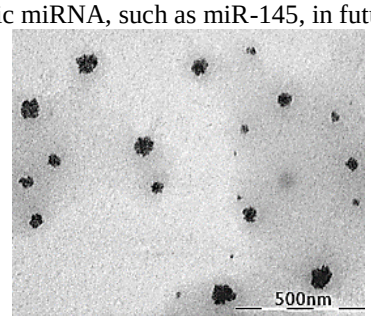


*Figure 1*. Transmission electron microscopy (TEM) image of micelleplexes at an N/P ratio 10/1.

**Acknowledgments**: This work was supported by the grant FCT PTDC/CTM-BIO/1518/2014 from the Portuguese Foundation for Science and Technology (FCT). The authors thank to UCQFarma and Nuno Ricardo Ferreira for FT-IR technical assistance. We also thank to NMR laboratory of Coimbra chemistry centre and Rui Carvalho for 1H-NMR technical assistance.
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**PC-74**

# Injectable Pluronic –Tetronic – α-Cyclodextrin supramolecular gels for bone regeneration in osteoporosis

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**Introduction:** Poloxamines (Tetronic®) form self-associate micellar and gel structures in response to their concentration, the pH and the temperature of the medium [1-2]. Hydrogels of Tetronic® (T908) and its combination with α-cyclodextrin (αCD) hydrogels were being explored for bone regeneration [3-4]. The osteogenic capacity together with the biocompatibility, injectability, and adaptability to multiple defect sizes and shapes made them promising syringeable systems that can contribute to regulate the local delivery of active substances. To improve mechanical properties of the Tetronic® gel an injectable *in-situ* gelling hydrogel of Pluronic®-Tetronic®-α Cyclodextrin (P-T-CD) is proposed as scaffolds containing β-estradiol as anti-bone resorption substance to enhance regeneration of bone defects in osteoporotic (OP) population. Optimization of the composition and characterization of the system in terms of rheological properties, cell viability, osteogenic differentiation and control release capacity of β-estradiol in PLA (Poly d,l-lactide) (Resomer® R203-S) microspheres included in the gel, was carried out.

**Materials and Methods:** Several combination of Pluronic® F-127 (P) and Tetronic® 1307 (T) and α-Cyclodextrin (αCD) under different conditions were assayed for gel preparation. P-T-CD gels were prepared using vortex, in an ice bath to maintain a liquid state, adding each component in the following sequence: Pluronic, Tetronic and αCD. Gel formation was determined after incubation at 37°C for 15 minutes by applying the inverted tube test. The 11/7/7 ratio was selected to be mixed with the β-estradiol microspheres to form the injectable drug delivery system. The PLA microspheres were prepared by the solvent evaporation method. β-estradiol was previously dissolved in methanol (MeOH) and mixed with the PLA in dichloromethane (DCM) in a final ratio of 1:4. Microspheres were characterized by SEM, and were sized by Mastersizer 2000. Encapsulation efficiency and *in vitro* β-estradiol released were determined spectrophotometrically at 280 nm. The viscoelastic behavior of the gel with and without microspheres was characterized using a Bohlin CVOD 100 rheometer equipped with a Peltier temperature control system and using a cone-plate geometry leaving a gap of 1 mm. Internal structure and porosity of the system, with and without microspheres, was evaluated on freshly prepared gels and after incubation in aqueous medium. SEM was used to observe the internal structure and porosity was calculated using the true (by helium pycnometry, ACCUPYC 1330) and apparent densities of the freeze-dried gels. Lastly, to seed Mesenchymal Stem cells of rats (rMSC), the freeze-dried hydrogel was reconstituted in cell culture medium. Cell viability was assessed by flow cytometry after cell staining with calcein-AM and propidium iodide (PI) (both from Sigma). To check the osteogenic differentiation of rMSCs in the hydrogel, alkaline phosphatase activity (ALP) was assessed in differentiation media after 7, 14 or 21 days.

**Results and Discussion:** The mean volume diameter of the microspheres was 79.7 µm. A biphasic *in vitro* release profile was observed; the incorporation of the microspheres into the hydrogel delayed the β-estradiol release. The rheological analysis of the P-T-CD showed that the gel forming process started before 30ºC and the maximum viscosity was located in the range of 35-45ºC. The addition of the microspheres showed an early increase of the viscosity moving the range to lower temperatures of 25-40ºC. Also, the porosity of the gel with microspheres was greater than without microspheres, but the slight increase in porosity observed after 4 weeks incubation was similar for both preparations. The flow cytometry analysis showed that 98% of the cells were alive after 5-day culture in the hydrogel. In addition, the number of positive ALP staining cells increased from 7 to 21 days after culture in the differentiation osteoblastic medium.

**Conclusions:** The P-T-CD (11/7/7) showed an adequate range of temperature for *in vitro* gel-forming, its biocompatibility was demonstrated as well as its capacity to modulate the release rate of the β-estradiol. Therefore P-T-CD prepared might be a good system for regeneration of bone defects in osteoporosis.

**Acknowledgments**: This work was supported by the Ministry of Economy, Industry and Competitiveness (MAT2014-55657-R).

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**PC-75**

# Biologically active and biomimetic 3D CaSO4/gelatin scaffolds for *in vivo* bone tissue engineering

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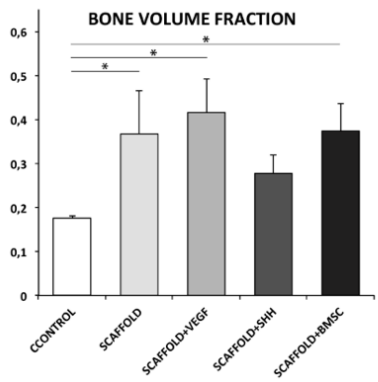
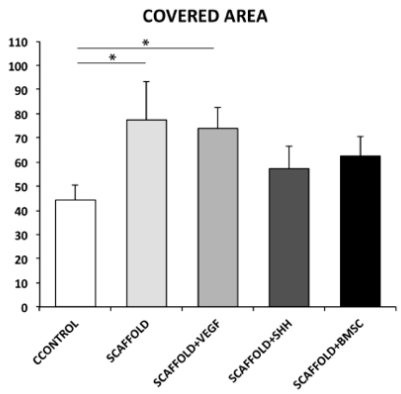
**Introduction:** The reconstruction of critical bone defects is one of the biggest challenges for orthopedic surgeons. Bone tissue engineering (BTE) which requires a biocompatible scaffold able to support osteoprogenitor cells and suitable microenvironment seems to be a promising way in recent years is booming [1,2]. A combination of hard/soft biomaterials could be an exciting approach to promote bone regeneration while releasing growth factors. Here, we report the design, fabrication and characterization of cross-linked three-dimensional (3D) CaSO4/gelatin scaffolds for bone tissue engineering. VEGF and sonic hedgehog (SHH) were used as model proteins and bone marrow stem cells (BMSC) as cell candidates to biologically fill the scaffolds. In addition, *in vivo* bone regeneration of the new 3D scaffolds was studied using the rat critical size calvarial defect model.

**Materials and Methods:** Type B gelatin powder was dissolved in DI water. Calcium sulfate was added to give a final concentration of 7.5%. 100 mg of genipin were dissolved in 10 ml of DI water to prepare 0.2% (w/v) genipin. Solutions were mixed at 40ºC and under stirring and then left crosslinking for 72 hours. Scaffolds were obtained by punching 8 mm-cylinders into ethanol 70% solution for 10 minutes and then freeze-drying. Swelling ratios were determined using a gravimetric method. Biomechanics include analyzing young´s modulus, Poisson distribution and permeability. Surface morphology was analyzed by SEM and toxicity by ISO 10993 guideline. Cell adhesion and morphology of D1-Mesenchymal stem cells (MSCs) and primary Bone Marrow Stem Cells (BMSC) was studied. Primary BMSC were obtained from 1 month Wistar rat femur. VEGF and SHH were used as model proteins for kinetic studies. Bone regeneration was tested in the rat critical size calvarial defect model. A total of 50 rats were used. A critical size bone defect of 8 mm was created using a low speed trephine under continuous irrigation of sterile saline. Subsequently, the defect was filled with the different formulations (control; empty scaffold; scaffold-VEGF: 300 ng; scaffold-SHH: 300 ng; scaffold-BMSCs) and the incision was closed using resorbable suture. The animals were sacrificed at 2 and 8 weeks to study the formation of new blood vessels and bone regeneration.

**Results and Discussion:** Several genipin cross-linked 3D gelatin prototypes were fabricated. Swelling ratio increased and young´s module decreased along with the concentration of genipin. All scaffolds were biocompatible according to the toxicity test. MSC adhesion improved in 0.2% of genipin. Results from the *in vitro* release assay revealed that both prototypes had the ability to retain both bioactive molecules, achieving in all cases encapsulation efficiencies of around 73% and 90% for SHH and VEGF respectively. Then, we fabricated 2% genipin-CaSO4 scaffolds. The latter resulted to be biocompatible and biomimetic as adhesion of D1-MSCs and BMSCs was confirmed. Bone regeneration capacity of all types of genipin-CaSO4 scaffolds was tested in the rat critical size calvarial defect model. Results demonstrated that bone covered area was higher for all them compared to control group, being the scaffold empty and the scaffold-VEGF the ones that reached statistical significance. Furthermore, bone volume fraction was significantly higher for all types of scaffolds when compared to the control group.




**Conclusions:** The new 3D genipin-CaSO4 scaffolds are biocompatible and functional both *in vitro* and *in vivo* as vehicles for the controlled delivery of growth factors and bone regeneration purposes.

**Acknowledgments**: Authors wish to thank project SAF2016-76150-R from the Spanish Ministry of Science.

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**PC-76**

# Design of theranostic nanoplatforms targeted to metastatic colorectal cancer cells

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## Introduction

Death related to cancer is due in almost all cases to the appearance of metastasis [1]. Therefore, trying to interrupt the metastatic cascade and thus the formation of the metatastasic niche would be an obvious improvement in the standard of living of patients. For this particular purpose, we aimed to develop a theranostic nanoplatform [2-4] based on oil in water (O/W) nanoemulsions (NE), that are directed specifically towards metastatic cells.

## Materials and Methods

NE were prepared by ethanol injection and characterized in terms of their physicochemical properties by Dynamic Light Scattering (DLS), Transmission Electronic Microscopy (TEM) and Atomic Forces Microscopy (AFM). NE were loaded with superparamagnetic iron oxide nanoparticles (SPIONs) and analyzed by magnetic resonance imaging (MRI). NE were decorated with PE-DTPA and $_{68}$Ga for positron emission tomography (PET). Several *in vitro* assays were performed using a colorectal cancer cell line SW620 (ATCC®, CCL-227™).

## Results and Discussion

NE were successfully decorated with a small organic molecule for selective targeting to a cell membrane receptor overexpressed in metastatic colorectal cancer cells. These formulations have a small coloidal size (136 ± 6 nm), a polydispersity index correspondent to a monodisperse population (0.17), and a negative surface charge (-58 ± 3 mV). Confocal microscopy and flow cytometry experiments confirmed that the presence of this ligand increases the interaction of the nanoemulsions with the targeted metastatic cells. The encapsulation of etoposide into the targeted nanoemulsions and further delivery to the cancer cells resulted in a marked decrease in cell viability. For the development of nanotheranostics for MRI imaging, hydrophobic SPIONs of manganese ferrite ($MnFe_2O_4$) were successfully incorporated into the oily nucleus of the nanoemulsions. TEM and AFM images allowed studying the morphology of the NE and confirming the association of SPIONs to the NE (Figure 1). Additionally, NE were radiolabelled with $^{68}$Ga for PET, upon incorporation of a chelating agent, PE-DTPA. The association of the radioisotope was efficient and it was possible to track the radiolabelled NE by PET after intravenous administration to mice.

## Conclusions

We have developed a promising nanoplatform that can be targeted to metastatic cancer cells and is highly versatile for the association of imaging agents and therapeutic agents, and therefore for the development of nanotheranostics.

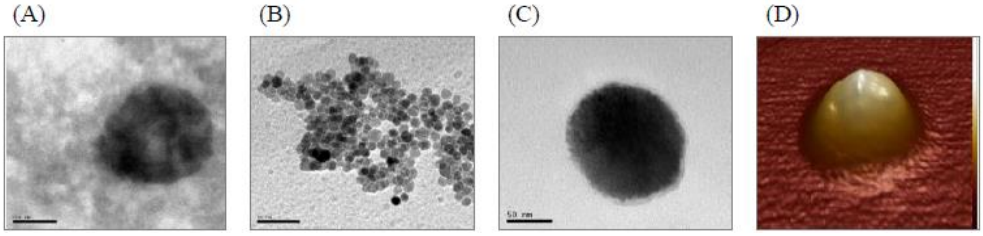


*Figure 1.* Morphological characterization of empty functionalized nanoemulsions (A) and loaded with SPIONs (C), observed by transmission electron microscopy (TEM). Hydrophobic SPIONs of manganese ferrite ($MnFe_2O_4$) in suspension (B) were also observed as controls. Functionalized nanoemulsions loaded with SPIONs were also analysed by Atomic Force Microscopy (AFM) (D).

**Acknowledgments**: This work was supported by the Carlos III Health Institute / FEDER funds (PI15/ 00828).

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**PC-77**

# Surface-decorated lipid nanoemulsions for targeting micrometastases

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## Introduction

Metastases are the major cause of death in cancer patients, mainly because of the ineffectiveness of therapies. Micrometastases of disseminated cells and circulating tumour cells (CTCs) are present in a relevant number of patients without clinical or even histopathological signs of metastases [1-2]. Nanoparticles tagged with ligands against specific tumour markers might be the 'magic bullet' for ultrasensitive micrometastases detection, giving a rapid and noninvasive tool to identify and eliminate these cells. Current research in liquid biopsy, based on the isolation of CTCs from patients with metastatic cancer [3-5], can be useful to find cell membrane receptors and biomarkers for the selective delivery of nanotheranostics. In this sense, we have identified three receptors of interest. Therefore, our aim was to decorate lipid nanoemulsions with ligands againts these receptors, and to determine the efficiency of the surfacedecorated nanoemulsions to interact with metastatic colorectal cancer cells.

## Materials and Methods

Nanoemulsions (NE), composed by natural lipids and prepared by ethanol injection, were functionalized with an amphiphilic ligand LCT (GalChimia S.L, Spain) and two peptides, URO and LAPI (China Peptides Co., Ltd., China), and characterized with a Nanosizer 2000® (Malvern Instruments, UK). NE were also loaded with the fluorophores Nile Red and DiD for study of their interaction with metastatic SW620 (ATCC®,CCL-227™) colorectal cancer cells, making use of a confocal microscope (Confocal Leica TCS SP8) and a flow cytometer (FACScalibur, BectonDickinson). NE were decorated with PE-DTPA, and $^{68}$Ga. PET/CT images were adquired with a Gemini TF-64 scanner (Philips Healthcare, Best, The Netherlands). Finally, different anticancer drugs (etoposide, paclitaxel and irinotecan) were encapsulated and the efficacy of the association determined by HPLC (1260 Infinity II, Agilent).

## Results and Discussion

NE were successfully decorated with the ligands. Smaller sizes and improved stabilities were observed in NE decorated with LCT, mainly due to its ability to accomodate at the colloidal interface and behave as an additional surfactant. Upon incubation of the surface-decorated drug-loaded NE with the metastatic cells, it was observed an improved therapeutic effect, with respect to the control formulations without ligand, a fact attributed to their improved cell uptake, as shown in Figure 1. Finally, NE were radiolabelled with $^{68}$Ga for PET imaging. First experiments in healthy mice showed adequate signal and highlighted the potential of this formulation for nanotheranostic applications. Next experiments will be performed in animal models of disseminated colorectal cancer.

## Conclusions

We have decorated NE with ligands against cel surface biomarkers overexpressed in metastatic cancer cells. In all cases, an improved accumulation of the functionalized NE was observed with respect to the control NE. We also proved the potential of our surface-decorated nanocarriers for application in cancer diagnosis by PET. We believe that the proposed strategy has a great potential for the management of micrometastatic disease.

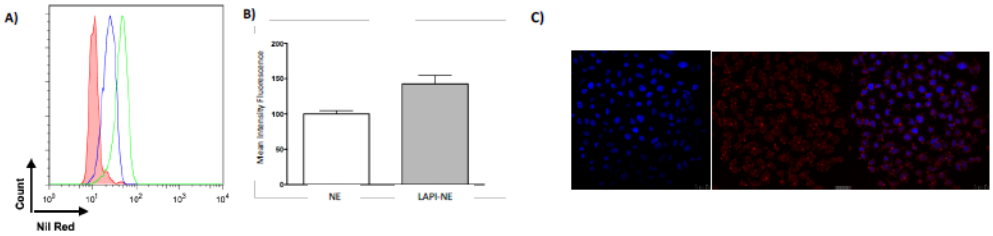


*Figure 1.* Cell uptake of surface-decorated NE in SW620 metastatic colorectal cancer cells. A) Distribution of positive cells by flow citometry for DiD-loaded nanoemulsions (NE, blue line) and surface-decorated-DiD-loaded nanoemulsions (LAPI-NE, green line) and B) mean intensity of fluorescence. C) Confocal microscope images that show the intracellular distribution of surface-decorated-DiD-loaded nanoemulsions (LAPI-NE, red) around the cell nuclei (blue) of the cells.

**Acknowledgments**: This work was supported by the Carlos III Health Institute / FEDER funds (PI15/ 00828).

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**PC-78**

# Magnetic nanocomposites as versatile theranostic agents

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**Introduction**: Magnetic hybrid nanocomposites (mNCs) have opened new perspectives in biomedical and environmental applications [1]. A range of different hybrid systems have been proposed within the scientific community as bioactive encapsulating agents and carriers due to their biocompatibility, low toxicity and ability to influence the delivery profile of pharmacological agents [2,3]. Hybrid organic-inorganic mNCs are being explored to synergistically combine the modified bioactive release provided by the organic encapsulation and the intrinsic physico-chemical properties from the inorganic counterpart [4]. In this context, we present the preparation of drug loaded magnetic nanocomposites showing good multifunctional performance as $T_2$-contrast agents in magnetic resonance imaging (MRI), heat generating sources in magnetic hyperthermia (MH) therapy, and responsive drug delivery vehicles.

**Materials and Methods**: mNCs were prepared following simple, versatile and scalable melt-emulsification methods. Obtained formulations were fully characterized not only from a physico-chemical point of view, but also in terms of cytotoxicity and functional performance in MRI, MH, and drug release. *In vitro/ex vivo* studies were performed to validate the potential of these formulations as theranostic drugs.

**Results and Discussion**: mNCs prepared presented a size, polydispersity index and stability suitable for biomedical applications. Also their encapsulation and loading efficiency of drugs and magnetic nanoparticles were optimized to allow a combined use as diagnostic and therapeutic probes. Measured values of relaxivity (over 400 $mM^{-1}s^{-1}$) and specific absorption rate (SAR, over 500 $Js^{-1}g^{-1}$) demonstrate the high potential of mNCs. *In vitro* results suggest a synergistic effect between heat generation and control drug delivery over cancer growth.

**Conclusions**: The high potential shown by mNCs in *in vitro* tests allow to propose them as next generation drugs for the diagnosis and therapy of cancer. The ability to encapsulate drugs and magnetic nanoparticles enables an external control over the release profile of the drugs and opens the door to personalized medicine through image-guided drug delivery.

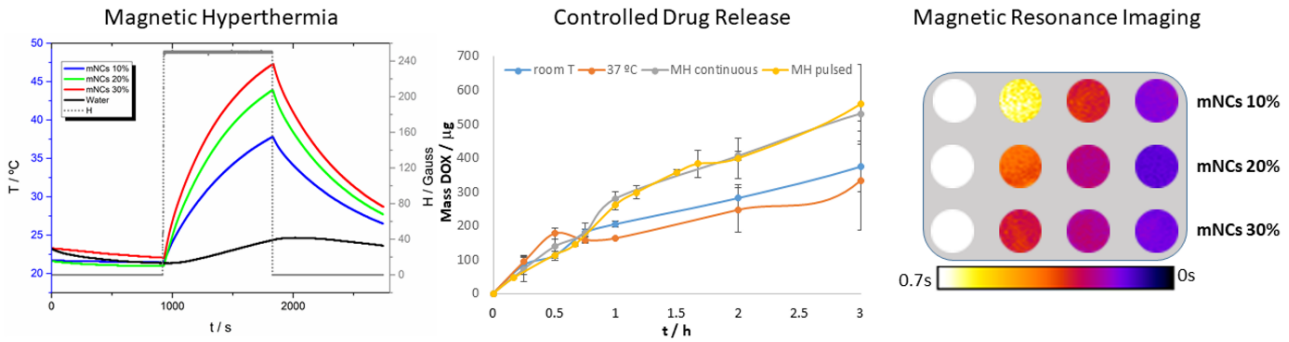


*Figure 1*. Performance of the mNCs in magnetic hyperthermia (left), drug delivery (center) and magnetic resonance imaging (right).

**Acknowledgments**: This work was partially supported by POCTEP (Co-operational Programme for Cross-border Cooperation Spain-Portugal) and by H2020 under project PANA, grant agreement contract number 686009.

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**PC-79**

# Tunable performance of manganese oxide nanostructures as MRI contrast agents

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**Introduction**: Magnetic resonance imaging (MRI) is a medical imaging technique perfectly suited for human healthcare applications. The lack of ionizing radiations, its high spatial/anatomical resolution and non-invasiveness make it an attractive tool for diagnostic purposes and to follow the progression of a disease/treatment [1]. To date the main limitation of MRI is its low sensitivity compared to radioactive-based and optical imaging modalities. To increase its sensitivity, contrast agents (CAs) are administered in about 50% of scans [2]. Clinically used CAs are based on a paramagnetic ion, $Gd^{3+}$, that due to its toxicity has to be administered strongly chelated to limit its interference with biological proceses [3]. Other species are being studied as potential substitutes for $Gd^{3+}$ chelates. $MnO_2$ nanostructures present several advantages over traditional Gd chelates. First, Mn is less toxic than Gd. Also, $MnO_2$ is not particularly active by MRI. This means that once administered it won´t produce significant changes in MR images. However, $MnO_2$ is very sensitive to biologically relevant conditions. For example, under deregulated redox conditions, $MnO_2$ will be easily reduced into $Mn^{2+}$ which is highly paramagnetic and significantly enhances $T_{1w}$ MR signal [4]. This OFF-ON MR behavior can be exploited for diagnostic purposes. In this talk the synthesis and functional characterization of several $Mn_xO_y$ nanostructures will be discussed as well as their combination with reporter molecules for other imaging techniques as a step further towards multimodal and unequivocal imaging diagnosis.

**Materials and Methods**: A green, scalable and simple protocol was used to explore the behavior of $Mn_xO_y$ nanostructures as MRI contrast agents. This method, based on the reduction of $KMnO_4$ under sonochemical conditions, allowed us to prepare a series of structurally related samples, that turned out to show completely different properties as MRI CAs. The characterization of $Mn_xO_y$ species is challenging due to the availability of oxidation states of Mn. Complementary techniques such as Raman, SAED and EPR were used to unequivocally assign the structure of each sample. Then the behavior of these samples as CAs was studied via relaxivity measurements and MR imaging at the two most relevant clinical fields (1.5 and 3.0T). The cytotoxicity and performance of these samples in cell culture was also explored.

**Results and Discussion**: The results show that three different species are available from this simple reaction, MnO, $MnO_2$ and $Mn_3O_4$. The predominance of each material can be traced back to the proportion of reducing agent used in the preparation of the samples. From relaxometric and MR imaging studies $Mn_3O_4$ was discarded as CA. the pther two species presented different performance in MRI. While MnO nanostructures behaved as classic CA creating signal from the beginning, $MnO_2$ nanostructures behaved as responsive CAs, failing to produce signal as synthesized but enhancing their performance under biologically relevant redox conditions.

**Conclusions**: A simple and versatile reaction allows to prepare different natures of manganese oxide nanostructures. Some of these structures behave as classic MRI CAs, while others behave as responsive CAs opening the door to next generation on demand imaging.

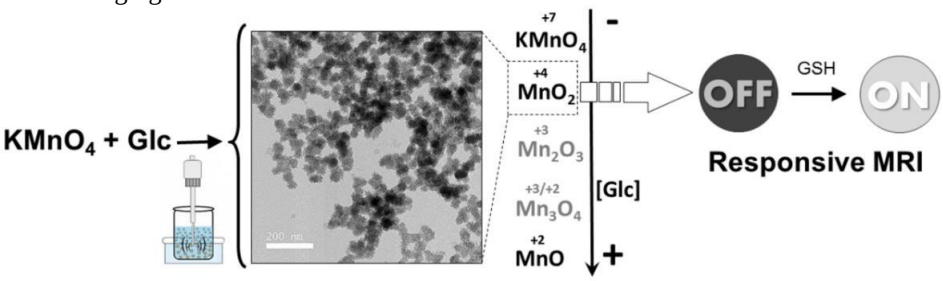


*Figure 1*. Scheme representation of the aim of this project.

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**PC-80**

# A nanotechnological approach for the serological diagnosis of Contagious Bovine Pleuropneumonia

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**Introduction**: Contagious bovine pleuropneumonia (CBPP) is a respiratory disease of cattle caused by *Mycoplasma mycoides* subsp. *mycoides* small colony type (*MmmSC*). It is currently one of the most important transboundary diseases, with great social-economic impact. The disease is endemic in Africa, having been eradicated elsewhere [1,2]. The complement fixation test (CFT) is used in the serological diagnosis of CBPP [2] and it is recommended by the OIE [3] as the reference test for surveillance and tracking of the disease, being INIAV an International Reference Laboratory for Contagious Bovine Pleuropneumonia diagnosis. However, its implementation is very laborious, time consuming and requires the use of several reagents from animal origin such as the complement (derived from guinea pig serum), the hemolytic system (red blood cells - RBC - and hemolysin from sheep and rabbits respectively), positive and negative test-controls (provided respectively from healthy bovines and bovines infected with CBPP) [3,4]. In addition, short storage-time and high differences from batch to batch of RBC are other disadvantages of CFT. In the present work, we have used a nanotechnological approach to innovate the CFT using liposomes for the serological diagnosis of CBPP. Liposomes, depending on the lipid composition, are able to promote extensive interactions with the complement system proteins which, in their natural environment, cause destruction of invading cells. Liposomes resemble very closely to cells and consequently are susceptible to be lysed by complement proteins, thus replacing the hemolytic system [2]. In the presence of a serum sample without *MmmSC* antibodies, the free complement will be activated by lipossomes and, thus, induce their disruption. On the the other hand, in case of a positive sample, the antigen/antibody and complement complex will unable the disruption of liposomes as the complement will not be free to react.

**Materials and Methods:** The liposomes used in the present work were prepared by the dehydration-rehydration method [5], followed by an extrusion step to reduce and homogenize their mean size. In order to achieve liposomes with specific physicochemical properties, namely thermotropic properties and surface charge, liposomes were prepared with different lipid constituints. The interaction of liposomes with complement serum proteins was assesed by spectrophotometry. The diagnosis of samples from animals with or without CBPP was performed by the classical CFT and using liposomes instead of hemolytic system comparatively.

**Results and Discussion:** Overall, results evidenced that among the liposomal formulations that were tested, negatively charged liposomes demonstrated higher complement activation (in comparation to neutral or positively charged liposomes), being this behavior influenced by the percentage of phosphatidyl glycerol (PG) used in the lipid composition. On the other hand, it was observed that the presence of cholesterol (CHOL) did not benefit the intended behavior. The influence of liposome mean size on the complement activation was also evaluated. Although guinea-pig complement activation has been found to be independent of lipid vesicle mean size, it was observed that lower mean size liposomes presenting fewer lipid bilayers are easily disrupted during the formation of the liposome-complement complex and so more adequate for replacing the hemolytic system used in the CFT.

**Conclusions**: The development of an alternative CFT replacing RBC and hemolysin by liposomes has been initiated. Small and negatively charged liposomes seem to be the best option to achieve our goal. The advantages of using liposomes with magnetic properties will constitute further studies.

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**PC-81**

# Targeted polymeric nanoengineered HBsAg DNA vaccine

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**Introduction:** Global Hepatitis B prevention through vaccination and the successful virus (HBV) clearance from the host are the most challenging achievements envisioning eradication [1,2]. Vaccination using plasmid DNA (pDNA) encoding HBV proteins is a hypotethical promising strategy, inducing both humoral and cellular mediated immunity, benefic for both approaches. Low cost of production and increased stability also renders it very appellative for developing countries, where hepatitis B is a major problem [3]. pDNA packaging and protection from degradation inside the host are easily overcome by cationic polymer complexation, such as poly[2-(dimethylamino) ethylmethacrylate] (PDMAEMA) and poly(β-amino ester) (PβAE) [4]. Antigen-specific immune response is frequently hard to achieve due to the difficulty to transfect antigen presenting cells. Thus, this study proposed the use of PDMAEMA:PβAE polyplexes as the vehicle of a pDNA vaccine encoding the hepatitis B surface antigen (HBsAg – pCMV-S) in combination with either a soluble (Glu) or a particulate (GPs) form of β-glucan, as a cell-specific targeting adjuvant.

**Materials and Methods:** The expression of luciferase and GFP reporter genes were used to determine the *in vitro* transfection activity in COS-7 cells and then confirmed in murine macrophages (RAW 267.4). Polyplex internalization was also evaluated in RAW 267.4 cell line. Vaccination studies were performed in C57BL/6 following four subcutaneous administrations of 40 µg of pCMV-S with or without 400 µg of either Glu or GPs. Serum HBsAg-specific IgG was evaluated.

**Results and Discussion:** PDMAEMA:PβAE polyplexes resulted in a luciferase activity 200 or 100-fold higher than the positive control (PEI polyplexes) in COS-7 and RAW 267.4 cells, respectively. Moreover, the presence of GPs induced even higher luciferase expression than polyplexes alone. Interestingly, although no differences were observed in polyplex internalization rate, the presence of GPs enhanced GFP transfection efficiency in RAW 267.4 macrophages. Subsequently, *in vivo* vaccination schedule was effective for 40 % of the mice.

**Conclusions:** PDMAEMA:PβAE polyplexes together with GPs have enhanced transfection activity in murine macrophages and resulted in an encouraging HBsAg seroconversion following subcutaneous vaccination. These results show the potential of this nanosystem to be used as a DNA vaccine, motivating further studies to evaluate the output of other delivery routes or pDNA.

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**PC-82**

# Combination of liposomes and serum albumin into albusomes, a green pharmaceutical vehicle suitable for vaccine formulation

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## Introduction

A rational design of vaccines requires the consideration of several aspects besides the adequate delivery of the corresponding antigen; vehicle safety and inmuno-stimulatory adjuvants are important. Liposomes have been proposed as potential carriers for vaccines due to biocompatibility and versatility and because these may be incorporated to parenteral and no parenteral dosages forms. On the other hand, albumin nanoparticles have gained considerable attention due to albumin transport capacity and these have been proposed for the development of multifunctional theranostic agents [1]. Combination of liposomes and albumin into micro/nanoparticles called albusomes seems to be an interesting strategy for vaccines formulation since this provides a multifunctional vehicle suitable for sustained release of antigen together with potential inmuno-stimulatory effects. The aim of this study was to preprare and characterize albusomes from liposomes that include dimethyldioctadecylammonium (DDA) or D-α-Tocopherol polyethylene glycol succinate (Vit E TPGS) and to compare them as potential vehicles for vacine formulations.

## Material and Methods

Two types of liposomes (A, B) were prepared by direct sonication of components according to a previously described method [2]. For type A liposomes, egg phosphatidycholine (EPC) and cholestyerol (CH) were mixed with a water solution of Vit E TPGS 0.25% w/w. For liposomes type B, EPC, CH and DDA were mixed with Milli-Q water. The mixtures were sonicated for 30 min in an ultrasonic bath (50 Hz) at 55 ± 2ºC. Sonicated samples were kept at room temperature for 60 min for liposomes stabilization. An aliquot of resulting samples was separated for liposomes characterization (size and zeta potential) and the rest was used to prepare albusomes. Liposomes type A and B were mixed with bovine serum albumin (BSA) and pH of resulting samples was adjusted to 4.0 and 7.0, respectively. The mixtures were kept for 20 h at 4ºC in a shaking water bath (Unitronic OR Selecta P). The resulting flocculates were redispersed by agitation; an aliquot of each sample was taken for albusomes characterization (size and zeta potential) and the rest was lyophilized.

## Results and Discussion

Liposomes type A exhibited a mean zeta potential value of -37.63±0.67 mV, while type B showed a mean zeta potential value of 62 ± 2 mV. Size analysis by dynamic light scattering revealed effective hydrodynamic diameters of 90.19 nm (PDI=0.33) and 57 nm (PDI=0.29), for anionic and cationic liposomes, respectively. Zeta potential of albusomes showed to be dependent of albumin amount. As albumin increases, liposomes charge is progressively neutralized by opposite charge of the protein. Albusomes showing zeta potential within a wide range were obtained. Effective hydrodynamic diameters showed mean values > 3 µm and high PDI in all cases. Characteristics of liposomes and albusomes obtained with 0.5% BSA are shown in table 1.

| | Liposomes type A | Liposomes type B | Albusomes type A | Albusomes type B |
|---|---|---|---|---|
| Size, nm (PDI) | 90.19 (0.33) | 57 (0.29) | >3000 | >3000 |
| Zeta potential, mV | -37.63 ±0.67 | 62 ±2.0 | 2.59±0.13 | 10.6 ±4.1 |
| Composition | EPC, CH, Vit E TPGS | EPC, CH, DDA | EPC, CH, Vit E TPGS , BSA | EPC, CH, DDA, BSA |

*Table 1.* Characteristics of liposomes and albusomes with 0.50% BSA

These albusomes might be selected as the most suitable due to the following: the moderate cationic charge mediates initial contact with the cell surface, while avoiding the toxicity produced by higher cationic charge [3]. The particles diameter >3 µm facilitates the depot effect and sustained release of entrapped liposomes, both mechanisms related to immune stimulation. Due to their multifunctional character, these particles may co-deliver antigen and adjuvants located at the liposomes core, lipid bilayer or albumin-bound. Optimum size and charge for the particles to elicit adaptive immune responses is controversial and likely depends on the type of vaccine. The proposed vehicle allows inclusion of different sized cationic or anionic liposomes into particles with positive or negative zeta potential.

## Conclusion

Tailored particles for achieving different immunity pattern may be obtained by combination of liposomes and albumin into albusomes, a green vehicle produced in absence of organic solvents and toxic chemical agents. *In vivo* assays to evaluate adaptive immunity and vehicle safety are, however, need to confirm or reject this hypothesis.

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**PC-83**

# Tailored lipidic nanoemulsions to increase the immunogenicity of pancreatic cancer

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## Introduction

Pancreatic cancer has a very poor prognosis, with more than one-half of cases diagnosed at a distant stage, for which 5-year survival is 2% [1]. Therapeutic strategies involving the manipulation of the immune system to defeat tumours are gaining momentum in cancer research, but they have failed to demonstrate any objective responses in patients with pancreatic cancer [2], since it is considered a non-immunogenic malignancy [3]. Stimulation of the immune system can be achieved by conventional antitumor agents able to promote an immunogenic cell death (ICD) [4-6]. We propose the development of lipidic self-emulsifying nanoemulsions for delivery of gemcitabine to pancreatic cancer cells, with the purpose of inducing ICD and stimulate the immune system to increase the immunogenicity of pancreatic tumors.

## Materials and Methods

Self-emulsifying nanoemulsions were synthesized upon adjusting the ratio of surfactant (Kolliphor® HS15) weight to oily phase weight (Labrafac® lipophile WL 1349), SOR, and the ratio of oily phase weight to total weight, SOWR, to 60% and 20% respectively. 4-(N)-lauroyl gemcitabine (GEMC12) was subsequently encapsulated, and the encapsulation efficiency determined by UPLC (Waters Acquity UPLC). Human pancreatic MiaPaca-2 cells were selected to determine calreticulin (CRT) exposure and HMGB-1 translocation, cardinal signs of immunogenic cell death, by confocal microscope, after incubation of the cells with our formulation. ATP release was also evaluated using ATP Determination Kit (Molecular Probes, Invitrogen). Finally, activation of dendritic cells, generated from monocytes isolated from healthy donors, was determined using conditioned cell culture medium collected from MiaPaCa-2 cells previously treated with the formulations.

## Results and Discussion

Nanoemulsions were prepared by self-emulsification, in absence of organic solvents, having a mean size of 50nm, a low polidispersity index (0.1), and a neutral zeta potential (-1,3mV). GEMC12 was successfully loaded into the nanoemulsions, with 100% association efficiency, leading to a final loading content of 5% with respect to the total weight of the components. The efficient internalization of the nanoemulsions in pancreatic MiaPaCa-2 cells was demonstrated upon incubation of fluorescently labeled nanoemulsions with the cells for 2h, and observation under the confocal microscope. MiaPaCa-2 cells treated with GEMC12-loaded nanoemulsiones, had high levels of exposed CRT, superior to that observed in untreated control cells and in cells treated with the positive control, the drug mitoxantrone. Similarly, translocation and release of the nuclear protein HMGB-1 was observed with higher intensity in cells treated with GEMC12-loaded nanoemulsiones, in relation to the negative and positive controls. With respect to ATP secretion, other of the DAMPs (Damage-Associated Molecular Patterns) related to ICD, results were cohereny with the previous findings. Most importantly, when dendritic cells were incubated with conditioned culture medium of pancreatic cancer cells treated with GEMC12-nanoemulsions, a significantly elevated expression of CD86 was induced, indicative of activation of the cells, a fact that proved the ability of the proposed nanosystems to initiate an immunre response.

## Conclusions

We have satisfactory developed a nanoformulation able to deliver a gemcitabine derivate to pancreatic cancer cells, induce immunogenic cell death, and stimulate the immune system. Next experiments will be durected to prove the potential of this approach in animal models of the disease.

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**PC-84**

# Chitosan-based Nanoparticles for Gene Delivery

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**Introduction:** Gene therapy presents great advantages for the vaccine field, since DNA vaccines are expected to generate both humoral and cellular immune responses, supporting prophylactic, as well as therapeutic vaccination strategies [1]. Comparatively to viral gene delivery systems, non-viral vectors are a safer alternative, with minimal side effects, highly stable and susceptible to physical/chemical modifications [2]. Among these gene carriers, the cationic polymer chitosan has gained attention as a non-viral gene delivery system due to its biodegradability, biocompatibility, low toxicity and ability to interact with the negatively charged DNA molecules, easily forming polyplexes [3,4]. Nevertheless, some authors claim that the interaction between the opposing charges of the amino groups of chitosan and the phosphate groups in the nucleic acid originates very stable complexes, precluding the unloading of the DNA and causing low transfection [2,5]. To overcome this limitation, we hypothesized that the addition of casein, as well as glucan, into the nanoparticle's structure would facilitate a proper gene transfer. The work herein presented aims to produce Chitosan-α-Casein Nanoparticles (ChiCas NPs) and to test their ability as a gene delivery system, using preliminary *in vitro* transfection studies.

**Materials and Methods:** The ChiCas NPs production was based on the electrostatic interaction between the two molecules, by adapting and optimizing a method previously decribed [6]. NP size and zeta potential were analyzed by Dynamic Light Scattering and Electrophoretic Light Scattering, respectively, and the NPs were also submitted to stability tests over the time and in different media. The DNA loading was achieved using two different techniques: the DNA adsorption to the NPs' surface and the incorporation of the DNA within the NPs. Similarly to ChiCas NPs, a physical characterization of the complexes was performed. The complexes were also submitted to a DNA complexation assay in agarose gel. Finally, to evaluate the suitability of the polyplexes to mediate gene transfer, preliminary *in vitro* transfection studies were carried out in COS-7 cells.

**Results and Discussion:** ChiCas NPs presented small and homogenous sizes (around 280 nm with polydispersity index < 0.23), matching the results obtained by other reports [6]. They also presented a highly positive zeta potential (near + 42 mV) and great long-term stability when in suspension, even at room temperature. Relatively to the complexes, they showed similar sizes to ChiCas NPs, with zeta potentials slightly lower (between + 31 mV and + 37 mV). The results from DNA complexation assays confirmed the successful production of NPs-DNA complexes, either with DNA adsorbed to the NPs surface, or with DNA incorporated within the NPs. Although the efficient production of NPs-DNA complexes, preliminary studies with several NPs-DNA formulations and ratios suggested a low transfection efficiency, comparatively to the positive control. Nonetheless, we could verify that the delivery systems with lower NPs:DNA ratios presented better transfection results than higher NPs:DNA ratios. We further verified that the addition of glucan or Tween 80® to the NPs' structure did not represent a meaningful improvement of transfection activity.

**Conclusions:** To conclude, chitosan nanoparticles have great technological potential to produce DNA-based vaccines. The results presented suggest that ChiCas NPs have high ability to form complexes with DNA, however, further investigation is needed to optimize ChiCas NPs: DNA ratios for successful plasmid DNA-based vaccination.

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**PC-85**

# Immune checkpoint blockade in melanoma by new targeted Dox immunoliposomes

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**Introduction:** Liposomes are known to be drug carriers with a potential application in drug and gene delivery, including anticancer agents [1]. However, the low bioavailability in tumor tissue limited the therapeutic efficacy. This can be overcomed with targeted liposomes, which present a specific affinity for antigens and receptors expressed in the membrane of tumor cells [2]. PD-L1 (Programmed cell Death Ligand 1) up-regulated in solid tumors, engages PD-1, a T cell receptor, representing an axis involved self-tolerance, but also in tumor resitance developments in immunotherapy cancer treatments [3]. The blockade of this axis using monoclonal antibodies (mAbs) against PD-1/PD-L1 may promote the activation of the immune system to inhibit tumor proliferation [4]. Hence, the combination of these mAbs with liposomes has led to the development of immunoliposomes [5], which are able to specifically reach cancer cells, delivering their content there. In that sense, Doxorubucin (Dox) acts mainly by intercalating DNA and inhibiting topoisomerase II, but its immunogenic properties contribute to enhance the antitumor efficacy. Moreover, its low release profile has led to the development of thermosensitive liposomes (TSL). This formulation releases rapidly the cargo atat 42ºC [6,7]. Therefore, the aim of this project was the development of PD-L1 targeted Dox TSL and its pharmacokinetic/pharmacodynamic evaluation in an *in vitro*/*in vivo* platform using a melanoma cell line.

**Materials and Methods:** Dox liposomes (LPDOX and TSLDOX) were prepared by the film-hydration method [8], combined with a pH gradient [9]. PD-L1 targeted Dox liposomes (LPDOXFab' and TSLDOXFab') were formulated following the post-insertion method. DSPE-PEG2000-Mal micelles were prepared in hepes buffer and incubated with anti-PD-L1-Fab' fragments. Afterwards, micelles Fab'-conjugated were incubated with preformed LPDOX and TSLDOX. Formulations were characterized according to particle size, PDI and Zeta, drug encapsulation and ligand coupling efficiency. Drug release profile at pH 7.4 was measured for 24h. *In vitro* studies were carried out in B16 OVA melanoma cell line. Cytotoxicity ($IC_{50}$) was obtained after 4 h exposure and Dox cell uptake was studied after 4, 8 and 24 h by FACS and confocal microscopy.

**Results and Discussion:** Post-insertion method allowed us to develop PD-L1 targeted Dox liposomes (LPDOXFab' and TSLDOXFab'). Particle size was approx. 130 nm associated with a low PDI. The EE was higher than 90% whereas ligand conjugation was around 40%. Accumulative drug release for both types of formulations was around 10% after 1h in FBS at 37ºC, while at 42ºC was higher than 90% for TSLDOX and TSLDOXFab'. Thermosensitive liposomes reached the higher cytotoxicity at 42ºC, which was in accordance with the results obtained with FACS and confocal microscopy at 42ºC. *In vivo* experiments, to evaluate the immune response activation have been done in order to explore the advantages that may provide the immune checkpoint targeting in combination with another drug acting immunogenic death and the hyperthermia.

**Conclusions:** PD-L1 targeted Dox liposomes have been successfully developed by the post-insertion method. *In vitro* studies revealed a higher activity of thermosensitive liposomes at 42ºC. Experiments studying the role of the immune response assayed in the *in vivo* model by blocking PD-L1 are currently ongoing.

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**PC-86**

# Immunotoxicity of chitosan salts and nanoparticles

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**Introduction** – Chitosan and its derivates have been investigated for many diverse medical applications. However, there is not yet a solid correlation between specific chitosan characteristics like the deacetylation degree (DD), molecular weight (MW) or size of the chitosan particles and its effects [1] on the immune system, nor a solid immunotoxicity profile determined by the ICH-S8 guideline. It has been claimed that chitosan is able to stimulate the cells from the immune system. Many pathways have been described, like the activation of dendritic cells and macrophages through the NLRP3 inflammasome [2] or the cGAS-STING [3], and also the activation of T cells with consequent secretion of cytokines [4].

**Material and Methods** – This is a review work, based on peer reviewed publications (publication date until 1997), using the following keywords: *immunotoxicity* AND *nanoparticles AND chitosan*. The inclusion criteria were publications that included information about, chitosan and chitosan particle characterization (DD, MW, particle zeta-potential, PDI, particle size) and purity. The inexistence of indication about the result of the LAL test (LPS contamination) and the inexistence of controls in the methods were exclusion criteria.

**Results and Discussion**– The last condition was the reason of the exclusion of a great part of the studies. Chitosan proved to be an activator of the inflammasome NLRP3 after a phagocytic step, via lysosome rupture, but only with a priming step with a TLR-agonist [2]. Chitosan associated with some antigens showed to be able to promote antigen-specific T cell differentiation in Th1, Th2 and Th17 with the consequent secretion of cytokines and immunoglobulins [4]. None of the studies claimed anti-inflammatory properties of chitosan.
Some results were discordant, especially the generation of a certain Th immune profile, but the discrepancies might be due to different types of chitosan used, administration routes, and more important, different antigens. There is still a lack in chitosan characterization that would allow them to be correlated with the effects in immune system. However, some parameters were well defined, namely the size of the particle, being the smallest size more effective; and the charge of the particles, being the positive the ones that activate the inflammasome, which means that a higher deacetylation degree induce a stronger response. The ICH-S8 guideline was never mentioned, but some tests from the articles were into its orientation, namely the gross pathology of lymph nodes, antibody responses and leucocytes total and differentiated count.

**Conclusion** – Chitosan showed to be a modulator of the immune system. There are still many studies to perform to reach a final conclusion, and they should be targeted to the ICH-S8 guideline. Future chitosan applications on pharmaceutical field should consider the possibility of the activation of the immune system and so allowing a safe-by-design of new medicines.

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**PC-87**

# Multifunctional polypeptide-based platform as an anti-cancer immunotherapeutic approach for melanoma

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**Introduction**: Melanoma is the most dangerous type of skin cancer and novel treatments are needed [1]. Therefore, alternative therapeutics should be devised isolated or in combination with targeted immunotherapies, to efficiently stimulate specific anti-tumor responses. Branched polypeptides exhibit advanced engineered complexity and unique structural properties inaccessible to linear polymers that make them ideal constructs to be employed as DDS with enhanced biological performance [2]. Branched nanoscale systems have the ability to activate immune cells, as dendritic cells (DC) and natural killer cells, constituting potential platforms to modulate the release profile of loaded molecules, including tumor associated antigens (TAA), adjuvants and drugs [3]. This work aims to evaluate the *in vivo* anti-tumor efficacy of peptide-1 -conjugated polypeptide (pept-1-BP), with special emphasis on their impact on the modulation of the immune cell function.

**Material and Methods**: BP were synthesized and conjugated with the peptide-1 (pept-1-BP) presenting a terminal cysteine via reductive-sensitive disulfide linker. To address *in vitro* and *in vivo* studies, Cy5.5 was conjugated to the platform. To evaluate the effect of the conjugate on melanoma tumor growth, B16.F10 cells were implanted subcutaneously into 8-week-old C57BL/6 mice. At day 7, animals were injected with two doses (1-week apart) of 100 µL of PBS, Toll-like receptor ligands CpG (20 µg/dose) and Poly I:C (40 µg/dose) in solution, BP backbone (575 µg/dose) and pept-1-BP (575 µg/dose) mixed with adjuvants. Every 2 days, weight of the animals and tumor growth was followed. At day 21, mice were sacrificed and tumor and lymph nodes were collected. A cell suspension from tumor cells and lymph nodes of each animal was prepared and analyzed for infiltrated lymphocytes (CD45.1, CD3e, CD8α, CD4, CD107, PD-1, CTLA-4) by flow cytometry.

**Results and Discussion**: The BP presented a size of 81.86 ± 1.63 nm and a zeta potential of -45.10 ± 1.72 mV, while pept-1-BP showed a mean average diameter of 104.1 ± 2.21 nm and a zeta potential of -24.8 ± 0.64 mV, with a pept-1 loading efficiency of 8.7% (w/w).
*In vivo* results showed a significant reduction of tumor size in conjugate treated mice compared with the other groups. In addition, the FACS analysis of infiltrating lymphocytes within tumor site evidenced an increased expression for CD4, CD8α and NK cells and no major differences for remaining markers.

**Conclusions**: Overall, our results support the promising use of this novel conjugate for the delivery of TAA, as an effective anti-tumor immune therapeutic strategy able to decrease and control of tumor growth.

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**PC-88**

# Chitosan nanoparticles as drug delivery systems: focus on immunotoxicological features

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**Introduction:** Nanoparticles (NPs) conquered an important role in many areas such as drug delivery. The number of studies that include them has grown in the last years [1]. Nonetheless, the correlation between their properties and their effects on the immune system is poorly understood. Furthermore, NPs can interfere with the traditional testing assays developed for testing the biological effects of chemicals, thereby additional attention should be given to the selection of appropriate methods and controls to avoid misinterpretations. So, it is necessary to develop trials and guidelines for the immunotoxicological evaluation of nanomaterials to develop safe-by-design (SbD) NPs, minimizing the probability of health and environmental risks associated with this innovative drug delivery systems.
Chitosan is a natural polymer that has proven to be a very versatile material, with interesting properties as stability, biocompatibility and biodegradability [2]. Therefore, chitosan has been explored regarding many therapeutic applications, including drug delivery vehicle. However, its immunotoxicological evaluation is poorly systematized and even some studies reveal contradictory results. Importantly, in several reports, different physical and chemical characteristics of the chitosan NPs, like the polymer molecular weight or deacetylation degree, the NPs size or endotoxin contamination are not reported, and they can directly influence the immunotoxicity of the delivery system. Considering all these variables and the extensive application of Chitosan NPs in the drug delivery field we consider important to study its immunotoxicity as a case study, to establish methods for testing immune function effects that can be adapted to other nanomaterials. In this study, we use two types of NPs, with a size of approximately 100 nm and different deacetylation degrees (DD) (70% and 95%). These will allow the further development of a set of methods for testing immunotoxicity of NPs, allowing a safe-by-design NP development.

**Materials and Methods:** Chitosan NPs are prepared using a coacervation method with Chitosan 0.1% and Sodium Tripolyphosphate (TPP) 0.16%, a method adapted and optimized from a protocol described by L. Qi and coworkers [3]. After production, NPs are isolated by centrifugation using Vivaspin 20 centrifugal concentrator (MWCO 300KD). Preliminary studies of toxicity were performed in peripheral blood mononuclear cells (PBMCs) isolated from human blood using a MTT cell viability assay.

**Results and Discussion:** The average size of the NPs obtained are 96.2 ± 2.2 nm and 133.0 ± 7.5 nm with a chitosan DD of 70% and 95%, respectively. The polydispersity indexes of 0.287 ± 0.009 and 0.283 ± 0.011 show a homogeneous size distribution of the NPs. The zeta potential of chitosan DD of 70% and 95% is 8.3 ± 0.7 mV and 11.1 ± 3.5 mV, respectively. Size and zeta potential of the Chitosan NPs were dependent on the chitosan DD. The more deacetylated chitosan yields NPs of larger mean size and higher zeta potential. After successfully isolating the NPs, cytotoxicity studies showed that at concentrations below 156.25 µg/mL the Chitosan NPs DD70% do not have cytotoxic effects in PBMCs. On its turn, in the case of chitosan nanoparticles DD95% there is no significant decrease in cellular viability at concentrations below 78.13 µg/mL. These results show that Chitosan NPs toxicity correlates with chitosan DD. Chitosan NPs with lower DD exhibits less cytotoxicity.

**Conclusions:** This work illustrates the influence of the chitosan NPs characteristics in the toxicity induced in immune system cells isolated from the blood of healthy donors. These results together with further studies will contribute to develop a knowledge base and guidelines to implement the Safe by Design approach for nanobiomaterials, with focus on polymeric drug delivery systems.

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**PC-89**

# Poly(anhydride) nanoparticles loaded with peanut extract aimed to treat peanut allergy

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## Introduction

Peanut allergy is an adverse reaction to peanut proteins, mainly Ara h1-16. This allergy is currently affecting 3-4% of global population [2] and there is no available treatment. As a result, great efforts are being made in order to find and effective treatment. Among others, allergen immunotherapy has been pointed. This therapy is based on the continuous administration of gradually increasing amounts of the allergen in order to promote tolerance to the allergens and provide protection against the allergic symptoms [3]. It has been pointed, that the use of polymer-based nanoparticles could act as a potential adjuvant due to their ability to be captured and internalized by cells of the GALT [4].

We propose the use of poly(anhydride) nanoparticles (NP) as a immunoadjuvant in oral therapy. These particles have been identified as innate immunity inducers in previous experiments, activating the complement system and Toll-like receptor (TLRs), mainly TLR2 [5].

## Material and Methods

Nanoparticles loaded with peanut extract (PE) were prepared by a solvent displacement method and dried by spray-drying and were characterised in terms of physico-chemical properties (mean size, PDI, zeta potential, surface hydrophobicity), *in vitro* release kinetics, and biodistribution. Moreoverm this nanoparticles were tested in a murine model of peanut anaphylaxis. Animals (CD-1 mice 20±1g) were sensitized against peanut by oral gavage with peanut proteins and cholera toxin. In order to increase the sensitization tape stripping and percutaneous sensitisation with peanut extract were performed. Once sensitised, animals were vaccinated with either free peanut extract or the nanoparticle based vaccine (NP-PE) and challenged to provoke an anaphylactic shock.

## Results and Discussion

It was observed that the encapsulation of PE in nanoparticles decreased significantly the mean size and negative zeta potential of the resulting nanocarriers. Furthermore, the PE loading was calculated to be close to 14 µg per mg nanoparticles, with an encapsulation efficiency of about 50%. It is worthy of notice that the hydrophobicity degree of the surface of PE-loaded nanoparticles strongly decreases, suggesting PE is at least partially bound to the surface of the nanoparticles.

Besides, the *in vivo* efficacy assay, NP-PE were able to diminish the anaphylaxis symptoms induced in mice by the ip administration of 2 mg PE (i.e. hypothermia, piloerection, low mobility) an protect the animals against the anaphylactic shock, as 40% of animals survive 60 minutes after challenge.

## Conclusions

Taken together, our findings indicate poly(anhydride) nanoparticles are able to load and carry a peanut extract, facilitating their biodistribution within the gut, including Peyer`s Patches. In addition, this nanoparticle-based formulation offers a promising protection against the effects induced by an anaphylactic shock in peanut sensitized animals, opening the door to a future clinical employment.

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**PC-90**

# Development of biodegradable rifampicin microparticles for the prevention of infections after orthopaedic replacement surgery

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**Introduction**.
The infection after the placement of prosthesis in orthopedic surgery is a devastating complication that implies an increase of the morbimortality of the patient. Recent studies warn of an incidence of more than 2% and will multiply by ten in the coming years. The cost of each revision ranges from 80,000 to 95,000 €, a circumstance that, together with the morbidity and mortality associated with this pathology, makes it necessary to develop new strategies that improve the effectiveness of current preventive treatments. The main responsible of the prosthetic infections is *Staphylococcus aureus* and rifampicin, in combination with other antibiotics, is considered the treatment of choice currently. The aim of the present study is to develop a formulation of microspheres of rifampicin that, after intra-articular administration, release the drug in a prolonged manner, and thus may be effective in preventing prosthetic infection.

**Materals and Methods.**
Microparticles were prepared by the solvent evaporation technique, using as polymer different types of poly-lactic-co-glicolic acid (PLGA): 502, 502 H, 504 y 504 H (Evonik Industries, Germany). Two different drug:polymer ratios, viscosities of the external aqueous phase, and organic solvents were tests in order to improve drug loading, keeping microparticle size between 10-50µm. Simulated synovial fluid was used to determine the release of rifampicin from microparticles. For the design of the release assay, the stability and the solubility of rifampicin at 37ºC in the release medium were previously evaluated. All rifampicin cuantifications were done by a previously validated HPLC method.

**Results and Discussion.**
With all the polymers and microencapsulation conditions rifampicin microparticles with mean diameter ranging from 20 to 35nm were obtained, suitable for their injection with a conventional needle and sufficiently large to avoid their fast clearance by macrophagous after their intra-articular administration. The H-serie polymers (with encapsulation efficiencies > 15%) are better encapsulation materials for rifampicin than non-H (with ecapsulation efficiencies < 5%), probably because of the ability of the drug to interact with the free carboxylic rests of the PLGA chains in those polymers. With respect to the Mw, higest encapsulation efficiencies (>40%) were obtained with the 502H polymer whereas with 504H polymer the EE was <20%. When dicloromethane was substituted by ethyl acetate as internal organic solvent, an increase of the EE of more than 10% was detected. However, the effect of the PVA concentration in the external aqueous phase on the EE, could not be clearly established. Finally, when the drug:polymer ratio was doubled, a slight decrease in the EE was observed. The best formulation of microparticles presented an EE of 57%, and mean diameter of 28µm with a polidispersión index of 0.33; being the process yield of 80%.
Acoording to the literature, rifampicin is poorly soluble in water. However, in PBS 7.4 with hyaluronic acid rifampicin showed a solubility of 1.2mg/mL, which allowed to performance the release assay with 10mg of microparticles in 5mL of medium, keeping sink conditions. Also according to the literature, rifampicin degrades to both basic and acid pHs, being the pH of maximum stability close to 7. Despite working at maximum stability pH, rifampicin underwent intense degradation in the release medium which led to the calculation of the amount of drug released from the data of rifampicin remaining into the microparticles. Rifampicin was released from the microparticles in a prolonged manner for 8 days.

**Conclusion.**
The formulation of microparticles of rifampicin developed shows characteristicas *in vitro* that make it a good alternative for the prevention of prosthetic infections. Next *in vivo* studies will have to confirm the efficacy of this new formulation.

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**PC-91**

# Moxifloxacin-loaded acrylic intraocular lenses for prevention of postoperative endophthalmitiss: *in vitro* and *in vivo* performance

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## Introduction

Conventional eye drops administration is commly prescribed for post-cataract surgery endophthalmitis prophylaxis. In this work, the main goal was to develop intraocular lenses (IOLs) loaded with moxifloxacin (MFX) that could ensure a controlled release of antibiotic for an adequate period of time and thus overcome the common constraints associated with the topical antibiotic administration after cataract surgery as uncertain patient compliance, drug wastage and side effects.

## Materials and Methods

Hydrophilic acrylic IOLs were loaded with MFX by soaking in a drug solution at 60ºC, autoclaved in the soaking solution and stored for 2 months. The lenses were characterized regarding surface morphology/topography by scanning electron microscopy (SEM) and atomic force microscopy (AFM), wettability, transparency, refractive index, dimensional control, haptic compression force and injectability with an IOL inserter. The MFX *in vitro* release profile was obtained using a microfluidic cell designed to mimic the hydrodynamic conditions of the aqueous humor. Solutions collected were tested against *S. aureus* and *S. epidermidis*. *In vivo* implantation was done in six 8-week-old New-Zealand rabbits, with clear lens extraction bilaterally performed through phacoemulsification. Rights eyes (RE) were systematically implanted with MXF-loaded hydrophilic acrylic IOLs. Left eyes (LE) were implanted with the unloaded same intraocular lens model. Eyedrops of dexamethasone were applied in both eyes during the experiment, while MFX eyedrops was only administered in the control eye (LE). Slit-lamp examination with scoring for intra inflammatory response was performed and an aqueous humor sample was collected after one week.

## Results and Discussion

The incorporation of the antibiotic in the IOLs produced negligible effect on the evaluated properties: transmittance in the 500-700 nm range decreased from 93 ± 2% to 90 ± 2%, the water contact angle from 44 ± 5° to 39 ± 4°. SEM and AFM images did not show significant changes in the surface morphology/topography. *In vitro* drug release experiments led to drug concentrations above the MICs for both microorganisms during more than 15 days and microbiological tests showed that the drug was active. Loaded IOLs showed to be stable, non-toxic when implanted, and did not lead to posterior capsular opacification. Scoring of inflammatory reaction was clinically low. A week after surgery, the MXF concentration in the aqueous humor was ~523 ng/mL and, thus, close to the value found in the eyes treated by conventional topical application (~488 ng/mL).

## Conclusions

The incorporation of the drug and the sterilization procedure did not impair the IOLs biomaterial properties. MFX-loaded IOLs prepared following the described methodology showed to be safe and as effective as the commercial topically applied MXF eyedrops on rabbit's eyes after clear lens extraction. The results indicate that the studied MFX-loaded IOLs are promising devices for the prophylaxis of endophthalmitis and encourage further research.

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**PC-92**

# Antibacterial LbL coatings to control drug release from ophthalmic lens

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## Introduction

The use of soft contact lenses (SCL) to correct vision problems has improved the quality of life of millions of patients. However, the surface of these devices is also prone to microbial pathogens adhesion which has a central role in causing infections [1]. One efficient way to reduce bacterial cell adhesion is to coat the surface of the ophthalmic lenses using the layer-by-layer (LbL) technique [2]. Recently, we described the use of chitosan (CHI) and sodium alginate (ALG) based multilayers to successfully control the drug release from ophthalmic lenses [3]. This work seaks to study the possibility of using the combination of different polyelectrolytes having antibacterial properties to form multilayers that may simultaneously control the drug release from SCL and ensure reduction of bacterial growth.

## Materials and Methods

In this work a diffusion barrier was created to control and retard the release of three ophthalmic drugs moxifloxacin hydrochloride (MXF), chlorhexidine diacetate (CHX), and diclofenac sodium salt (DIC). A silicone based hydrogel (TRIS/NVP/HEMA, 40:40:20 w/w) was produced and coated by a layer-by-layer surface modification process with antibacterial polyelectrolytes. Three coatings showed interesting characteristics, which involved the combination of alginate (ALG), chitosan (CHI), hyaluronate (HA) and poly-l-lysine (PLL), using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) as a cross-linking agent. The coatings consisting in two double layer of ALG/PLL(EDC), HA(EDC)CHI and HA/PLL(EDC)+Drug. Release kinetics was investigated in sink conditions in NaCl solution. Material properties such as wettability, surface roughness and optical properties (transmittance and refractive index) were studied. Additionally the hydrogel – lachrymal proteins interaction was evaluated by quartz-crystal microbalance with dissipation (QCM-D). The antibacterial activity of the most promising coating was tested against *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aerugonosa*).

## Results and Discussion

The three coatings revealed a controlled release for DIC, while maintaining the physical properties necessary for contact lenses application. In contrast, the release of MXF and CHX was not retarded by those coatings. The specificity of the barrier effect of these LbL films for DIC may be attributed to the formation of reversible bonds between the DIC molecule and the polyelectrolyte chains. QCM-D data revealed possible film degradation of the coatings by lysozyme. A new top layer of HA was added to the ALG/PLL(EDC) coating, which offered a protection against proteins, maintained the properties and the controlled release of DIC. The resulting coating ALG/PLL(EDC)//HA demonstrated to be able to control the release of DIC, to resist the action of the tear proteins and to reduce the growth of *S. aureus* and *P. aeruginosa*.

## Conclusions

Controlled release was achieved for DIC, through the establishment of molecular interactions (H-bonds, hydrophobic or physical entrapment) between the drug molecule and the polyelectrolytes, leading to the formation of reversible complexes. Film degradation by lacrimal proteins was overcome by an addition of a new top layer of HA, which showed controlled release of DIC and reduce bacterial growth.

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**PC-93**

# Co-culture of cystic fibrosis human bronchial epithelia and macrophages to evaluate host-responses and efficacy of anti-infective formulations on *P. aeruginosa* infection

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**Introduction**. Following the establishmnet of chronic infection and biofilm, *Pseudomonas aeruginosa* is recognized as the main pathogen causing impaired lung function in cystic fibrosis (CF) patients. In the infected lung, *P. aeruginosa* can transit from planktonic form into biofilm, contributing to antibiotic resistance. To fight pulmonary pathogens, phagocytes are recruited and transmigrate across the bronchial epithelium, also inducing a strong immune response. To assess host response and efficacy of new anti-infectives we established a 3D *in vitro* co-culture model of the CF infected airways, expectantly also suitable for aerosol deposition.

**Materials and Methods**. CFBE41o- cells (cystic fibrosis patient-derived derived cells with ΔF508-homozygous) were seeded on 12 well-Transwell® inserts (pore size 3 µm) with MEM medium. Cells were grown for 10 days at air-liquid interface (ALI), reaching a transepithelial resistance (TEER) of >1000 Ω.cm². THP-1 monocytes were differentiated into macrophages-like cells with 10 ng/mL of phorbol-12-myristate 13-acetate (PMA) for 2 days. Inserts with previously grown CFBE41o- cells were turned upside down and THP-1 were seeded at the basolateral side. *P. aeruginosa* (PAO1GFP) was added at OD600 of 0.1 (CFU/ml ~ $2x10^9$), which correspond to a multiplicity of infection (MOI) of 1:20 (epithelial cell: bacteria). Tobramycin (1mg/mL) was used in co-cultures as well as in planktonic bacteria.

**Results**. To mimick air-exposition in the lungs, CFBE41o- cells were lifted to the ALI after 3 days in culture. Once reaching a tight epithelial barrier (day 10), a co-culture with THP-1 macrophages was established for 24h, followed by infection with PAO1GFP. After 6h of infection, both epithelial cells (red) and macrophages (yellow) were still viable. Fluorescent *P. aeruginosa* grows on top of the epithelium, and tends to form biofilm-like aggregates (Figure 1B). On a cross-section (Figure 1A), THP-1 macrophage migrated to the apical side to reach the bacteria. Such migration only occurred upon infection, and was observed after 3 to 6 hours of infection. Bacteria survival upon Tobramycin treatment was compared by growing PAO1GFP in different substrats: plastic culture plates or on host cells. Tobramycin efficacy reduced the number of viable bacteria grown in absence of host cells in 10 log while the efficacy on bacteria grown on host cells reached 5 log reduction.

**Conclusion**. We set-up a 3D air-liquid interface co-culture model of CFBE41o- cells and THP-1 macrophages infected with *P. aeruginosa*, that will further allow to study host responses (e.g. inflammation). The susceptibility to Tobramycin was reduced for bacteria growing on top of cells when compared to bacteria monocultures. The 3D co-culture system offers interesting perspectives for evaluating the safety and efficacy of novel pulmonary anti-infectives.

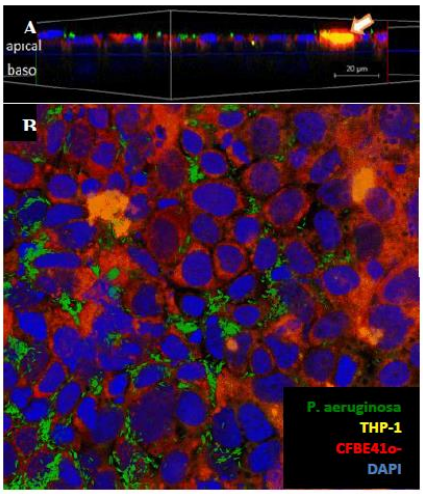


*Figure 1*. Cross-section and apical view of Co-culture (6h infection). *Arrow*: Macrophage transmigration to apical/bacteria side.

**Acknowledgments**: This work was supported by the ITN-Nabba (Design and Development of advanced NAnomedicines to overcome Biological BArriers and to treat severe) from the European Union's HORIZON 2020 Programme for research, technological development and demonstration under grant agreement no. 642028 H2020-MSCA-ITN-2014.

**PC-94**

# Photoreceptors rescue by novel PLGA-loaded microspheres of GDNF and melatonin in rhodopsin knockout mouse experimental model

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**Introduction:** Multiple inherited, trauma and age-related retinal disorders are characterized by irreversible loss of photoreceptors [1-3]. Neuroprotection, a mutation-independent approach to protect photoreceptor cells, has been used as an effective therapeutic strategy for these retinal degenerations among others [4]. In this sense, we propose a novel PLGA [poly (lactic-co-glycolic acid)] microparticulate delivery system able to provide simultaneous sustained release of two neuroprotective agents: GDNF (Glial-derived neurotrophic factor) and melatonin during long term. The aim of this work is to characterize a formulation based on GDNF/melatonin microspheres (MSs) as well as to explore the therapeutic potential of this PLGA-based slow release system to rescue photoreceptors using an experimental mouse model of retinal degeneration.

**Materials and Methods:** GDNF and the ELISA (enzyme-linked inmunosorbant assay) kit for GDNF quantification were purchased from R&D (Minneapolis, MN USA). Melatonin was supplied by (Sigma-Aldrich (St. Louis Mo., USA)). Poly-(D,L-lactide-co-glycolide) 50:50 (Resomer® 503) was supplied by Boehringer Ingelheim (Pharma Co., Germany) and α-tocopherol acetate (Vit E) was obtained from Sigma-Aldrich (Schnelldorf, Germany). Rhodopsin-null mice (Rho-/-, Peter Humphries, Trinity College, Dublin) were used for the *in vivo* studies. Animal experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic Vision Research. GDNF/melatonin-loaded microspheres were prepared using a solid-in-oil-in-water emulsion solvent extraction-evaporation technique. The microspheres were characterized in terms of morphology, particle size, encapsulation efficiency and *in vitro* release studies.After a preliminary study of the *in vitro* rescue of hRPC from peroxide-induced death conducted to determine the optimal microspheres concentration, the GDNF/melatonin MSs and blank MSs were intravitreally administered to 3-week old rhodopsin knockout mice and the neuroprotective effect was assessed by electroretinography (function) at 3, 6 and 9 weeks after the injection. The rescue of structure was determined by photoreceptor quantification at 9 weeks after MSs administration. Immunohistochemistry for photoreceptor, glia and proliferative markers was also performed.

**Results and Discussion:** GDNF/melatonin-loaded microspheres ranged from 20-40µm in size and were spherical in shape with porous surfaces. The encapsulation efficiency was 22.1 ± 0.8 ng GDNF/mg MSs and 32.8 ± 0.2 µg melatonin/mg MSs. The microspheres were able to co-deliver GDNF and melatonin in a sustained fashion up to 63 days. The intravitreal injection of GDNF/Melatonin-loaded MSs led to partial rescue of photoreceptors on both functional and structural level compared to blank microspheres and vehicle. No significant intraocular inflammatory reaction was observed after the intravitreal injection of the microspheres.

**Conclusions:** Sustained release of GDNF and melatonin from PLGA microspheres promoted a rescue of the photoreceptors in rho (-/-) mice.

**Acknowledgments:** Spanish Ministry of Economy and Competitiveness for financial support (MAT 2013-42127-R), RETICs 65288, UCM Research Group 920415 (GR3/14).

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**PC-95**

# Pharmacokinetics of Nimesulide nanoparticles after intraprostate injection

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## Introduction

The use of anti-inflammatory drugs in the coadjuvant treatment of epithelial cancers such as those affecting the prostate or breast is the focus of several research groups [1,2]. However, the doses needed are much higher than those employed in anti-inflammatory therapy with the potential to raise concerns about the efficacy profile of the conventional drug products already on the market. Therefore, the design of novel injectable formulations that might be administered intratumorally might be interesting in order to obtain high drug concentrations at the tumor site thus reducing systemic adverse effects. Among the different alternatives, nanoparticles (NP) are versatile colloidal suspensions easily administered by parenteral routes. In addition, they can control drug release kinetics and target drugs to certain organs and tissues if properly designed. However, they still face challenges to reach massively their target after systemic administration. The objective of this research was the pharmacokinetic evaluation of biodegradable NP containing nimesulide after intratumoral injection for the coadjuvant treatment of prostate cancer.

## Materials and Methods

Nimesulide loaded NP (NSNP) were obtained by the emulsion evaporation method from 100 mg PLGA and 10 mg NS. After NP characterization, volumes corresponding to 1mg NS/kg body weight were administered into the prostate of fasted Wistar rats weighing 250-500 g. The study protocol was approved by the ethical committee of UAH (OH-UAH 2017/005/000). A NS solution (5 mg/ml) in a mixture of EtOH:PEG 300:PBS 7.4 (20:54:26) was used as reference formulation. Blood samples (150 µl) were withdrawn over 20µl sodium heparine (5%) at pre-dose and predetermined time points post-administration. Plasma was obtained by centrifugation at 3500 rpm for 15 min. Samples were processed and analyzed by HPLC according to a previously described method [3]. Pharmacokinetic (PK) analysis was carried out on individual data sets by a non-compartmental approach and the corresponding parameters were calculated by using standard equations.

## Results and Discussion

Figure 1 shows the concentration-time curves corresponding to NS (A) and its main circulating metabolite 4-OHNS (B) after intraprostatic injection of NSNP. The PK parameters associated to these curves show $C_{max}$ for NS is 2.42 µg/ml and 0.5 µg/ml for the metabolite at 480 min. The elimination rate constants are the same regardless of the formulation injected (0.0012 $min^{-1}$ for NP and 0.0017 $min^{-1}$ for the NS solution) and similar to IV dosing. Drug Cl (0.222-0.239 ml/min/kg) and $V_d$ (140-204 ml/kg) are also within the same range. In all cases, the elimination rate constant for 4-OHNS is one half of parent drug. MRT values after intraprostatic administration (566 and 670 for NS solution and NP, respectively) suggest the contribution of an input process. When using the drug solution $C_{max}$ 3,5 µg/ml and $t_{max}$ 240 min demonstrate faster drug absorption than NP and higher bioavailability (F=0.82 vs 0.66).

## Conclusions

NP have the ability to reduce the systemic bioavailability of anti-inflammatory drugs such as NS when administered as an intraprostate injection.

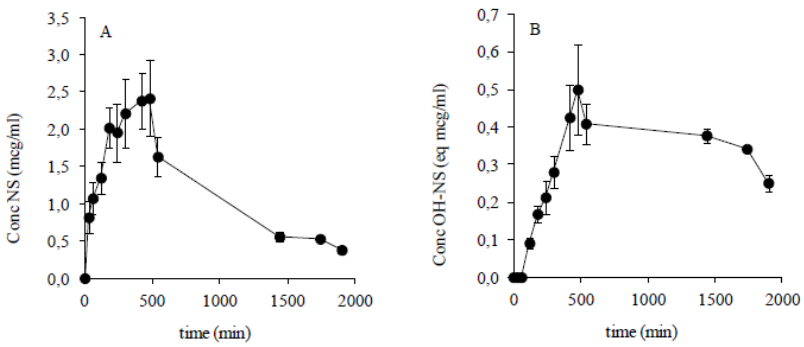


*Figure 1.* NS (A) (µg/ml) and 4-OHNS (B) (eq. µg/ml) concentrations in rat plasma after IPr administration of 1mg/kg NSNP. (mean ± s.e.m.) n=5. Concentrations in some animals have been normalized to the referred dose.

**Acknowledgments**: The authors are grateful to the staff of CEA for helping with animal handling.

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**PC-96**

# Development of Nimesulide-loaded nanoparticles coated with chitosan and hyaluronic acid

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## Introduction

Nimesulide (NS) is a NSAID drug with preferential inhibition of COX-2 isoform (1). Several clinical studies have demonstrated the therapeutic potential of NS but also its idiosyncratic hepatotoxicity responsible for its withdrawal from several markets (2). It has been shown that the COX-2 isoform is over-expressed in different epithelial cancers (3). The objective of this work was the development and characterization of NS-loaded nanoparticles (NPs) in order to achieve high local drug concentrations in the tumor area avoiding systemic exposure to the drug and minimizing the risk of hepatotoxicity. In addition NPs coating with hyaluronic acid (HA) could improve its internalization by tumor cells trough interactions with the CD44 receptor over-expressed on their surface (4).

## Materials and methods

NPs were obtained by the emulsion-solvent evaporation method. Then, they were coated using a layer by layer deposition method using two counter-charged polymer solutions, chitosan (CS) 0.1% and HA 0.05% in order to obtain NPCSHA.

## Results and discussion

Drug encapsulation was assessed by UV-Vis spectrometry at 230 nm. The yield was determined by weighing the dry residues obtained after NPs centrifugation at 20000 for 15 min. The morphology of NPs was evaluated by transmission electron microscopy (TEM, JEOL JEM-2000 FX). Particle size and zeta potential was measured in a Microtrac UPA and Zetasizer Nano ZS, respectively. Retention ability and drug release in pH 7.4 PBS were studied at room temperature and 37ºC, respectively.

Figure 1 shows the main parameters calculated for the characterization of the nanoparticles. Encapsulation efficiency was above 90% regardless of the coating but more than 98% of the drug remained entrapped into the NPs after resuspension. The release of NS loaded inside NPCSHA under *sink* conditions was complete after the first hour of the study; however, uncoated NP released 80% approximately of the encapsulated drug in the same time period. Its ability to retain the entrapped drug with a 99%dilution in pH 7.4 PBS decreases more than 60% with the addition of CS and HA coatings.

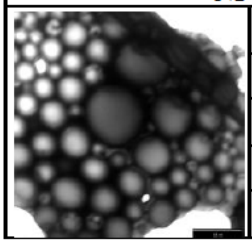
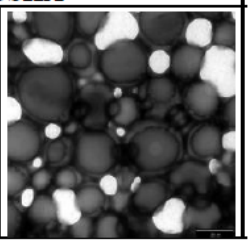
| NP | | | NPCSHA | |
|---|---|---|---|---|
|  | 433±35.4 | **Size (nm)** | 560±19.1 |  |
| | (-30.1)±0.55 | **Z Potential (mV)** | (-32.13)±0.96 | |
| | 95.86±1.76 | **Enc. Eff. (%)** | 92.21±4.11 | |
| | 97.22±10.28 | **Recovery (%)** | 88.34±9 | |
| | 87.35±6.61 | **Yield (%)** | 91.32±3.88 | |

*Figure 1.* Main characterization parameters and TEM images of NP and NPCSHA

## Conclusion

Spherical NPs coated with HA and particle sizes lower than 0.6 µm have been obtained. The NS is efficiently incorporated into the NP and shows a retention ability depending on the employed dilution and the structure and composition of the developed nanocarriers.

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**PC-97**

# SLNs as gene delivery systems to treat corneal inflammation with IL-10

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**Introduction:** Gene therapy is a promising strategy to treat corneal inflammatory diseases by releasing anti-inflammatory molecules such as interleukin-10 (IL-10) [1]. Nevertheless, corneal cells are hard to transfect, despite the numerous vectors, delivery methods and strategies developed. The aim of this study is to evaluate non-viral vectors based on Solid Lipid Nanoparticles (SLNs) combined with protamine (P) and a polysaccharide (chitosan (CH), hyaluronic acid (HA) or dextran (DX)) to deliver a plasmid that encodes IL-10 in human corneal epithelial cells (HCE-2).

**Materials and Methods.**
Preparation of SLNs and vectors: SLNs were prepared by two different techniques: solvent evaporation/emulsification (SLNs1) [2] and coacervation (SLNs2) [3]. Not only the technique, but also the composition of the SLNs was different (figure 1A). To prepare SLN1-based vectors, the plasmid was firstly complexed with P and DX or HA and then, SLNs1 were incorporated to the complexes. In the case of SLNs2, which contain CH, the plasmid was only complexed with P, and then with the nanoparticles. Characterization of the vectors: Size, polidispersity index (PI) and Z potential of the SLNs and the vectors were determined by using the ZetaSizer Nano (Malvern). Binding, protection and release capacity of the plasmid in the vectors was also studied by agarose gel electrophoresis. Intracellular distribution of the vectors: Vectors prepared with the plasmid labeled with ethidium monoazide (EMA) were added to HCE-2 culture cells, and at different times (4h, 12h and 24h) the intracellular disposition of the DNA was analyzed by fluorescence microscopy. Quantification of IL-10: The secreted IL-10 in HCE-2 cells was quantified by ELISA 72h after addition of vectors.

**Results and Discussion:** Vectors presented particle size in the nanometer range and positive surface charge (figure 1A). In the agarose gel electrophoresis, it was observed that all the vectors were able to bind, and protect the pIL-10; however, in the case of P-SLN2, the plasmid was not fully released from the vectors.
Regarding the intracellular disposition (fig 1B), the condensation of DNA (red dots) was higher in the case of P-SLN2 vectors, even 24h after addition of the formulations, which correlates with the hampered plasmid released from the CH vectors observed in the electrophoresis gel. In the DX-SLN1 and HA-SLN1 vectors, DNA descondensation (diffused red) was observed along time, and the highest descondesation degree was observed in HA-SLN1 vectors.
DX-SLN1 produced the highest level of IL-10 (10.42±1.52 ng), followed by HA-SLN1 (3.68±0.52 ng), and lastly, P-SLN2 (0.09±0.05 ng). Differences in the transfection efficay may be related to the different intracellular disposition of the DNA provided for the different formulations. The plasmid must be realeased in the cytoplasm to enter into the nucleus, but it must be also protected enough to avoid the degradation by intracellular components. On the one hand, P-SLN2 vectors hardly release the DNA along the time, and on the other hand, the rapid decondesation from HA-SLN1 vectors, exposes the DNA to higher degradation.

**Conclusions:** This work shows the potential of non-viral vector based on SLNs as a therapeutic strategy for the treatment of corneal diseases that course with inflammation.

A

| | Composition | Size (nm) | PI | Z pot (mV) |
|---|---|---|---|---|
| SLN1 | Precirol ATO 5, DOTAP, Tween 80 | 341.9 ± 22.5 | 0.32 ± 0.03 | 54.8 ± 0.6 |
| SLN2 | Sodium behenate, PVA 9000, glycol chitosan | 365.4 ± 34.4 | 0.33 ± 0,00 | 21.0 ± 0.8 |
| DX-SLN1 | | 233.1 ± 81.7 | 0.36 ± 0.10 | 36.1 ± 2.2 |
| HA-SLN1 | | 242.1 ± 38.0 | 0.35 ± 0.04 | 29.6 ± 0.8 |
| P-SLN2 | | 510.7 ± 81.2 | 0.44 ± 0.03 | 29.5 ± 2.2 |

B

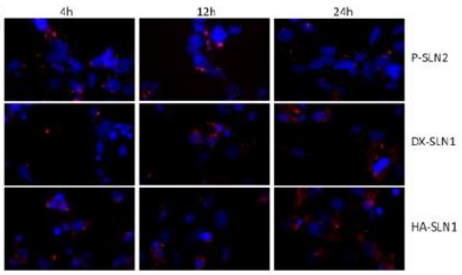


*Figure 1*. A) Characterization of SLNs and vectors; B) Intracellular disposition of EMA-labeled plasmid.

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**PC-98**

# Potential application of solid lipid nanoparticles for the treatment of herpetic keratitis infection through RNA interference

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## Introduction

One of the most distressing lesions that herpes simplex virus (HSV) could cause in cornea is vision impairment and blindness .These conditions result from a chronic immunoinflammatory reaction in the corneal stroma that starts with neovascularization. Matrix metalloproteinase-9 (MMP9) is involved in this process [1] and, therefore, downregulating MMP9 protein levels involved in the inflammatory process may be an ideal approach to treat this corneal infection. Solid Lipid Nanoparticles (SLN) hold especial promise for the treatment of eye diseases by gene therapy [2]. Thus, the aim of this work was to develop and characterize SLN-based non-viral vectors containing interference RNA (iRNA) against MMP9 as a potential gene therapy for herpetic queratitis.

## Materials and Methods

Vectors were prepared with SLN [3], protamine (P), hyaluronic acid (HA) or dextran (Dx), and a plasmid (MMP9GFP) containing two genes: short-hairpin RNA (shRNA) targeted to MMP9 and the gene encoding the green fluoresecent protein (GFP), as a reporter gene. Different fomulations were prepared by varying the proportion of P and SLN. The size of the vectors was determined by photon correlation spectroscopy (PCS) and Z potential was measured by laser Doppler velocimetry (LDV). The capacity of the vector to join, to protect and to release MMP9GFP from SLN, was evaluated by electrophoresis on a 0.4% agarose gel. The efficacy of transfection was evaluated *in vitro* in the HCE-2 cell line (human corneal epithelial cells). Seventy-two hours aftere cell treatment with vectors, the GFP produced was detected by microscopy and quantified using a fluorometric assay. Cell internalization was analized by flow cytometry, labeling the SLN with Nile Red.

## Results and Discussion

The vectors prepared with HA presented higher particle size (266-415 nm) and lower surface charge (27-31 mV) than those prepared with Dx (149-171 nm and 41-47 mV). All of them were able to completely bind the plasmid, to protect it from nuclease action and to release it after treatment with SDS. A high and rapid internalization in HCE-2 cell was observed. The treatment of HCE-2 cells with the vectors induced the expression of the GFP protein (and therefore, the production of shRNA against MMP9), which is indicative of the transfection process. Free DNA was not able to transfect the cells. Figure 1 shows the GFP expression obtained with all the formulations. As it can be seen, the vectors prepared with HA induced a higher amount of GFP than those prepared with Dx. Regarding HA vectors, the transfection was higher when the proportion of protamine and SLN in the vector was higher. This is due to the protection of the plasmid provided by protamine and the cationic lipid used to form the SLNs.

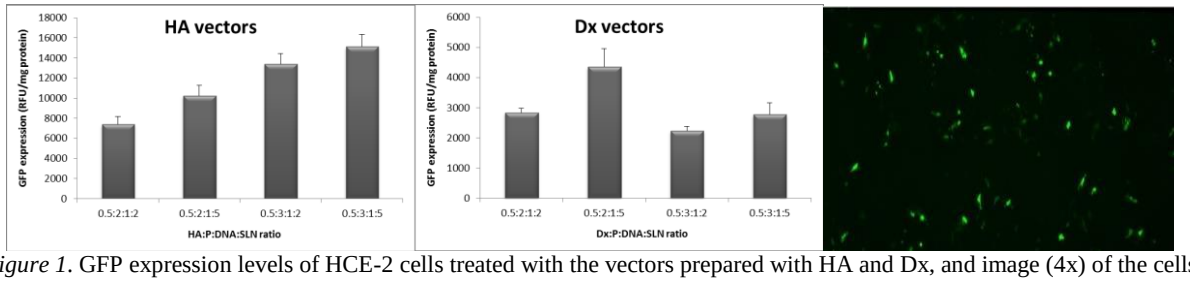


*Figure 1*. GFP expression levels of HCE-2 cells treated with the vectors prepared with HA and Dx, and image (4x) of the cells expressing GFP.

## Conclussions

This study demonstrates the abiliy of SLN based vectors to transfect human corneal cells. More studies should be done to confirm the ability of the vectors to downregulate the MMP9 protein.

**Acknowledgments**: This work was supported by the Spanish Ministerio de Economía y Competitividad (SAF2014-53092-R), by FEDER funds from the EU and by the UPV/EHU (PPG17/65). J Torrecilla thanks UPV/EHU for her research grant.

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**PC-99**

# A new drug delivery system for glaucoma patients: poly(ε-caprolactone) biodegradable intravitreal implants

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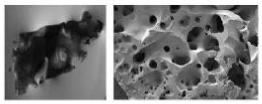
Glaucoma is a chronic disease characterized by a progressive loss of retinal ganglion cells (RGCs) and optic nerve degeneration, represented by changes in the optic disc and progressive visual field loss. Elevated intraocular pressure (IOP) is a major risk factor to develop glaucoma [1]. Current treatments are directed towards lowering IOP, but the disease progresses in several patients despite IOP control [2]. Therefore, new and more effective treatments are needed, and neuroprotection of RGCs may offer potential as an additional strategy. Biodegradable implants may serve as drug release systems for the treatment of retinal diseases, including glaucoma. Therefore, we developed highly porous poly(ε-caprolactone) (PCL) intraocular implants using supercritical $CO_2$ foaming/mixing method (Figure 1) and evaluated their safety for the retina.

In retinal primary neural cell cultures the presence of the PCL implant for 6 days did not increase cell death, as assessed by TUNEL assay, and did not decrease the number of neurons (NeuN-immunoreactive cells) in the culture. Moreover, the incubation of retinal organotypic cultures with PCL implant for 24h, 48h or 72h did not reduce the number of Brn3a-immunoreactive RGCs in culture. PCL implants were surgically inserted in the vitreous of Wistar rats and IOP was measured regularly with a rebound tonometer (Tonolab). The presence of the implants in the vitreous did not cause alterations in the IOP. The electrical activity and structure of the retina were assessed by electroretinography (ERG) and optical coherence tomography (OCT), respectively, at baseline and at 1 and 2 months after implantation. The presence of the PCL implant did not change the retinal response to flash lights nor the structure of the retina. Moreover, the total number of Brn3a-immunoreactive RGCs was assessed in whole-mounted retinas, and PCL implant did not change the number of RGCs.

Taking into consideration the lack of retinal toxicity of PCL implants, we can envisage that PCL implants can be used as drug delivery systems targeting the neuroprotection of RGCs against glaucomatous damage.



**Highly porous poly (ε-caprolactone) release systems**

**PCL implants**

*Figure 1*. PCL implants prepared by $scCO_2$-assisted melting/mixing/foaming. Implants were processed at 45ºC and 200 bar, for 2h. Depressurization was performed at 20 bar/min. Optical microscopy images (left) and Scanning Electron microscopy (SEM, right) images of PCL implants.

**Acknowledgments**: FCT (PD/BD/114115/2015, Grant PTDC/NEU-OSD/3123/2014 and Strategic Project-UID/NEU/04539/2013), FEDER-COMPETE (FCOMP-01-0124-FEDER-028417 and POCI-01-0145-FEDER-007440) and Centro 2020 Regional Operational Programme (CENTRO-01-0145-FEDER-000008: BrainHealth 2020), Portugal.

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**PC-100**

# Diclofenac eluting intraocular lens: effect of loading time and temperature on the drug release behavior

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**Introduction:** Nowadays, cataracts are the main cause of blindness worldwide. Their treatment involves a surgery for remotion of the opacified natural lens, followed by implantation of an intraocular lens (IOL). In the post-surgery period, anti-inflammatories and antibiotics are prescribed for prophylaxis of complications such as endophthalmitis. Diclofenac (DFN), for example, is usually administered in the form of eye drops, during an extended period of time (about 2-3 weeks). This drug delivery method is not very effective, since more than 95% of the drug is lost due to protective eye mechanisms (e.g. blinking, tearing) and systemic absorption [1]. It also requires patient compliance and ability to correctly instill the drops. The use of drug-loaded IOLs is an interesting alternative to overcome these problems. However, generally, the burst experimented in the beginning of the release and the inadequate kinetics is a problem [2]. The objective of this work was to optimize the loading conditions (time and temperature) of IOLs to obtain a controlled and extended delivery of DFN.

**Materials and Methods:** Discs (5 mm diameter, 1 mm thickness) of the material CI26Y for IOLs (Contamac UK) were loaded with diclofenac sodium salt (DFN, Sigma-Aldrich) by soaking in 3 mL of drug solution at 4°C, for 4 days, 2 weeks and 1, 2 and 3 months and, at 60°C, for 4 days and 1 and 2 weeks. Drug release experiments were carried out in closed tubes containing 3 mL of PBS, at 36ºC, with stirring at 180 rpm. The concentration of the drug released was determined by UV-Vis spectroscopy. To understand the effect of the temperature, a fixed loading time of 2 weeks was chosen at temperatures of 4°C and 60°C, to produce samples for NMR and DSC analysis. Young's modulus was determined for these samples from tensile tests. Transmittance of the DFN loaded material that led to the best release profile was determined with a UV-Vis spectrophotometer.

**Results and Discussion:** The release of DFN loaded at 4ºC was affected by the loading time, being the maximum loading achieved only after 2 months. However, at 60ºC, the different loading times tested did not have any effect on the release, being the maximum loading achieved after 4 days. The release was higher and more controlled for the samples loaded at 60ºC. With a fixed loading time of 2 weeks, the increase of the loading temperature led to an increase in the released amount and release duration of DFN. The NMR, DSC and Young's modulus studies demonstrated the existence of reversible interactions between the polymer chains and DFN, more significant at 60ºC, which explain the differences in the drug release behaviour. Best loading conditions were achieved when the material was loaded at 4°C for 2 months. These conditions allow achieving a sustained release of DFN for at least 15 days. The transmittance of these samples was above 90% above 550 nm.

**Conclusions:** Temperature and time of loading may be adjusted to improve the drug release behaviour from IOLs. The establishment of interactions between the polymeric matrix and the loaded drug helps to achieve sustained drug release profiles. In the present work, specific loading conditions (time and temperature) allowed to obtain a release of DFN for 2 weeks from an acrylic based material for IOLs.

**Acknowledgements:** The authors acknowledge funding from Fundação para a Ciência e Tecnologia (FCT) [projects UID/QUI/00100/2013 and PTDC/CTM-BIO/3640/2014] and support from PhysIOL for providing the materials for IOLs.

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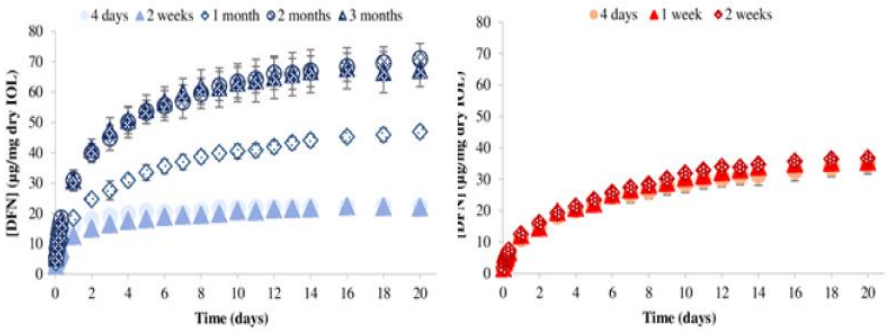


*Figure 1.* Cumulative release profiles of DFN from CI26Y hydrogels loaded at 4 °C (left) and 60 °C (right) during different periods of time.

**PC-101**

# NRG1 loaded PLGA microparticles do not alter global inflammatory response in the heart after myocardial infarction

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**Introduction**: Incorporation of growth factors into poly (lactic-co-glycolic acid) microparticles (PLGA MPs) has improved the efficacy of protein therapy for treating myocardial infarction (MI) [1]. For instance, the growth factor neuregulin-1 (NRG1) significantly increased heart function as well as angiogenesis and arteriogenesis when administered encapsulated into PLGA MPs [2]. However, total heart recovering after MI has not been achieved yet, in partly due to lack of understanding of heart mechanisms ruling cardiac positive remodeling. Among these processes, inflammation has been identified as one of the major and critical heart responses to MI [3]. Switching inflammatory response from proinflammatory to regenerative process may result in better heart outcomes. Thus, the objective of this work is to assess the interactions between NRG1 PLGA MPs and the inflammatory response of the heart after MI.

**Materials and Methods**: PLGA MPs containing NRG1 were prepared through a double emulsion solvent extraction/evaporation method using the Total Recirculation One Machine System (TROMS®). For MI induction, C57/BL/6J mice underwent a left thoracotomy and left anterior descending coronary artery was permanently blocked. Animals received treatment (1.2 mg of NRG1 PLGA MPs or resuspension medium (sham)) 24, 72 or 168 hours after MI induction via intramyocardial injection and were sacrificed 2 or 5 days after treatment administration. Inflammatory response was assessed by immunofluorescence against $CD68^+$ (general macrophages), $B7\text{-}2^+$ (inflammatory macrophages) and $CD206^+$ (regenerative macrophages) cells.

**Results and Discussion**: MPs were formulated with a final size of 7.58±0.96 µm and encapsulation efficiency of NRG1 PLGA MPs was 84.83±7% (corresponding to 845 ng NRG1 per 1 mg of polymer). NRG1 PLGA MPs induced the expression of CXCL1 cytokine *in vitro* in J774A1 cells, whereas other proinflammatory proteins were not affected (Figure 1.A). Animals treated with NRG1 PLGA MPs showed a similar number of $B7\text{-}2^+$ and $CD206^+$ macrophages than sham animals (Figure 1.B). Importantly, time of treatment administration did not affect inflammation process, and NRG1 PLGA MPs treated animals followed a normal inflammatory evolution curve. Since ErbB4 biological receptor for NRG1 is expressed in immune cells [4], and PLGA degradation in the tissue lowers the local pH environment and that causes inflammation [5], NRG1 PLGA MPs may affect inflammatory response close to the injection track.

**Conclusions**: NRG1 PLGA MPs did not affect global macrophage populations in the heart after a MI nor induce inflammation, thus PLGA MPs might be a suitable vehicle for the delivery of therapeutics into the heart.

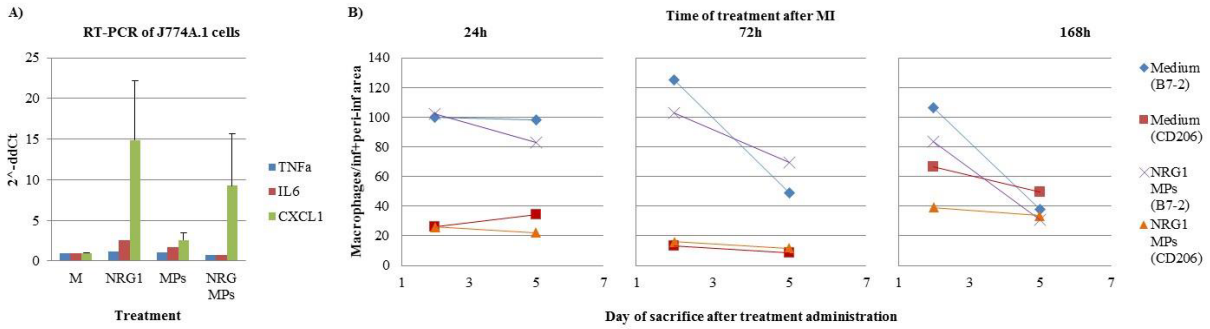


*Figure 1*. Effects of NRG1 PLGA MPs on inflammation. A) RT-PCR for TNFα, IL6 and CXCL1 of J774A1 cells treated with NRG1 PLGA MPs. B) Macrophage quantification of infarcted C57/BL/6J hearts treated with NRG PLGA MPs or resuspension medium.

**Acknowledgments:**
This work was supported by the Spanish Ministry of Economy and Competitiveness (SAF2013-42528-R and Programa FPI BES-2014-067838).

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**PC-102**

# Glucan particles to liver-targeted delivery of curcumin

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**Introduction:** The incidence of primary liver cancer is increasing in several developed countries. Curcumin, extracted from *Curcuma longa* has several therapeutic properties, including antimicrobial, anti-inflammatory, antioxidant and anticarcinogenic activity. However, despite being a powerful bioactive agent, curcumin presents low water solubility, low bioavailability and so high doses would be necessary to obtain therapeutic effects [1,2]. One way to overcome these problems and enhance curcumin medicinal relevance may involve the targeted delivery of the drug. β-glucans are complex polysaccharides that occur naturally, and are found in the cell walls of bacteria, fungi, yeasts, algae, lichens and also some plants (e.g. oats and barley) [3]. β-glucan particles (GPs), with a diameter of about 2 µm to 4 µm, derived from *Saccharomyces cerevisiae* cell walls are commonly called shells and mainly consist of β-1,3-Glucan. The inner hollow cavity of these shells can be loaded with different compounds and delivered to macrophages and dendritic cells through interaction with dectin-1, a receptor highly expressed on the surface of these cells [4]. The main objective of this work was to optimize the preparation method for obtaining curcumin-loaded glucan shells, characterize the formulation and test its efficacy in macrophages and liver cells.

**Materials and Methods:** Glucan particles were obtained by a series of alkaline and acid treatments and extractions with solvents. The encapsulation of curcumin was carried out using a method optimized in our laboratory; briefly, curcumin was solubilized in acetone at a final concentration of 2 mg/mL, and 100 µL of this solution were added to 10 mg of glucan particles. The eppendorfs with shells and curcumin were vortexed, so that all the powder was wetted with the solution, and maintained in the refrigerator (4°C) for 2 h. After that, they were placed open at room temperature for acetone to evaporate. This same procedure was repeated until curcumin-loaded shells were obtained with several loading cycles. The curcumin loading efficiency and particle loading capacity were determined after the quantification of the unencapsulated curcumin, dissolved in acetone and measurement at 421 nm in a spectrophotometer. For the assessment of anti-inflammatory activity and cytotoxicity, the mouse macrophage cell line, Raw 264.7, was used. $3\times10_5$ macrophages were plated in each well of a 48-well plate and 24 h later, the following formulations were added: empty shells, curcumin-loaded shells and free curcumin. After 1 h, LPS was added and the cells were incubated at 37°C for 24 h. Then, the supernatant was collected to analyze the production of nitric oxide assessed as the amount of nitrites accumulated by the Griess reaction. The cytotoxicity was assessed using the resazurin reduction assay. The same tests were performed in the hepatocyte cell line, HepG2.

**Results and Discussion:** The curcumin encapsulation efficiency was superior to 92%, and the loading capacity increased with the number of loading cycles. TEM study showed that the particles have an oval shape and confirmed that they have an average size of 2-4 µm. The cell viability tests performed demonstrated that either empty glucan shells or those loaded with curcumin in concentrations up to 40 µM had no cytotoxicity to Raw 264.7 and HepG2 cell lines. Curcumin delivered in glucan shells was as effective as free curcumin in reducing nitric oxide production induced by LPS.

**Conclusions:** The encapsulation of curcumin into glucan particles was effective in eliciting its anti-inflammatory effect. Further testing will be needed to find out how curcumin is released from shells and to confirm its pharmacological efficacy both *in vitro* and *in vivo*.

**Acknowledgments**: This work was funded by FEDER funds through the Operational Programme Competitiveness Factors - COMPETE 2020 and national funds by FCT - Foundation for Science and Technology under the project PROSAFE/0001/2016 and strategic projects POCI-01-0145-FEDER-007440 and CENTRO-01-0145-FEDER-000012-HealthyAging2020.

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**PC-103**

# Albumin nanoparticles for the ocular delivery of bevacizumab

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## Introduction

Albumin nanoparticles have been studied as drug delivery systems because they are biocompatible, biodegradable, non-toxic and they can incorporate a large number of drugs. Bevacizumab is a humanized monoclonal antibody that targets VEGF and its receptors to inhibit angiogenesis [1]. It has been used in ophthalmology to treat several ocular pathologies, such as diabetic retinopathy and age-related macular degeneration [2,3]. The aim of this study was to prepare and characterize human serum albumin nanoparticles for the ocular delivery of bevacizumab.

## Materials and methods

Bevacizumab nanoparticles (B-NP) were prepared by a desolvation process of 100 mg HSA in 8 mL purified water by the continuous addition of 16 mL ethanol under continuous stirring. Then, nanoparticles were freeze-dried using sucrose as cryoprotectant. The resulting nanoparticles were characterized by measuring the size, polydispersity index, zeta potential and morphology. The amount of protein transformed into nanoparticles was calculated by etiher micro-BCA or microfluidic electrophoresis. Furthermore, the bevacizumab payload in the nanoparticles was quantified with a specific immunoassay (Shikari Q-Beva). On the other hand, cytotoxicity studies were carried out in human retinal pigment epithelial (RPE) cells from the ARPE-19 cell line. B-NP were radiolabeled with $^{99}$mTc and administered as eye drops to male Wistar rats. Images were taken in a Symbia T2 Truepoint SPECT-CT system (Siemens). As controls, $^{99}$mTc and $^{99}$mTc-HSA in a commercial colirium (Acuolens®) containing 3 mg/mL hydroxypropyl methylcellulose were used.

## Results and Discussion

B-NP displayed a size of about 310 nm, a zeta potential of about –15 mV and a drug loading of 13%. Likely, these nanoparticles were stable and did not need to be stabilized with glutaraldehyde or any other cross-linking strategy.

The *in vitro* release of bevacizumab from albumin nanoparticles (B-NP) in PBS presented a biphasic profile, characterized by an initial release (burst effect) followed by a slower and more sustained release rate (6 µg/h) for at least 24h.

The *in vitro* cytotoxicity studies were carried out in ARPE cells with a single dose up to 72 hours and with repeated doses over a 5-day period. Neither bevacizumab nor B-NP altered cells viability even when repeated doses were used.

Radioactivity associated with $^{99}$mTc-B-NP remained in the eye for at least 4 hours, and dissapeared from the administration point gradually into the GI tract. On the contrary, free $^{99}mTcO_4^-$ and $^{99}$mTc-HSA were rapidly drained from the administration point.

## Conclusions

In summary, albumin nanoparticles obtained by a desolvation method could be an appropiate candidate for the ocular delivery of bevacizumab.

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**PC-104**

# PLGA nanoparticles are an effective formulation to control the colonic release and absorption on ibuprofen

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## Introduction

The oral controlled release (CR) formulations have become more important in recent years [1]. Among them, the polymeric nanoparticles are being very studied during the last decades; consequently they are used and studied for several applications and drugs [2], [3]. The objective of this research was to develop polymeric nanoparticles (NPs) of ibuprofen with poly(lactic-co-glycolic) acid (PLGA) as polymer, and to test their applicability for oral CR formulations development.

## Materials and Methods

Different proportions of drug/polymer were employed to develop the ibuprofen NPs and their *in vitro* release profiles were analysed. The *in vitro* segmental permeability of ibuprofen was tested in Wistar rat and demonstrated the high permeability of ibuprofen in rat colon [4]. In addition, *in vivo* assays were performed in Wistar rat to study the plasma concentration-time profiles after oral administration of encapsulated versus non-encapsulated ibuprofen.

## Results and Discussion

The *in vitro* studies results showed that the release of the ibuprofen from the NPs was pH-dependent and, consequently, higher at colonic pH. According to *in vitro* assays, the ibuprofen is a high permeability drug, so there will be no absorption problems when the drug is released in colon. Moreover, the plasma concentration-time profiles reveal a controlled release from the ibuprofen NPs, and an increase in the total rat gastrointestinal transit time can be observed.

## Conclusion

Therefore, the ibuprofen PLGA-NPs will be a good CR formulation to achieve a controlled release targeted to the colon, where the release rate of the drug from the NPs will be the limiting factor for the absorption process.

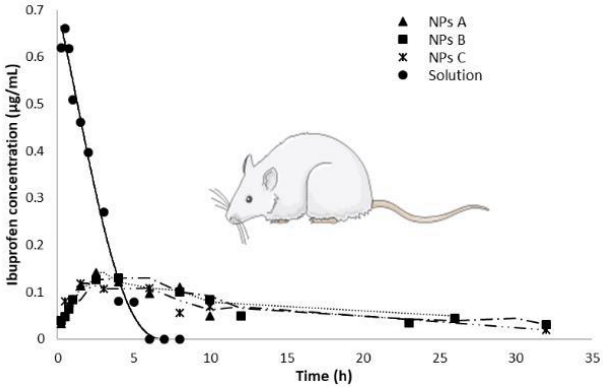


*Figure 1*. Ibuprofen plasma concentration-time profile obtained after oral administration of ibuprofen solution and ibuprofen NPs.

**Acknowledgments**: This work was supported by Ministry of Education, Culture and Sports of Spain (FPU 2012-00280 and EST 15/00083); Fundo Europeo de Desenvolvimento Regional trough the Operacional Programme for Competitiveness and Internationalisation, Portugal 2020 (POCI-01-0145-FEDER-007274); Fundação para a Ciência e a Tecnologia in the framework of the project "Institute for Research and Innovation in Health Sciences" and with the financial support: SFRH/BD/87016/2012; and by the Spanish Ministry of Economy, Industry snd Competitiveness with the partial financial support to the project SAF 2016-78756.

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**PC-105**

# Covalently crosslinked organophosphorous derivatives-chitosan hydrogel as a drug delivery system for oral administration of camptothecin

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## Introduction

Hydrogels are macromolecular three-dimensional crosslinked networks of polymers with high water or biological fluids content. The physicochemical and biological features of hydrogels have attracted the interest of a broad range of biomedical applications such as tissue engineering and controlled drug delivery systems [1]. In this work we propose the employment of tetrakis(hydroxymethyl)phosphonium chloride as crosslinking agent to obtain covalent hydrogels based on chitosan. This is an inexpensive, simple, and rapid method to prepare hydrogels using chitosan and THPC as basic components.

## Materials and Methods

These hydrogels are obtained by Mannich reaction between the amino groups of chitosan with the hydroxymethyl groups of the crosslinker molecule. The hydrogel network was built through the formation of covalent bonding between amine groups of chitosan backbone and hydroxymethyl groups of THPC [2].

## Results and Discussion

They show a pH sensitive second order swelling kinetic, have low toxicity, are biocompatible, mucoadhesive and allow a modified release of the encapsulated drug, camptothecin, for 48 hours..

## Conclusion

This antitumor drug has been studied as a drug of interest to develop oral chemotherapy administration strategies. This polimeric material could be very useful as controlled drug delivery system for oral administration in order to get constant plasmatic levels during much more time thanks to its mucoadhesivity properties. According to the obtained results, oral administration of camptothecin through hydrogels would provide low concentrations of drug at the absorption site, avoiding carrier saturation and reducing its intestinal toxicity.

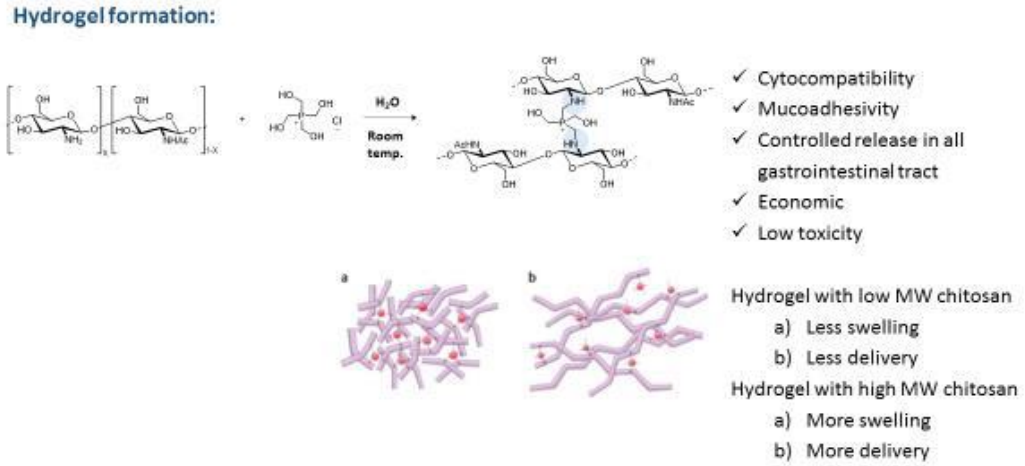


*Figure 1*. Hidrogel formation of covalent bonding between amine groups of chitosan backbone and hydroxymethyl groups of THPC.

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**PC-106**

# HET-CAM assay and bioadhesivity

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## Introduction

Sustained-release tablets contain special excipients in order to extend the time of release of the active ingredient, looking for increased dosing interval and therefore better adherence of treatments. Hydrogels are used to control drug release rate after administration. Their three-dimensional structure based on long main chains linked together by shorter molecules, provides characteristics of both, solid and liquid compounds [1]. One type of these hydrogels are the acrylic acid derivatives, that are used as excipients in pharmaceutical technology due to their high biocompatibility, their ability to swell and their mucoadhesiveness.

In this work we study the biocompatibility, by an irritant power assay, and the mucoadhesive properties of tablets elaborated with PAMgA, a new hydrogel synthesized from acrylic acid, in two different concentrations of PSA (precursor): 5mM and 40 mM.

## Materials and Methods

HET-CAM was used to estimate the irritant potential of PAMgA polymer. Fertilized chicken eggs were incubated for 9 days. Tests were performed on the chorioallantoic membrane formed, adding to its surface 100µL of a 0.5% solution of each polymer [2]. The presence of injuries such as hemorrhage, lysis and coagulation was observed. Validation of the assay was done by using as control an aqueous solution of sodium chloride 0.9% (negative control), and an aqueous solution of sodium hydroxide 0.1N (positive control). Each assay was performed in triplicate. Reference photographs for all endpoints were taken.

Tablets were prepared in an eccentric tableting machine BE-30 (J. Bonals) with PAMgA polymer as unique component.

Mucoadhesive strength analysis was performed using a TA-XT Plus texturer. Different substrates were used: bovine vaginal mucosa, and pig stomach and duodenum mucosa. Biorrelevant media [3] were prepared: fasted state simulated gastric fluid (FaSSGF), fed state simulated intestinal fluid, simulated vaginal fluid (SVF). Substrates were cleaned and moisturized with the biorrelevante media for 5 minutes at 37ºC. A force of 0.5 N was applied for 60 seconds. The probe was withdrawn at a constant speed of 0.1 cm/s. [3]. All measurements were performed six times.

## Results and Discussion

PAMgA polymer showed good compression characteristics. Tablets obtained were flat-faced, rounded and unscored, with 65N of hardness.

Formulations were classified as nonirritant in the HET-CAM test. The reactions on the CAM were observed over a period of 300 seconds. The hemorrhage (bleeding from the vessels), lysis (blood vessel disintegration) and coagulation (intra- and extra-vascular protein denaturation) appeared only in positive controls with NaOH 0.1N solution.

With respect to the mucoadhesiveness, the elastic component of the formulations promoted the contact of the tablet and mucosa, what increased the mechanical resistance of bioadhesive layer avoing the fracture of the formulation. Values of bioadhesion work indicated that in the conditions assayed, only slightly differences with both polymers in the different substrates were observed, showing slightly higher values for the tablets prepared from PAMgA 5 that with those prepared from PAMgA40. Values for bioadhesion work were higher in pig mucosa at pH 1.2 than in the intestinal conditions with duodenum mucosa. The values for the vaginal mucosa also showed a lower adhesion. The maximum values of detached force were low in all cases because of the elasticity of the substrates used.

## Conclusions

These results show the excellent compression and biocompatibility characteristics of PAMgA polymer. Its mucoadhesive properties allow extending the release of drugs beyond the gastrointestinal transit time, being an interesting tool to the development of prolonged release tablets for oral administration.

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**PC-107**

# Development of mucoadhesive buccal hydrogels containning mucopenetrating NLC

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Since the development of nanosystems, novel drug delivery systems have been studied in order to overcome the disadvantages of the oral route [1]. Transmucosal administration, as is the case of buccal route, is rather promising allowing the use of nanosystems such as Nanostructured Lipid Carriers (NLC). NLC can be, therefore, incorporated into semisolid systems such as hydrogels. However, buccal administration can be challenging because the pharmaceutical dosage form must be mucoadhesive, so that the retention time in the mucosa increases, while at the same time NLC must have the ability to permeate the mucus, in order to endorse the drug permeation through the mucus layer. These two approaches allow a better pharmacological response, since both promote an increase of drug bioavailability [2]. Coating the nanoparticles with hydrophilic polymers has also proven to be a promising strategy to improve mucus penetration [3].

Thus, the aim of this work was to develop and characterize mucoadhesive gels containing mucopenetrating nanoparticles. For this purpose, dispersions of mucopenentrating NLC were obtained using Precirol® ATO 5 and Miglyol® 812 as lipids and Pluronic® F127, as a hydrophilic polymer. The obtained dispersions were then incorporated into Carbopol® 980 hydrogels, in order to obtain a mucoadhesive preparation [4].
NLC dispersions were produced by High Shear Homogenization (HSH) followed by sonication and incorporated in the hydrogels by mechanical stirring. The developed dispersions of NLC were analysed for size, polydispersity index, zeta potential and entrapment efficiency. The hydrogels were also submitted to rheological, texture, pH, mucoadhesiveness and *in vitro* release analysis.

In general, the results showed NLC with nanometric size, low polydispersity index, and good entrapment efficiency of ibuprofen, the model drug used in this work [5]. The hydrogels showed good rheological, texture, pH and mucoadhesive properties. The *in vitro* release studies displayed good results, showing a sustained release of ibuprofen [4]. This result highlighted the importance of the incorporation of NLC in a system with good mucoadhesiveness, as a combined approach to improve the bioavailability of drugs administered by buccal route, improving their therapeutic efficacy.

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**PC-108**

# Engineered albumin variants are effective ligands to target insulin loaded nanoparticles to intestinal FcRn

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**Introduction** Most of current treatments for type 1 diabetes are invasive, painful injections and suffer from poor patient compliance, which contribute to be a leading cause of mortality and morbidity [1]. Oral administration beyond avoids these situations, also decreases risk of contamination, and it is a way to delivery exogenous insulin, mimicking the physiological pathway undergoing first hepatic bypass and provides better glucose homeostasis [2,3]. Functionalization of nanoparticles (NPs) is a strategy to endure the harsh environment of the gastrointestinal tract and cross epithelial barriers efficiently. With this, the ligands can bind specifically to the surface receptors and improve the delivery of pharmaceuticals through biological barriers [4]. The neonatal Fc receptor (FcRn) is a cellular receptor that binds and rescues albumin and IgG antibodies from intracellular degradation, extending their half-life [5]. In addition, FcRn binds both its ligands in a strict pH dependent manner, binding at acidic pH (6.5-5.0) and release at neutral pH. At mucosal surfaces, FcRn is predominantly located within acidified endosomes where it has been shown to transport IgG and albumin through a pH gradient of endosomal pathway. Upon exposure to physiological pH of basolateral surface, IgG and albumin are released. Thus, FcRn-mediated transcytosis holds a tremendous promise to drugs surpass the intestinal barrier [6]. Herein, we aimed to explore whether polymeric nanoparticles (NPs) conjugated with engineered human albumin variants for enhanced FcRn binding could be an attractive strategy for delivery of encapsulated drugs across mucosal barriers.

**Materials and Methods** To address this, we have designed polymeric NPs using double emulsion/evaporation technology where engineered human albumin was site-specifically conjugated to the polymers. This was done by utilizing a free cysteine residue within albumin domain I, which is located distally from the core interaction site for FcRn. NPs quantification was done using 2-way ant-HSA and FcRn-binding ELISAs. Also, recycling and uptake were evaluated with Human endothelial cell-based recycling assay (HERA).

**Results and Discussion** By the use of maleimide chemistry, engineered albumin was conjugated to the surface of the polymer, and the designed NPs were shown to bind human FcRn with expected binding hierarchy. According to the 2-way anti-HSA ELISA, albumin is detected on the surface of the NPs after conjugation. Also, with FcRn binding ELISA at pH 5.5, FcRn-binding properties of HSA is retained post conjugation and the HSA-NPs bind with expected (comparing with naked albumin) binding hierarchy to hFcRn in ELISA: HSA K500A (weak binder) < HSA WT < HSA K573P (12-fold stronger binder than WT) < HSA KPEQTM (even stronger binder than KP). In addition, results from HERA suggest that NPs with conjugated albumin is recycled in an FcRn-dependent manner being HSA KP-NPs, which binds strongly to FcRn, recycled more efficiently than HSA KA-NPs, which has weak affinity for FcRn.

Next, we will explore the use of the NPs using an *in vitro* transcytosis assay as well as state-of-the-art human FcRn transgenic mouse model.

**Conclusions** The FcRn-targeted approach may pave the way for more efficient delivery of NP-encapsulated drugs.

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**PC-109**

# Evaluation of long term stability of peptide-loaded protamine nanocapsules for transmucosal delivery

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**Introduction:** Polymeric nanocarriers have shown to be promising vehicles for transmucosal delivery, due to their capacity to encapsulate, protect and release high amounts of drugs and because of their ability to interact with biological barriers. Given the known penetration enhancing properties of protamine, we hypothesized that protamine nanocapsules (NCs) would be potential carriers for oral peptide delivery [1]. Our previous studies have indicated the capacity of these nanocapsules to load high amounts of peptide drugs, protect them from degradation and release them in a controlled manner. In addition, we have also shown that nanocapsules can interact efficiently with the intestinal mucosa without inducing toxic effects [2].

**Objectives**: In the present work, our objective has been to evaluate the long-term stability of peptide-loaded protamine nanocapsules after their storage as suspension at 4ºC or as freeze-dried powder formulations at room temperature.

**Methods:** Protamine nanocapsules were prepared by the solvent displacement technique [2]. The nanocapsules were lyophilized in presence of 5% (w/v) trehalose as cryoprotectant. The freeze-dried formulations were re-suspended in ultrapure water by manual shaking and their physicochemical characteristics were evaluated with regard to their size, zeta potential, morphology, peptide encapsulation and release.

**Results:** Initially, protamine NCs had a hydrodynamic diameter of ~ 400 nm with positive zeta potential. Both the storage at 4ºC and the freeze-drying process allowed to store the formulation for long periods, after which, there were no changes observed in size or surface charge. With regard to the peptide loading, significant leakage of the associated peptide was observed after 3 months storage in suspension. On the contrary, freeze drying prevented the loss and/or degradation of the encapsulated peptide as the encapsulation efficiency was preserved for more than 6 months upon storage at room temperature. (Figure 1).

**Conclusion:** These results support that freeze-drying process provides optimal conditions for preserving the stability and integrity of peptide-loaded protamine nanocapsules, which are advantages sought by employing the concept of nanomedicine.

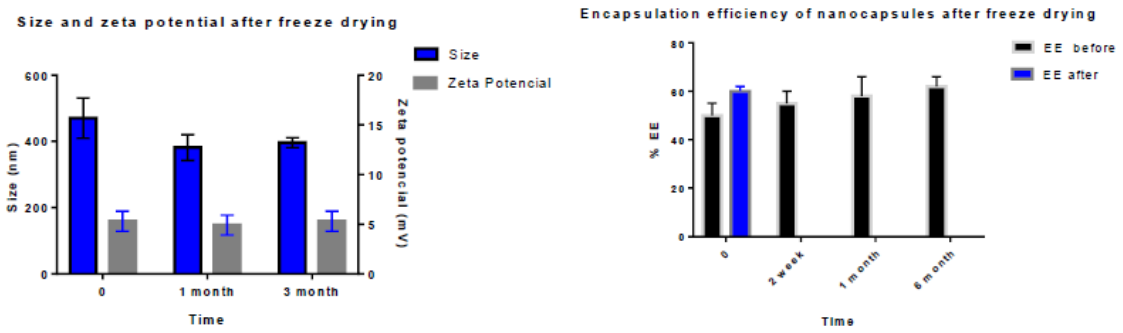


*Figure 1*. Physicochemical characteristics of protamine nanocapsules after freeze drying cycle. (EE: encapsulation efficiency)

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**PC-110**

# Mucoadhesive assessment of different antifungal nanoformulations for the Treatment of Yeast Infections in oral mucosa

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**Introduction:** Oral candidiasis is an important opportunistic fungal infection and polyens and azoles are still the most used antifungal agents. However, the majority of those treatments present a poor oral absorption and, consequently, a very high frequency of administrations. Therefore, the major challenge to focuse on may now be the improvement of the absorption of the antifungal agents in oral mucosa, and the encapsulation technology may be considered as a possible strategy to achieve this objective. Several types of mucoadhesive nanoparticles (NPs) will be prepared using nystatin (Nys) as model drug, and then drug-loaded NPs will be included in toothpaste (TP), Oral gel (OG) and film patches or oral films (OF).

**Materials and Methods:** For the development of the NPs, alginate from brown algae, acquired from Sigma-Aldrich (St. Louis, USA), PLGA and PLA both obtained from Purac (Gorinchem, Netherlands), were applied as encapsulation materials. The mean particle size, polydispersity index (PI) and zeta potential of the NPs were measured with a Coulter Nanosizer Delsa Nano™C (Fullerton, CA, USA). The interaction between the mucin and the formulations containing NPs was determined using a TA-XT2i Texture Analyser (Stabel Micro Systems, Surrey, England) [1]. The *in vitro* muchoadhesion was assessed using mucus producing HT29-MTX cells as mucosal surface in a biorelevant oral cavity model [2].

**Results and Discussion:** The results showed that the interaction between the mucin and the formulation is more evident in the mucoadhesive formulations loaded with the NPs where the peak force obtained was arrond 4.90, 3.43 and 2.94 Newtons for the OF, OG and TP, respectively, when compared with the values of the formulations without the NPs (Figure 1). The mucin interation with Nys loaded NPs was supported by *in vitro* test using HT29-MTX cells. Nys solution showed an interaction with the mucosal surface around 3.94±2.96% after 2 hours under simulated saliva flow (1.6 mL/min). For drug-loaded formulations, this percentage increased up to ten-fold factor.

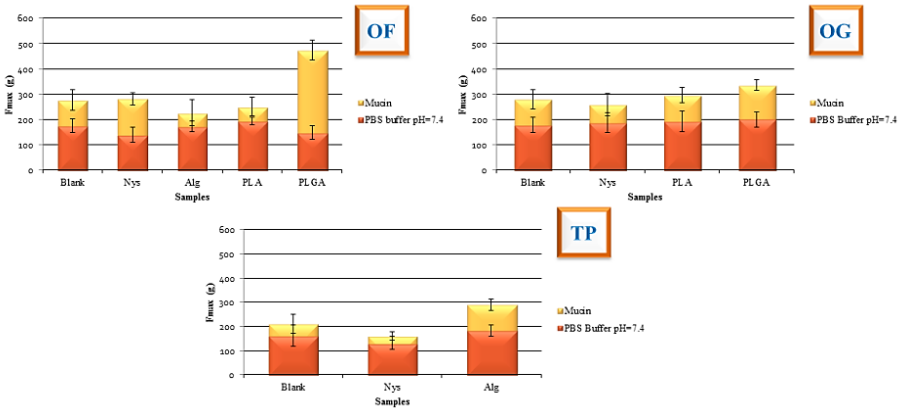


*Figure 1*. The max force obtained from the *in vitro* detachment tests for the formulations *versus* buffer (unspecific adhesion-the orange columns) and mucin dispersion (the estimated mucin interaction-yellow columns) (n= 10).

**Conclusions:** Based on analysis of all samples, we observed that the best formulation in terms of the mucoadhesion was the oral film loaded with the PLGA NPs followed by the oral gel with PLGA NPs and finaly the toothpaste with alfinate NPs. This fact was also confirmed in the *in vitro* test using HT29-MTX cell in the oral cavity model. Further studies will include *in vivo* testing using animal models.

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**PC-111**

# Development of Polymer Therapeutics for the Treatment of Skin Diseases

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## Introduction

Psoriasis is a chronic inflammatory skin disease and affects over 125 million people around the world. The disease is characterized by scaly skin, erythematous skin plaques, inflammatory cell infiltration and an incomplete differentiation of keratinocytes [1]. The cause of psoriasis is unknown, although it appears to be an autoimmune disease with the likelihood of genetic predisposition. Although the skin provides a wide accessible surface for the adsorption of therapeutic agents, topical administration of drugs has some limitations. Polymer-drug conjugates are used as an appealing platform for drug delivery in different pathologies with demonstrated clinical benefit [2]. Polymers have the property of enhancing dermal penetration, but not many references are found with polypeptide-based materials in this field [3]. Additionally, the use of polymer-drug conjugates in topical administrations has been so far limited to wound healing. In this study, we have investigated the feasibility of polypeptides to cross the skin or to be localized within the skin layers for topical treatment of skin diseases, such as psoriasis.

## Materials and Methods

PGA-drug conjugates have been synthesized by well stablish synthetic procedures by means of a pH-labile ester bond in organic solvents. The synthetic strategy followed yielded the desired conjugates with good purity as revealed via $_1$H NMR experiments. Synthetic methodology was optimized and proven to be reproducible and scalable (up to g scale), yielding the desired products with high purity. The synthesized conjugates were characterized through a complete battery of physico-chemical techniques to ensure identity, purity total drug loading and free drug content, including $^1$H-NMR, size-exclusion chromatography, size and z-potential by Dynamic Light Scattering, Circular Dichroism, UV-Vis for drug loading and High-performance liquid chromatography for free drug content and drug release kinetics.

The PGA-drug conjugates have been tested *in vitro* in relevant cell lines (Hacat, fibroblasts and macrophages Raw264.7). MTS assay was used for cell viability studies. The internalization of the conjugates, labeled with a fluorescence probe, in keratinocytes has been studied by flow cytometry and confocal microscopy. In terms of activity, the capability of the conjugate to reduce the proliferation of the inflammation after the treatment with 5ng/ml of LPS from *E. coli* in Raw264.7 was evaluated by Luminex® Multiplex Immunoassay after 72h of treatment.

Finally, ex-vivo skin permeation studies of PGA-drug conjugates in human skin have been performed in Franz Diffusion Cells and also in a human skin organotypic culture for 24 and 72h.

## Results and Discussion

The conjugate has been successfully synthesized and fully characterized. Cell viability against keratinocytes and fibroblasts has been tested, and we have also showed the capability of the conjugate to inhibit the release of cytokines from macrophages, demonstrating that the drug maintains the anti-inflammatory activity when is conjugated to the polypeptidic backbone. By confocal microscopy, we have shown that the conjugate is able to be uptaken by endocytic mechanism and co-localizes in the lysosomes. Drug release kinetics suggests that at pH 7.4 the release is slightly faster than at pH 5. Moreover, in the presence of cathepsin B a 20% release of the drug is obtained within 72 h. Ex-vivo skin permeation studies in human skin have been also performed, and the confocal fluorescence results suggest that the conjugate is able to reach the epidermis after 24h of treatment.

An *in vivo* psoriasis model was established by daily application of a specified dose of Imiquimod cream for 5 consecutive days after which they were divided into 5 groups receiving once daily treatment for 10 days. Histological examination of the skin showed significant reduction in the epidermal layer thickness when the conjugated drug was used in comparison with the parent free treatment.

## Conclusions

In this study, we have proven the capability of PGA-drug conjugates synthesis to be reproducible and scalable, being able to control parameters such as self-assembly behavior, size and drug loading.

Our results suggest that we have developed a topical therapy based on rationally designed polypeptide-drug conjugates, to be able to modulate the permeability of the drug to specific layers of the skin and so treat different skin diseases.

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**PC-112**

# PLGA Nanoparticles loaded with *Sambucus nigra* L. extracts for skin delivery

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## Introduction

*Sambucus nigra* L., common named European elder, belongs to an Adoxaceae specie and it is widely used because its medicinal properties such as anti-inflammatory and immune-stimulatory properties [1,2]. In this study, fresh and dried elderberries extracts were obtained by supercritical fluid extraction and accessed their *in vitro* antioxidant capacity, toxicity and inhibitory effect on several modulating skin enzymes (collagenase, elastase, tyrosinase and acetylcholinesterase). The most promising extract was then encapsulated into nanocarriers in order to inprove in the next future skin permeation of the loaded extract and to prevent or treat skin problems.

## Materials and Methods

The antioxidant activity (AA) was evaluated by DPPH method as described in previous studies [3]. Each plant extract was tested for collagenase (col.), elastase, tyrosinase (tyr.) and acetylcholinesterase (AChE) inhibition by measuring its effects on the activity of those enzymes using spectrophotometric methods. The toxicity of the extracts was assessed by *Artemia salina* L., brine shrimp, lethality bioassay [4]. PLGA nanoparticles (NPs) were prepared by emulsification/solvent diffusion method [5] and their mean particle size/polydispersity index (PI) and pH over the time were measured by quasi-elastic laser light and potenciometry, respectively. Particle morphology was observed by scanning electron microscopy (SEM).

## Results and Discussion

Table 1 summarizes the results obtained with extract A (dried berries with absolute ethanol), extract B (dried berries ethanol 96%), extract C (dried berries ethanol 70%) and extract D (fresh berries ethanol 96%).

*Table 1* - AA, enzyme inhibition of the extracts obtained from fresh and dried *S. nigra* berries under different extraction conditions (50 µg of extract/mL) and Screening of extracts for toxicity at 10mg/mL using *A. salina* test 1 - Quercetin, 2 - EGCG, 3 – Ursolic Acid, 4 - Kojic Acid, 5 - Tacrine and 6 - Saline medium – reference compounds used as positive controls (mean ± SD, n=3).

| Number Extract | AA (%) | Enzyme Inhibition (%) | | | | Dead larvae (%) |
|---|---|---|---|---|---|---|
| | | Col. | Elastase | Tyr. | AChE | |
| **A** | **18.9±2.49** | **84.7±3.67** | <9.07 | 32.6±2.50 | <16.0 | 27.2 ± 0.41 |
| **B** | 6.66±2.87 | 72.8±2.91 | 31.6 ± 1.36 | <10.2 | 31.0±4.22 | ----- |
| **C** | 1.49±0.69 | 45.4±0.13 | **77.3± 3.56** | <10.2 | **62.7±1.85** | 20.3 ± 1.02 |
| **D** | 1.16±0.61 | 59.1±1.70 | 17.7± 0.25 | **51.9±3.10** | 37.2±3.44 | ----- |
| **Positive Control** | 95.3±0.20[1] | 84.4±0.91[2] | 69.9±3.65[3] | 97.7±2.95[4] | 97.9±0.78[5] | 14.6 ± 0.93[6] |
| **Negative Control** | 0.09±0.02 | 8.26±0.81 | 9.07±0.82 | 10.2±2.53 | 16.0±0.00 | 17.2 ± 1.33 |

--- means not tested.

Considering the high col. inhibition (84.68 ± 3.67%, similar to positive control) and moderate AA (18.9 ± 2.49%), extract A was selected for encapsulation in PLGA NPs. By SEM, it was possible to observe that small particles were produced. Empty PLGA NPs had a rounded shape but seemed somewhat collapsed, while loaded NPs showed a spherical shape with a smooth surface. The encapsulation process produced a slight increase in the NPs size (201.57 ± 3.96nm versus 272.5 ± 2.92nm). They present monodisperse populations with a PI varying from 0.069 ± 0.013 (empty NPs) to 0.238 ± 0.013 (loaded NPs). The value of pH increased when the extract was encapsulated, changing from 3.98 ± 0.10 in empty NPs to 5.01 ± 0.01 in loaded NPs.

## Conclusions

This study suggested that *Sambucus nigra* fruits can be considered as a potential source of bioactive molecules with future application for prevention and treatment of skin disorders. Futher studies will be required to carry out in order to evaluate the efficiency of nanocarrier and their permeation through the skin.

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**PC-113**

# Thymol Nanostructured Lipid Carriers for Psoriasis Topical Therapy

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**Introduction:** Several topical formulation strategies have been developed for the treatment of psoriasis. However, there is a search for alternative less toxic therapeutics [1]. The use of natural compounds is an attractive approach for treating inflammatory disorders [2,3]. The objective was to develop a hydrogel composed of thymol-loaded Nanostructured Lipid Carriers (Thy-NLC) and to evaluate its potential in imiquimod-induced psoriasis model to ameliorate symptoms of psoriasis.

**Materials and methods:** Thy-NLC were prepared by hot-homogenization and sonication method. Particle size, polydispersity index (PDI) and entrapment efficiency were selected as critical quality attributes. Antipsoriatic potential of Thy-NLC-gel was evaluated by Psoriatic Area and Severity Index (PASI) score and histopathological examination in the Imiquimod-induced psoriasis-like inflammation model [4].

**Results and discussion:** Optimized Thy-NLC presented 105.8 ± 2.1 nm average particle size (polydispersity index of 0.224 ± 0.004), zeta potential of -12.5 ± 1.1 mV and 89 ± 1% of thymol encapsulation efficiency. Furthermore, Thy-NLC-gel significantly reduced the PASI score (Figure 1) and mice treated with thymol-NLC-gel presented a delay in the development of inflammation.

**Conclusion:** The developed Thy-NLC-gel formulation can be a promising alternative to existing topical formulations in treating psoriasis.

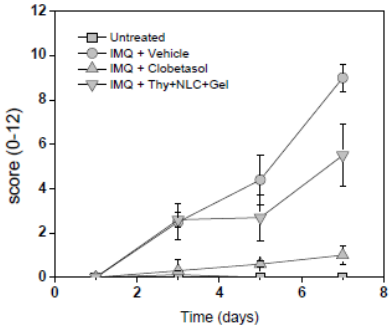


*Figure 1.* Cumulative score according to PASI regarding, erythema, thickening and scaling development with imiquimod (IMQ) challenge and treatment with vehicle, clobetasol, or thymol-NLC-gel. Untreated animals were used as IMQ treatment control.

**Acknowledgments**: This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), grants # 2013/24413-2 and #2016/07596-4 and iMed.ULisboa (UID/DTP/04138/2013) from Fundação para a Ciência e a Tecnologia (FCT), Portugal.

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**PC-114**

# Optimization of production of nanostructured lipid carriers by solvent evaporation double emulsion technique for topical drug delivery

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**Introduction:** Nanostructured lipid carriers (NLC) are the second generation of lipid nanoparticles, developed to overcome some problems of solid lipid nanoparticles (SLN) [1]. NLCs are composed by a mixture of solid and liquid lipids, usually in a ratio up to 70:30 [2]. The addition of a liquid lipid in the formulation promotes changes in the structure of the nanoparticle, leading to higher drug loading than SLN [2]. There are several techniques to produce NLC, but most of them use heating in the process making it unfeasible to encapsulate thermolabile drugs. Thus, the aim of this study was to produce NLC by a modified solvent-evaporation double emulsion technique.

**Materials and Methods:** NLCs were produced using a modified solvent evaporation method based on a w/o/w, double emulsion technique described by Fonte et al [3], by placing the liquid lipid together with the solid lipid in the organic phase. An experimental design was performed to evaluate the capacity of several lipids and surfactants to obtain particles in the nanosized range, with low polydispersity, good colloidal stability and high association efficiency (AE) of lidocaine chlorohidrate, used as a model drug. The diameter, polydispersity index, zeta potencial and AE of the drug were characterized following an appropriate methodology [4]. All measurements were performed in triplicate.

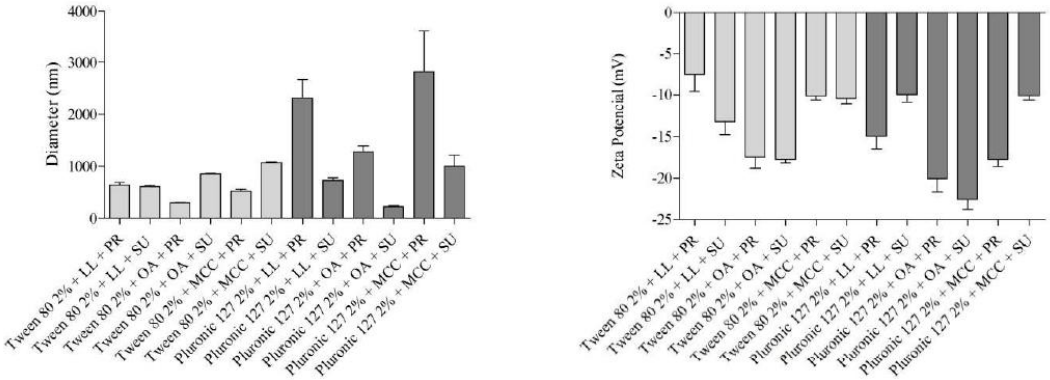


*Figure 1*. Particle size (left graph) and zeta potential (right graph) characterization of NLC formulations. LL: Labrafac Lipophile WL 1349; OA: Oleic Acid; MCC: Maisine CC; PR: Precirol ATO 5; SU: Suppocire DM Pallets.

**Results and Discussion:** The nanoparticles diameter may be affected by many factors, but the most important parameters include the amount and type of lipid and surfactant concentration [3]. The criteria to choose the best formulation were to obtain that with the lower diameter, good zeta potencial, formulation homogeneity and higher drug association efficiency. Thus, the best formulation was the one combining Tween 80® 2% (w/v) and Precirol® ATO 5 and Oleic Acid (Figure 1). The AE was higher than 70% and the polidispersity index of formulations ranged from 0.18 to 0.84.

**Conclusions:** The developed protocol allowed to produce NLC with 294.2 nm without necessity of heating, and with good colloidal stability and high association efficiency of the model drug. It is foreseen that this formulation may be applied to thermolabile drugs, such as therapeutic proteins and peptides.

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**PC-115**

# Expanding Transdermal Delivery with Lipid Nanoparticles: A New Drug-in-NLC-in-Adhesive Design

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## Introduction

Nanostructured lipid carriers (NLC) are nanosystems (40-1000 nm) considered a second and smart generation of lipid nanoparticles, consisting of a matrix composed of a blend of solid and liquid lipids (oils), stabilized by an aqueous emulsifier solution [1]. In the present work, NLC were used to simultaneously convey olanzapine (OL) and simvastatin (SV). OL is an atypical antipsychotic drug, extensively used in the treatment of schizophrenia and bipolar disorder, but long-term treatment is associated with metabolic adverse effects, in particular the alterations in the lipid profile. Its association with simvastatin would prevent dyslipidemia and reduce cardiovascular risk [2]. The aim of this work was the development of a monolithic drug-in-NLC-in-adhesive transdermal patch, with a novel design, for codelivery of olanzapine (OL) and simvastatin (SV). Nanostructured lipid carriers (NLC) and enhancers were used as passive strategies, while the pretreatment of the skin with Dermaroller, a microneedle device, was tested as an active approach.

## Materials and Methods

The NLC were prepared by the hot high pressure homogenization (HPH) technique, following an optimized procedure, previously reported [3]. In the drug loaded formulation, Combo-NLC, the addition of OL and SV was carried out in the initial lipid molten phase. Transdermal patches were manufactured by the solvent evaporation technique. For that, NLC were dispersed in different adhesives and ethanol as cosolvent. Different enhancers, including propylene glycol, transcutol, limonene and menthol, were tested at a concentration of 3.77% (w/w). They were dissolved in adhesive and added to NLC dispersions. The components were placed in a backing layer, followed by solvent evaporation in an oven at 35°C for 48 h. The coating weight of the optimal dried patch was 3.84 g, corresponding to 13.6% of the total initial weight. The characterization of transdermal patches was carried out in terms of adhesion and bioadhesion, *in vitro* permeation and cellular cytotoxicity. Molecular dynamics simulations were also performed to investigate the impact of enhancers on a model lipid bilayer.

## Results and Discussion

The formulation was optimized for composition in a quality by design basis, in terms of enhancer and adhesive, with focus on permeation behavior, adhesion properties, and cytotoxicity. Propylene glycol promoted the best permeation rate for both drugs, with enhancement ratios of 8.05 and 12.89 for OL and SV, respectively, relative to the corresponding Combo-NLC patch without enhancer. Molecular dynamics results provided a rationale for these observations. The adhesive type displayed an important role in skin permeation, reinforced by the presence of the enhancer. The permeation rates obtained for the optimized transdermal patch largely exceed (by factors of 4.4 and 12.3, for OL and SV, respectively) the target values to ensure therapeutic drug concentrations. Finally, Dermaroller pretreatment did not promote a significant improvement in permeation, which highlights the role of the combination of NLC with chemical enhancer in the transdermal patch as the main driving force in the process. It is also observed that NLC are able to reduce cytotoxicity, especially that associated with SV.

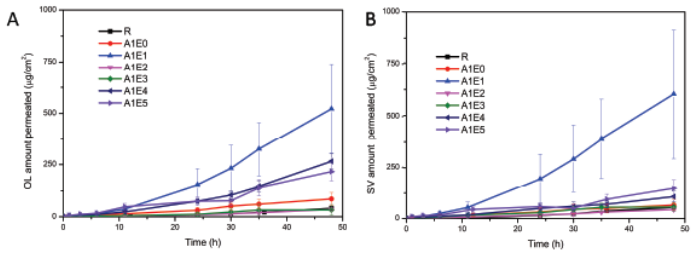


*Figure 1. In vitro* permeation profiles of the Combo-NLC-in-adhesive patches, with different permeation enhancers (mean ± SEM, $4 \leq n \leq 9$) for (A) OL and (B) SV. Key: A1= Adhesive; E1 = Propylene glycol; E2 = Transcutol; E3 = Limonene; E4 = Mentol; E5 = Propylene glycol:Mentol

## Conclusions

In conclusion, this work allowed demonstration of the versatility in the application of NLC for transdermal administration, in an innovative transdermal design, which could be a valuable basis for a future *in vivo* study.

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**PC-116**

# Controlled delivery of exosomes for chronic wound healing

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Worldwide, there are 382 million patients with diabetes and is estimated that this number will rise up to 592 million by 2035 [1,2]. Approximately 15% of these individuals have or will develop diabetic foot ulcer [2]. The rising tendency of chronic wounds incidence and prevalence demands the development of more efficient therapies. In the current work, we have investigated the therapeutic effect of exosomes isolated from umbilical cord blood mononuclear cells in diabetic chronic wounds. Exosomes are vesicles with 50 to 200 nm in diameter that transport bioactive proteins, lipids and RNA [2, 3]. Exosomes were characterized for their morphology, surface marker expression and miRNA composition by RNA deep sequencing. Our *in vitro* results indicate that exosomes are bioactive against skin cells including keratinocytes, endothelial cells and fibroblasts, increasing cell proliferation, enhancing cell survival under ischemic conditions and promoting cell migration in a *in vitro* wound healing assay. Exosome administration in wounds of diabetic and non-diabetic mice lead to enhanced wound healing kinetics as compared to control groups and an advanced therapy approved by FDA (PDGFBB). Our results further show that miR-150-5p mediates in part the bioactivity of the exosomes, as validated by *in vitro* and *in vivo* tests. Moreover, we developed a photo-sensible hydrogel based on hyaluronic acid that allows the controlled release of exosomes in the wound site promoting accelerated wound closure, enhanced vascularization and better wound healing in diabetic mouse model.

In summary, a new platform to delivery therapeutic exosomes was successfully developed and represents a promising tool for the treatment of chronic wounds.

**Acknowledgments**: This work was supported by FCT (SFRH/BDE/103512/2014; MITP-TB/ECE/0013/2013; EXCL/DTP-PIC/0069/2012; UID/NEU/04539/2013); QREN-COMPETE funding (FCOMP-01-0202-FEDER-038631; Project "ExoCord" promoted by Stemlab, SA and Biocant) and Portugal 2020-COMPETE funding (Project "Stem cell based platforms for Regenerative and Therapeutic Medicine", Centro-07-ST24-FEDER-002008; Project "StrokeTherapy" co-promoted by Stemlab, Rovisco Pais and Universidade de Coimbra).

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**PC-117**

# Passive and active dermal delivery of a new photosensitizer for photodynamic therapy

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**Introduction**. Most clinically approved photosensitizers for photodynamic therapy (PDT) are tetrapyrrolic macrocycles [1] and their percutaneous permeation is very slow. This led to the topical administration of precursors of endogenous photosensitizers (e.g. aminolevulinic acid or methyl aminolevulinate), rather than the use of pre-formed photosensitizers, to treat dermatological disorders. Although such precursors may lead to the accumulation of Protoporphyrin IX (PpIX) in target cells and enable PDT 3 to 4 hours after the topical administration. PpIX is a poor photosensitizer, with low light absorption in the red, and PDT with PpIX is limited to superficial lesions. Progress in PDT of skin lesions depends on the availability of more potent photosensitizers that rapidly and efficiently permeate the skin, and of methods that promote the dermal delivery of such photosensitizers.
Physical methods, such as sonophoresis, iontophoresis or microneedles, actively deliver molecules through skin using external forces. More recently, piezoporation using high-frequency ultrasound was proposed as a painless and efficient active method to permeabilise the skin, with the advantage of allowing the skin barrier to recover within minutes in the end of the procedure [2]. This work describes piezoporation of skin to increase the dermal deliver of a low molecular weight and potent photosensitizer (LUZ51B). Piezoporation was used to reduce the time to achieve therapeutic amounts of LUZ51B in epidermis.

**Materials and Methods** The test substance, 5-methylamide-10,20-bis(2,6-difluorophenyl)bacteriochlorin, or LUZ51B, was prepared by Luzitin SA, and made available for these studies.
Cytotoxicity and phototoxicity studies were performed in colon adenocarcinoma cells and keratinocytes cells. Studies with bacteria employed gram-positive Propionibacterium acnes.
Skin permeation studies were performed by the topical application of a gel based formulation containing 1% photosensitizer in minipig skin. Active permeation was achieved by photoacoustic waves generation with a nanosecond Nd:YAG laser and piezophotonic materials made available by LaserLeap Technologies SA.
The evaluation of the penetration depth of the photosensitizer was performed by confocal microscopy (LSM 510 Meta, Carl Zeiss, Jena, Germany) analysis of cryo preserved skin slices.

**Results and Discussion** We show that a new carboxamide bacteriochlorin with molecular weight below 600 Da has a low cytotoxicity in the dark but in the presence of ca. 750 nm light is able to generate both singlet oxygen and superoxide anion, showing unprecedented sub-nanomolar phototoxicity in cancer cell lines. The colonies of gram-positive Propionibacterium acnes are reduced by 8 log units when incubated with 2 μM LUZ51B and illuminated with a 10 J/cm$^2$ light dose.
Piezoporation was used as an active method to deliver LUZ51B through skin, increasing significantly the initial fluxes through the stratum corneum and enhance photosensitizer delivery when compared with passive delivery over the same period of time. Confocal microscopy showed a depth of photosensitizer diffusion of approx. 50 μm which confirmes the presence of the compound in epidermis.

**Conclusions** Our results show that this carboxamide bacteriochlorin is an excellent candidate for PDT of skin lesions. It is particularly relevant for the clinical translational potential to note that the amount of photosensitizer available in skin epidermis 30 min after skin exposure to piezoporation is identical to the amount obatined with 2 h of passive diffusion.

**Acknowledgments** This work was financially supported by the Portuguese Science Foundation (007630UID/QUI/00313/2013, PTDC/QEQ-MED/3521/2014, Roteiro/0152/2013/022124) and by QREN/30218. We acknowledge EC support through Laserlab-Europe, H2020 EC-GA 654148. Helder T. Soares thanks FCT for grant SFRH/BD/119058/2016. We thank Centro de Neurociências e Biologia Celular, and specially Luísa Cortes, for the assistance with confocal microscopy studies.

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**PC-118**

# Laser piezoporation: Safe interaction with human skin and proof-of-concept of intraepidermal delivery

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**Introduction:** Pressure waves can be used to permeabilize biological barriers, such as the outer layers of skin or cellular membranes, with a reversibility that allows skin to recover its protective function and cells to remain viable [1]. The outer layer of the skin – the *stratum corneum* – with a dozen of layers of hardly packed nonliving corneocyte cells embedded in a mixture of lipids with high spatial organization, is the main contributor to the skin impermeability. Piezoporation is based on pressure waves generated by irradiation of highly absorbing thin films [1-4] with nanosecond laser pulses that generate steep pressure gradients. The pressure gradients perturb the *stratum corneum* structure, expanding the extracellular domains and promoting dermal delivery [1,4]. Piezoporation is a non-invasive and painless alternative method to transiently permeabilize the skin enabling molecules to diffuse more rapidly through the *stratum corneum* [1,3,4].

**Materials and Methods:** Laser piezoporation of skin is based on the interaction between high-frequency ultrasound and the *stratum corneum*, acoustically coupled to the skin by the topical formulation containing the drug. Production of pressure waves capable of transiently perturb the stractum corneum was acheived by 10 ns Nd:YAG lasers pulsed irradiation of a thin piezophotonic film [2]. The drugs studied in this work were prepared in topical gel formulations.

**Results and Discussion:** Evidence for safe and painless application of piezoporation in humans is presented. It is widely recognized that transepidermal water loss (TEWL) is a measure of the skin barrier function and permeability. An average increase of TEWL from 14 to 30 g/($cm_2$ h) was observed in 21 human volunteers aged 18–25 years after 2 minutes exposure to piezoporation, thus evidencing skin barrier perturbation. The TEWL values exponentially return to normal values with a half-life of 40 seconds, demonstrating that the perturbation is transient [1]. Erythema was measured at the site of exposure before and after piezoporation and the observed increase is barely perceptive to the view and resolved in a few minutes. Thus we conclude that piezoporation interacts with skin with a reversibility that allows skin to recover its protective function within a few minutes and without any aesthetic footprint. Piezoporation was successfully applied to promote the efficient intraepidermal delivery into *ex-vivo* and *in-vivo* pig skin of three photosensitizers used in photodynamic therapy, with relatively high molecular weights (600 Da to 1.1 kDa) and distinct physicochemical properties [1]. The proof of concept on delivery of macromolecules is shown by the intraepidermal delivery of green fluorescence protein (28 kDa) and high molecular weight hyaluronic acid (800 kDa) [1,4].

**Conclusions:** Piezoporation with high frequency pressure waves generated by nanoseconds laser pulses reversibility permeabilizes the skin, which recovers its protective function and original appearance in a few minutes. The process is transient and painless. Proof-of-concept of fast intraepidermal delivery of photosensitizers and macromolecules is presented.

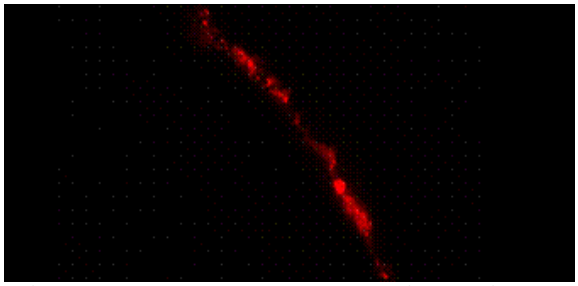

*Figure 1*. Fluorescence microscopy image of pig skin biopsies intraepidermal delivery of temoporfin in 0.5% (w/w) formulation; 2 min piezoporation plus 20 min passive diffusion**.**

**Acknowledgments**: Work supported by European Regional Development Fund (COMPETE 2020) and by national funds (FCT): PTDC/QEQ-MED/3521/2014; 007630UID/QUI/00313/2013; RNEI/0152/2013/022124; Helder T. Soares thanks FCT for grant SFRH/BD/119058/2016. EC support through Laserlab-Europe H2020 EC-GA 654148.

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