CRS
Spanish-Portuguese
Local Chapter

XIIIth Spanish-Portuguese Conference on Controlled Drug Delivery

Santiago de Compostela, Spain

22-24, January 2020

Abstract Book

INDEX

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42º52'57.1"N 8º32'43.5"W
(42.882539, -8.545429)

WELCOME MESSAGE

Dear participants,

On behalf of the Board of the Spanish-Portuguese Local Chapter of the Controlled Release Society (SPLC-CRS), it is my pleasure to welcome you to the **XIIIth Conference of our chapter**. This year, on the occasion of our **25th anniversary**, we have tried our best to organize a very special Conference.

The conference will last for 3 days, on which we will spend a very intense program together containing:

- 5 Plenary Talks presented by distinguished experts in the area of Drug Delivery, and 9 sessions:

  - In the first of these, representatives of the European CRS Local Chapters will give us an overview on their research interests.

  - In the 6 scientific sessions in which we have structured the Program, we will have keynote speakers and short talks focusing on various areas of Drug Delivery, such as: Transdermal and transmucosal delivery, Infectious diseases, Regenerative medicine, Targeted delivery, Delivery of biomacromolecules and oligonucleotides, and Oral delivery.

  - Finally, a group of European industry experts will analyze and discuss the current state of the art of Nanomedicine.

I would also like to highlight the session dedicated to the “25th Anniversary” of the SPLC-CRS, with testimonies from a few past Presidents.

During the Conference we will award a prize for the best thesis of the last two years. The best oral communication and poster presented during the meeting will also be recognized thanks to the financial support of the international CRS. At this moment, we would like to thank all the sponsors for their financial support to help the organization of the Conference
We hope that you can all enjoy the atmosphere of this meeting, not only from the scientific point of view, but especially from the point of view of networking.

I also hope that you have the opportunity to enjoy Santiago, its ancient stones, its typical "ruas" and also its special student atmosphere.

We look forward to welcoming you to Santiago!

Dolores Torres

President of the SPLC-CRS

SCIENTIFIC PROGRAM

Wednesday 22nd January

12.00-14.00 Registration and Posters placement

14.00-14.15 Welcome: USC authorities

Dolores Torres (SPLC President)

María José Alonso (CRS Past President)

14.15-15.00 PLENARY LECTURE 1

Alexander Florence

Emeritus Professor, University College of London, UK

"A « nano » trajectory and the complexities of targeting"

15.00-15.30 **Coffee and refreshments • Posters viewing**

15.30-19.30 SESSION 1. The Future of the CRS Local Chapters in Europe

Session Chair: Avi Schroeder

15.30 United Kingdom-Ireland Local Chapter

Katie Ryan, University College Cork, Ireland

'Biomaterial drug-delivery systems to support bone tissue regeneration"

16.00 Nordic Local Chapter

Bente Steffansen, Leo Pharma, Denmark

"Paracellular permeability across Caco-2 cell monolayer: in vitro studies and modeling"

16.30 BeNeLux & France Chapter

Giovanna Lollo, Lyon University, France

"Tailoring structural properties of nanocomposite scaffolds for sustained intestinal delivery"

17.00 Germany Local Chapter

Cornelia M. Keck, Philipps University Marburg, Germany

"Smart nanocarriers for improved drug delivery"

17.30-18.00 **Coffee and refreshments • Posters viewing**

18.00 Italian Local Chapter

Paolo Decuzzi, IIT Central Research Labs, Genova, Italy,

"Mechano-Pharmacological Properties of Drug Delivery Systems in Brain Diseases"

18.30 Turkish Local Chapter

Sevgi Güngör, University of Istanbul, Turkey

"Follicular targeting of topical nano-carriers for the treatment of dermatological diseases"

19.00 Greece Local Chapter

Sophia Antimisiaris, Patras University, Greece

"Extracellular vesicles, cellular vesicles or liposomes for targeted delivery of drugs?"

19:30 PhD Thesis Award

20:30 **Welcome Cocktail**

Thursday 23th January

08.30-09.00 Registration-Posters placement and presentations upload

09.00-09.45 PLENARY LECTURE 2

Mark Prausnitz

Director of the Center for Drug Design, Development and Delivery at Georgia Institute of Technology, USA

"Microneedle patch technology for long-acting contraception and dermal interstitial fluid diagnostics"

09.45-11.15 SESSION 2. Transdermal and Transmucosal Delivery

09.45 Richard Guy

Professor of Pharmaceutical Sciences. University of Bath

"Prediction and Optimisation of Drug Delivery into and through the Skin"

10.15 Marcelo Calderón

Head of Responsive Polymer Therapeutics GroupPolymat, San Sebastian, Spain

"Crossing biological barriers with responsive nanogels to improve drug delivery Performance"

10.45 Short Talks selected from abstracts

11.15-11.45 **Coffee and refreshments • Posters viewing**

11.45-13.00 SESSION 3. Therapy and prevention of infectious diseases

11.45 Juan Manuel Irache

Professor of Pharmacy and Pharmaceutical Technology. University of Navarra, Spain

"Nanoparticles as adjuvants for mucosal vaccination"

12.15 Short Talks selected from abstracts

13.00-14.00 **Lunch**

14.00-14.45 PLENARY LECTURE 3

Steven R. Litlle

Chairman of the Department of Chemical and Petroleum. University of Pittsburgh. McGowan Institute for regenerative Medicine, USA

"Controlling "Controlled Release" to Make Medicine That Imitates Life"

14.45-16.00 SESSION 4. Regenerative medicine and cell therapy

14.45 Miguel Oliveira

3B´s Research Group, Institute 3Bs, University of Minho, Portugal.

"From Micro- and Nano-technologies to Nanomedicines: Applications in tissue engineering and regenerative medicine"

15.15 Short Talks selected from abstracts

16.00-16.30 **Coffee and refreshments • Posters viewing**

16.30-18.15 SESSION 5. Targeted Drug Delivery and Combination therapies

16.30 Avi Schroeder

Head of the GroupTechnion - Israel Institute of Technology.

"Personalized barcoded nano-medicines indicate primary breast tumors respond to different drugs than brain metastasis"

17.00 Hélder Santos

Head of Nanomedicines and Biomedical Engineering Group, University of Helsinki, Finland.

"Nanobiostructured systems for biomedical applications: From design to cancer therapy"

17.30 Short Talks selected from abstracts

18.15 SESSION 6. The 25 Anniversary of the 1st SPLC-CRS Conference

Testimonies of Past Presidents

María José Alonso (University of Santiago de Compostela)

Eugenia Cruz (Retired from University of Lisboa)

Joao Nuno Moreira (University of Coimbra)

José Luis Pedraz (University of Basque Country)

María Jesús Vicent (Principe Felipe Research Center)

Friday 24th January

08.30-09.00 Registration-Presentations upload

09.00-09.45 PLENARY LECTURE 4

Ignacio Melero

Director of the Immunology and Immunotherapy department of the CIMA. University of Navarra.

"On translatable local approaches for cancer immunotherapy"

09.45-11.00 SESSION 7. Delivery of Biomacromolecules and oligonucleotides

09.45 Javier Montenegro

CIQUS, University of Santiago de Compostela, Spain

"New Chemical and Supramolecular Concepts for Nucleic Acid and Macromolecular Cytosolic Delivery"

10.15 Short Talks selected from abstracts

11.00-11.30 Coffee and refreshments • Posters viewing

11.30-12.15 PLENARY LECTURE 5

Luis Liz Marzán

Scientific Director of CIC biomaGUNE, San Sebastian, Spain

"Diagnosis and Therapy Based on Gold Nanoparticles"

12.15-13.15 SESSION 8. Oral Delivery of drugs and other active ingredients

12.15 Noemi Csaba

Associate Professor, CIMUS, University of Santiago de Compostela, Spain

"Bioinspired delivery systems for oral peptide therapeutics"

12.45 Short Talks selected from abstracts

13.15-14.15 Lunch

14.15-15.45 SESSION 9. Organized by the SPLC-CRS Young Section

14.15 Flash communications selected from posters

15.00 Interactive session with Alexander Florence (University College of London, UK)

15.45-17.15 SESSION 10. Discussion Panel: The perspective of the Industry in the Nanomedicine

Moderator: María José Alonso (University of Santiago de Compostela)

Participants:

Nazende Günday-Türeli (MyBiotech GmbH, Überherrn, Germany)

Maria Teresa Peracchia (Sanofi Aventis, Paris, France)

Dario N. Carrara (FERRING Galenisches Labor AG, Allschwill, Switzerland)

Marisol Quintero (Bioncotech Therapeutics, Valencia, Spain)

Pilar Calvo (Pharmamar S.A., Madrid, Spain)

17.15-17.45 Coffee and refreshments

17.45-18.15 Awards for the best oral communication and poster. Closing ceremony

18.15-19.15 General Assembly of the SPLC-CRS

21:00 Gala Dinner

PLENARY LECTURES

A "nano" trajectory and the complexities of targeting

Alexander T. Florence *

Professor Emeritus, UCL School of Pharmacy, London, UK

*e-mail: *ataylorflorence@aol.com*

Our life as individuals has been described as a parabolic trajectory: some events are the result of chance or coincidence. So too are the trajectories of the nanosystems we administer in the hope of targeting them to specific endpoints. They are affected individually and in concert by stochastic events. This presentation will emphasise some of these phenomena based on our findings over a number of years, a reflection of what was once known as the field of colloids or "the world of neglected dimensions," but now part of the expanding nanoworld. It reflects on transition from studies on surfactant micelles (~5-15nm in diameter), systems such as emulsions, microparticles (100μm) and nanoparticulates including dendrimers (5nm), which in terms of dimensions was a parabolic trajectory. Each system revealed some characteristics relevant to the behaviour of nanosystems designed for drug delivery. It involved application of physical chemistry of a variety of systems to nanosystem trajectories in vivo, understanding of the stability of these colloidal systems, and lessons from our failed attempts to adapt Nature's nanoparticles such as low density lipoproteins as drug carrier systems. It led us to confront the complexities of the interactions of both microparticles and nanosystems with the biological environment both in vitro and in cells and tissues, a reminder that biology cannot escape the laws of physics. Amongst the relevant topics to be discussed briefly will be:

- Particle diffusion in complex media;
- Obstruction effects: diffusion inhibited by objects in their way such as actin threads in cells;
- Particle flow behaviour in vessels and channels;
- Percolation in complex 3D structures;
- Brownian motion influencing the movement of particles, not least in the vicinity of absorbing surfaces such as Peyer's patches in the gut; and
- The vital importance of the physical properties of the drugs and their intrinsic potency.

All these are aspects of the complexity of targeting. Close nanometric proximity of nanosytems and target receptors is required to achieve any ambition of targeting, better defined as final interaction with the target. It is a challenge: finding complexity even in interpretation of gathered data does not mean failure. In cancer chemotherapy the tumour targets are often heterogeneous and change their 3D structure so the target is a dynamic system. Herein lies the impossibility of generalisations. Unexpected findings can be valuable, as two examples from our work might show: the finding that a dendrimer we had synthesised possessed intrinsic fluorescence, which allowed us to follow its trajectory in vivo. Another was the finding that a polylysine dendrimer formed a complex with heparin, conferring on the complex anti-angiogenic (and hence anticancer) activity. While findings such as these may be modest, we must look for clues in cognate literature searching for analogies in often disparate work and we must certainly not ignore the older literature. Our work on the oral delivery of nanoparticles which commenced in 1987 followed from our reading of a 1936 book on Absorption from the Intestine by F. Verzár, for us one rather long trajectory!

Microneedle patch technology for long-acting contraception and dermal interstitial fluid diagnostics

M.R. Prausnitz

School of Chemical & Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA, USA

e-mail: prausnitz@gatech.edu

Microneedle patches enable minimally invasive access to the body interior. This access can be used to administer drug formulations that would otherwise require injection, and can be used to create pathways for flow of interstitial fluid from the skin. Two applications of microneedle patch technology will be discussed.

Our first project is motivated by the need for many women to have improved access to contraception. Barrier methods have low acceptance and a high failure rate, mostly due to incorrect use, which can result in unplanned pregnancies. Sustained-release formulations of contraceptive hormones are available, yet typically require their administration by trained personnel. To address this need, we designed a microneedle patch with rapidly separable biodegradable polylactic acid and poly(lactic-co-glycolic acid) needles, and its application for the continuous release of levonorgestrel, a contraceptive hormone. Bubble structures between each microneedle and the patch backing allow the microneedles to efficiently penetrate into skin under compression, and to snap off under shear within five seconds after patch administration. In rats, the microneedle patch was well tolerated, leaving little visible evidence of use, and maintained plasma concentrations of the hormone above the human therapeutic level for one month. Further development of the rapidly separable microneedle patch for self-administered, long-acting contraception could enable women to better control their fertility.

Our second project is motivated by an interest in sampling tissue interstitial fluid (ISF) as a novel source of biomarkers that complements conventional sources like blood and urine. However, ISF has received limited attention due largely to lack of simple collection methods.To address this need, we developed a method to sample ISF from human skin in a relatively simple manner with minimally invasive microneedles, that was well tolerated by study participants. Using a microneedle patch to create an array of micropores in skin coupled with mild suction, we sampled ISF from 21 human participants and identified valuable and sometimes unique biomarkers in ISF when compared to companion plasma samples based on mass spectrometry analysis. Many biomarkers used in research and current clinical practice were common to ISF and plasma. Because ISF does not clot, these biomarkers could be continuously monitored in ISF like current continuous glucose monitors, but without an indwelling subcutaneous sensor. Biomarkers unique to ISF included molecules associated with systemic and dermatological physiology as well as exogenous compounds from environmental exposures. We also determined that pharmacokinetics of caffeine (a model biomarker in healthy adults) and pharmacodynamics of glucose (in diabetic children and young adults) were similar in ISF and plasma, indicating that dynamic information of biomarkers could also be captured using ISF. Overall, these studies provide a minimally invasive method to sample dermal ISF using microneedles and demonstrate human ISF as a source of biomarkers that with further optimization can enable research and translation for future clinical applications.

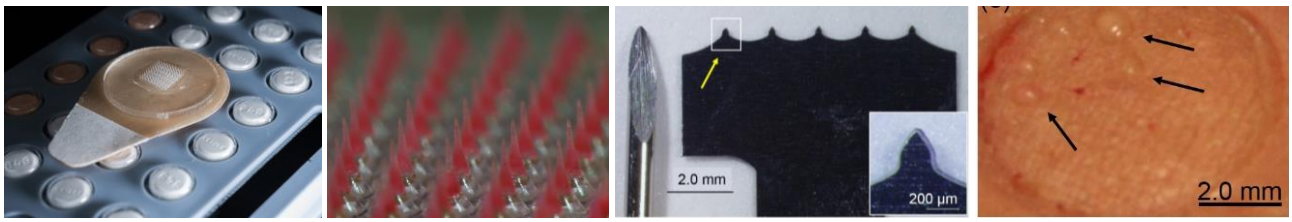

Figure 1. Microneedles for long-acting contraception and dermal interstitial fluid diagnostics. Microneedle patch shown with oralcontraceptive tablets (far left). Magnified view of microneedles containing red dye to indicate the site of contraceptive hormone encapsulation (center left). Metal microneedle array for collection of interstitial fluid from skin (center right). Interstitial fluid droplets (arrows) extracted from the skin using microneedles and suction.

Acknowledgments: This work was supported in part by the U.S. National Institutes of Health (U2CES026560, P30ES020953, R01ES023485, P30ES019776, S10OD018006) and Children's Healthcare of Atlanta. This was also supported by a subcontract funded by Family Health International (FHI 360) under Co¬operative Agreement No. AID-OAA-15-00045 funded by the U.S. Agency for International Development (USAID). The content of this publication does not necessarily reflect the views, analysis or policies of FHI 360 or USAID, nor does any mention of trade names, commercial products, or organizations imply endorsement by FHI 360 or USAID.

MRP is an inventor of patents licensed to companies developing microneedle-based products, a paid advisor to companies developing microneedle-based products and a founder/shareholder of companies developing microneedle-based products (Micron Biomedical). This potential conflict of interest has been disclosed and is managed by Georgia Tech and Emory University.

Controlling "Controlled Release" to Make Medicine That Imitates Life

S. Little

Department of Chemical and Petroleum Engineering, The Swanson School of Engineering, University of Pittsburgh, Pennsylvania, United States

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Although the field of controlled release (as applied to release of drugs, cosmetic agents, fertilizers, etc…) has existed for over 40 years, it is still non-trivial to formulate (or engineer) a system to produce a target release behavior (duration of release and rate of release). Over the last 12 years, our research group has revealed fundamental phenomena in how the most widely-used degradable polymers degrade/erode and how this impacts release behavior from systems comprised of these polymers. These discoveries allow for more precise (even in silicon) design of controlled release formulations that meet the specific needs of the customer. It also permits, for the first time, design of "biomimetic" controlled release systems that reproduce the basic spatio-temporal information transfer that naturally occurs between the cells in our body, with the goal of inducing and/or regulation key biological processes. Such is currently out of the reach of modern medicine. As just one example, simple temporal control over the release of specific growth factors can induce robust formation of specific tissues that naturally regenerate via stage-wise processes. This is possible using recent advances in the precise design of controlled release formulations. In the same way, this concept can also be used to reproduce spatial information that cells (and even tumors) employ to manipulate immunological responses. Collectively, these new tools can effectively reproduce biological context and have already shown significant promise as next-generation medical treatments in a variety of disease models where current medical treatments have no answer.

On translatable local approaches for cancer immunotherapy

Ignacio Melero Bermejo[1,2]*

[1] *Program of Immunology and Immunotherapy, Cima Universidad de Navarra, Pamplona, Spain*
[2] *Department of Immunology and Immunotherapy, Clínica Universidad de Navarra, Pamplona, Spain*

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Cancer immunotherapy has become standard of care based on checkpoint inhibitor monoclonal antibodies targeting PD-1 and CTLA-4 as well as on adoptive cell transfer with CAR engineered T cells. This form of therapies are delivered through systemic routes. Alternatively, strategies of intratumoral of locoregional administration can have been tested and are under clinical development to maximize efficacy and limit systemic side effects. Preclinical and clinical experience through intratumoral routes will be presented for the nanoplexed dsRNA analogue BO112. Preclinical results for repurposing the yellow fever vaccine and for intratumoral adoptive T cell transfer. In the latter case, anti-tumor T cells transiently engineered by IL-12 and CD137 mRNA electroporation achieve unprecedent results in terms of efficacy. The talk will include background information regarding other agents being developed and opinions and insights into the future of the strategy.

Diagnosis and Therapy Based on Gold Nanoparticles

Luis M. Liz-Marzán[1,2]*

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[2]*Ikerbasque, Basque Foundation for Science, 48013 Bilbao, Spain*

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Metal nanoparticles display very interesting optical properties, related to localized surface plasmon resonances (LSPR), which give rise to well-defined absorption and scattering peaks in the visible and near-IR spectral range. Such resonances can be tuned through the size and shape of the nanoparticles, and are highly sensitive towards dielectric changes around the particles and to their specific organization within assemblies. Therefore, metal nanoparticles have been proposed as ideal candidates for biosensing and bioimaging applications.

In this communication, we present several examples of novel strategies to employ nanostructured materials comprising gold nanoparticles, as substrates for ultrasensitive detection and imaging of biorelevant molecules, based on different plasmonic phenomena such as surface enhanced Raman scattering and plasmonic chirality.

An additional consequence of the LSPR phenomenon is the release of absorbed light in the form of heat, in a highly localized fashion. Therefore, gold nanoparticles are also promising candidates for photothermal therapy, which however requires careful design of the morphology and surface chemistry of the nanoparticles, as well as the laser irradiation conditions. Recent advances in photothermal effects coupled to in situ nanothermometry will be presented.

Acknowledgments: This work was supported by the European Research Council (ERC AdG and ERC AdG)

[1] D. Jimenez de Aberasturi et al., *Chem. Mater.* 2016, **28,** 6799.
[2] G. Bodelón et al., *Nat. Mater.* 2016, **15**, 1203.
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[4] M. Quintanilla et al., *Theranostics* 2019, **9**, 7298.

INVITED LECTURES AND ORAL COMMUNICATIONS

Session 1: The Future of the CRS Local Chapters in Europe

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Biomaterial drug-delivery systems to support bone tissue regeneration
Katie B. Ryan, D. Kenendy, Z. Sartawi, A. Doozo and C.W. Waeber

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Paracellular permeability across Caco-2 cell monolayer: in vitro studies and modeling
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Mechano-Pharmacological Properties of Drug Delivery Systems in Brain Diseases
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Follicular targeting of topical nano-carriers for the treatment of dermatological diseases
S. Güngör

IL1.7 Greece Local Chapter (p.32)
Extracellular vesicles, cellular vesicles or liposomes for targeted delivery of drugs?
S.G.Antimisiaris, A. Marazioti, S. Mourtas

Biomaterial drug-delivery systems to support bone tissue regeneration

Katie B. Ryan*[1], D. Kenendy[1], Z. Sartawi[1], A. Doozo[1] and C.W. Waeber[1;2]

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[2]*Department of Pharmacology and Therapeutics, University College Cork, Cork, Ireland, TI2YN60.*

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Introduction

Repair of critical size bone defects typically involves autograft and allograft procedures, however these are associated with numerous limitations including supply problems, donor-site morbidity and infection-risk that are well documented. In order to address these problems, current research is focusing on developing bioactive, load-bearing, bone graft substitutes that promote bone healing *in vivo*. A wide range of materials including polymers, ceramics and bioactive constitutents have been investigated to achieve this goal [1]. Our work to date has focused on investigating composite polymer scaffolds to enhance biocompatibility and osteconductive properties to promote the regeneration of bone tissue by combining biocompatible polymers with bioactive glasses, bone morphogenetic proteins (BMP-2) and osteogenic constituents (stem cells). More recently, the low molecular weight lipid mediator sphingosine 1-phosphate (S1P) has been of increasing interest in the context of bone regeneration [2]. S1P receptor modulation has been shown to improve outcomes in murine studies of bone defects, ostensibly by supporting vascularization and chemotaxis of specific cellular contributors to bone repair [3,4]. S1P and the S1P receptor modulator fingolimod have previously been prepared for localised delivery using PLGA-based scaffold methodologies [5,6]. While several S1P receptor subtypes seem to play a role in bone physiology, there is evidence that $S1P_1$ receptor modulation, may be advantageous in the context of bone repair [2]. As part of our work, we are investigating the the newly developed $S1P_{1/5}$ selective agent Siponimod for its potential role in bone regeneration. Sepcifically, the goal of this present study was to exploit the mechanical stiffness of hydroxyapatite (HA), and the biocompatibility and biodegradability of PLG polymer to create a composite scaffold, which acts to control the presentation of a novel, bioactive lipid, Siponimod. Samples were characterised in terms of the physical, chemical and biological propensity to support bone regeneration.

Materials and Methods

Samples were prepared by electrospraying the polymer containing the Siponimod drug. Porous organic-inorganic composites were prepared by combining different ratios of hydroxyapatite and Siponimod-loaded poly(lactic-co-glycolic acid) (PLGA) (85:15) polymeric microspheres with the porogen, sodium chloride. The components were then compressed into a 6 mm disk, followed by particle-foaming using carbon dioxide. The porogen was then leached from the scaffold by immersion in water.

The porous structure and morphology were characterised using a JEOL JSM-5510 Scanning Electron Microscope (SEM). The physical and chemical proeprties of the samples were characterised in terms of differential scaning calorimetry, Fourier Transform Infrared (FTIR) spectroscopy, contact angle gonimetry and tensile strength testing. Siponimod stability in PBS (pH 7.4) was monitored over 90 days at 2-8 °C and at 37 °C. Drug release studies were conducted over a 90-day period and drug content was determined using HPLC analysis. Drug loading efficiency of the samples was determined and was used to calculate percentage release over the 90-day period. The viability of hFOB pre-osteoblast cells seeded on the composite surface was measured using resazurin and was used as an indicator of cytocompatability and osteoconductivity.

Results and Discussion

The architecture of 3D porous scaffolds offers the support to support bone formation through provision of mechanical support and an environment that allows cellular infiltration and colonisation [7]. In this study, scaffolds prepared by an electrosprayed and CO_2 foaming process produced structures with porosity values between 70-84%, which is necessary to support cell infiltration and nutrient/waste exchange. Siponimod drug encapsulation efficiency ranged between 35-48.5%, which is considerably lower than encapsulation achieved through electrospinning methods (80-94%) employed in our lab. Consequently, we believe the reduction was likely affected by the porogen-leaching step employed in this fabrication method. Drug-loading efficiency was not significantly altered by the inclusion of HA. The scaffold system acts to control release of the Siponimod, with < 50% drug release over the 90-day timeframe. The addition of siponimod did not adversely effect hFob cell viability over 7 days, whereas inclusion of HA resulted in statistically significant increases in cell viability in those samples over the 7-day cell culture period.

Conclusions

To improve osteoconductive properties, natural components of bone (collagen and hydroxyapatite) can be combined with biocompatible polymers to generate composites with enhanced bioactive properties. To our knowledge, this is one

of the first studies to investigate the formulation and controlled release of Siponimod for its potential use in bone regeneration. In this study, we have fabricated a bioactive, porous scaffold that supports cell growth over 7-days. Current studies are investigating the osteoinductive potential of the scaffold.

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Paracellular permeability across Caco-2 cell monolayer: *in vitro* studies and modeling

B. Steffansen[1, 2]

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Introduction: The CRS Nordic Chapter will be outlined. Furthermore, to examplify collaborative work in the Nordforsk/Nordic POP and CRS Nordic Chapter network, the following recent published work is presented [1].

During drug development, fraction absorbed (F_a) in humans of permeability limited compounds are often estimated from apparent permeabilities (P_{app}), measured across filter-grown Caco-2 cell monolayers. However, estimates of such F_a for paracellular permeating compounds are challenging compared to compounds permeating by transcellular passive diffusion. The hypothesis behind this study is that estimating paracellular permeability across Caco-2 cells can be improved by refinement of the existing models described by Linnankoski et al. [2] and by Avdeef [3]. The aim was therefore to apply such refined model by investigating the relative contributions of paracellular and transcellular permeabilities to P_{app} of the permeability limited drug acamprosate. **Methods:** The P_{app} of four paracellular marker molecules were used to determine paracellular pore radius, pore capacity and potential drop of the Caco-2 cell monolayer (system-specific parameters). These parameters were then applied to refine the model for determining the modelled permeability ($P_{modelled}$) of the marker compounds and of acamprosate. **Results and Discussion:** Apical to basolateral (A-B) acamprosate P_{app} was measured to $1.56 \pm 0.28 \times 10^{-7}$ cm·s^{-1}, which is similar to the measured P_{app} of the three paracellular markers, for which the P_{app} were $2.72 \pm 0.24 \times 10^{-7}$ cm·s^{-1} (mannitol); $1.80 \pm 0.35 \times 10^{-7}$ cm·s^{-1} (lucifer yellow); $2.10 \pm 0.28 \times 10^{-7}$ cm·s^{-1} (fluorescein), but lower than P_{app} for atenolol, which was $7.32 \pm 0.60 \times 10^{-7}$ cm·s^{-1} (mean ± SEM, n = 3-6) [1].

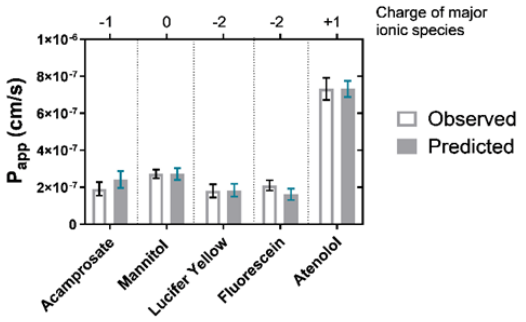

Figure 1. Experimentally determined P_{app} (cm·s^{-1}) expressed as mean ± SEM (N=6-12, pooled data from both A-B and B-A) of P_{app} (white bars, observedThe Figure is from reference [1].

For each compound, the P_{app} in the A-B direction were not signigficantly differet from that in the B-A direction [1]. The $P_{modelled}$ for all the paracellular markers and acamprosate was dominated by the P_{para} component. As shown in Figure 1, the $P_{modelled}$ values were similar to the experimentally obtained P_{app} [1]. **Conclusion:** The refined model for paracellular permeability estimated well, the experimental P_{app} values of all the paracellular markers. Regarding the $P_{modelled}$ for acamprosate, the paracellular permeation appears to be the determining mechanism for acamprosate P_{app} across the Caco-2 cells.

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Tailoring structural properties of nanocomposite scaffolds for sustained intestinal delivery

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Introduction

Precise and controlled release rate of drugs from nanosystems in specific part of the body has numerous advantages over conventional formulations, as enhanced bioavailability and minimized side effects. These benefits are even more important for patients suffering from chronic diseases that require multiple oral dosage regimes [1]. To investigate the improvement of localized treatment and to elucidate the mechanisms that control the passage of nanosystem in the systemic circulation from the intestinal wall, an innovative oral drug platform based on a tailored nanoemulsion doped into a biodegradable scaffold was developed.

Materials and Methods

Nanoemulsion (NE) formulation was obtained by emulsion phase inversion (EPI) technique and optimized by a mixture design. The crystallinity and fluidity of the NE shell were investigated for NE both in colloidal suspension or in dried state. Then, scaffolds made of chitosan (CS) doped with NE were prepared by the freeze-casting technique [2, 3] and structural analysis using scanning electron microscopy (SEM) and optical images was performed. *In vitro* release studies were carried out in biorelevant GI fluid (FaSSIF-V2) to evaluate the modulation of the release rate of NE associated to chitosan scaffold. Finally, to evaluate the intestinal residence time of the systems, fluorescent-labeled NE and CS-NE were administered by oral gavage to healthy mice.

Results and Discussion

NE were optimized and loaded with the model drug tacrolimus by an experimental design. NE converted into dried powders using both spray drying and freeze-drying techniques recovered their initial properties following reconstitution in water. Structural analysis suggested that when in dry state the NE shell became crystalline while amorphous in colloidal suspension [4]. Then, CS scaffolds were prepared and loaded with NE. By SEM and optical images it was observed that CS scaffolds presented a cell-walls structure and the presence of NE improved the their mechanical strength due to interactions occurring between particles and polymer chains. Scaffolds were rehydrated in biorelevant intestinal fluids (FaSSIF-V2) to investigate the impact of the CH matrix on NE release kinetics. The NE release rate *in vitro* was highly dependent on scaffold structure and composition. A sustained NE release was achieved from scaffolds at high CH concentrations (28% in 2 h followed by a plateau at 50% after 8 h). *In vivo* fluorescent imaging study evidenced the increased retention of the CS-NE in comparison to NE in the gastrointestinal tract upon oral administration.

Conclusions

Overall, our results have demonstrated the potential of developed nanocomposite scaffolds as a versatile tool for sustained intestinal drug delivery.

Acknowledgement.

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SmartFilms® and tablets made from paper for improved drug delivery

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Introduction: Drug delivery aims to transport active pharmaceutical ingredients (API) to the desired site of action. If classical pharmaceutical dosage forms fail to reach this target, more sophisticated drug delivery systems are required to reach this goal. In case of BSC class II drugs nanocarriers, i.e. nanocrystals, lipid nanoparticles, solid dispersions or liposomes can be used to overcome poor solubility and bioavailability of these actives. A novel drug delivery system to overcome poor solubility is the smartFilm®-technology. The technology is simple but smart. It uses ordinary paper in which poorly soluble APIs can be loaded in amorphous state [1].

Materials and Methods: The antioxidants rutin and curcumin were used as model drugs and were loaded into the pores of different commercially available papers. The crystalline state, solubility in artificial body fluids, drug release and dermal penetration were determined. In order to allow for a more convenient oral application of the smartFilms®, the drug loaded papers were transferred into pellets and tablets made from paper. The pharmaceutical properties were determined according to the European Pharmacopoeia (Pharm. Eu.).

Results and Discussion: The BCS class II drugs curcumin and rutin were loaded into the pores of commercially available paper and remained in amorphous state, leading to a pronounced increase in dissolution rate and kinetic solubility, when compared to larger sized bulk material [1-3]. Passive diffusion of curcumin from smartFilms® was determined by applying the API loaded smartFilms® on fresh porcine ears (Fig. 1A, B). After 6h of penetration the amount penetrated was analysed by observing the autofluorescence of curcumin from punch biopsies. Results showed a pronounced transdermal penetration, hence pronounced passive diffusion of the API into the viable dermis, from the smartFilms®, whereas bulk material and even nanocrystals with a size of about 200nm, failed to do so. In the next step paper was transferred into pellets and into tablets made from paper. After optimization of the production parameters, the tablets made from paper appeared shiny and with a smooth surface (Fig. 1C). Also, the resulting pellets and tablets fulfilled all pharmaceutical criteria, i.e. mass variation, content uniformity, resistance to crushing, disintegration and drug release according to the Pharm. Eu. [3,4]. Drug release of the poorly soluble actives was increased when compared to bulk material and could be modified by coating of the tablets.

Conclusions: SmartFilms® are a novel drug delivery principle. They can be used for dermal and oral application to improve the drug delivery of poorly soluble actives. In case of oral application, the smartFilms® can be transferred into pellets or tablets to allow for a more convenient swallowing of the smartFilms®. Loading of paper can be done in large scale but also individually. This enables the universal use of smartFilms® in industrial drug products but also for the tailor-made production of precision medicines.

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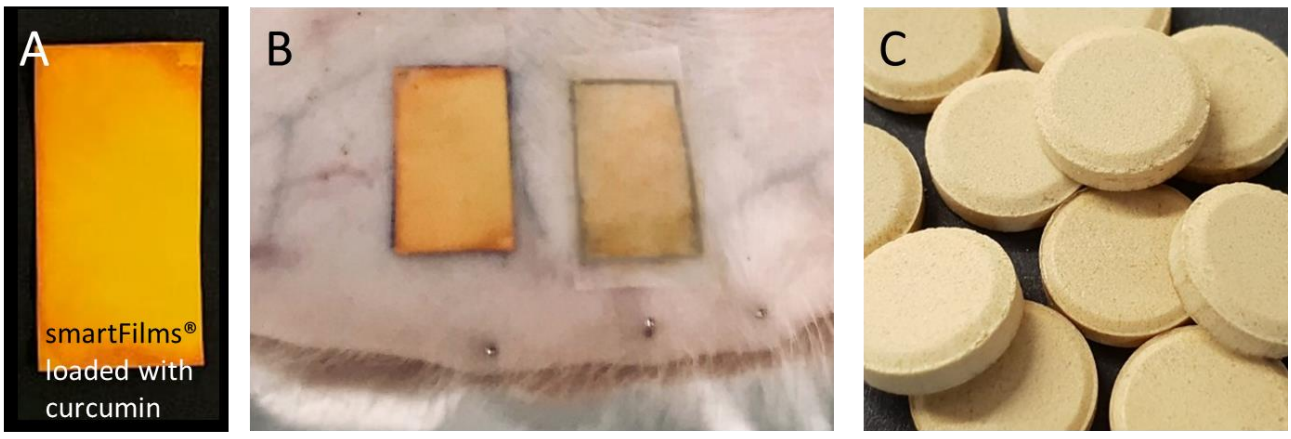

Figure 1. SmartFilms® are composed of paper in which APIs are loaded in amorphous state (A). They can be used for improved drug delivery of poorly soluble actives, either for dermal application (B) or – after transfer into pellets or tablets (C) – for oral application.

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Mechano-Pharmacological Properties of Drug Delivery Systems in Brain Diseases

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Multifunctional nanoconstructs are particle-based nano-scale systems designed for the 'smart' delivery of therapeutic and imaging agents. The Laboratory of Nanotechnology for Precision Medicine at IIT-GE synthesizes multifunctional polymeric nanoconstructs with different *sizes*, ranging from a few tens of nanometers to a few microns; *shapes*, including spherical, cubical and discoidal; *surface* properties, with positive, negative, neutral coatings; and mechanical *stiffness*, varying from that of cells to rigid, inorganic materials, such as iron oxide (Figure 1). These are the *4S parameters* – size, shape, surface, stiffness – which can be precisely tuned in the synthesis process enabling disease- and patient-specific designs of multifunctional nanoconstructs. In this lecture, the role of manipulating these 4S parameters over different temporal and length scales will be elucidated in the context of future nanomedicines with applications in cancer, cardiovascular and anti-inflammatory diseases.[1],[2] Particular attention will be given to the role of particle deformability in modulating US-triggered drug release, vascular transport, mechanical support and the therapeutic efficacy of implantable drug delivery systems.[3]

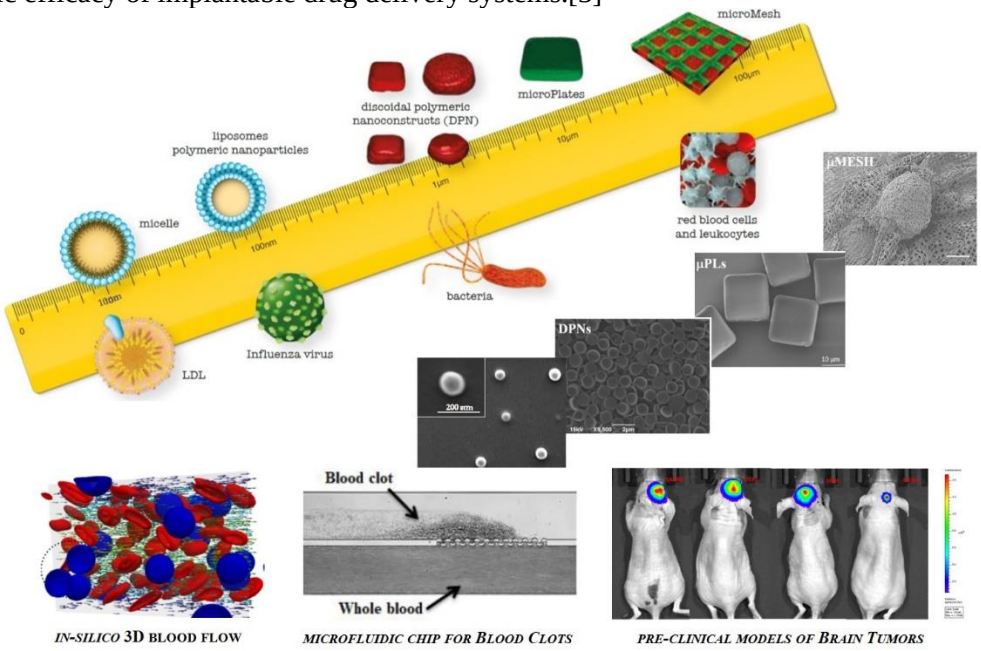

Figure 1. Drug Delivery Systems with different geometrical attributes and mechanical properties

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Follicular targeting of topical nano-carriers for the treatment of dermatological diseases

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Introduction

Topical therapy is an attractive choice for the treatment of dermatologic diseases due to its advantageous such as reduction of risk of systemic side effects. It also offers a potential approach for the targeting of drugs to the site of disease. However, human skin is a well-designed unique membrane, particularly *stratum corneum*, the outermost layer of epidermis, is an extremely effective barrier for the penetration of most drugs due to its excellent structure. It behaves as a rate-limiting barrier for diffusion of almost all drugs due to its well-ordered morphological structure. The conventional dosage forms such as creams, gels could be unsatisfactory in the topical treatment due to the poor penetration of drugs into targeted layers of skin and inadequately deposition in the skin, resulting in low topical bioavailability. Thus, the delivery of drugs into skin layers to achieve the required drug concentrations in the target cutaneous tissues is challenging. Targeting of drugs into hair follicles *via* nanocarriers has also been considered as a promising strategy for improving the topical treatment efficiency of some dermatological diseases such as acne and other inflammatory diseases [1-3].

Methods

Different types of nanocarriers of drugs used in the treatment of dermatological diseases such as acne, atopic dermatitis and psoriasis were optimized. The characterization studies consisting of their particle size and distribution, zeta potential, drug loading efficiency were carried out. Then, the feasibility of their dermal targeting ability of selected these nanocarriers were examined by *in vitro* skin penetration studies. Following skin penetration studies. The localization of drugs into skin layers were determined by tape stripping technique, and the follicular deposition of drugs were also quantified. Skin penetration depth and the deposition of the nanocarriers in hair follicles was also visualised by confocal laser microscopy analyzes.

Results and Discussion

Nnaocarriers of drugs used in the treatment of acne and atopic dermatitis improve the skin deposition efficacy of topically applie. Confocal laser microscopy images indicates the follicular pathway appears to be quite reasonable for the localization of nanocarriers in the skin. The nanocarriers seems to be promising for selective, targeted drug delivery to the hair follicles.

Conclusion

Nanocarriers could improve cutaneous penetration of drugs, and localization of drugs in the target layers of the skin. Thus, the dose required to obtain therapeutic efficacy could be decreased and, then possible side effects of drugs could be minimized. In this respect, nanocarriers systems seem to have potential for targeting drugs to hair follicles as a topical skin delivery strategy. However, it should also be confirmed the potential of the nanocarrier-based dermal delivery with *in vivo* studies in depth.

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Extracellular vesicles, cellular vesicles or liposomes for targeted delivery of drugs?

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In the last 8 years we have studied a number of targeted liposomes, as nanoparticle based drug delivery systems for brain-located pathologies, and especially for Alzheimers Disease (AD) [1-9]. For in vitro evaluation BBB targeting potential of targeted liposomes, we used the hCMEC/D3 cellular model of the BBB. In vivo studies were carried out by live animal imaging after iv administration of DiR-labelled liposomes, in normal mice and APP/PS1 double transgenic mice and their wild-type (WT) littermates. Interesting conclusions have been drawn, however the liposomes targeting potential was not increased more than 4 times (compared to plain pegylated liposomes), even in the case of multi-targeted nanocarriers.

Recently, cellular vesicles have been proposed as alternative of exosomes or extracellular vesicles as targeted drug carriers [10]. We have prepared various types of cellular vesicles, were constructed using liposome-technology engineering methodologies, and were tested (in vitro and in vivo) under identical conditions with those used before for the targeted liposomes. The results of these latter studies confirm that exploitation of the targeting components of cellular vesicles may potentially lead to construction of superior types of drug-carriers for theragnosis of brain localized diseases [11].

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INVITED LECTURES AND ORAL COMMUNICATIONS

Session 2: Transdermal and Transmucosal Delivery

IL2.1 Prediction and optimisation of drug delivery into and through the skin. (p.35)
Richard H. Guy

IL2.2 Crossing biological barriers with responsive nanogels to improve drug delivery performance. (p.36)
Marcelo Calderón

OC2.1 Photothermal therapy of basal cell carcinoma using gold nanorods-loaded microarray patches. (p.37)
A.S. Cordeiro, Á. Carcamo-Martínez, M.T. Rahman, S.E.J. Bell and R.F. Donnelly

OC2.2 Functionalized hydrogels for transferulic acid loading and release. (p.38)
A. Varela-Garcia, C. Alvarez-Lorenzo, A. Concheiro

Prediction and optimisation of drug delivery into and through the skin

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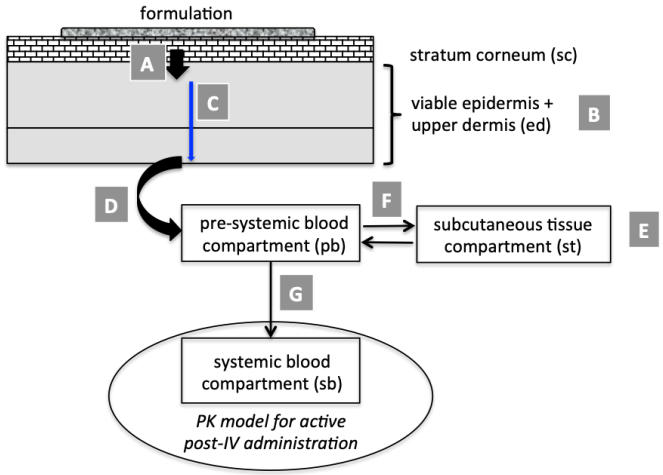
A relevant and testable skin absorption and physiologically-based pharmacokinetic (PBPK) model should be able to capture and shed light upon the complex interplay between topical drug product attributes and *in vivo* outcomes. The development, validation and application of such a model – an example of which is illustratrd below - would facilitate the design of high-quality and efficient formulations for application to the skin.



A. Input function of active from SC into viable skin.

B. Viable epidermis and upper dermis combined.

C. Passive diffusion from SC to dermal microcirculation.

D. Skin "clearance" (blood flow, 'extraction coefficient').

E. Subcutaneous tissue compartment.

F. Extent of distribution into subcutaneous tissue.

G. 'Elimination' of active from skin into blood.

The strategy adopted recognizes 'up-front' that topical products are complex, multicomponent systems, the properties of which change profoundly post-application to the skin. Consequently, the absorption kinetics of the active moiety are difficult to simulate with a simple mathematical construct. To address this challenge, the "input process" of the active compound is being characterized experimentally using the innovative application of spectroscopic, microscopic and stratum corneum sampling techniques [1-5]. Further innovation in the approach postulates that the kinetic and distribution parameters, which describe the disposition of the active within the model's physiologically-based but experimentally inaccessible structures between the 'input function' and the systemic blood, are correlated with and predictable from key physicochemical properties of the active. Validation, development and refinement of these uniquely novel strategies for the PBPK modeling and simulation of dermal absorption has, at its core, the goal of a pragmatic approach that is no more complicated than is needed to perform its task

Ultimately, the research aims to link judiciously chosen experimentation to *in vivo* outcomes, and to illustrate that the physicochemical properties of a topical product (i.e., the active molecule + its delivery system) can be manipulated during the iterative development process of design and testing to direct the rational and efficient optimisation of high-performance formulations. Realization of this objective translates very clearly into a significant reduction, or even elimination, of the dependency (for example) on animal models and human studies, accelerating the product development process, and bringing safe and effective topical medicines to market more rapidly and economically.

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Crossing biological barriers with responsive nanogels to improve drug delivery performance

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The term nanogel (NG) refers to nanometer-sized crosslinked polymeric networks that reveal intrinsic properties ideal for biomedical applications, i.e. high water content, soft nature, cell and tissue compatibility, and excellent water dispersability/solubility. Therefore, NGs are commonly developed as drug carriers which shrink or swell significantly by expelling or absorbing large amounts of water, and selectively release their cargo in response to external stimuli. Among such stimuli, temperature is attractive because of its simple accessibility and easy applicability, both in vitro and in a biological environment.[1] In this context, our group has developed several thermoresponsive NGs (tNGs) and nanocapsules as drug delivery systems based on dendritic polyglycerol as a macro-crosslinker and different thermoresponsive polymers such as poly(N-isopropylacrylamide),[2] poly(oligo ethylene glycol methacrylate),[3] and thermoresponsive poly(glycidil ether).[4] Their biocompatibility, together with the possibility of fine tuning their size and responsive modality, makes them ideal candidates for various therapeutic and diagnostic approaches, particularly for topical drug delivery.

Topical administration permits targeted, sustained delivery of therapeutics to human skin and mucose. Delivery to the skin however is limited to lipophilic molecules with molecular weight of typically < 500 Da, capable of crossing the stratum corneum. Nevertheless, the investigated tNGs were found to enhance the penetration of fluorescent dyes, used as model drug, without any sign of toxicity.[5] To further evaluate the potential of the thermoresponsive nanogels for the loading and delivery of therapeutic moieties on inflamed skin models, we effectively adapted the synthetic methodologies to enable the encapsulation of either a highly hydrophobic drug, i.e. Dexamethasone, or a biologically active protein, i.e. Etanercept. We could demonstrate the high potential of such nanocarrier systems, using excised human skin and reconstructed human skin models.[4] For the encapsulation and delivery of Dexamethasone, a synthetic methodology for the introduction of β-cyclodextrin to the nanogels surface was developed. This approach showed to be advantageous to exploit the complexation of β-cyclodextrin with Dexamethasone, along with its role as a cutaneous penetration enhancer. Furthermore, the compatibility of the thermonanoprecipitation synthesis with in situ encapsulated proteins could be achieved by a water-in-water nanoprecipitation approach. Etanercept, an anti-TNF fusion protein, could be delivered into reconstructed skin equivalents providing the first evidence for its efficient non-invasive efficacy. Moreover, it could be shown that the delivery of the protein into the viable epidermis occurred explicitly upon its temperature triggered release, also for Transglutaminase [1,2b] and Ovalbumin [6] for protein replacement therapy and needle-free vaccination, respectively. Furthermore, providing the tNG network with disulfide functionalities improved their ability for mucosal penetration and adhesion along with a slow degradation over time, which was used as a trigger to deliver a therapeutic active to submucosal layers.

The examples hereby described highlight the great potential that tNGs hold, which are able to incorporate multiple functionalities and target several skin and mucosal diseases.

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Photothermal therapy of basal cell carcinoma using gold nanorods-loaded microarray patches

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Basal cell carcinoma (BCC) is the most common form of non-melanoma skin cancer, with an incidence of 77 to 158 cases per 100,000 people per year in Europe. Although BCC is usually non-fatal, with limited metastatic abilities, it leads to severe local destruction and considerable disfigurement, mainly in the face and upper body. BCC lesions are typically treated by surgical excision, which is an expensive procedure and may result in functional and cosmetic defects [1]. Plasmonic photothermal therapy (PPTT) proposes the use of particles with plasmonic properties, such as gold nanorods (GNR), stimulated by laser radiation to induce local hyperthermia in the lesions, leading to cell death and tumour ablation [2]. In this work, we investigate the use of GNR-loaded microarray patches (MAPs), as a potentially more cost-effective alternative for scar-free removal of deep nodular BCC lesions.

GNRs were obtained through an optimized seed-mediated growth method [3] and imaged by transmission and scanning electron microscopy. To confirm that the maximum wavelength of UV absorbance was within the required range, the UV spectrum was recorded for each batch. Hydrogel-forming 5x5 MAPs (with needles measuring 1.2 mm in height and 500 µm in base width) were prepared from aqueous blends of 25% w/w Gantrez® S-97, 10% w/w poly(ethylene glycol) and 27.5% w/w aqueous GNR suspension. The aqueous blends were degassed and cast into MN moulds in two steps to allow for GNR accumulation in the needle tips and dried at room temperature before cross-linking *via* esterification at 80ºC. To ensure the adequate mechanical properties of the developed MAPs, their resistance to compression and insertion into skin models were evaluated. As a proof-of-concept of the potential of this approach to induce local hyperthermia in tissue models, MAPs (in air or inserted into agar blocks) were irradiated with a 2 W laser at 809 nm and temperature changes were recorded using a thermal camera.

The seed-mediated growth synthesis method led produced GNRs with an adequate size ratio (approximately 60 nm in length and 15 nm in width), as required to achieve maximum UV absorption at 760-780 nm. Once incorporated into the hydrogel formulation, similar UV absorption peaks were observed following laser irradiation, indicating the stability of the GNRs when combined with the hydrogel. A 2-step casting process was implemented and this successfully allowed for the incorporation of the GNRs into the needle tips of the MAP, without affecting the MAP mechanical properties. Approximately 85% of the needle height was inserted in Parafilm M®, used here as a skin model, showing the ability of these MAPs to penetrate deeply into the tumour lesions. Following laser irradiation, an increase of 38ºC in the temperature of the needle tips was observed. However, when performing the same experiment with the MAPs inserted into an agar block (used as a tissue model), the temperature increase was only moderate (8ºC), as required for tumour ablation.

These preliminary results show the potential of this approach to generate a temperature increase in the tumour lesion that should be sufficient to promote tumour ablation as required for PPTT-based BCC treatment. Further studies are now ongoing regarding the diffusion of heat through skin models and the *in vivo* efficacy of this strategy.

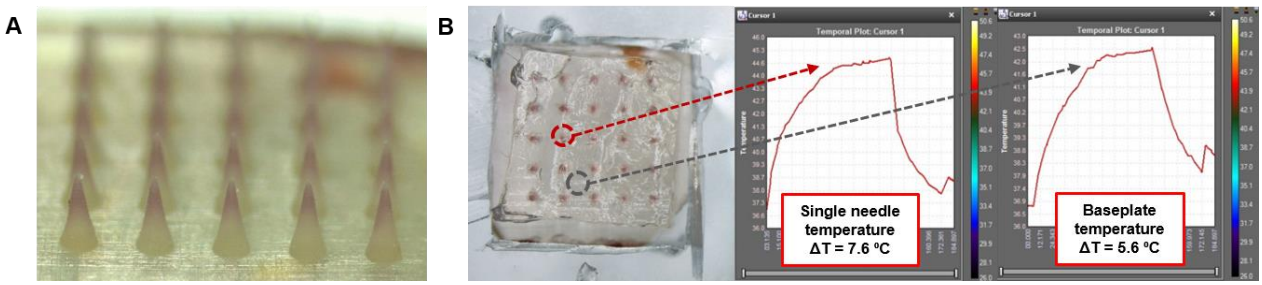

Figure 1. (**A**) Optical microscopy (16x magnification) of a 5x5 microarray patch (MAP) loaded with gold nanorods (GNRs). (**B**) Summary of the tissue heating assay, with a 5x5 GNR-loaded MAP inserted into an agar block, used as a tissue model, and irradiated with a 2W laser at 809 nm for 1 min. The temperature changes (shown in the graphs) were recorded with a thermal camera.

Acknowledgments: This work was supported by the Engineering and Physical Sciences Research Council (EPSRC).

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Functionalized hydrogels for transferulic acid loading and release

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Introduction

Classic eye treatments with antioxidant agents, like eye drops, have disadvantages such as the low bioavailability of the drug, due to the structure of the eye that acts as an efficient barrier, as well as the physical processes that occur after instillation, i.e. blinking, tearing or naso-lacrimal drainage. Soft contact lenses (CLs) can be a good alternative for the administration of drugs in a controlled and painless way [1]. The objective of this work was the functionalization with cytosine of acrylic hydrogels to reinforce the affinity for the antioxidant agent and thus improve the loading and release properties. The hypothesis was that cytosine can act as a binding monomer for transferulic acid (TA), which presents a complementary structure to DNA or RNA in terms of hydrogen bonding and stacking capacity.

Materials and Methods

The hydrogels were prepared by mixing 8 mL of HEMA (2-hydroxyethyl methacrylate) and EGDMA (8 mM) with GMA (glycidyl methacrylate) 0, 100, 200, 400 or 600 mM and EGPEM (ethyleneglycolphenylether methacrylate) 0, 100 or 200 mM. Finally, AIBN 10 mM (2,2'-azobiobisobutyronitrile) was added as polymerization initiating agent. The mixture was injected into pre-assembled moulds 0.45 mm thick, and incubated for 12 hours at 50 ºC and 24 hours at 70 ºC. Next, the hydrogel sheets were immersed in 100 mL of water:dioxane solution (1:1) with 1.11 g of cytosine, at 80 ºC for 24 h. Finally, they are cut into discs with a diameter of 10 mm. [2]

A physicochemical characterization of the hydrogels was carried out, analyzing the FTIR spectrum, swelling, transmittance and mechanical behavior. Next, the hydrogels were loaded in aqueous solution of TA (10 µg/mL) by soaking of individualized discs in 5 mL of solution during 48h at 25 ºC, under agitation. The loaded amount was determined spectrophotometrically (320 nm). Drug release was then monitored in 5 mL of simulated lacrimal fluid (SLF). The biocompatibility of the loaded hydrogels was evaluated with the HET-CAM test, the cytocompatibility with human corneal epithelial cells (ATCC® PCS-700-010™) and the antioxidant activity by means of the ORAC test (Oxygen Radical Absorption Capacity). The permeability of AT through the cornea and sclera, and the accumulation in them was evaluated following a previously described protocol. [3]

Results and Discussion

The inclusion of cytosine into the hydrogel was confirmed under UV light (monitoring fluorescence) and recording FTIR spectra where the characteristic carbonyl amide group was observed. All hydrogels had a similar degree of swelling, between 45 and 65%. Young's modulus was close to 0.50 MPa. Transmittance properties were higher than 80% in all cases and the biocompatibility and cytocompatibility tests were positive.

The differences in load between functionalised hydrogels with cytosine and non-functionalised hydrogels were notable. For example, non-functionalized hydrogels showed load values of 0.17 (s.d. 0.02) µg of TA per gram of dry net, while those functionalized with cytosine showed higher values of 0.41 (s.d. 0.02) µg of TA per gram. In addition, functionalized hydrogels maintained the release of TA for 24 hours and that amount of drug released retained its high antioxidant capacity. The coefficients of permeability through the cornea and sclera of the drug contained in the functionalized hydrogels were as high as those recorded for the aqueous solution of TA.

Conclusions

Acrylic hydrogels functionalized with cytosine showed a greater affinity for TA, particularly when combined with EGPEM 200 mM. The addition of GMA as a bridge to immobilize the cytosine was effective. The physicochemical properties of hydrogel remained stable after functionalization. Therefore cytosine-functionalized hydrogels can be considered a good prototype to host drugs and release them in a sustained manner.

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INVITED LECTURES AND ORAL COMMUNICATIONS

Session 3: Therapy and prevention of infectious diseases

Nanoparticles as adjuvants for mucosal vaccination

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Vaccination is one of the most cost-effective ways of improving living standards, health and economic prospects. Traditional vaccines usually include the whole microorganism (attenuated or inactivated) and a parenteral route of administration. Although many of them have been central to control different infectious diseases (particularly attenuated vaccines), they show some safety concerns for different groups of population (i.e., pediatric and immunocompromised individuals). The design of vaccines based on microbial elements (i.e, OMVs, proteins, polysaccharides, DNA) has been proposed to offer safer medicines. However, these subunit vaccines are poorly immunogenic, requiring adjuvants to boost their efficacy. On the other hand, mucosal surfaces are the major portal of entry for many pathogens and, in principle, vaccines capable of eliciting mucosal immune responses may fortify defenses at mucosal front lines and protect against infection. Nevertheless, the large majority of the approved adjuvants are inneficient when administered by an enteral route.

One alternative to solve these drawbacks may be the use of polymeric nanoparticles as adjuvant for mucosal immunization. These carriers offer protection against the degradation of the loaded antigen and are, in general, capable of controlling its release. However, conventional nanoparticles usually display poor targeting abilities for the mucosal lymphoid tissues and/or dendritic cells underling the epithelium, being rapidly eliminated by physiological clearance mechanisms (i.e., mucus, peristalsis, ciliary movements, blinking, etc.). Therefore, in some cases, the immune response elicited with these antigen carriers is usually not as high as required to offer the adequate degree of protection to the host. In order to overcome these drawbacks and render nanoparticles more efficient as adjuvants, one possible strategy can be their association with compounds or ligands capable to specifically interact with receptors located in the mucosa, including compounds involved in the colonization and invasion processes of microorganisms.

Gantrez-AN or poly(anhydride)-based nanoparticles can be adequate materials to prepare mucosal adjuvants. These nanoparticles act as agonists of various Toll-like receptors (TLRs) (TLR2, -4, and -5), triggering a Th1-profile cytokine release (gamma-interferon and interleukin-12) and, after incubation with dendritic cells (DCs), inducing a significant increase of CD54 and CD86 co-stimulatory molecules expression [1]. In addition, these nanoparticles can be easily modified by simple incubation with different excipients or ligands in order to modify their distribution within the gut or/and their bioadhesive potential as well as their capability to promote specific immune responses.

The aim of this lecture is to summarize the properties and capabilities of different types of nanoparticles (based on the use of Gantrez AN) as adjuvants. Some examples of their ability for vaccination purposes are also presented.

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Hyaluronic acid-Amphotericin B Nanocomplexes: a Promising Anti-leishmanial Targeted Drug Delivery System

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Leishmaniasis has been classified as one of the most neglected tropical diseases, causing 50 thousand deaths and 1.5 to 2 million new cases every year. This disease, promoted by protozoan parasites of the genus Leishmania, has a high incidence affecting 89 countries worldwide [1]. Nowadays, current treatment strategies still rely on the antifungal agent amphotericin B (AmB) [2] but are rather inadequate due to the high prevalence of the disease within low-income population of sub-developed regions, the intracellular location of the parasite and the emergence of parasite resistance. Thus, other strategies have been pursued to improve the therapeutic efficacy and to reduce the toxicity of AmB such as the use of biocompatible polysaccharides as carriers [3]. In this work, a simple and inexpensive production process using hyaluronic acid (HA, 50 kDa) was used in order to develop water-soluble hyaluronic acid-amphotericin B nanocomplex (HA-AmB). HA is the main ligand of CD44 receptor, thus being favorably internalized by macrophages that overexpress this receptor upon infection [4]. Therefore, HA arises as a suitable polysaccharide to target the AmB delivery to the leishmania-infected macrophages.

The nanocomplex, obtained by simply processing the mixture of the polysaccharide with the drug in a nanospray dryer (HA-AmB SD), was characterized in terms of size/zeta potential (DLS) and morphology (SEM and Cryo-SEM). Furthermore, an HPLC-MS detection method was optimized and used to determine the AmB content in the nanocomplex. Also, to ascertain the interaction between AmB and the HA, FTIR, DSC and PXRD analysis were performed. Cytotoxic and hemolytic effects were assessed on different cell lines through the resazurin test and in dog's blood, respectively. Anti-leishmanial activity was assessed *in vitro* in axenic cultures of Leishmania by resazurin and in infected bone marrow-derived macrophages (BMMΦ) stained with different fluorescent probes using high-content microscopy.

Our results shown that the produced material has a spherical morphology in aqueous solution with a mean hydrodynamic diameter of 318.4 ± 34.7 nm and low polydispersity (0.239 ± 0.02). Moreover, this material that presents an AmB content of 13.56 ± 3.49 %, has a good colloidal stability due to the highly negative surface charge (-39.45 ± 1.12 mV). DSC and PXRD analysis strongly suggested the formation of an amorphous inclusion complex between AmB and the complex polysaccharide chain networks, explaining the high solubility of the drug in water. The *in vitro* assays showed that compared to free-AmB, the nanocomplex had significantly less cytotoxicity against BMMΦ and HEK293T cell lines, significant less hemolytic effect and inhibited the infection in the Leishmania-infected BMMΦ. Exploratory *in vivo* assays are being conducted in mice. In conclusion, this work has shown that the hyaluronic acid-AmB nanocomplex is a promising system for the treatment of leishmaniasis, possessing similar effects to the free-AmB against leishmania-infected macrophages and leishmania axenic cultures, with reduced cytotoxicity. Given the affordability, simplicity, low-toxicity and facile scale up of the developed formulation, the hyaluronic acid-AmB nanocomplex may represent an alternative to the expensive nanoformulations available.

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Influence of Nanoparticle Design on the Immune Response against an HIV Peptide Antigen

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Introduction

HIV continues to be one of the most threating infectious diseases, with almost 38 million people infected worldwide. Unfortunatedly, only six clinical trials have been completed to this date, and none of them has offered encouraging results [1]. As an alternative to the full protein antigens *Env*, *Gag* or *Pol*, traditionally investigated for HIV vaccines, the peptide sequences surrounding the HIV protease cleavage sites (PCS) could be potential antigens to generate protective immune responses. On the other hand, although antigen carriers have been shown to boost immune responses [2], there is still much to be known about how the design of the carrier influences the final immune response. The main goal of this work was to elucidate the role of antigen conjugation and the presence of the adjuvant poly(I:C), in the humoral and cellular responses against an HIV peptide antigen (PCS5).

Materials and Methods

The peptide antigen PCS5 was conjugated to the polysaccharides either by a thioether or an oxime bond, or associated by ionic interactions. Nanoparticles were prepared by ionic complexation. NMR, dynamic light scattering, X-Ray photoelectron spectrometry and electron microscopy were employed for the physicochemical characterization of the nanosystems. BALC/c mice were intramuscularly vaccinated with each formulation at 0, 4 and 8 weeks. Antibody levels were measured from the peripheral blood samples. Cellular activation was quantified from splenocytes by flow cytometry.

Results and Discussion

All developed nanoparticles presented sizes around 200 nm, with low polydispersity values (Figure 1). While the oxime bond was expected to only release the peptide after been process by the antigen-presenting cells, the thioether bond was engineered to release the peptide antigen only in the presence of high amounts of free thiols. Additionally, all particles were freeze-dryable and stable for up to 18 months under storage. *In vivo*, all nanosystems elicited robust immune responses, with 3-times higher levels of antibodies against PCS5 than naïve mice, at week 16. Furthermore, both the conjugation of the antigen and the presence of poly(I:C) generated higher numbers of activated antigen-presenting cells than the nanosystem based on ionic interactions. Interestingly, effector T cells presented different TNFα and IL-2 secretion patterns depending on the type of nanosystem, and thus of antigen attachement, employed [3].

Conclusions

Overall, both, the type of antigen attachment and the presence of adjuvants, may influence the type and extent of the immune response. Hence, they are important design aspects that need to be considered when developing nanovaccines.

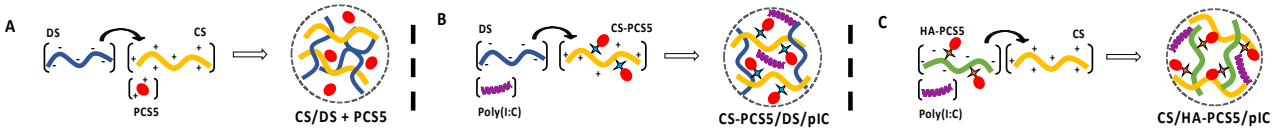

Figure 1. Composition of the different nanoparticles developed. (A) CS/DS + PCS5 NPs, (B) CS−PCS5/DS/pIC NPs, and (C) CS/HA−PCS5/pIC NPs. Key: CS, chitosan; DS, dextran sulfate; PCS5, protease cleavage site 5; NPs, nanoparticles; pIC, poly(I:C); HA, hyaluronic acid.

Acknowledgments: This work was supported by the National Institute of Allergy and Infectious Diseases of the NIH (Award N0. R01AI11805, Subaward No. 41795-02), by the European Union's Horizon 2020 research program (NanoPilot project, Grant Agreement No. 646142) and by Xunta de Galicia (Competitive Reference Groups, Ref: ED431C 2017/09). T.G.D. also acknowledges a predoctoral FPU grant from the Spanish Ministry of Education, Culture and Sports (Grant No. FPU14/05866).

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Polymeric films: a novel proposal to improve the management of eye infections.

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Introduction:

Eye infections usually require an immediate treatment, as they can easily trigger visual impairment [1]. The ability of a drug to access the anterior parts of the eye is almost non-existent after systemic administration. Therefore, different approaches have been used to treat these infections [2], such as eye drops, ointments and ocular injections. Topically applied substances to the cornea suffer a fast clearance before they can be absorbed, due to tear production and blinking. On the other hand, injections require application by expert staff and cause pain, and ointments cause discomfort in patients and blurred vision [3]. For these reasons, intense efforts for developing efficient new topical formulations have been made, such us the use of hydrogels. Ocular films could improve the efficacy of eye-conditions treatments due to their ability to increase the time permanence of drugs and control their delivery [4].

Objective:

The main purpose of the work is the improvement of corneal and conjunctival permeation of antibiotics using a self-dissolving Ciprofloxacin-loaded polymeric film. This is a wide spectrum antibiotic used in the treatment against a large range of bacteria responsible for the most common eye infections.

Materials and Methods:

Drug-loaded polymeric films were prepared using the direct dissolution-method. The method consists in preparing drug solutions in water or PBS at specific proportions and then mix them in a polymeric water solution. After homogenization, the drug-loaded dispersion is spread in a petri dish with specific dimensions, which defines the final thickness of the film, and placed in an oven at 40°C for at least 8 hours to remove the water content. Around 50 formulations with different proportions of components were assayed until the desired properties were achieved. Organoleptic properties and *in-vitro* studies were performed to ensure that the drug release, absorption profiles and antimicrobial activity were optimal for ocular delivery. Physical and chemical stability were also checked.

Results and Discussion:

Soluplus®/PVA in rate 3.3:6.6 - Propilenglicol 20% w/w and Soluplus®/PVA in rate 5:10 - Propilenglicol 20% w/w demonstrated the best organoleptic properties and high efficiency. The *in-vitro* drug release profile from drug-loaded films showed a sustained release of the drug for up to 24h. Permeability tests showed the efficacy of the formulations, as significant amounts could be found in the receptor compartment after six hours using rabbit cornea. Microbiological tests confirmed the pharmacological properties of the films after the manufacturing process. Film stability was maintained over the 2 months measured after formulation.

Conclusions:

Self-dissolving films can improve the current treatments for eye infections because they are able to deliver the drug with a sustained release profile, achieving an adequate absorption, providing a pharmacological activity against common types of microorganisms that cause ocular infections and presenting adequate organoleptic properties for the patient's acceptance.

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INVITED LECTURES AND ORAL COMMUNICATIONS

Session 4: Regenerative medicine and cell therapy

IL4.1 From Micro- and Nano-technologies to Nanomedicines: Applications in tissue engineering and regenerative medicine. (p.47)
Miguel Oliveira

OC4.1 Interpenetrating polymer networks with adjustable mechanical properties provide efficient transfection and prime mesenchymal cells for chondrogenesis. (p.48)
Marcos Garcia-Fuentes, Adriana M. Ledo, Kyle H. Vining, Maria J. Alonso, David J. Mooney

OC4.2 Overcoming the inflammatory stage of non-healing wounds: in vitro mechanism of action of Negatively Charged Microspheres (NCM). (p.49)
Edorta Santos-Vizcaino, Aiala Salvador, Claudia Vairo, Luis Correa, Rosa Maria Hernandez, Silvia Villullas, Garazi Gainza, Manoli Igartua

OC4.3 Controlled delivery of non-coding RNA in wound healing via photodegradable polymeric nanoparticles. (p. 50)
V. Francisco, J. Blersch1, C. Rebelo, A. Jimenez-Balsa, H. Antunes, C. Gonzato, S. Pinto, S. Simões, K. Liedl, K. Haupt, L. Ferreira

From Micro- and Nano-technologies to Nanomedicines: Applications in tissue engineering and regenerative medicine

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Tissue Engineering and regenerative medicine (TERM) strategies have been showing a great promise for tackling different diseases/disorders. By its turn, the convergence of TRME with emerging technologies such as microfluidics and nanotechnologies can offer new regenerative possibilities which can greatly impact Human health. In this lecture, I will discuss the recent developments related to advanced engineering approaches combining (nano)biomaterials and in vitro 3D models on a chip for personalised medicine. A personal perspective of the future directions of the field will be also presented. In summary, we aim to develop a novel class of multimodal nanobiomaterials and 3D in vitro models that can open new possibilities in pharma and personalized medicine.

Interpenetrating polymer networks with adjustable mechanical properties provide efficient transfection and prime mesenchymal cells for chondrogenesis

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Introduction: Gene delivery within hydrogel matrices can prime mesenchymal stem cells (MSCs) towards a chondrogenic fate and promote the regeneration of cartilage [1]. Here, we investigate whether the mechanical properties of the gene-activated hydrogel can affect transfection and chondrogenic priming of primary human bone marrow-derived MSCs [2]. To study this, we developed a set of interpenetrating polymer networks (IPNs) where stiffness can be adjusted without significantly changing polymer composition, gel architechture and adhesion ligand density.

Materials and Methods: We developed collagen-I-alginate IPNs where stiffness could be modulated by changing the amount of CaO_3 nanoparticles added, and cell-binding ligand density could be tuned by the selection of the ratio between both polymers. IPNs were activated with 3DFectin complexes of pDNA or mRNA, using polynucleotide sequences coding for the chondrogenic transcription factor SOX9. Cell transfection was quantified by qRT-PCR. Intracelular trafficking was investigated by fluorescence-activated cell sorting and confocal scanning microscopy. Chondrogenic priming was characterized by qRT-PCR and immunohistochemistry.

Results and Discussion: The transfection of MSCs was regulated by the biomechanical characteristics of the IPNs. The highest transfection was observed in IPNs that were the stiffest and with the highest density of cell binding sites. This effect was attributed to the higher profiferation rate of the cells and higher nanopacomplex cell uptake in these IPNs. MSCs cultivated in gene-activated IPNs showed higher expression of chondrogenic markers compared to the same cells transfected in 2D and then encapsulated in blank IPNs, showing the importance of a 3D environment during cell priming. MSCs cultured in condrogenic media in the presence of SOX9-activated IPNs resulted in similarly high levels of chondrogenesis as a positive control, but significantly reduced levels of the hyperthrophic marker collagen type-X. Since tissue hyperthrophy is a major problem in chondrogenesis, we hypothesize that these gene-activated IPNs could represent important technologies for future therapies aimed at reparing articular cartilage.

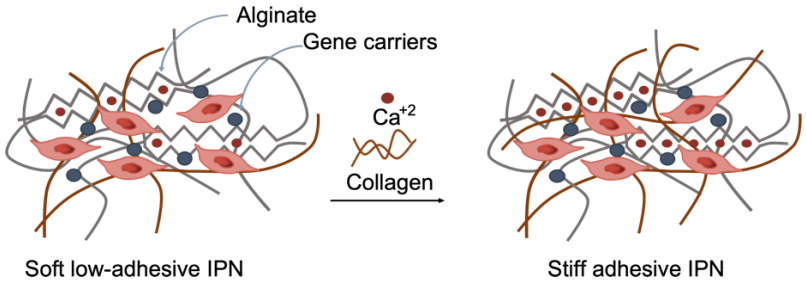

Figure 1. Concept for the design of gene activated IPNs. IPN stiffness could be regulated by Ca^{+2} addition. IPN ligand adhesion could be regulated by a higher proportion of collagen type-I to the hydrogels.

Conclusions: Matrix stiffness and ligand density are important factors in regulating gene delivery. MSCs cultivated in collagen-I-Alginate IPNs activated with SOX9 are primed towards a chondrogenenic phate and show low hypertrophy levels. These results suggest the interest of matrices combining SOX9 activation and optimized biomechanical properties as devices for cartilage repair.

Acknowledgments: This work was financed by Ministerio de Economía y Competitividad (MAT2017-84361-R, ERDF).

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Overcoming the inflammatory stage of non-healing wounds: *in vitro* mechanism of action of Negatively Charged Microspheres (NCM)

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Introduction

Chronic wounds are characterized by an impairment of the healing process. An interesting treatment approach consist of strategies that combine keratinocyte and fibroblast proliferation and skewing macrophages to a non-inflammatory phenotype. In this work, we have used Negatively Charged Microspheres (NCM) for such purpose.

Materials and methods

Experiments were carried out with NCM extracted from a commercial microsphere formulation (PolyHeal® Micro), consisting in polystyrene microspheres suspended in glycerol and phosphate buffer. Cytotoxicity was assessed in human keratinocytes (HaCaT), fibroblasts (HDFa) and primary macrophages. Attachment and proliferation were evaluated in both HaCaT and HDFa, while phagocytosis and polarization was investigated in macrophages.

Results and discussion

NCM were not cytotoxic at most of the studied concentrations. Based on these results, the following NCM to cell ratios were chosen: 50 and 10 NCM/cell for HaCaT and HDFa, and 10 and 5 NCM/cell for macrophages.

Fluorescent NCM were used to assess cell attachement. After 24 h, NCM were attached to the HaCaT clusters (fig.1a), as well as HDFa aggregates (fig.1b), and NCM started to accumulate therein ($p<0.001$). This is noteworthy, since that contact can trigger cell proliferation and thus, wound healing [1].

An increase in the proliferation rate of HDFa was observed after 24 h with the lowest dose of NCM ($p < 0.001$). Regarding HaCaT, we observed a statistically significant increase in cell proliferation after 72 h of treatment with both doses ($p < 0.05$). These results are important since keratinocytes and fibroblasts are key cells involved in tissue repair in wound healing [2].

Uptake of NCM by M0, M1 and M2 macrophages after 48h of incubation was studied by observation under inverted contrast phase microscope. NCM were always located in the area occupied by macrophages, and not in their surrounding area, indicating that NCM were internalized. In addition, SEM images showed the cell membrane surrounding the NCM (fig.1c). Remarkably, particle accumulation into macrophages is considered advantageous for skewing cell phenotype [3].

Regarding the macrophage phenotype, M0 and M1 macrophages showed and increased CD206 expression and a decrease in the CD64 expression after NCM treatment. In addition, M1 macrophages also expressed less CD83, showing the ability of the NCM to skew macrophages towards a non-inflammatory phenotype (M2). This ability results crucial in chronic wounds, and can explain NCM mechanism of action. In fact, M2 macrophages produce collagen precursors and factors that can stimulate fibroblasts, play an important role in ECM regeneration, and favour angiogenesis by secretion of PDGF (platelet derived growth factor) [4].

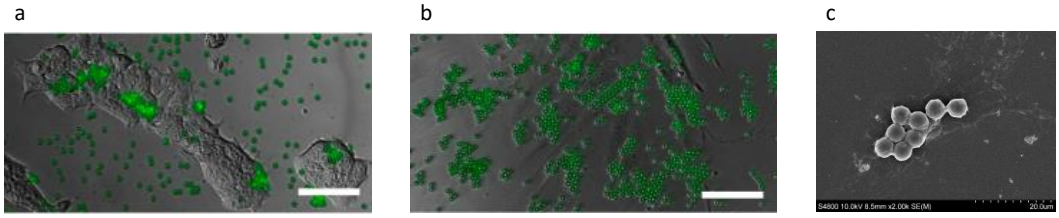

Figure 1. Attachment assay by using FYG-NCM in HaCaT (a) and HDFa (b). NCM taken up by macrophages (c)

Conclusions

Based on these results, we proved that the mechanism of action of NCM to induce wound healing is mainly due to the capability of skin cells to attach to NCM, and their capacity to induce cell proliferation and macrophage differentiation.

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Controlled delivery of non-coding RNA in wound healing via photodegradable polymeric nanoparticles

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Introduction: Non-coding RNAs have emerged as an attractive tool for regulating gene expression and can be used to treat a variety of diseases, such as cancers, neurodegenerative diseases, virus infections and wound healing [1-4]. However, several drawbacks need to be addressed before the clinical translation of RNA-based therapeutics, such as the control over when and where gene release will occur. To this end, it is crucial to develop formulations that enable their delivery to a target cell, with potential off-target effects and simultaneously increase their efficacy in the intracellular delivery. The goal of the current work is to obtain biocompatible light-activatable nanoparticles (NPs), allowing precise control of the timing and spatial release of the RNA molecules, in order to accelerate the translation of these therapies.

Materials and Methods: By Michael-type addition chemistry we produce polymers with chemical diversity. Then, the polymers were conjugated with a light sensitive molecules to increase their hydrophobicity as well as confer light responsiveness properties. The polymers obtained were then precipitated in water to forms NPs and then complexed with siRNA. The NPs library was characterized for size, zeta potential, light disassembly, siRNA complexation, cellular internalization and gene knockdown activity.

Results and Discussion: Herein, we design a nanoparticle library composed by 270 possible formulations, with a variety of physico-chemical properties and responsiveness to UV light. Most of the polymers (90 %) were able to form NPs by nanoprecipitation. 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 selected candidates for subsequent studies 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.

Conclusions: We have synthesized a light responsive library of polymeric NPs and screened their performance for the intracellular siRNA delivery. We showed distinct cellular uptake for the same formulation into different cell lines with minimal cytoxicity. Moreover, functional studies exhibited the impact of controlling the nanoformulation disassembly in the delivery of non-conding RNAs. These photo-triggerable formulations offer a new strategy to deliver topically non-coding RNAs.

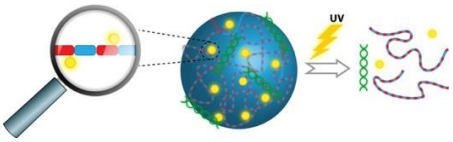

Figure 1. Schematic representation of the light disassembly of the NPs.

Acknowledgments: The authors would like to thank the financial support of ERA Chair project (ERA@UC, ref:669088) through EU Horizon 2020 program, the POCI-01-0145-FEDER-016390 (acronym: CANCEL STEM) and POCI-01-0145-FEDER-029414 (acronym: LIghtBRARY) projects through Compete 2020 and FCT programs.

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INVITED LECTURES AND ORAL COMMUNICATIONS

Session 5: Targeted Drug Delivery and Combination therapies

Barcoded Nanoparticles for Precision Cancer Medicine: The primary tumor and metastasis are sensitive to different medicines

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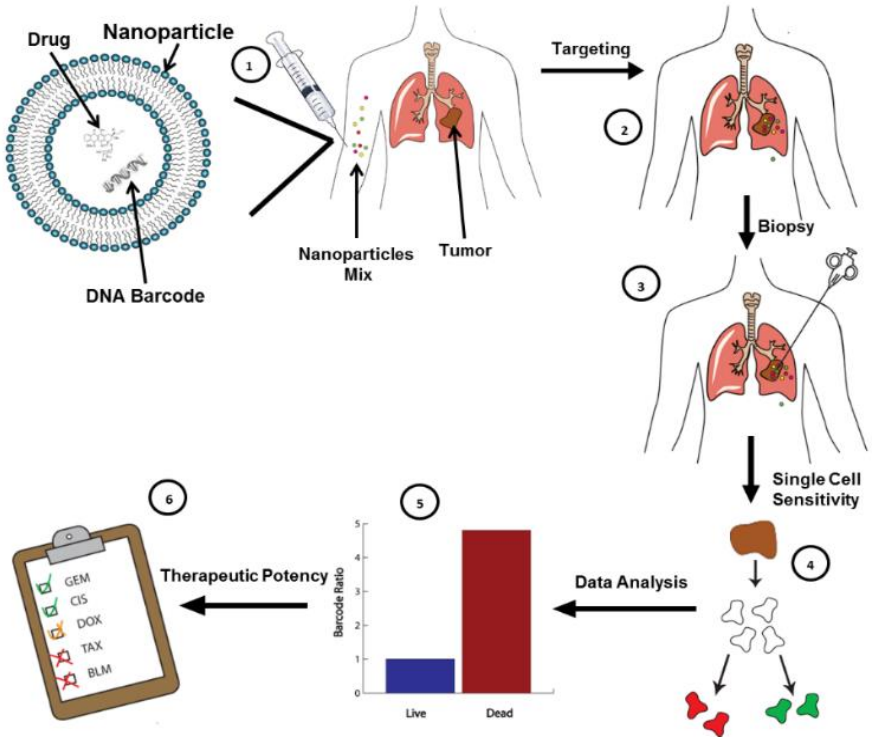
Medicine is taking its first steps towards patient-specific cancer care. Nanoparticles have many potential benefits for treating cancer, including the ability to transport complex molecular cargoes including siRNA and protein, as well as targeting to specific cell populations.

The talk will discuss 'barcoded nanoparticles' that target sites of cancer where they perform a programmed therapeutic task. Specifically, liposomes that diagnose the tumor and metastasis for their sensitivity to different medications, providing patient-specific drug activity information that can be used to improve the medication choice. The talk will also describe how the liposomal lipid composition affects its ability to internalize into triple-negative breast cancer cells, looking at the different segments of the lipid molecule, and how this can be leveraged to induce an anti-tumor immune response.

The evolution of drug delivery systems into *synthetic cells*, programmed nanoparticles that have an autonomous capacity to synthesize diagnostic and therapeutic proteins inside the body, and their promise for treating cancer and immunotherapy, will be discussed.



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Nanobiostructured Systems for Biomedical Applications: From Design to Cancer Therapy

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Introduction

The recent cutting-edge advances on nanomaterials is anticipated to overcome some of the therapeutic window and clinical applicability of many drug/peptide molecules and can also act as innovative theranostic platform and tool for the clinic in the future [1-4]. In the last decade, research on cancer immunotherapy resulted in a new set of potential treatments with promising results in the clinics [5-8]. Among these, immune checkpoint inhibitors are one of the few immunotherapies that have been clinically validated, yet with variable results, ranging from complete responses to hyperprogression. Amongst the different experimental treatments, active cancer immunotherapy hold great promises for the future.

Materials and Methods

In this work, prominent nanosystems, such as biohybrid nanocomposites made of different nanoparticles (porous silicon and oncolytic virus) and cancer cell-based membrane materials are presented and discussed as potential platforms for the individualization of medical intervention and cancer immunotherapy applications.

Results and Discussion

Examples on how these biohybrid nanomaterials can be prepared and scaled-up, as well as how they can be used to enhance the drug's targetability, intracellular drug delivery for both cancer chemo- and immune-therapy applications, will be highlighted and discussed.

Conclusions

Overall, our results suggest that biohybrid nanomaterials are a versatile and advanced platform for cancer treatment with an interesting potential for present and future clinical impact given its easy tailorability to each patient, choosing a suitable inorganic or virus and obtaining cancer cells from biopsy.

Acknowledgments: This work was supported by the Sigrid Jusélius Foundation, the HiLIFE Research Funds, the Academy of Finland (decision no. 317042), the Faculty of Pharmacy–Helsinki University, and the European Research Council Proof-of-Concept Research Grant (grant no. 825020).

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Self-illuminating nanoparticles can activate the photosensitizer verteporfin for photodynamic therapy in Pancreatic Ductal Adenocarcinoma (PDAC)

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Introduction: The morbidity of pancreatic cancer does not present this type of cancer as very common. However, the mortality of this tumor is very high, even with the palliative chemotherapy and surgical resection, patients only survive 10-20 months. Therefore, the development of new strategies to address this tumor are essential. Photodynamic Therapy (PDT) has proven to be efficient for treating external lesions such as melanoma. Besides, this therapy provides a new approach to its treatment and has some proven advantages compared to chemotherapy, radiotherapy and inmunotherapy [1]. Nevertheless, due to the poor penetration of light, conventional PDT is not adequate for treating internal organs. For this particular purpose, we aim to determine the potential of self-illuminating nanoparticles, to mediate a localized and controlled antitumoral response in pancreatic cancer using verteporfin as a photosensitizer. Verteporfin is a second-generation photosensitizer with an increased excretation from human body and its strong absorption at 690 nm is very suitable for treating internal organs like the pancreas [2].

Materials and Methods: Self-illuminating nanoparticles were developed by the conjugation of a mutant version of *Renilla reniformis luciferase* (RLuc8) to quantum dots (QDots). The functionality of RLuc8, after its purification, was determined by measuring its bioluminescence intensity after adding its substrate (coelenterazine), showing robust activity. The conjugation of the protein to the quantum dots (RLuc8-QDots) was performed by EDC coupling, forming an amide group between the primary amine group of the protein and the carboxylic groups set on the surface of the QDots [3]. Conjugates were characterized by different methods of gel electrophoresis and measuring its bioluminiscence spectral scanning for calculation of BRET. The stability of this nanosystem was also tested through the time, measuring its BRET kinetics. The interaction of the conjugates with pancreatic cancer cells was analyzed by confocal microscopy. The distribution of verteporfin in the cytosol was also tested in pancreatic cancer cell lines.

Results and Discussion: The conjugation of RLuc8 to QDots was successfully achieved, as demonstrated by agarose electrophoresis; while free QDots freely migrate through the gel, the conjugates were stuck at the loading wells. A second analysis by polyacrylamide gel electrophoresis confirmed the absence of free unreacted RLuc8. Additionally, the functionality of the conjugates was evidenced by determining their BRET ratio after the addition of the substrate (BRET ratio= 5.05). The nanosystem was stable up to seven days. Physical characterization upholds that this approach theoretically works measuring the emission spectra of the nanosystem and the excitation spectra of the verteporfin. Citotoxicity assays with the Verteporfin were performed in order to obtain optimal work conditions in different pancreatic cancer cell lines. Confocal microscopy confirmed the goodly internalization of our developed nanosystem and the photosensitizer (Verteporfin), which is positive for the realization of further *in vitro* experiements. The formation of Reactive Oxygen Species (ROS) after addition of RLuc8-QDots (Nanosystem) and Coelenterazine (substrate), to activate the photosensitive drug verteporfin, is currently under study.

Conclusion: In conclusion, we have characterized self-illuminating nanoparticles that are efficiently internalized into pancreatic cancer cells. Because of this efficient internalization, considering the optimal BRET properties of the conjugates and the suitable use of verteporfin for PDAC, we expect that they will efficiently activate this photosensitizer, generating ROS and driving cancer cells into apoptosis by photodynamic therapy.

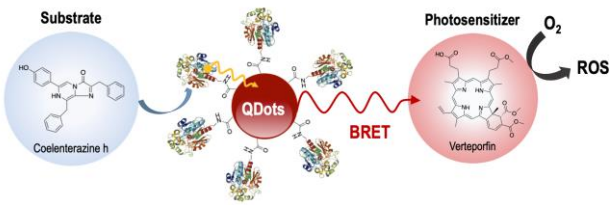

Figure 1. Figure title

Acknowledgments: This work was supported by the Carlos III Health Institute / Semilla ONC18PI04-NATBIL

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Combinatorial nanomedicine for treating pediatric osteosarcoma

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Introduction: Nanotechnology has been widely studied in recent decades with the aim of enhancing the therapeutic potential of antitumor drugs. However, these therapies are mainly available in adult cancer protocols but not for their use in the pediatric population [1]. Our research focuses on pediatric osteosarcoma, whose importance resides in its poor prognosis. Squalenoyl nanoassemblies (NA) have obtained encouraging outcomes in a great variety of tumors [2,3]. Among these, the prodrug squalenoyl-gemcitabine (SQ-gem) has been chosen to construct a novel multidrug nanosystem in combination with edelfosine (EF), an alkyl-lysophopholipid with proven anticancer activity [4]. Given their amphiphilic nature, we hypothesized that both anticancer compounds, with complementary molecular targets, could lead to the formation of a new anticancer nanomedicine. The nanomedicines were tested for efficacy in metastatic patient-derived pediatric osteosarcoma cells and animal models of the disease.

Methods: The synthesis squalenoyl-gemcitabine was performed as previously reported [3] and the NAs were formulated by the nanoprecipitation method. The colloidal properties and structure of the new co-assembly were analyzed by DLS, T.E.M., UHPLC/MS/MS and X-ray photoelectron spectroscopy. EF hemolysis activity was studied using human blood erythrocytes. NAs cell uptake and its anticancer activity were tested in metastatic patient-derived pediatric osteosarcoma cells. Pharmacokinetic studies were performed after a single *i.v*. administrationn of SQ-gem/EF NAs. For the *in vivo* efficacy tests, athymic nude mice were treated twice per week following an intratibial injection of P1.15 osteosarcoma cells. Tumor bioluminiscence images were taken with a PhotonIMAGER™ imaging system.

Results: The physical mixture of EF and SQ-gem at equimolecular concentrations led to the formation of stable and monodisperse NAs of 50 ± 4 nm in a surfactant/polymer free-aqueous suspension. This combination resulted in smaller particle size and a new supramolecular conformation, with higher stability and drug content in comparison with SQ-gem NAs only. SQ-gem/EF NAs presented only 6% of hemolysis and thus were able to protect erythrocytes from EF associated hemolysis. *In vitro* experiments revealed that SQ-gem/EF NAs were succesfully uptaken by metastatic patient-derived pediatric osteosarcoma cells and presented a better antitumor profile than SQ-gem NAs. The pharmacokinetic profile of the NAs showed controlled release behaviour by gemcitabine and diminished plasma peak concentrations of EF in comparison with the free drugs. Finally, multiple administrations of SQ-gem/EF NAs given via retro-orbital plexus to osteosarcoma bearing mice were reported to be safe. The hindlimb bioluminiscence corresponding to the presence of PI.15 cells measured in these tumorized mice showed that SQ-gem/EF NAs were able to detain tumor growth from day 21 of treatment.

Conclusion: The in-depth physico-chemical characterization demonstrated the successful design and formulation of this novel multidrug nanomedicine. Its improved safety and efficacy observed in *in vitro* and *in vivo* models of pediatric osteosarcoma make it a suitable candidate for childhood cancer therapy.

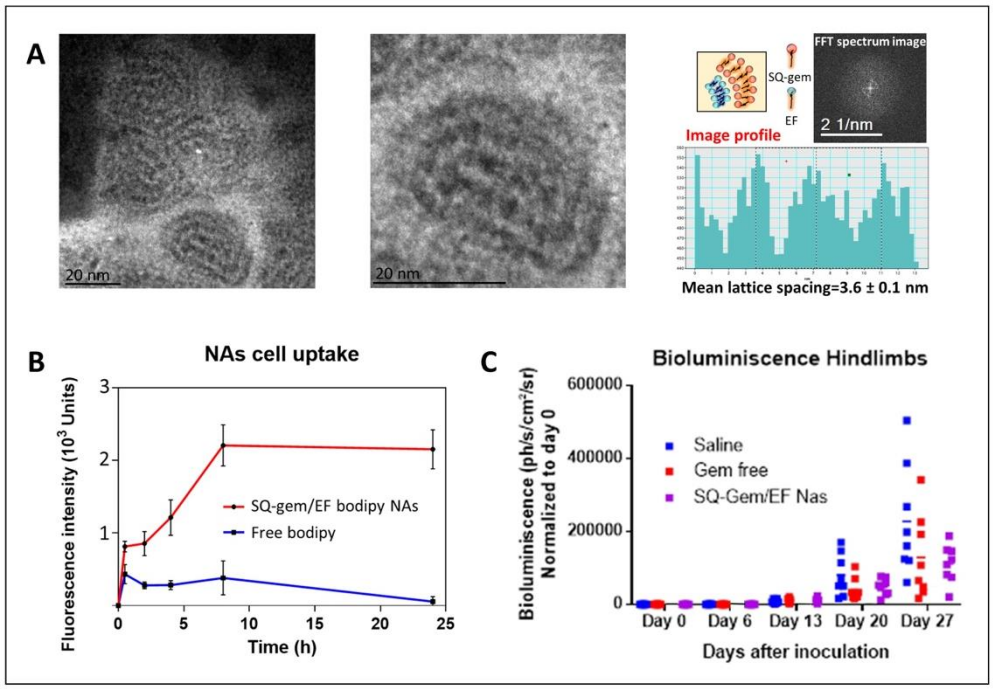

Figure 1. (A) High angle annular dark field (HAADF) scanning transmission electron microscopy (STEM), Fourier transform image spectrum analysis and mean lattice spacing measurement of SQ-gem/EF NAs. (B) Osteosarcoma cell uptake of fluorescently labelled SQ-gem/EF NAs. (C) Bioluminiscence hindlimbs of pediatric osteosarcoma bearing mice treated with SQ-gem/EF NAs.

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Synthesis of biologic-responsive targeting polymeric conjugates to manufacture docetaxel-loaded nanoparticles for glioblastoma chemotherapy

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Glioblastoma is the most lethal brain cancer, with a median survival time of only 15 months. Docetaxel is one of the most effective chemotherapeutics against glioblastoma, although it presents pharmacokinetic constraints mainly due to its low solubility and poor blood-brain barrier (BBB) permeation. This project proposes a targeted, biologic-responsive nanomedicine to circumvent these inadequacies based on docetaxel-loaded nanoparticles for glioblastoma treatment. The developed nanomedicine comprises a poly(lactic-co-glycolic) acid (PLGA) core and a polyethylene glycol (PEG) shielding of long- and short-length. The long-length PEG possesses an Angiopep-2 moiety for BBB targeting (binding to the low-density lipoprotein receptor) and is able to dissociate in the acidic pH of BBB endosomes, hence sterically de-protecting the short-length PEG coupled with L-histidine for further glioblastoma targeting (binding to the L-type amino acid transporter 1) upon brain arrival. The overall concept is schematized in Figure 1.

Chemical strategies based on carbodiimide, hydrazone formation via Schiff base reaction and Thiol-Michael addition were employed to synthesize the **PLGA-acid-cleavable, long-length PEG-Angiopep-2** and **PLGA-short-length PEG-L-Histidine** polymeric conjugates that will constitute the nanoparticles matrix. These polymeric conjugates were further characterized by different techniques such as nuclear magnetic resonance (NMR), Fourier-transform infrared spectroscopy (FTIR) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Preliminary work has focused the production of docetaxel-loaded nanoparticles post-polymer synthesis through a scale-up microfluidic manufacturing technique using 80% of plain PLGA core polymer and 10% of each targeted, biologic-responsive polymeric conjugate.

The chemical synthesis achieved a total conjugation efficiency value of around 70% and 90% for the **PLGA-acid-cleavable, long-length PEG-Angiopep-2** and **PLGA-short-length PEG-L-Histidine** polymeric conjugates, respectively, as demonstrated by NMR calculations. FTIR confirmed the successful reactions by elucidating the formation of intermediary bonds between the constituents of the polymeric conjugates, and MALDI-TOF confirmed the different ionization behaviors and proved the presence of PLGA and PEG monomers in the structure of the polymeric conjugates. The physicochemical characterization of docetaxel-loaded nanoparticles manufactured through the microfluidic technique demonstrated around 100 nm average size, 0.1 polydispersity index and 60% association efficiency.

Overall, this work has allowed, so far, the synthesis of targeted, biologic-responsive polymeric conjugates with high conjugation efficiency (>70%) and suitable to manufacture low size, monodispersed and highly loaded docetaxel-loaded nanoparticles in a microfluidic technique with potential to scale-up the batch size. Future work will be dedicated to test the efficacy of the developed targeted, biologic-responsive nanomedicine *in vitro* and *in vivo*. The current need to accelerate drug delivery to glioblastoma, bypassing the BBB and targeting tumor tissue of brain, places this system in a privileged position in the field of translational nanomedicines. This work also lays foundation for future targeted, biologic-responsive delivery of other therapeutics to a range of pathologies.

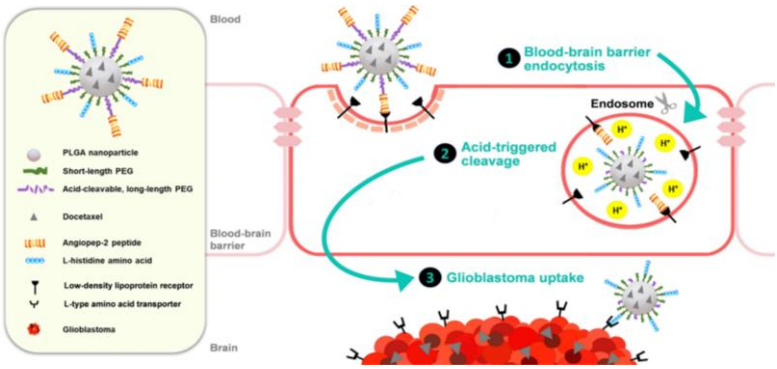

Figure 1. Graphical abstract.

Acknowledgments: This work is a result of the project NORTE-01-0145-FEDER-000012, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). The work was also financed by FEDER – Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 – Operational Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT – Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior in the framework of the project "Institute for Research and Innovation in Health Sciences" (POCI-01-0145-FEDER-007274). CM gratefully acknowledges FCT for financial support (grant SFRH/BD/137946/2018).

INVITED LECTURES AND ORAL COMMUNICATIONS

Session 7: Delivery of Biomacromolecules and oligonucleotides.

Peptides and Supramolecular Strutures for Gene Delivery

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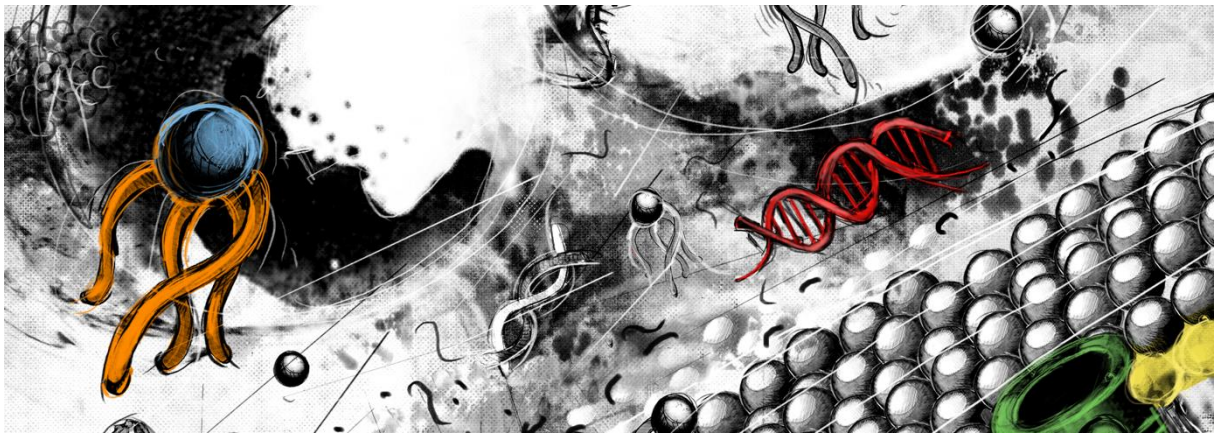
Our research group is interested in the application of supramolecular chemistry to understand and manipulate biology. [1,2] This is because the weak and non covalent forces that control the shape and the topology of biomolecules are governed by the principles described by supramolecular chemistry. This topology is ultimately responsible of bimolecular properties and by modulating the shape we can control and improve functional behaviour.

With focus in supramolecular interactions for artificial membranes vectors we have investigated the construction of amphiphilic peptide libraries for gene and protein delivery. [3-5]



Acknowledgments: This work was supported by AEI: [SAF2017-89890-R], the Xunta de Galicia (ED431C 2017/25, 2016-AD031 and 2016–2019, ED431G/09) and the ERDF). Ramón y Cajal (RYC-2013-13784), an ERC Starting Investigator Grant (DYNAP-677786) and a YIG from the Human Frontier Science Research Program (RGY0066/2017).

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Precision Nanomedicine-based Therapeutics to Regulate Immunosuppression Against Melanoma

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Introduction: The immune checkpoint therapies revolutionized the treatment of metastatic melanoma. Even though, a low percentage of patients responds to these therapies and severe side effects are commonly reported. Thus, new therapeutic options are urgently need. To that end, we focused on the development of a dual precision nanosystem-based therapeutic to target dendritic cells (DC) and successfully modulate melanoma-vascular-immune cell interactions by expanding the range of targeted cells in a site-specific manner.

Materials and Methods: Poly(lactic acid) (PLA) and Poly(lactic-co-glycol) (PLGA)-based nanoparticles (NP) were prepared by a modified double emulsion (w/o/w) solvent evaporation method, using mannose-grafted polymers to deliver combinations of melanoma neoantigens, toll-like receptor ligands and regulators of the PD-L1/PD-1 pathway. NP physico-chemical properties were fully characterized, including the mean average size, surface charge and morphology, by Dynamic Light Scattering, Laser Doppler Electrophoresis, and Atomic Force Microscopy, respectively. The amount of melanoma antigens and regulators of immunosuppression entrapped within NP was determined by distinct methods. Immature DC (iDC) were used to evaluate the impact of NP on cell viability (propidium iodide assay), and to assess NP uptake kinetics by flow cytometry. The anti-tumor immune-mediated effect was evaluated *in vivo* in two melanoma-bearing mouse models (B16F10 and B16MO5), which included the immune profiling within tumor site, before and after treatment.

Results and Discussion: Mannosylated NP (man-NP) presented an average diameter of 180 nm, narrow polydispersity index, surface charge close to neutrality, spherical morphology, and high loadings of the antigens and the other immune function regulators. No cytotoxic effect was observed on iDC up to 48 h. Cy5.5-labeled NP were extensively internalized by DC. Man-NP triggered the activation of DC *in vivo*, leading to the expression of significantly higher levels of co-stimulatory molecules CD80, CD86 and CD40, when compared with non-mannosylated carriers. Man-NP successfully induced a potent immune-mediated anti-tumor response, which was the strongest when the dual nanosystem comprised the vaccine delivery and the modulation of the PD-L1/PD-1 signalling pathway.

Conclusion: The dual precision nanomedicine-based system is a promising multifunctional platform able to associate the induction of strong antigen-specific immune response with the regulation of immunosuppressive mechanisms within tumor milieu.

Acknowledgments: This work was supported by FCT under the Programme grants SAICTPAC/0019/2015 and UID/DTP/04138/2013, and the PhD grants PD/BD/113959/2015, PD/BD/128238/2016 and SFRH/BD/131969/2017. The MultiNano@MBM project was supported by The Israeli Ministry of Health and FCT-MCTES under the frame of EuroNanoMed-II (ENMed/0051/2016). Partially funded by la Caixa" Health Research 2018 Call the Project proposal HR18-00589.

Hyaluronic acid enveloped metallic nanocomplexes for RNA delivery

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Introduction: RNA interference (RNAi)-based therapies have a high potential for the treatment of different diseases [1]. Unfortunately, their high potential is hindered by the multiple biological barriers that they need to overcome before reaching their target [2].

Nanosystems involving metal cations have been used for the complexation of nucleic acids, because of their capacity to form coordination complexes via electrostatic interactions [3]. Cationic surfactants have also gained a lot of attention as complexing agentes for the delivery of RNA [4].

The **objective** of this work was to develop nanocomplexes, based on the cationic metal ions iron (Fe^{3+}) and zinc (Zn^{2+}), and the cationic surfactant ethyl lauroyl arginate (LAE). Fe^{3+} and Zn^{2+} are able to condensate the negatively charged nucleic acids through interactions with the phosphate groups or by chelation via guanine atoms. LAE is known for its optimal self-assembly properties, low toxicity profile, high biodegradability and ability to permeate lipid membranes [5]. These nanocomplexes were enveloped with hyaluronic acid (HA), with the purpose of increasing their stability and reduce the risk of opsonization and RES recognition [6].

Materials and Methods: Transfer Ribonucleic acid (tRNA) from baker's yeast (S. cerevisiae) and zinc acetate dihydrate were purchased from Sigma Aldrich (St. Luis, USA). *Silencer*™ GFP (eGFP) siRNA, *Silencer*™ Negative Control No. 1 siRNA and Fetal Bovine Serum (FBS) were purchased from Thermo Fisher Scientific (USA). Ferric chloride hexahydrate was provided by Acofarma (Spain). Ethyl Lauroyl Arginate Hydrochloride (LAE) was kindly gifted by Vedqsa (Barcelona, Spain). Sodium hyaluronic acid (HA) was purchased from LifeCore Biomedical. Phosphate-Buffered Saline (PBS) was purchased from Fisher Scientific (USA).

Results and Discussion: Two different types of nanocomplexes were prepared by a simple two-step assembly process. The first step allows the complexation of the cationic core with the anionic RNA, forming a nanometric complex with a positive charge. In the second step, the nanocomplexes were enveloped with the anionic polymer, leading to a negative surface charge.

The first prototype consisted of a nanometric complex of Fe^{3+}-LAE-RNA enveloped by HA, with an average size of 163.1 ± 2.85 nm and a surface charge of -13.07 ± 0.47 mV. The second prototype consisted of a nanometric complex of Fe^{3+}-Zn^{2+}-RNA enveloped by HA, presenting an average size of 162.37 ± 6.92 and a surface charge of -16.83 ± 3.62 mV. In both cases, the nanocomplexes presented an RNA final concentration of 20 µg/mL. Both prototypes presented stability at least 24 hours in PBS supplemented with FBS, being a relevant simulated media for further administration of the nanosystems. Cell viability of the nanocomplexes was tested and the results indicated that none of the prototypes were toxic for HeLa cells at the concentrations tested (from 25 to 500 nM of RNA). The silencing capacity of these nanocomplexes is now being tested in vitro using a GFP siRNA,

Conclusions: Two different metal-RNA nanocomplexes were developed and characterized. Their highly efficient RNA incorporation and their non-toxic in vitro profile of these nanocomplexes are attractive properties to further pursue their in vitro/in vivo evaluation.

Acknowledgments: This work was supported by the European B-Smart Consortium, which received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 721058. This work was also supported by the Competitive Reference Groups, from the Consellería de Educación e Ordenación Universitaria, Xunta de Galicia, with reference number ED431C 2017/09.

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INVITED LECTURES AND ORAL COMMUNICATIONS

Session 8: Oral Delivery of drugs and other active ingredients

Bioinspired delivery systems for oral peptide therapeutics

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Introduction: Polymeric nanocarriers have been extensively investigated as a promising strategy for the transmucosal administration of biomacromolecules such as protein and peptide drugs. There have been considerable advances over the last two decades regarding the rational design of such nanosystems, showing the critical influence of particle size, surface charge, and chemical composition on their efficacy.[1,2] Despite these advances, premature elimination and/or degradation before establishing optimal contact with the mucosal surface still continues to be an important limitation of these otherwise promising technologies.

Herein we present a possible startegy to overcome such limitations by using a biomimetic multi-stage delivery platform. This platform is based on natural, pollen-derived biomaterials and is conceived to improve nanocarrier stability, while its specific 3D surface morphology is intended to enable efficient and prolonged interaction with the intestinal mucosa upon oral administration.

Methods: Prior to nanocarrier association, natural pollen grains were purified to remove all potential allergenic components. Association efficacy and release of different non-biodegradable and biodegradable nanocarriers were studied by fluorimetry, FT-IR, elemental analysis, etc. Physical and biological stability were evaluated in simulated gastric and intestinal media. Mucointeraction was evaluated ex vivo in a non-everted intestinal sac model and in vivo upon oral gavage to healthy rats.

Results: The optimized purification process has enabled the production of allergen-free hollow pollen prototypes with a porous, echinate 3D external structure. These vehicles allow the association of remarkably high amounts of nanocarriers (up to 120% w/w) (Fig.1). Ex vivo mechanistic studies showed a positive effect for nanocarriers integrated into the pollen prototypes, which largely enhanced the mucointeraction in a non-everted rat intestinal sac model. This effect was also observed in vivo, following oral administration, where the intact vehicles could be detected together with a high number of associated nanocarriers in the duodenal and jejunal regions of the intestine for extended periods of time. Histological examination showed that the integrity of the intestinal tissue was preserved throughout all experiments.

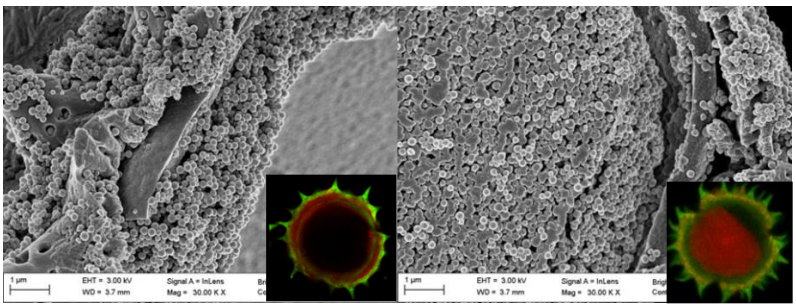

Figure 1. SEM and confocal microscopy images of different nanocarrier-loaded pollen platforms

Conclusions: The developed delivery platforms show improved mucointeraction performance and represent a promising strategy for the oral delivery of biopharmaceuticals.

Acknowledgments: This work was supported by the Ministry of Economy and Competitiveness – FEDER (SAF2016-79230-R and 2018-PN018) and the Xunta de Galicia (GAIN Oportunius Program and Funding for Competitive Reference Groups).

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Polymeric micelles obtained by a novel chitosan derivative as potential delivery nanosystem of hydrophobic drugs

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Introduction: Chitosan (CS) is a biocompatible and biodegradable polymer extensively explored in the development of drug delivery systems. The synthesis of new CS derivatives with amphiphilic properties allows the production of polymeric micelles, which have the ability to encapsulate hydrophobic drugs [1]. Once encapsulated, the drug is protected from the body fluids and pH and, its aqueous solubility is increased as well as, its cellular uptake and therapeutic activity due to the use of micelles as delivery vehicle [2].

Materials and methods: CS was chemically modified with O-methyl-O′-succinylpolyethylene glycol (mPEG-COOH) and with oleic acid (OA) by carbodiimide reaction and an amphiphilic graft copolymer with ability to produce micelles by self-assembly was obtained. Characterization of the copolymer included ^{1}H NMR, FTIR and GPC and a complementary physico-chemical characterization was performed by DSC/TGA, XRD and contact angle evaluation, as well as its substitution degree by ^{1}H NMR. Moreover, critical micelle concentration (CMC) was determined by surface tension and DLS. Micelles were prepared by self-assembly and size and zeta potential were determined also by DLS and micelles morphology was assessed by TEM. The association efficiency of the anticancer drug camptothecin (CPT) was determined by HPLC as well as the CPT in vitro release performed in simulated gastrointestinal fluids and the evaluation of CPT lactone ring protection from hydrolysis. Cytotoxic studies were performed against Caco-2 and HT29-MTX intestinal cel lines during 4 and 24 h of incubation.

Results and discussion: The success of the synthesis and the purity of the new polymer were demonstrated by ^{1}H NMR, FTIR and GPC and, after OA grafting, the polymer showed an increase on its thermal stability and crystallinity. The substitution degree obtained for mPEG-COOH was 4.3 ± 0.2 % and 5.8 ± 2.9 % in the case of OA grafting. The contact angle showed decrease or increase on copolymer hydrophobicity when CS was grafted with mPEG-COOH and OA, respectively. The CMC reveals the stability of the system after dilution obtaining a value of 0.076 and 0.065 mg/mL for DLS and surface tension determination, respectively. An average size of 140 nm, polydispersity index of 0.230, a positive superficial charge ranging from + 33.7 to + 41.8 mV and an association efficiency of 78% for a drug loading of 5% were obtained. TEM analysis demonstrated a round and smooth shape for both empty and CPT-loaded micelles and a smaller size comapred with DLS data. The in vitro intestinal release showed a low release in gastric media and a controlled release in intestinal fluids, suggesting a pH-dependent behaviour. These micelles were able to protect the drug up to 75% of its initial lactone form, exhibiting a good system stability. Moreover, CS micelles maintained its size and polydispersity during one week at 4 ºC and also after freeze-drying with 0.5 % (w/v) trehalose. Regarding the safety profile, copolymer did not presented a cytotoxic effect against Caco-2 and HT29-MTX cell lines in concentrations equal or bellow 1 mg/mL.

Conclusions: The design of this new CS amphiphilic system with amphiphilic properties emerged to have great potential loading hydrophobic drugs with enhanced the solubility and stability. Polymeric micelles showed a safe profile against intestinal colorectal cancer cell lines suggesting to be useful as therapeutic strategy for cancer treatment as delivery vehicle of anticancer drugs.

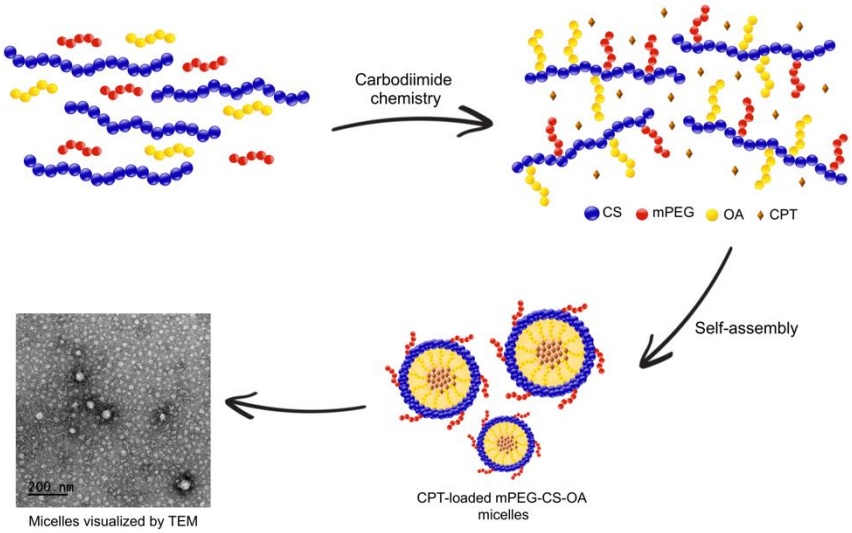

Figure 1. Schematic representation of the CS modification to its assemble into polymeric micelles and CPT encapsulation.

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Potential of nanoemulsions stabilised by ascorbyl-dipalmitate to increase the interaction with the intestinal barrier via the SVCT-1 transporter

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Introduction: Nanotechnology has significantly contributed to the oral delivery of drugs with poor stability or low permeability [1]. In this sense, the design of novel structures that interact with the gastrointestinal tract and overcome the barriers associated to the oral route is highly desirable. Size and surface charge are some of the factors that affect to this interaction; additionally, the composition of nanocarriers and their possible functionalisation may promote the targeting to specific cell populations. Enterocytes present different receptors and transporters that mediate their function, like the intrinsic factor receptor or the epidermal growth factor receptor, which have been used as targets for different nanocarriers [2,3]. The ascorbic acid transporter SVCT-1 expressed at the apical membrane of enterocytes is involved in the maintenance of ascorbic acid homeostasis. Therefore, it has recently been proposed by Luo Q. et al. as a novel target for promoting the interaction of nanocarriers with the enterocytes [4]. We have previously reported that ascorbyl-dipalmitate nanoemulsions (NEE-ADP) facilitate the intracellular accumulation of curcumin when incubated in Caco-2 monolayer and that this behaviour is determined by the presence of the ascorbyl derivatives on the surface of the systems [5]. In this work, we present preliminary results that suggest the role of the SVCT-1 transporter on the uptake of these formulations when incubated with Caco-2 cells.

Materials and Methods: NEE-ADP were formulated by the solvent displacement technique at two different ADP ratios (18% and 29% w/w), and were fluorescently labelled by the incorporation of the tracer DiD (8 µL/mL) to the oil phase. The physicochemical properties of NEE-ADP were assessed by Dynamic Light Scattering. For transport studies, Caco-2 cells were seeded in 12 well culture inserts (1 µm pore diameter, Corning Costar®) at a density of 5 x 10^5 cells/well. After 21 days, Caco-2 cells formed a monolayer and were incubated with NEE-ADP (50% v/v) for 2 h. Additionally, Caco-2 cells monolayers were preincubated with a solution of ascorbate (100 µM) for 24 h; afterwards, NEE-ADP were incubated with the cells to determine the role of SVCT-1 on the internalization of the formulations. Fixation was achieved by the incubation of the inserts in 4% (v/v) PFA and gently washed in PBS. Incubation with 200 µL of rhodamin-phalloidin (1:100) in HBSS + 0.2 % (v/v) Triton X-100 allowed the staining of actin. Inserts were incubated for 30 min in the dark, washed with PBS, cut and mounted on glass slides with Vectashield® mounting medium with DAPI [6]. Images were captured using LSM710 Zeiss confocal laser scaning microscopy (CLSM) and analysed with the ZEN (black edition 2.1) software to obtain x-y, x-z and y-z views of the Caco-2 monolayers.

Results and Discussion: The incubation of the fluorescent NEE-ADP led to strong DiD signal within the Caco-2 monolayers for both ADP ratios. This indicates that the nanoemulsions are taken up by the cells and accumulated, which is in accordance with previous results where curcumin encapsulated in NEE-ADP was accumulated in the cells [5]. Intracellular DiD signal depended on the presence of ascorbyl dipalmitate in the formulation, as the nanoemulsion control stabilised by lecithin instead of the ascorbyl derivative, showed no DiD signal. Preincubation of Caco-2 cells with ascorbate did not affect to the integrity of the monolayer, which was further confirmed by the transepithelial resistance measurements, thus indicating the suitability of the experimental procedure. In addition, ascorbate preincubation induced a remarkable decrease of the fluorescent signal when NEE-ADP formulations were in contact with the Caco-2 cells, suggesting that the SVCT-1 transporter may be involved on the uptake of the formulations.

Conclusions: These preliminary results suggest that the uptake of NEE-ADP in Caco-2 cells could be mediated by the SVCT-1 transporter. Nevertheless, further research is still required to confirm these results in order to propose ascorbic acid as a promising ligand to enhance the interaction of nanoemulsions with the enterocytes.

Acknowledgments: UCLM (OE154 and 2018/152504), Spain. Fonds de la Recherche Scientifique-FNRS (Belgium).

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POSTER SESSIONS

Session 2: Transdermal and Transmucosal Delivery

P01.2 A Polymer-based Intranasal Drug Delivery Platform Towards the Safe and Effective Treatment of Pediatric Glioblastoma (FLASH PRESENTATION) (p.73)
T. Melnyk, I. Conejos-Sánchez, I. Dolz-Pérez, E. Masiá, H.F. Florindo, A.M. Carcaboso, M.J. Vicent

P02.2 Alopurinol-loaded Nanostructured Lipid Carriers for Topical Application: in vitro and in vivo evaluation (p.74)
M. Carvalheiro, C.G. Varrica, C. Faria-Silva; G. Sandri and S. Simões

P03.2 Design of bioinspired hydrogel contact lenses with affinity for statins (p.75)
A. F. Pereira-da-Mota, M. Vivero-Lopez, A. Concheiro and C. Alvarez-Lorenzo

P04.2 Development of a new 3D intestinal model as a more reliable platform to perform in vitro drug permeability studies (p.76)
M. H. Macedo, E. Martínez, C. Barrias and B. Sarmento

P05.2 Effect of oil content on the physical-chemical properties and in vitro release profile of microemulsions (p.78)
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A Polymer-based Intranasal Drug Delivery Platform Towards the Safe and Effective Treatment of Pediatric Glioblastoma

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Introduction: Brain tumors are the second leading cause of cancer death in children after leukemia, with malignant gliomas accounting for 75% of all cases[1]. The low levels of drug delivery through the blood-brain barrier (BBB) and the associated poor survival rate (12-15 months) of glioblastoma (GBM) patients highlight the nesessity for new treatment approaches for this unmet clinical need. Intranasal administration represents a promising non-invasive strategy with several advantages, which include minimum patient discomfort, the bypass of hepato-gastrointestinal metabolism and the BBB, thereby enabling direct nose-to-brain delivery. Our research focuses on (i) the development of a novel rationally designed polymer-CDK4/6 inhibitor conjugate and (ii) the development of a safe and efficient intranasal platform for targeted delivery and release in the brain as treatments for pediatric glioblastomas. Nose-to-brain transport can be facilitated by polymeric formulations that enhance solubility, stability, and permeation through mucosa and systemic exposure when compared to the free drug administration. Our studies have demonstrated that polyglutamate-based polymers (PGA) represent excellent candidates for brain delivery due to their biodegradability and multivalency, which allows combination therapy or the introduction of targeting moieties to enhance mucosal permeation[2]. The modulation of mucoadhesivity, mucodiffusion, polymeric size and shape, as well as the optimization of drug ratios, linking chemistry, and release kinetics can combine to overcome the limitations associated with the intranasal route.

Materials and Methods: PGA-based nanocarriers were synthesized by NCA-ROP polimerization[3], and the CDK4/6 inhibitor conjugated using stimuli-responsive linkers. Size and conformation were determined by DLS, CD, and SAXS. Drug release studies in the presence of DTT/cathepsin B was analyzed by RP-HPLC. Cell studies were performed in patient-derived pediatric tumorsphere cultures (GBM-glioblastoma and DIPG-diffuse intrinsic pontine glioma). The selection of OG-labeled polymeric nanocarriers was carried out in a sheep mucosa *ex vivo* model. Preliminary *in vivo* biodistribution was conducted on anesthetized healthy mice following intranasal administration of Cy5.5-labeled polymer-drug conjugates, with fluorescent imaging performed using the IVIS system.

Results, Discussion, and Conclusions: We obtained well-defined linear[2] and star-shaped PGA[4] systems with the conjugation of fluorescence probes and the CDK4/6 inhibitor performed following established protocols[2-4]. To select the candidates for mucosa permeation, we established an intranasal screening platform based on permeation kinetic studies in Franz-cells (**Fig. 1A**) using sheep mucosa. The results (**Fig. 1B**) highlighted the relevancy of mucodiffusive ligand exposure (lower exposure promotes aggregation and decreases passage) and size/shape (stPGA displayed enhanced permeation compared to the linear analog). St-PGA incorporated into a hyaluronic crosslinked hydrogel as a vehicle (stPGA-HACP) exhibited a delayed permeation pattern compare to stPGA alone, a finding confirmed with preliminary *in vivo* biodistribution experiments on healthy mice. We designed two stimuli-responsive linkers (GHS responsive disulfide linker and a Cathepsin B responsive peptidic linker) for the conjugation of the CDK4/6 inhibitor to PGA to allow drug release in the tumor microenvironment. Cell viability analysis (**Fig. 1C**) displayed the expected effect of the free *vs.* conjugated drug against glioma cells (DIPG) due to altered cell trafficking and enhanced drug release kinetics. We selected a stPGA-CDK4/6 inhibitor for subsequent in vivo experiments in a DIPG-PDX model using intranasal administration. Overall, our in vitro studies have demonstrated the potential of PGA-based nanocarriers as an effective and efficient treatment for pediatric glioblastoma.

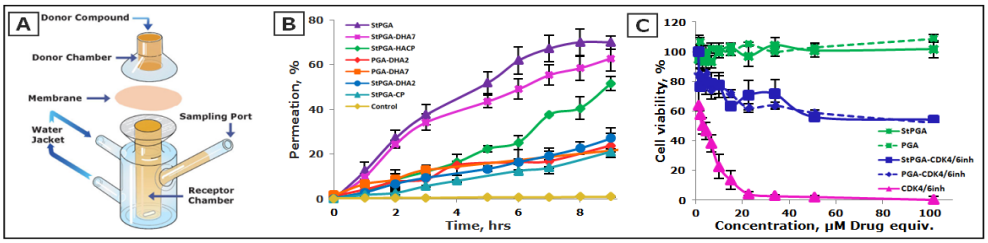

Figure 1. **A**) *Ex vivo* intranasal model, **B**) permeation kinetic studies of nanocarriers, and **C**) DIPG cell viability (MTS 72h)

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Alopurinol-loaded Nanostructured Lipid Carriers for Topical Application: *in vitro* and *in vivo* evaluation

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Introduction Topical drug delivery systems provide valuable strategies to formulate and deliver therapeutics and other substances to and via the skin. The major advantage is that they allow a direct and localized treatment within the different skin layers reducing systemic side effects. They can also contribute to decrease the dosage retaining the drug at the site of action. Several topical drug delivery systems have been developed to enhance either drug permeation properties or drug targeting to a specific layer of the skin [1]. Nanostructured lipid carrier (NLC) are particulate systems made up of physiological, biodegradable and biocompatible lipid materials and surfactants that have been widely studied as delivery systems for a variety of routes, including the skin [2]. Allopurinol is used for the treatment of Canine Leishmaniasis and in combination therapy for Cutaneous Leishmaniasis [3]. It is characterized by low water solubility and high melting point which has hampered its use to the topical route. In this work Allopurinol (AP) was incorporated in an NLC formulation to enhance drug absorption as a topical approach to treat CL skin lesions.

Materials and Methods NLC were prepared by the emulsion and sonication method using glyceryl distearate as solid lipid, caprylic/capric triglyceride as liquid lipid and Tween 80 as surfactant. The Allopurinol-NLC (AP-NLC) system was characterized in terms of size, charge, rheological behaviour, and permeation through newborn pig skin using Franz diffusion cells. The *in vitro* cytotoxicity was evaluated using HaCaT and THP-1 cells and the anti-leishmania activity was assessed in *L. major* parasites using appropriated assays. A protocol for the evaluation of the efficacy of the AP-NLC formulation on animal skin lesions was established using male Wistar rats.

Results and Discussion The AP-NLC-formulation exhibited a homogeneous, translucent fluid gel-like aspect and a white colour. These macroscopic characteristics were suitable for topical administration. This nanoparticulated system presented a mean size of 259 nm with a PdI of 0.563, zeta potencial values around -49.6 mV and encapsulation efficiency of 49.38%. Permeation and penetration assays revealed that AP-NLC formulation has an adequate profile to be used in a topical formulation since epidermal and dermal drug retention was achieved. No reduction in HaCaT and THP-1 cell viability was observed at the tested concentrations (AP < 10 μg/mL). Treatment of infected THP-1 with *L. major* parasites resulted in around 50% growth inhibition demonstrating that AP-NLC present activity against intracellular amastigotes. The *in vivo* application of the AP-NLC formulation resulted in the decrease of a rat skin lesion when compared with non-treated controls.

Conclusions In this work, NLC were chosen as systems with the ability to incorporate and deliver AP into the skin. The results obtained suggest that the proposed novel formulation presents suitability for dermal delivery of AP. *In vivo* studies have proved that AP-NLC formulation has a potential to be used in the regeneration of skin lesions. Further experiments with a cutaneous leishmaniasis animal model still need to be performed.

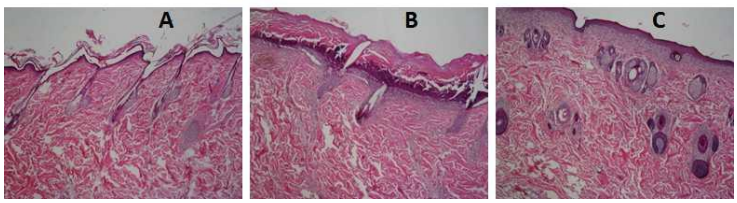

Figure 1. Microphotographs of the skin of rats stained with haematoxylin and eosin (200 x magnification). A – naïve control rat skin; B – rat skin treated topically with SDS gel; C – rat skin treated topically with SDS gel followed by topical application of AP-NLC. Histology analysis showed that the application of 40% SDS gel on rat skin for 5 consecutive days resulted in extensive and severe dermatitis lesions with epidermal necrosis (B). Skin treated with AP-NLC presenting macroscopic signs of healing showed microscopically advanced healing of dermatitis lesions (C).

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Design of bioinspired hydrogel contact lenses with affinity for statins

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Introduction

Statins are receiving increasing attention in the ophthalmic field [1]. Their activity as 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors is clinically used to regulate cholesterol levels, but also leads to pleiotropic effects. Their anti-inflammatory, neuroprotective and antiproliferative properties together with corneal wound healing make statins suitable candidates for the treatment of a variety of ocular pathologies including dry eye, diabetic retinopathy and macular degeneration, among several others [2]. In parallel, contact lenses (CLs) are being positioned as advantageous platforms for sustained release of therapeutic doses on the ocular surface [3]. The present work relies on the hypothesis of that hydrogels that can mimic the active site of HMG-CoA [4] might exhibit enhanced affinity for statins. Thus, the aim of the work was to design CLs using functional monomers that bear chemical groups that ressemble those present in HMG-CoA and to evaluate the loading and release of atorvastatin sodium.

Materials and Methods

Hydrogels were prepared mixing 2-hydroxyethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EGDMA) with the functional monomers ethylene glycol phenyl ether methacrylate (EGPEM), 2-aminoethyl methacrylate hydrochloride (AEMA) or N-(3-aminopropyl) methacrylamide hydrochloride (APMA), and polymerized at 50 °C for 12 h and 70 °C for 24 h. Hydrogels were boiled, cut as discs, and washed in ultrapure water and 0.9% NaCl to remove non-reacted monomers. Atorvastatin sodium loading was carried out by soaking of dried discs in 5 mL of drug solution (ethanol:water 20:80 v/v; 0.02 mg/mL) kept under magnetic stirring (150 rpm) at room temperature. Release experiments were carried out in 5 mL of simulated lachrymal fluid (SLF) pH 7.5 at 37º C and 150 rpm. The vials were protected from the light and the amount loaded and released was measured from the absorbance recorded at 242 nm at preestablished periods of time. The swelling degree was monitored as the increase in weight of dried discs with atorvastatin solution (ethanol:water, 20:80 v/v) and SLF at room temperature. The transmittance was measured with fully hydrated discs in atorvastatin solution. The experiments were carried out in quadruplicate.

Results and Discussion

Designed hydrogels showed adequate properties to be used as the basis of contact lenses. The swelling degree in atorvastatin solution was higher (approx. 85%) than in SLF (approx. 50%). All hydrogels were transparent in the visible range (200-800 nm), with light transmission above 80%. Non-functionalized hydrogels did not uptake any atorvastatin. The amount loaded of atorvastatin was remarkably higher for hydrogels prepared with AEMA or APMA (approx. 3.0 mg/g). These hydrogels showed sustained release for several hours.

Conclusions

Incorporation of AEMA or APMA as functional monomers notably increases atorvastatin loading capacity to therapeutically useful levels while retaining appropriate release characteristics. Future experiments will focus on the optic and mechanical properties of the hydrogels and the optimization of the release profiles to address specific clinical needs.

Acknowledgments: This work was supported by MINECO (SAF2017-83118-R), AEI Spain, Xunta de Galicia (ED431C 2016/008, ED431E 2018/08), and FEDER.

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Development of a new 3D intestinal model as a more reliable platform to perform *in vitro* drug permeability studies

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Introduction: The small intestine is the primary site of drug absorption after oral administration. *In vitro* models are extremely important in preclinical drug development, since they are less laborious, cost-intensive, and raise less ethical considerations compared to in vivo studies [1]. The Caco-2 model is the "gold-standard" of *in vitro* intestinal models, although this model presents several drawbacks, representing only one layer of the small intestine and one cell type. Besides, it has an over expression of tight junction proteins, hampering the passage of drugs [2]. We have developed a new 3D intestinal model, comprised of a collagen layer with human intestinal fibroblasts (HIF) embedded, mimicking the intestinal lamina propria, providing the 3D support for the epithelium (Fig. 1). **Materials and Methods:** The optimization of the collagen layer with HIF was performed testing different collagen concentrations (5 and 6 mg/mL) and different HIF seeding densities (1, 5, 10 and $20x10^5$ HIF/mL). The metabolic activity of fibroblasts, as well as their morphology and fibronectin deposition inside the collagen hydrogel was assessed. Epithelial cells Caco-2 have been seeded on top of the collagen hydrogel. The transepithelial electric resistance (TEER) of the cultures was assessed during 21 days and a permeability assay with FITC-dextran 4kDa, a compound that is absorbed paracellularly, was performed. **Results and Discussion:** The optimization of the collagen layer using a collagen concentration of 5mg/mL and different HIF seeding densities revealed that cells were able to contract the hydrogel and a higher initial cell density resulted in higher contraction. In a second optimization, the lowest HIF seeding density ($1x10^5$ HIF/mL) was used and the collagen concentration was increased to 6mg/mL. This increase prevented the contraction of the gel by the fibroblasts. The metabolic activity of the fibroblasts inside the gel increased along the time in culture. Regarding HIF morphology and fibronectin deposition, it was possible to observe that cells presented their normal elongated shape and were able to remodel the collagen matrix, producing fibronectin. Regarding the behavior of epithelial cells on the model, it was observed that TEER decreased when Caco-2 cells were seeded on top of the collagen layer compared to the 2D control, what was expected according to literature [4]. This might be due to the fact that cells sense an environment that is more similar to the *in vivo* situation so they behave in a more realistic manner, decreasing the tightness of TJs [4]. Permeability with FITC-dextran showed that absorption of the compound is higher in the 3D model, comparing to the 2D model. This is probably related with the lower TEER values, which indicate a looser monolayer, making it easir for the compound to cross paracellularly. **Conclusions:** It was possible to optimize the collagen layer obtaining a compromise between HIF proliferation and concentration of collagen to obtain a layer that mantains its integrity during the time in culture, which is extremely important when performing permeability assays. Permeability with FITC-dextran showed that the 3D intestinal model can be promising when testing the permeability of compounds that are paracellularly absorbed, being more similar to what is observed in vivo [5]. This new model can be an important tool regarding drug development, giving more trustable results and contributing for the 3R's policy.

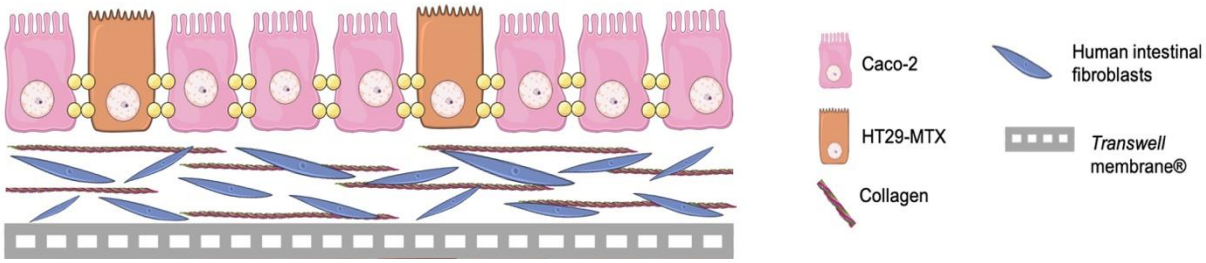

Figure 1. Configuration of the 3D intestinal model. The model is comprised of a collagen layer with fibroblasts embedded, with an epithelial layer on top.

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Effect of oil content on the physical-chemical properties and *in vitro* release profile of microemulsions

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Microemulsions have been widely studied as an approach for the cutaneous delivery of hydrophilic and lipophilic substances in the pharmaceutical and cosmetic industry. These systems present the advantage of having easy and low cost methods of preparation, and being thermodynamically stable when compared with other topical formulations. A microemulsion is usually composed by a blend of oil, water, surfactant and co-surfactant, forming a spontaneous, clear and isotropic system with an internal droplet size lower than 100 nm [1,2].

This work aims to explore the influence of the amount of oily phase in the physical-chemical properties of microemulsions as well as, in the *in vitro* release profile of lipophilic substances.

A pseudoternary phase diagram was initially constructed (Figure 1), by selecting monoacylglycerol and diacylglycerol as the oily phase, and a mixture of polyoxyglycerides and glycol in a ratio of 1:1, as the components of surfactant/co-surfactant phase. Ranging the proportions of oily phase/surfactant and co-surfactant phase from 1:9 to 9:1, the mixtures were titrated with constat proportions of water, and the aspect (clear, translucent or cloudy) was further evaluated. Based on the pseudoternary phase diagram, three different formulations with different amounts of oil, but with a clear aspect and without phase separation, were selected and characterized. The mean droplet size of internal phase and polydispersity index (PdI) was assessed by Dynamic Light Scattering and the transparency was also evaluated by measuring the transmittance trough UV-VIS spectrophotometer.

After the construction of a pseudoternary phase diagram, three different formulations with 50% of surfactant/co-surfactant in a ratio of 1:1, but with different proportions of oil (10%, 25% and 40%), were selected. All formulations presented a fluid and clear aspect (transmittance close to 100% when assessed by spectrophotometry). Microemulsions with 10% and 25% of oily phase presented an internal droplet size between 4 and 11 nm and a PdI between 0.2-0.3, while microemulsions with higher amount of oil (40%) presented an internal droplet size higher than 100 nm and PdI higher than 0.5.

Although the microemulsions selected presented a clear aspect, its physical-chemical properties were influenced by the amount of oil in the microemulsion**.**

Curcumin, a lipophilic fluorescent probe, will be incorporated into the oily phase of microemulsions and the *in vitro* release profile of different microemulsions will be assessed through Franz Cell diffusion assay. The results obtained will confirm the influence of the oil content in release and permeation of curcumin from the developed microemulsions.

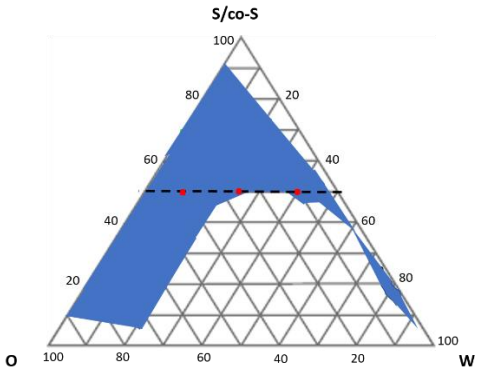

Figure 1. Pseudoternary phase diagram of mixtures constituted by monoacylglycerol and diacylglycerol (O), water (W) and a blend of polyoxyglycerides and glycol (S/co-S) in a ratio of 1:1. Blue areas correspond to the formulations with a clear or translucent aspect and red dots correspond the selected formulations.

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Enfuvirtide-Loaded Deformable Lipid Vesicles for Transdermal Delivery

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Introduction: This work aimed to innovate the route of administering enfuvirtide (T-20), an antiretroviral drug to HIV-1 that acts in the early phase of the replicative cycle, preventing the entry of the virus into CD4 + T lymphocytes, by inhibiting the function of the transmembrane glycoprotein gp 41. This synthetic peptide presents a unique mechanism of action among the drugs currently approved for clinical use [1], however, its administration requires b.i.d. subcutaneous injection. In addition to cause lesions at the site of application after continued use, this invasive method has low therapeutic acceptance, especially in the pediatric and geriatric populations. Thus, the goal of this work was to construct T-20-loaded deformable vesicles (Transfersomes (TR) and Transethosomes (TE)) able to be applied on the skin to deliver T-20 systemically.

Materials and Methods: The formulation was set up from soybean phosphatidylcoline (SPC)-based systems, especially designed to be deformable containing T-20. Vesicles were prepared according to classical methods and downsized by filtration under pressure. T-20 loaded vesicles were characterized to confirm the potential of the new system as follows: mean diameter, polydispersity index (PdI) and zeta potential by dynamic light scattering (DLS), and elasticity by atomic force microscopy (AFM). Quantification of T-20 was carried out on both formulations using liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS), following a method previously described [2].

Results and Discussion: Both the vesicles size and PdI increased after T-20 addition, especially for TE. Although the mean size of T-20 TR and T-20 TE were acceptable for the proposed application, the PdI was higher than 0.3 for both formulations as T-20 molecules might bound to the vesicle surface rather than encapsulated due to their previously characterized peptide-lipid interactions [3]. These parameters remained stable over 30 days for all formulations, except for T-20 TE. This difference was observed by comparison with the respective control (empty TE). The zeta potential of the final formulations was almost neutral and in the same range to those obtained for controls (empty vesicles) which is in accordance to the null formal charge of T-20 molecule and to the use of a nonionic surfactant in the formulation (Tween 80) as well as a neutral or zwitterionic phospholipid (SPC) over a pH range. The incorporation of T-20 reduced the deformability of TR and TE as expected. Finally, HPLC-MS/MS revealed to be a suitable technique to quantify this drug.

Conclusions: The results supported the extemporaneous preparation T-20 formulations. Future *in vivo* pharmacokinetic studies will be performed to provide information on the bioavailability of the molecule applied to the skin and delivered by these nanosystems. In addition, *in vitro* efficacy will be demonstrated by experimental assays with HIV-1 viral isolates.

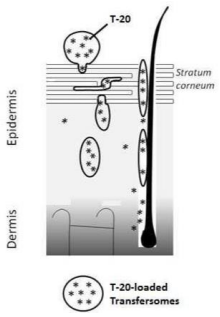

Figure 1. T-20 vesicles applied on the skin.

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Ex Vivo permeation of progesterone from a microemulsion drug delivery system

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Introduction: Retinitis pigmentosa (RP) is the most frequent hereditary degenerative disorder affecting visual photoreceptors which, in advanced stages, may produce blindness. An effective treatment has not been found yet, but some authors described that progesterone (PG) could be used to slow the progression of the disease [1]. The study of release systems for RP is interesting and microemulsions (ME) can play an important role in addressing this issue. ME are thermodynamically stable systems manufactured with oil, water and surfactant (and/or co-surfactant) mixtures. The wide range of oil-water-surfactant components in ME vehicles enables solubilization of lipophilic drugs [2], as is the case with PG. The aim of our research is to evaluate the suitability of ME for the ocular delivery of PG.

Materials and Methods: Several empty ME were formulated and the one with best characteristics was selected. ME was elaborated by mixing of surfactant/cosurfactant (labrasol/transcutol HP (4:1), 29.1%), oil phase (lauroglycol FCC, 3.98%) and PG (0.69%). Finally, was incorporated an aqueous phase (water, 66.23%). PG stability in ME was studied over a period of 15 days at room temperature and at 8ºC. The diffusion studies of ME were performed with Franz cells (0.567 cm^2 permeation available area) using rabbit cornea and sclera as membranes. PG in the samples was quantified by HPLC-UV using a validated method. [Column C18 (150 x 4.6mm), Mobile phase Acetonitrile:water (80:20), flux 1mL/min, volume injection 50 µL, UV detection 240 nm].

Results and Discussion: The saturation solubility of PG in ME formulations revealed high-solubilizing power of the ME systems compared to that of the simple hydroalcoholic or aqueous propyleneglycol systems. ME loaded with PG performed well in stability studies (99.7 ± 0.2%). The permeability coefficients obtained for the PG in ME were 0.45 ± $0.06 \cdot 10^{-3}$ cm/h and 0.24 ± $0.09 \cdot 10^{-3}$ cm/h for cornea and sclera respectively (n=7). Better results were obtained in the diffusion of PG from ME in cornea than sclera (Figure 1). The amount of drug that permeated through the cornea was approximately double than through sclera. Differences in the amount of PG accumulated in cornea (69.1 ± 18.2µg) and sclera (185.7 ± 57.9µg) were found to be statistically significant ($p < 0.05$).

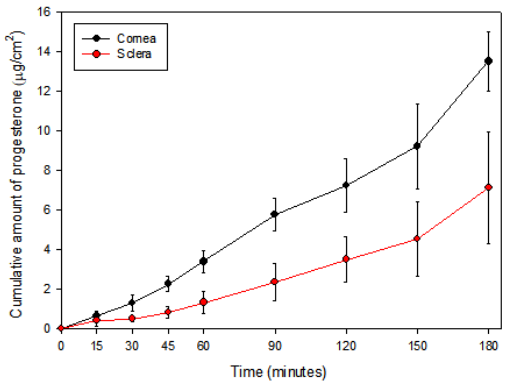

Figure 1. Accumulated amount of PG in the receptor compartment (µg/cm^2) (n= 7) versus time (minutes) obtained from samples of ex vivo trans-corneal and trans-sclera diffusion of ME in the donor compartment.

Conclusions: PG remains stable for at least 15 days in ME. Statistically significant differences in the diffusion of PG between both ocular structures, being higher in corneas than scleras. On the other hand, a greater amount of PG accumulated in sclera than in corneas.

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Hydrocortisone controlled release hydrogels for topical application

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Introduction

Hydrogels are three-dimensional polymer networks formed by hydrophilic polymers, which can absorb and retain a significant amount of water, without being dissolved due to the crosslinking of polymer chains. One of the most widely used polymers in pharmaceutical applications is chitosan due to its biocompatibility, biodegradability, non toxicity, mucoadhesivity, antibacterial and antifungal activity and so on. Chitosan is an aminopolysaccharide obtained by N-deacetylation of chitin which is part of the exoskeleton of crustaceans and insects. This polymer has an amino group, a primary hydroxyl and a secondary hydroxyl. The amino group may be acetylated or deacetylated; the molar fraction of deacetylated units versus total is known as the degree of deacetylation. Different types of chitosan result in hydrogels with slightly different properties. The amino group has a pKa of 6.3 so at pHs lower than this value this group protonates, conferring solubility on the polymer. On the other hand, at neutral pH and alkaline and in organic solvents the quitosan is insoluble.

The application of hydrogels to the skin has allowed to create drug release systems, wound dressings and even polymeric films to improve the appearance of the skin. In this study has been studied the release of hydrocortisone from an ion hydrogel consisting of chitosan and a reticulating agent with phosphate groups

Materials and Methods

For the synthesis of the hydrogel, a 2% chitosan solution in HCl 0.1 N was prepared and different volumes of a stock solution of linker in water were added. The formulations were freeze-dried by obtaining dry hydrogels in the form of disc; this product can be easily liophilizated and can be rehydrated in aqueous medium forming an easily extendable viscous hydrogel. The hydrogels obtained have been characterized by different techniques and their release profile at pH= 5,5 have been determined in order to know the properties of the system.

Results and Discussion

The formation of the hydrogel is based on the ionization states of the components. The chitosan dissolved in acid has a pH of 1.4 and the reticulant dissolved in water a pH 8.5, however, the resulting product has neutral pH. Extensibility, compatibility, non-irritation and non-toxicity of the rehydrated hydrogels after liophilization have been previously demonstrated. The rehydrated hydrogel can be easily applied on the skin for topical administration of hydrocortisone because of their mechanical properties. Results of hydrocortisone release assays indicate that the release of hydrocortisone contained in the hydrogel may be prolonged for more than 24 hours although 70% of the content is released during the first 10 hours of exposure.

Conclusions

Ionic chitosan hydrogel is an excellent option for topical hydrocortisone administration and allows a controlled release of the drug during more than 24h

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New user-friendly software enables the accurate characterization of the diffusion pattern followed by nanocarriers in complex biological media

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Introduction: Particle tracking (PT) is a powerful microscopy technique that enables to quantify the diffusion of single particles in complex biological media, such as the mucus, the brain extracellular matrix or the cytoplasm [1,2]. The raw data of PT is based on time-lapse videos. The analysis of the specific *x* and *y* position over time allows the calculation of the mean-square displacement as $MSD=\langle \Delta x^2 + \Delta y^2 \rangle$. The effective diffusion coefficient (D_{eff}) of the particles depends on the MSD as $MSD=4D_{eff}\,\tau^{\alpha}$, where τ is the time scale and α gives information about the diffusion mode of the particle. Linear modelling of MSD as a function of τ allows the calculation of D_{eff}.

PT thus arises as a promising tool for the development of novel nanocarriers based on their biological behaviour. Nevertheless, this technique entails the processing of large data sets, what might be tedious and make it difficult to obtain reliable results for the non-specialist. Furthermore, there is a lack of homogeneicity in the selection of parameters used to screen erratic trajectories. In this sense, the goodness of the linear fit is usually evaluated as R^2, considering only those results with a R^2 value in the 0.75-1 range [1]. However, MSD of non-diffusing particles presents a negligible dependence on τ, which leads to $R^2 \sim 0$. This results in a clear overestimation of the diffusion capacity of the nanoparticles under study. In this scenario, we have developed a novel PT software which efficiently screens trajectories and turns the raw data derived from PT experiments into trustworthy and easy-comprehensive parameters related to diffusion, such as α and D_{eff}.

Materials and Methods: we obtained raw data derived from *ex vivo* PT experiments. Concretely, we performed PT in porcine intestinal mucus, which was obtained from a local slaughterhouse. We carried out PT with unmodified and PEGylated polystyrene nanoparticles, which were used as mucoadhesive and mucodiffusive controls, respectively. For each sample, 20-30 movies of 800 frames were recorded at a frame rate of 100 fps, obtaining more than 100 trajectories per movie. For the development of the novel PT software we used the most recent version of R Statistical Software and Programming Language (currently R 3.6.1) [3].

Results and Discussion: We obtained a novel PT software which covers the whole analysis cycle, from data import to interactive visualisation of results. The R scripts are implemented in a Shiny [4] application that allows user interactivity, reactive programming, and responsive design. Theoretical diffusion models are represented and simulated given a configurable set of parameters. PT input files are spreadsheets with thousands of rows and hundreds of columns with the particles MSDs that are loaded in the software workspace in tidy format. The videos and particles can be browsed and represented for exploratory purposes, including a so-called "prototype particle" for each video. The power of R takes off in the modelling stage, when thousands of linearized models are to be fitted. For each particle, Least Squares Estimators are obtained, and, given the model residuals, R^2 is computed, for both the non-linear model and the linearized model. Along with other error measurements, e.g., RMSE, and further input from the analyst, the application leads to a data-driven decision making about the D_{eff} of the particle, beyond the current state of the art.

We have widely validated this PT software, which has turned to be accurate and extremely time-saving. [5,6].

Conclusion: we have developed a comprehensive yet simple software which efficiently screens erratic trajectories and fastly transforms large and complex raw data derived from PT experiments into a complete characterization of the diffusion pattern followed by nanocarriers. Additionally, this PT software is free, public-accessible and user-friendly, so it is a new tool that might provide insightful help in the development of novel nanocarriers based on their real behaviour in biological samples.

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Layer-by-layer polymeric microneedles for cancer chemo-photothermal therapy

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Cancer is one of the leading causes of death worldwide and its incidence has been increasing in the last decades [1]. In this field, the combination of photothermal and chemo therapeutic approaches presents a high potential to increase the cancer treatments efficacy [2-3]. Additionally, in last years, researchers have been re-exploring the application of macroscale delivery systems (e.g. microneedles and hydrogels) directly in the tumor to control the therapeutics' pharmakocinetics and avoid the complications associated to the systemic delivery [4].

In this work, a new layered polyvinylpyrrolidone (PVP) microneedle system loaded with doxorubicin and gold-core silica shell nanorods (Dox@MicroN) was developed to be used in the cancer combinatorial therapy. For that purpose, a PVP solution was deposited on a PDMS mold and allowed to dry at room temperature. Then, the PVP microneedle rigid base layer was coated with a layer of doxorubicin/chitosan followed by a layer of gold-core silica shell nanorods/poly (vinyl alcohol) using the electrospraying technique. The physicochemical characterization of the microneedles demonstrated that the electrospraying technique can be used to produce a layer-by-layer microneedle coating consisting in doxorubicin loaded chitosan and AuMSS enriched poly (vinyl alcohol) layers with 20.63±8.33 and 26.66±3.91 µm, respectively. Further, the Dox@MicroN patches presented a good photothermal capacity leading to a temperature increase of 12ºC under near-infrared irradiation (808 nm, 1.7W/cm^2 for 5 min), which in conjugation with the chitosan' pH sensitivity could be used to control the doxorubicin release. In fact, the doxorubicin release occurred in a pH dependent manner, i.e. at pH 7.4 reached the 12% whereas this value increased to 56% at pH 5.6, which could be enhanced with the 5 min of NIR laser irradiation, 20.05% at pH 7.4 and 59.55% at pH 5.6. Moreover, the microneedles were able to penetrate the tumor-mimicking agarose gel and promote a layer dependent drug release. Additionally, the Dox@MicroN patches capacity to simultaneously mediate the chemo- and photothermal-therapies rendered a superior cytotoxic effect against the cervical cancer cells.

Overall, the Dox@MicroN patches demonstrated to be a simple macroscale delivery device that can be used to mediate the local administration of new drug-photothermal combinations, avoiding all the issues related with the systemic administration of anti-cancer therapeutics.

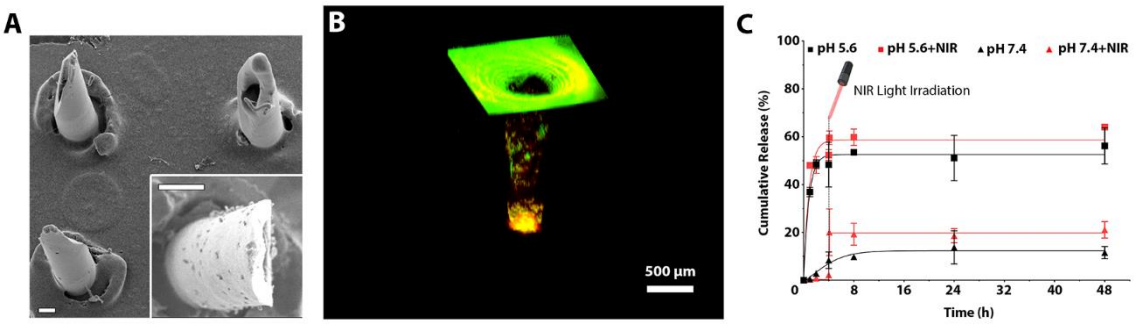

Figure 1. (A) SEM images of the microneedle patches. (B) Confocal image of the layer-by-layer organization of the microneedles (green channel: doxorubicin loaded chitosan layer; red channel: Wheat Germ Agglutinin, Alexa Fluor™ 594 Conjugate stained poly (vinyl alcohol) layer). (C) Analysis of the doxorubicin release from the microneedles at different pHs and in the presence or absence of NIR laser irradiation.

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Predicting topical bioavailability of dermatological products

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Introduction: Predicting local bioavailability of topically applied products remains a challenging issue. Better predictions would enable developers to select formulations with improved drug delivery profiles for further development and clinical testing but, while a range of methods are available for this purpose, none is without limitations. Traditional *in vitro* permeation tests have good predictability but lack a functioning microcirculation and are unable to test multiple applications simulating a chronic treatment. *In vivo* test methods account for skin circulation effects and include: (a) systemic pharmacokinetics for actives with measurable blood levels; (b) microdialysis and open flow microperfusion sample the viable skin but are technically demanding and have a limited test duration, and (c) *in vivo* tape-stripping, which samples only the stratum corneum (SC) but allows testing over an extended time including the application of multiple doses. Also, and critically important, is the development of metrics based on the data provided by these methods that enable local bioavailability to be characterised. Our long-term aim is to use data derived from tape stripping experiments to predict local biovailability.

Materials and Methods: A simple compartmental model depicts the transit of drugs delivered to the SC by a formulation to the deeper, viable skin compartments (Fig.1). Transfer to deeper tissues is characterized by the first-order rate constant β and by the flux J estimated as:

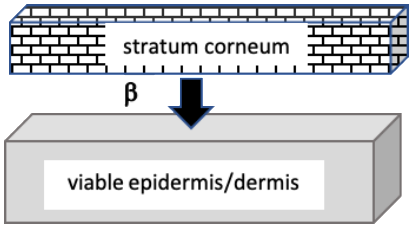

Figure 1. Drug input to viable skin is controlled by the 1st-order rate constant β

$$\beta = \frac{\ln\left(Q_{uptake}/Q_{clearance}\right)}{\Delta time} \text{ and } J = \frac{Q_{uptake} - Q_{clearance}}{\Delta time}$$

where Q_{uptake} and $Q_{clearance}$ are the amounts of drug present in the SC after a given exposure time to a formulation and after a given exposure + clearance combined time, respectively.

Following ethics approval, a series of studies were performed in heathy adult volunteers who provided informed consent. In the betamethasone valerate experiments, Betnovate 0.1% cream was applied (2 and 5 mg/cm^2) to the ventral forearms of 12 healthy volunteers. Q_{uptake} and $Q_{clearance}$ were measured following tape-stripping (a) after a 4-hour 'uptake' period, and (b) after removal, subsequent to a further 6-hour 'clearance'. Experimental details for the other experiments are described elsewhere [1-2].

Results and Discussion: J and β derived from tape striping experiments conducted with three drugs and seven marketed products are summarized in Table I. The slowest and fastest β were observed for acyclovir and betamethasone valerate, with 0.3-2.8% and 6-7% of the drug in the SC being delivered to deeper tissues per hour, respectively. The diclofenac study illustrates formulation effects that modify the uptake of a drug by the SC and flux through the SC without altering β. Increasing betamethasone dosing caused small, non-significant, increases in J without altering β.

Table I. β and J values (mean ±90% CI) in healthy volunteers derived from SxN experiments (number of studies x number of subjects per study). Superscripts indicate significantly different values ($p < 0.05$).

Drug	**Acyclovir**			**Diclofenac**			**Betamethasone valerate Betnovate 0.1%**	
Product (S x N)	**Zovirax USA (2x10)**	**Zovirax UK (1x10)**	**Aciclovir 1A Pharma (2x10)**	**Pennsaid 2% (1x 14)**	**Voltaren 1% (1x 14)**	**Solaraze 3% (1x 14)**	**2 mg/cm^2 (1x12)**	**5 mg/cm^2 (1x12)**
10^2 x β (h^{-1})	2.3 ± 1.2	2.8 ± 2.2	0.3 ± 1.2	3.0 ± 2.2	3.6 ± 1.9	3.3 ± 2.2	6.12 ± 4.21	7.16 ± 2.72
J (ng/h.cm^2)	14 ± 8.6	9.6 ± 8.0	9.6 ± 24	694^{a,b} ± 312	97^{a} ± 59	128^{b} ± 93	3.05 ± 1.81	5.78 ± 2.14

Conclusions: J is a model-independent parameter reporting on the quantity of drug that enters the viable skin layers below the SC over a certain time period, and is expected to be formulation and dose-dependent. In contrast, β is a fitted parameter to a pre-defined model (for which full validation is required), representing the transfer rate to the viable skin layers independent of drug uptake into the SC.

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Rationally Designed Polymer Therapeutics for the Topical Treatment of Psoriasis

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Introduction Psoriasis, a chronic inflammatory skin disease that affects over 125 million people, is characterized by erythematous skin plaques, inflammatory cell infiltration, and incomplete differentiation of keratinocytes [1]. Although the skin provides a wide accessible surface for the adsorption of therapeutic agents, topical administration of drugs has some limitations. Polymer-drug conjugates represent an appealing platform for drug delivery in different pathologies with demonstrated clinical benefit [2]. Polymer conjugation enhances dermal penetration of drugs, although there exist very few studies explicitly using 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 investigated the feasibility of polypeptide-conjugation to allow an anti-inflammatory drug to cross the skin barrier for the advanced topical treatment of skin diseases, such as psoriasis.

Materials and Methods
Polymer-drug conjugates were synthesized by well-established synthetic procedures using a pH-labile ester bond. The conjugates were characterized through an exhaustive physico-chemical characterization to ensure identity, purity, total drug loading, and free drug content. Conjugates were tested *in vitro* in various cell lines (keratinocytes, fibroblasts, and macrophages). The internalization of the conjugates labeled with a fluorescence probe in keratinocytes was studied by flow cytometry and confocal microscopy. The activity of the conjugate (72h of treatment) was tested by assessing the reduction in the release of pro-inflammatory cytokines from macrophages previously treated with LPS. *Ex-vivo* skin penetration studies in human skin were performed in Franz diffusion cells and human skin organ cultures for 24h.
Finally, an *in vivo* psoriatic mice model was established by the daily application of imiquimod cream for seven consecutive days, after which, mice were divided into different groups receiving daily treatment for five days.

Results and Discussion
We have successfully synthesized and characterized a polymer-drug conjugate employing a wide range of techniques. Cell viability assays demonstrated that the conjugate maintains or improves cell viability up to the concentrations tested. The conjugate also inhibited the release of inflammatory cytokines, suggesting that the drug retains its anti-inflammatory activity following incorporation into the polypeptidic backbone. By confocal microscopy, we established the endocytic uptake of the conjugate and colocalization with the lysosomes. *Ex vivo* human skin permeation studies using Franz diffusion cells indicated the presence of the conjugate within the epidermis 24h after treatment. Finally, in an *in vivo* psoriatic mice model, the histological examination revealed a significant reduction in epidermal layer thickness following treatment with the conjugated drug in comparison with treatment with the free form of the drug.

Conclusions
We have developed a topical therapy for the treatment of skin diseases based on a rationally designed polypeptide-drug conjugate that modulates drug permeability and penetration, and targets specific layers of the skin.

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Sustained release of resveratrol from antifouling contact lenses

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Introduction

Contact lenses (CLs) are widely used to correct vision problems, but the inherent risk of ocular infections limits their use [1]. SCLs are prone to the formation of bacterial biofilms, which can remain on their surface for long periods of time releasing planktonic cells regularly and bacterial products like endotoxins that damage the corneal epithelium and produce infections. The aim of this work was to develop poly(hydroxyethyl methacrylate) (pHEMA) and silicone-based hydrogels holding the antibiofouling monomer 2-methacryloyloxyethyl phosphorylcholine (MPC), a polar and biocompatible monomer with high capacity to prevent bacterial adhesion and protein adsorption [2] and loaded with resveratrol, a natural polyphenol with antioxidant and antimicrobial properties due to its performance as quorum sensing inhibitor [3]. The loading and release profiles may differ depending on the CL backbone. Furthermore, the release profile may also depend on the testing conditions. Thus, resveratrol release in a large volume of medium under gently shaking and in a microfluidic chamber that mimics the physiological tear fluid removal [4] were compared.

Materials and methods

Polymerization of pHEMA and silicone-based monomer solutions with and without MPC was carried out in molds at 50 ºC for 12 h and 70 ºC for 24 h. Then, the hydrogels were boiled in water, cut as discs, and washed until the complete removal of unreacted monomers. To evaluate the loading, dried discs were inmersed in resveratrol solution (5 mL, 12-30 µg/mL) at 36 ºC, 180 rpm, protected from light. The amount loaded was quantified from the absorbance recorded at 305 nm. Release experiments were carried out in (a) tubes with 3 mL of NaCl 0.9% at 36 °C and 180 rpm replacing the samples took at given time intervals for quantifying the amount released with the same volume of NaCl 0.9%; and (b) a microfluidic cell at 36 °C under continuous flow (3 µL/min) of NaCl 0.9%. At preset times, the output stream solution was collected, and its absorbance was measured at 305 nm. HET-CAM test was carried out with hydrogel pieces swollen in NaCl 0.9%.

Results and Discussion

Physical properties of all hydrogels were adequate for CLs. The amount loaded of resveratrol was similar for both types of hydrogels (approx. 2.5 mg/g) and it was slightly increased by the presence of MPC (approx. 3 mg/g). Nevertheless, the silicone hydrogels strongly retained resveratrol and released less than 5% amount loaded in three weeks, while pHEMA hydrogels provided sustained release of 20% in the first 24 h. pHEMA hydrogels tested in the microfluidic chamber led to constant release rates, which were slightly faster for hydrogels containing MPC. Tested compositions can be considered as non-irritant since changes in the CAM were not recorded.

Conclusions

Therapeutically remarkable amounts of resveratrol can be incorporated into the designed hydrogels, silicone hydrogels showing slower release rate. Moreover, the release conditions strongly determined the release rate profiles, which should be considered during the in vitro testing of drug-eluting CLs. Future experiments will focus on the effects of the different release rates on the antioxidant performances.

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Terahertz spectroscopy for crystallinity investigation of smartFilms®

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Introduction: SmartFilms® represent a new formulation principle to improve the dissolution rate and kinetic solubility of poorly water-soluble actives [1]. SmartFilms® consist of a cellulose matrix, e.g. commercially available paper, in which the active can be loaded in amorphous state. Subsequently, the smartFilms® can be filled into capsules or compressed into tablets without any further additives [2]. To investigate the crystallinr state of the incorporated active, differential scanning calorimetry (DSC) and X-ray powder diffraction can be used. A major drawback of both methods is that smartFilms® have to be destroyed prior to analysis and a measurement of a whole sample is not possible. Terahertz spectroscopy (THzS) is another method to detect changes in the crystalline state of chemicals. THzS is non-destructive and capable to measure specific points/areas or an entire smartFilm®. Aim of this study, was an investigation of the suitability of THzS to determine the crystalline state of incorporated actives in smartFilms®. As comparison and proof of concept DSC measurements were performed.

Materials and Methods: Coffee filter (Melitta Europa GmbH & C. KG, Germany) was used as paper base to produce smartFilms®. The model drug used in this study was tartaric acid (TA), as it is known to result in a prominent peak at 1.19 THz [3]. Paper cut-outs were loaded with different amounts of a tartaric acid solution, resulting in smartFilms® loaded with 1, 2, 3, 4 and 5 mg TA/cm^2, respectively. To validate that the methods are capable to detect the incorporated amount of active, physical mixtures with the same paper:TA ratio as in the smartFilms® were produced and analysed.

Results and Discussion: THzS of TA crystalline bulk material led to a spectrum with a characteristic peak at 1.19 THz. Analysis of the smartFilms® showed no crystalline TA peak for smartFilms® up to a loading of 2 mg TA/cm², higher loadings resulted in a significant peak. These results seemed reasonable, as it is known that the amount of active that can be incorporated in an amorphous state is limited and recrystallization must be expected at a certain concentration of the active. For all physical mixtures crystalline TA could be reliably detected.

DSC analysis of pure TA resulted in a peak at around 175 °C. Regardless of the loading. All smartFilms® showed no peak at 175 °C. Surprisingly, the analysis of the corresponding physical mixtures also did not result in a TA peak. Possible explanations for the failure of DSC to detect crystalline TA even in the physical mixtures are for example the detection limit of the device or an impact of the paper matrix which might mask the TA peak.

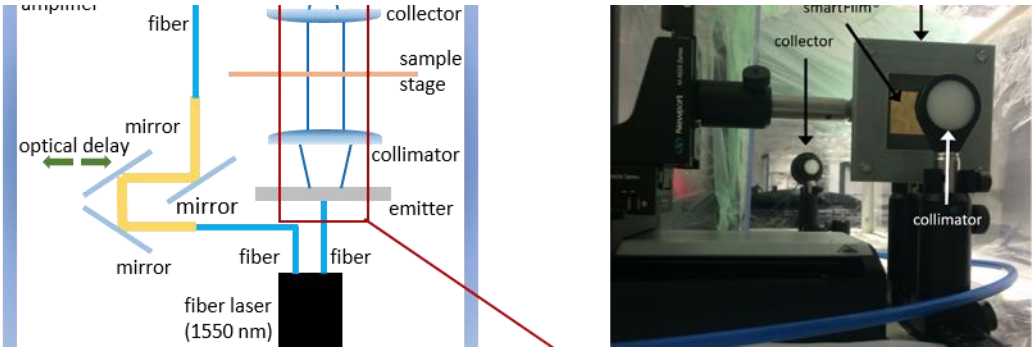

Figure 1. Measurement setup for transmission based THzS.

Conclusion: Analysis of the crystalline state of the active loaded smartFilms® is a crucial part for the production and development of smartFilms®. First experiments seem very promising, as THzS was able to detect changes in the crystallinity depending on the loading, whereas DSC failed its purpose and would have led to false conclusions. In fact, THzS wa found to be a promising method to determine the crystalline state of actives in smartFilms®. Additional studies have to be performed to investigate this promising method further.

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Thermoresponsive nanocapsules for skin hydration

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Introduction

The skin is a multifunctional organ, some of its most notorious features include: the prevention of loss of fluids and salts as well as the regulation of the body temperature; however, its most important function is to act as the first protective layer of the human body. Skin diseases (e.g. atopic dermatitis or psoriasis) often diminish the barrier efficiency of the skin, allowing the penetration of slightly hydrophobic drugs (e.g Dexamethasone). However, these drugs have only a brief residence time at the targeted site, resulting in poor therapeutic efficiency.[1] Penetration enhancers (chemical or physical) have proven effective in aiding the penetration of diverse substances by disrupting the skin barrier. Nevertheless, their use poses great risks, as they often lead to permanent damage on the skin, enabling the intrusion of harmful substances. Water is one of the most accepted penetration enhancers, as the skin is able to quickly recover to its exposure and no long-term damage is caused.[1-2] Nanoparticle based, dermal and transdermal drug delivery were found to be suitable approaches for the delivery of drugs into the skin, while reducing or eliminating side effects. Moreover, soft nanoparticles have been found to induce skin hydration.[2] Owing to their high surface area, easy functionalization and large encapsulation capacities, Nanocapsules (NCs) (defined as hollow nanoparticles composed of a cross-linked shell surrounding a core forming space) represent a promising strategy for topical drug delivery. In this study, the skin hydration properties of thermoresponsive nanocapsules (tNCs) will be investigated, as well as their derived penetration enhancement properties.

Materials and Methods

tNCs were synthesized using silica nanoparticles as sacrificial templates, as well as different ratios of poly(*N*-isopropylacrylamide) (pNIPAM) and poly(*N*-isopropylmethacrylamide) (pNIPMAM) and dendritic Polyglycerol (dPG) as macrocross-linker. Varying the ratio between pNIPAM and pNIPMAM, the volume phase transition temperature (temperature at which the particles change from a swollen state to a collapsed state) (VPTT), as well as the shell density of the tNCs, could be controlled. Moreover, the interactions and effects of tNCs on the stratum corneum were investigated using fluorescence microscopy, high resolution microscopy, and stimulated Raman spectromicroscopy.

Results and Discussion

Novel tNCs with various VPTTs and shell densities were synthesized. It could be shown that tNCs induced skin hydration, which upon applying a thermal trigger could be increased. Furthermore, the tNCs were found to enhance the penetration the high molecular weight dye ATTO OXA (used as a model for high molecular compounds).

Conclusions

These results highlighting nanocapsule excellent hydatrion properties and suggest their possible use for future drug delivery applications.

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Investigation of Rapamycin-loaded Core Multi-Shell Nanocarriers using a Skin Inflammatory Model based on Ex Vivo Skin Co-Cultures with Jurkat T Cells

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Introduction. Rapamycin is an immune suppressive drug used to prevent organ transplant rejection. The drug inhibits mTOR activity and exerts anti-proliferative effects towards different cell types including immune cells, keratinocytes and endothelial cells. For this reason, it is a promising compound for the treatment of hyperproliferative and inflammatory skin conditions, ideally by topical application [1]. However, due to high molecular weight (MW = 914.172 g/mol) and lipophilicity (log P = 4.3) [2], its penetration across the skin barrier is low. In addition, the severe side effects make it necessary to improve the selectivity of its delivery to diseased skin.

Materials and Methods. In this study, core multi-shell (CMS) nanocarriers were used to encapsulate rapamycin and enhance its dermal delivery. Redox-sensitive properties were obtained by introducing sulfide groups in the chemical architecture on the nanocarriers. Recently, to investigate the efficacy of immunesuppressive drug formulations, a skin model based on *ex vivo* human skin co-cultered with T cells in a trans-well set-up has been developed [3]. Here, we further developed this model. To introduce characteristics of inflamed skin conditions, serine protease was used to degrade corneodesmosomes (the protein complexes interconnecting corneocytes) and induce an impairment of the stratum corneum barrier. In addition, to activate T cells and test the immunosuppressive efficacy of the tested formulations, the culture medium was enriched with phytohaemagglutinin (PHA) and Th17 cytokines (IL-17 and IL-22). Scanning transmission x-ray microscopy (STXM) and atomic force microscope infrared spectroscopy (AFM-IR) were used as label-free methods to measure the spatial distribution of rapamycin penetrated across untreated and serine protease-treated skin, whereas inflammatory cytokines were monitored to test drug efficacy.

Results. The *ex vivo* skin-based inflammatory model enhabled to study both drug dermal delivery and anti-inflammatory efficacy. The spectro-microscopical analyses showed that the drug penetration strongly depends on the barrier integrity (serine protease pre-treatment) and on the drug formulation. Rapamycin formulated in CMS nanocarriers efficiently reduced the expression of inflammatory cytokines. Effects on pro-inflammatory cytokine IL-6 and IL-8 were measured in epidermis and dermis, while IL-2 suppression was measured in Jurkat cells.

Conclusions. Redox-sensitive CMS nanocarriers resulted to be effective drug formulations for the selective delivery of rapamycin to inflamed skin.

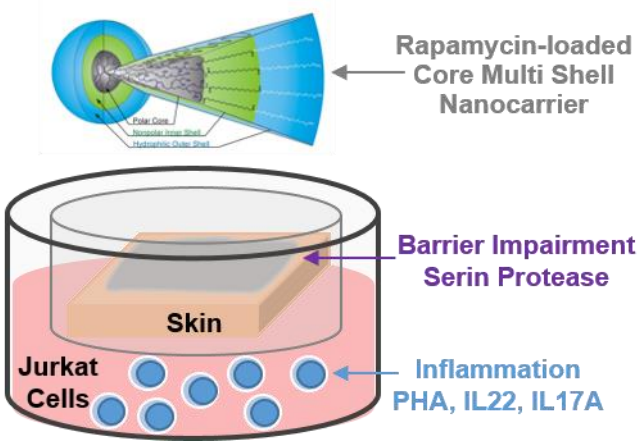

Figure 1. Schematic representation of the CMS nanocarrier and of the inflammatory skin model

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POSTER SESSIONS

Session 3: Therapy and prevention of infectious diseases

Aerodynamic characterisation of konjac glucomannan microcarriers targeted to the alveolar region through inhalation

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Introduction: Tuberculosis (TB) remains a major cause of death worldwide [1]. Despite the success of conventional TB treatment, the side-effects associated with the oral administration of antitubercular drugs and the development of multidrug-resistant TB, due to the long period of the treatment, demand new approaches to treat this disease [2]. In this work, an inhalable system was developed to target the alveolar macrophages, where *Mycobacterium tuberculosis* is located. Due to the presence of D-mannose on the konjac glucomannan (KGM) molecule, this natural polymer was chosen as the matrix material of the carriers. Mannose units are recognised by the infected alveolar macrophages and may potentiate the phagocytosis of KGM particles through the mannose receptors present on macrophage surface [3]. Isoniazid (INH) and rifabutin (RFB) were used as model drugs and were incorporated into KGM microcarriers by spray-drying. The resultant microparticles were characterised regarding their size and shape, and the aerodynamic properties of the particles were studied using a Next Generation Impactor (NGI).

Material and Methods: After an acid partial hydrolysis to reduce viscosity, KGM (1.5% w/v, Chemos, Germany) was solubilised in ultrapure water at 70 ºC. INH (Sigma-Aldrich, Germany) and RFB (Chemos, Germany) were added to the KGM solution, after their solubilisation in water and 0.01M hydrochloric acid, respectively. KGM/INH/RFB (10/1/1 w/w) microparticles were produced using a mini spray-dryer (Buchi Labortechnik AG, Switzerland) equipped with a high-performance cyclone, at a spray flow rate of 473 L/h. Inlet temperature of 170 ºC, aspirator at 90% and a flow rate of 0.8 mL/min were set for the atomisation. The microparticles were characterised by Morphologi 4® (Malvern Instruments Limited, Malvern, UK) and scanning electron microscopy (FESEM Ultra Plus, Zeiss, Germany). The association efficiency for INH and RFB was quantified by high performance liquid chromatography (HPLC, Agilent 1100 series, Concord, Germany) after microparticle solubilisation in a mixture of methanol/water (60/40 v/v). The aerodynamic properties of drug-loaded KGM microparticles were studied using a NGI (Copley Scientific, Nottingham, UK), where the cut-off diameters of the NGI stages ranged between 8.06 and 0.14 µm (flow rate of 60 L/min). The content of three gelatine capsules loaded with KGM/INH/RFB microparticles (30 mg) was discharged using an RS01 device (IFR = 0.033 kPa0.5/LPM, Plastiape, Lecco, Italy). The test duration was 4 s. The NGI stages were rinsed with a mixture of methanol/water (60/40 v/v) and the samples analysed by HPLC.

Results and Discussion: KGM/INH/RFB microparticles were successfully produced by spray-drying with a yield of 80%. According to the data obtained from Morphologi 4®, KGM particles had a geometric diameter of 2.06 ± 2.15 µm and a spherical, slightly convex, morphology. Previous works have shown that macrophages prefer small and spherical particles for phagocytosis [4], which suggests the suitability of KGM/INH/RFB microparticles for the uptake by alveolar macrophages. Although both drugs were effectively associated with KGM microparticles, the association efficiency of INH (85%) was significantly higher than that of RFB (73%, $p < 0.05$). The characterisation of the aerodynamic properties indicated that more than 90% of the particles were emitted from the device, and around 60% of these constituted the fine particle fraction which would be expected to reach the deeper zone of the lung. Moreover, the mass median aerodynamic diameter and the geometric standard deviation of particles were around 3 µm. The deposition of INH and RFB in the NGI stages reflected the ratio of drugs in the formulation, which supports our approach for co-delivery of drugs using a novel macrophage-targeted combinatory therapy for pulmonary TB.

Conclusion: The suitable size, morphology and aerodynamic characteristics revealed by KGM/INH/RFB microparticles, indicate that this inhalable approach is a promising new option towards the treatment of pulmonary TB.

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Antibiotic-loaded Liposomes with High Potential Towards *S. aureus* Infections

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Introduction: *Staphylococcus aureus* is the most infectious nosocomial pathogen in many hospitals worldwide, being responsible for a wide range of clinical infections, namely bacteremia, infective endocarditis, as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections. In addition, the ability of this bacterium to form biofilms associated to emergence of multi-drug resistant strains, turns the infection eradication a huge challenge. *S. aureus* biofilms provides protection to bacteria tolerating higher concentrations of antibiotics (ABs) than their planktonic counterparts [1]. A promising solution, liposomes, constitute an innovative and alternative strategy to improve the therapy of this pathology, enabling a preferential accumulation at the infected sites, higher interaction with *S. aureus* biofilms and subsequent release of incorporated ABs. In the present work, three ABs were selected: rifabutin (RFB), levofloxacin (LEV) and vancomycin (VCM).

Materials and Methods: As a first step, susceptibility assays of methicillin susceptible *S. aureus* strain ATCC®25923 (MSSA) to ABs in free and liposomal forms against planktonic and biofilm organized bacteria were performed. Minimal inhibitory concentrations (MIC_{95}) were determined by broth microdilution method followed by turbidity evaluation, MTS and MTT reduction assays. Minimal biofilm inhibitory concentrations ($MBIC_{50}$) were assessed by broth microdilution method followed by MTT reduction assay and confirmed by colony-forming unit (CFU) counts. Biofilm biomass was also monitored by CV staining assay. All ABs were incorporated in nanoliposomes using the dehydration-rehydration method, followed by an extrusion step to reduce and homogenize liposomes mean size [2]. Negative and positively charged lipid compositions were tested aiming to maximize ABs loadings. Moreover, the presence of the fusogenic phospholipid, dioleoyl phosphatidyl ethanolamine (DOPE) was also evaluated. These nanoformulations were characterized in terms of incorporation parameters, mean size and superficial charge. ABs and lipid contents were quantified spectrophotometrically. Liposome-biofilm interactions were evaluated by spectrofluorimetry and confocal microscopy using rhodamine as a lipid fluorescent biomarker [3].

Results and Discussion: Among the ABs under study, RFB displayed the highest antibacterial effect both in planktonic and biofilm forms: 0.02 and < 0.3 µg/mL, respectively. For LEV and VCM the obtained values were 0.18 and 1.54 µg/mL (MIC_{95}), and 0.03 and 46.17 µg/mL ($MBIC_{50}$), respectively. The ABs were incorporated in lipid-based systems aiming to evaluate the advantages of liposomes on biofilm interaction. Higher Incorporation Efficiencies (I.E.) were obtained for VCM and RFB, ranging from 32 to 88% and AB loading between 23 and 47 µg/µmol of lipid, respectively. Fusogenic and negatively charged RFB liposomes presented the lowest $MBIC_{50}$ values (<0.3 µg/mL), equal to the result obtained for RFB in free form (<0.3 µg/mL), while for the positive charged RFB liposomes a higher value was achieved (1.58 µg/mL). The CFU counts and the biofilm biomass determinations corroborated the bacteria susceptibility results obtained. The internalization of RFB nanoliposomes, within *S. aureus* biofilm assessed by spectrofluorimetry proved that lipid-based systems were able to interact with bacteria being this effect lipid composition dependent. These results were also confirmed by confocal microscopy. *In vivo* studies, in a systemic *S. aureus* murine model of infection are now on course to validate these results.

Conclusions: Overall, RFB-loaded liposomes constitute a highly promising approach to improve treatment of *S. aureus* infections.

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Characterization of a novel 3D-polymeric scaffold as a co-delivery system

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Introduction

Osteomyelitis is an inflammation of bone caused by infection, leading to necrosis and tissue destruction. It can cause persistent morbidity and become a chronic disease, being one of the infectious diseases most difficult to manage. [1]

Staphylococcus aureus is the most usual causative pathogen in osteomyelitis, and bacterial infections are often complicated by concomitant fungal infections, *Candida* sp. being the most common. [2,3]

Co-encapsulation of drugs provides a convenient mean for administration of multiple drugs directed at commonly associated diseases. Three-dimensional scaffolds have become a crucial element of bone tissue engineering and regenerative medicine, being designed to provide an ideal environment for bone formation. [4]

Thus, this work aims to develop a new local drug-delivery system for the modulation of polimicrobial activity in bone infections, through the co-delivery of minocycline and voriconazole to the local site of infection, while fostering bone repair.

Materials and Methods

PDLLA porous scaffolds were produced by solvent casting technique and functionalized with bioglass and collagen. The produced scaffolds were loaded with 0.1 mg/mL of minocycline (MH) (antibiotic with positive effect on bone tissue healing and on bone formation) and 0.1 mg/mL of voriconazole (VOR) (antifungal triazole agent commonly used on fungal osteomyelitis) in the same system for drug co-delivery.

Drug-loaded scaffolds were characterized by Scanning Electron Microscopy (SEM) and Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). Drug release studies in Hepes 10 mM solution at pH 7.4 (37ºC, 7 days) were also performed. MH was quantified by UV-spectrophotomety (λ = 350 nm) and VOR by reverse-phase HPLC (λ = 260 nm; flow = 1 mL/min; mobile phase = acetonitrile/water).

Results and Discussion

SEM analysis revealed a porous structure of all drug-loaded PDLLA scaffolds that is suitable for drug release from the polymeric matrix. FTIR-ATR studies showed some spectral modifications in the drug loaded scaffolds spectra suggesting slight interactions between the polymer and the drugs.

The obtained results for minocycline release suggest a two-phase stage release profile, with an initial burst release of approximately 45% of MH in the first 15 minutes, followed by a sustained release.

Regarding voriconazole release profile, the obtained results also suggest a two-phase stage release profile, with an initial burst release of approximately 30% of VOR in the first 15 minutes, followed by a sustained release.

The 7 days release assay showed that the co-delivery platform maintains its integrity over time.

Conclusions

In conclusion, due to the obtained profile of high-dose burst release, the developed PDLLA scaffolds seem a promising strategy for drug co-delivery for the treatment of acute bone infections. *In vitro* cell studies and antimicrobial assays are ongoing for further characterization of this promising co-delivery platform.

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Delivery of nucleic acid mimics to target *Helicobacter pylori* infections – the effect of biological barriers

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Introduction: Antimicrobial resistance is predicted to cause 10 million deaths in 2050 [1], if no solution is found. Nucleic acid mimics (NAMs) able to hybridize to and thus inhibit the expression of selected bacterial genes, have the potential to become such solution. Bacterial infections are usually associated with mucus, as mucus presents the body's first protective barrier against the external environment. Therefore, the NAMs need to be able to cross the mucus to reach the target bacteria. In addition, in order to access their target gene in the bacterial cytosol, the NAMs must overcome the bacterial envelope that poses a stringent barrier to the internalization of nucleic acids [2]. This work focuses on these two major barriers for NAMs therapy, the mucus and the bacterial envelope, directed at the gastric *Helicobacter pylori* (*H. pylori*) infection. As model NAMs, locked nucleic acids and 2'-OMethylRNA, either containing phosdiester internucleotide linkages (PO) or phosphorothioate linakges (PS), were used to hybridize to *H. pylori* rRNA.

Materials and Methods: The diffusion of NAMs through native mucus collected from the stomach of pigs was first studied, using fluorescence recovery after photobleaching (FRAP) [3]. The ability of the NAMs to hybridize in *H. pylori*, in the presence of gastric mucus was also evaluated, using Fluorescence *in situ* hybridization (FISH) [3]. The NAMs were then complexed to DOTAP-DOPE liposomes (Lp) and post-PEGylated with DSPE PEG (DSPE Lp). The lipoplexes diffusion in gastric mucus was characterized by single particle tracking (SPT) and their ability to deliver the NAMs in *H. pylori*, in the presence of mucus, was finally determined via FISH [4].

Results and Discussion: We found that the NAMs diffuse fast through gastric mucus (Figure 1a) [3]. However, binding interactions with mucus hampered their efficient hybridization in *H. pylori* (Figure 1b) [3]. In order to protect the NAMs from mucus interactions and to overcome the challenging bacterial envelope, the NAMs were formulated into fusogenic liposomes (Lp) and post-PEGylated (DSPE Lp). The DSPE Lp promoted intracellular delivery of the NAMs in *H. pylori* [4]. In the presence of mucus, DSPE Lp showed improved mobility compared to cationic Lp (Figure 1c), and led to enhanced hybridization of the NAMs in *H. pylori* (Figure 1d) [4].

Conclusions: NAMs hold promise to be used as novel antibacterial agents, if they are protected from interactions with mucus and delivered in bacterial cells. We have shown that DSPE Lp meet these requirements for *H. pylori* infection.

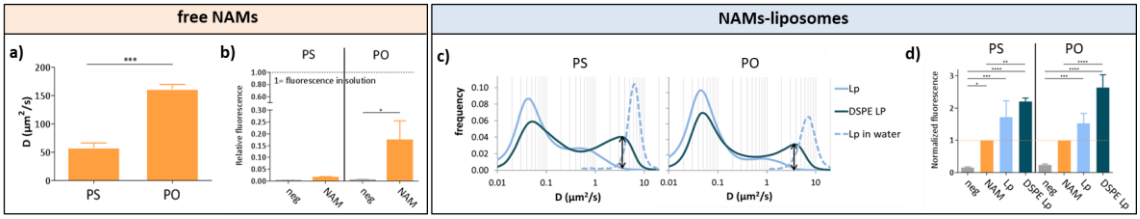

Figure 1. a) Diffusion of free NAMs in mucus, by FRAP; b) Hybridization of free NAMs in *H. pylori* within mucus, using FISH; c) Distribution of diffusion coefficients of DSPE Lp in mucus, compared to cationic Lp in mucus and in water, obtained by SPT; d) Hybridization of DSPE Lp in *H. pylori* within mucus, compared to Lp and free NAMs, via FISH. The NAMs were fluorescently labelled.

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Development of Antimicrobial Chitosan Nanoparticles loaded with Rhamnolipids Biosurfactants

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Introduction: Current failure and abuse of antibiotic therapy is increasing problems and finding new strategies for infection prevention is mandatory, specially against bacterial biofilms. Biosurfactants as rhamnolipids (RLs), glycolipid surfactants produced by Gram-negative bacteria such as *Pseudomonas aeruginosa* emerge as one possible solution due to environmentally friendly properties and the lack of resistance development [1]. Using nanoparticles as delivery systems for these compounds may be a promising strategy in the context of antibiofilm infections management. As such, the aim of this study was to develop RLs loaded chitosan nanoparticles (RLs-NPs) and then test their antimicrobial activity towards *Staphylococcus aureus*, a common pathogen associated to biofilm-related medical infections. Moreover, their biocompatibility profile was also evaluated (Figure 1).

Materials and Methods: Blank nanoparticles (b-NPs) and RLs-NPs were prepared by ionic gelation using sodium tripolyphosphate as crosslinking agent. Size distribution, zeta potential and encapsulation efficiency of the produced nanoparticles were assessed. The antimicrobial properties (minimal inhibitory concentration and biofilm inhibition) were evaluated using *S. aureus* (ATCC 25923). The cytocompatibility of the nanoparticles was assayed *in vitro* by the tetrazolium dye assay (MTT test) with a mouse fibroblastic cell line (L929, ECACC General Collection Catalogue No.: 85011425), in a concentration range of 1 – 500 µg/mL.

Results and Discussion: RLs-NPs presented an encapsulation efficiency of 74.2 ± 1.3%, a zeta potential of 37 ± 1 mV, a size ranging from 300 to 400 nm. The minimum inhibitory concentration of RLs-NPs that was able to inhibit the growth of *S. aureus* was 0.13 mg/mL. A biofilm inhibition of 50% was achieved with RLs-NPs meaning that their antimicrobial activity is also effective towards sessile bacteria. As comparing to control, cell cultures grown in the presence of b-NPs presented no significant differences regarding the MTT reduction values, at the assayed time points (up to 11 days), suggesting that the addition of NPs up to 500 µg/mL did not significantly interfere with the cells' viability and proliferation.

Conclusion: the results of this study demonstrated that the rhamnolipids incorporated in chitosan nanoparticles inhibited bacterial growth showing adequade cytocompatibility and might become after additional studies a possible approach to fight *S. aureus* biofilm associated infections.

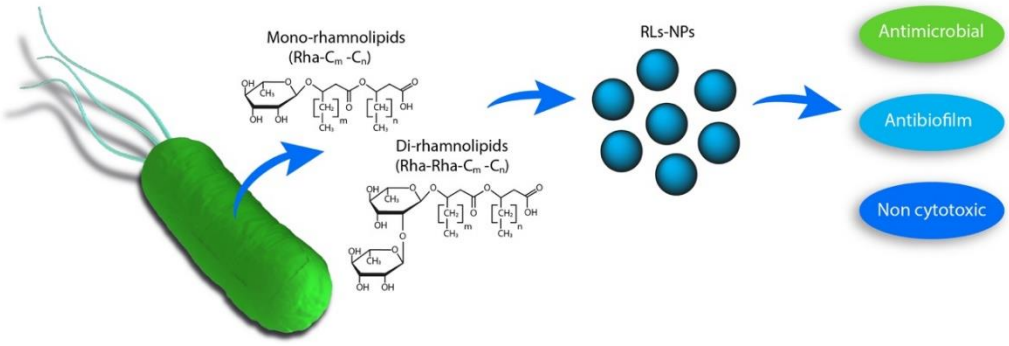

Figure 1. The obtention of RLs-NPs and their bioactivity

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Dually crosslinked chitosan nanoparticles block quorum sensing and affect cell growth on a controlled, cell-density dependent manner

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Introduction. Quorum sensing (QS) is a cell-to-cell signaling mechanism mediated by exocellular chemical compounds which act as autoinducers. Gram-negative bacteria mainly use acyl-homoserine lactones (AHLs) as autoinducers[1]. Since QS is deeply involved in the development of biofilms and pathogenicity in many Gram-negative bacteria, the search for anti-QS strategies, or quorum quenching (QQ), is a growing field of interest[2]. Chitosan (CS) has been shown to interfere with biofilm formation and QS[3,4]. However, no studies on the QQ effect of CS-based nanomaterials were reported until very recently[5]. In this study, we investigate ultrastructural features determining the QQ and antibacterial activities of CS nanoparticles.

Materials and Methods. We have investigated two mechanisms to fabricate chitosan nanoparticles dually crosslinked with genipin (GNP) and sodium tripolyphosphate (TPP). Procedure (A) involves the fabrication of *i*onically *c*rosslinked parental *NPs* (IC-NPs) via ionic gelation[6], followed by covalent crosslinking with GNP[7]. Procedure (B) involves covalent pre-crosslinking of CS with GNP, followed by the formation of CS NPs by ionic gelation with TPP. The resulting nanoparticles were physichochemically characterized by non-invasive back scattering (DLS-NIBS; size and polydispersity) and by phase-analysis light scattering (M3-PALS; zeta potential). The kinetics of CS crosslinking with GNP was characterized by UV/VIS spectroscopy, small deformation oscillatory rheology, and DLS-NIBS. The presence of a GNP-induced core-shell structure in CC-NPs was studied by synchrotron SAXS[7]. The antibacterial and QQ activities of nanoparticles were tested with a *E. coli* QS biosensor that can respond to AHL by expressing GFP under LuxR-AHL regulation[8].

Results and Discussion. Covalent co-crosslinking of parental IC-NPs yielded monodisperse CC-NPs in the size range of ~200 nm, with enhanced colloidal stability, likely due to the presence of a stable core-shell structure. CC-NPs had lower toxicity and lower QQ activity than IC-NPs. To improve the physicochemical properties of IC-NPs while maintaining their QQ activity, we explored a new method for the fabrication of GNP-crosslinked NPs (procedure (B)). By using a straightforward procedure, we obtained a new kind of NPs that we named PC-NPs, that displayed improved physico-chemical properties (diameter ~130 nm, ξ potential +30 Mv) and higher stability in biological medium relative to IC-NPs and CC-NPs. Importantly, PC-NPs strongly reduced the QS response of the *E. coli* biosensor. The mode of action of all our NPs is consistent with the existence of a "stoichiometric ratio" of NP/bacterium, at which the negative potential of the bacterial envelope is counteracted by the positive charge of the NPs. Strikingly, the cell density at which the "stoichiometric ratio" is established displays clear dose dependence relative to PC-NP concentration.

Conclusions. The ultrastructure of CS nanoparticles strongly defines their colloidal and biological properties and determines their application spectrum. CC-NPs could be used to develop smart drug delivery nanocarriers under conditions in which QQ is not desired. The low stability of IC-NPs limits, in principle, our ability to exploit their QQ activity. This problem has been solved with PC-NPs, which combine the superior physico-chemical features of CC-NPs with the QQ activity of IC-NPs. Thus, PC-NPs constitute promising candidates for a new generation of materials with QQ activity suitable for novel antimicrobial strategies. PC-NPs could be ideal for applications in which the targeting of bacterial populations at specific cell densities are desired. For example, PC-NPs could be engineered for the precise release of bioactive molecules at a certain stage of bacterial growth.

Acknowledgments: This work was supported by Xunta de Galicia grants (Competitive Reference Groups, ED43LC2017/09-FEDER, 2014/043-FEDER), EU FP7 IIF Marie Curie (BioNanoSmart_DDS, Contract No. 221111), Spanish Government grants (Competitive Reference Groups; REF. 2010/18; Strategic Health Action, FIS PSI14/00059), ESRF grant (CH-3386). CVS's fellowships: pre-doctoral (Xunta de Galicia), FPU (Spanish Government), DAAD (Germany), Fundación Pedro Barrié de la Maza (Spain).

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Econazole and Voriconazole controlled-release ophthalmic hydrogels for fungal keratitis treatment

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Introduction: Fungal keratitis (FK) is a severe disease of difficult treatment that can lead to loss of vision. FK can be caused by filamentous fungi such *Fusarium* and *Aspergillus* spp. or yeasts such *Candida albicans*. Its treatment is complex because it is necessary to use the drugs for a prolonged period of time and frequent instillations. Polyene drugs such as natamycin and amphotericin B are the most common treatment, but azole drugs are also a good alternative. Currently, voriconazole is the most popular drug because of its broad activity spectrum (yeasts and filamentous fungi) [1]. Econazole has shown a similar efficacy than natamycin [2] so it could be used as an alternative to topical natamycin in countries where it is not available or in natamycin refractory infections. The aim of the present work is to compare the *in vitro* release and opthalmic biopermanence of different formulations containing econazole and voriconazole by Franz's cells and Positron Emission Tomography/ Computericed Tomography (PET/CT) images.

Materials and Methods: Ophthalmic hydrogels were prepared by adding different polymers (sodium hyaluronate for mucoadhesives hydrogels (HAH); gellam gum and κ-carrageenan for ion-sensitive hydrogels (ISH)) to a drug-cyclodextrin inclusion complexes aqueous solution .
In vitro release from the hydrogel systems (ISH and HAH) and the control drug solution (VCN and ECN) in contact with simulated lacrimal fluid (SLF) was estimated by using Franz diffusion cells and 0.22 μm cellulose acetate membranes (0.784 cm^2 membrane surface area). Ophtalmic biopermanence studies were carried out in male Sprague-Dawley rats (250 g). Anesthetized animals were positioned into the PET/CT imaging bed and 7.5 μL of each formulation, labelled with ^{18}F-fluorodeoxyglucose (^{18}F-FDG), were instilled into the conjunctival sac eye. After the instillation, static PET frames at different times were acquired. Two animals (4 eyes) were tested for each formulation.

Results and Discussion: The results obtained in the *in vitro* release assay have shown that both ion-sensitive and mucoadhesive hydrogels control drug release for more than 24 hours. Mucoadhesive hydrogels showed a faster release than ion-sensitive hydrogels due to the fact that the ISH reveal high viscosity values, reducing inclusion complexes diffusivity. PET/CT images confirm the hydrogels high retention time compared with the one of the drug solution used as control. Elimination constant, halflife time and mean residence time were also evaluated. All results showed both hydrogels (ISH and HAH) have an adecuate consistence in order to improve the ocular surface biopermanence.

Conclusion: All results reveal the interest on these formulations for their controlled drug release in the eye. This biopermanence improvement could lead to an increse in the effect's duration, reducing the number of instillations and enhancing the patient's adherence-to-treatment.

Acknowledgments: X.G-O acknowledges the financial support of the Health Research Institute Foundation of Santiago de Compostela (FIDIS) (Predoctoral research fellowship).

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Insights into the Intracellular Trafficking of Chitosan Nanoparticles

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Introduction: Chitosan, a naturally derived polymer, is often selected as a carrier for nanoparticles preparation due to its high degree of biocompatibility and potential therapeutic application on infections management [1]. In particularly, intracellular infections are highly challenging to treat as internalized bacteria can impair cell functionality, altering migration, cell cycle regulation and viability, being further protected from the host's immune surveillance and conventional therapeutic approaches. In this context, the aim of the present study was to evaluate if chitosan nanoparticles were able to be uptaken by a model cell and understand their intracellular trafficking.

Materials and Methods: Chitosan nanoparticles (NPs) were prepared by an ionic gelation technique [1]. FITC-labeled NPs were produced with a fluorochrome using Oregon Green 488 reactive dye. Physico-chemical characterization of the NPs included the morphology and size distribution assessed by transmission electron microscopy (TEM) as well as the zeta potential by electrophoretic light scattering. Uptake studies and intracellular trafficking in human gingival fibroblasts (ATCC® PCS-201-018™) were performed using both fluorescent imaging and TEM. Mitochondria specific tracking dye was MitoSpyTM Red CMXRos (250 nM, BioLegend) and Lysosome Staining kit was obtained from AAT Bioquest, Inc.

Results and Discussion: NPs showed a spherical-like shape and an average diameter of 50±17 nm. Zeta potential measurements, demonstrated that NPs presented a positive charge in water as well as in cell media (29 mV).

In cellular uptake studies, NPs were found to organize into small aggregates that interacted with the cell membrane. Moreover, NPs induced the formation of distinct membrane perturbations associated with the activation of two endocytic pathways: macropinocytosis and clathrin-mediated endocytosis. Additionally, NPs were found to induce cell autophagic activity, as suggested by the high number of multi-membranous autophagic vacuoles (Fig. 1), resulting from the fusion of autophagosomes with endosomes, further evidencing remnants of endocyted NPs. This ability to modulate autophagy could further enhance the therapeutic effectiveness of drug loaded NPs, conjoining the antibiotic delivery within the endosomal/lysosomal pathway with the increased autophagic activation, targeting intracellular pathogens.

Conclusion: Overall, it was demonstrated that chitosan-nanoparticles may serve as a carrier for the intracellular delivery of antibiotics as they were successfully internalized by the cells by macropinocytosis or clathrin-based endocytosis and may follow a similar intracellular trafficking pathway as bacterial pathogens.

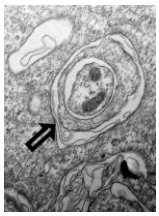

Figure 1. Intracellular trafficking of NPs in human gingival fibroblasts, addressed by TEM. Note the formation of autophagosomes (black arrow).

Acknowledgments: Support for this work was provided by FCT through Portuguese government, PTDC/BTM-SAL/29335/2017 and Pest-UID/DTP/04138/2019.

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Interaction of cationic liposomes with Gram-negative and Gram-positive bacteria

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Introduction: Bacterial infectious diseases have reemerged as a serious worldwide health problem due to antibiotic resistant bacteria. Therefore, new therapies are required to replace the antibiotics that are no longer effective [1,2]. The very selective bacterial envelopes constitute an efficient permeability barrier, limiting the potential of alternative antibacterials acting on the bacterial cytosol such as oligonucleotides [3]. A few reports have demonstrated the potential of fusogenic cationic liposomes to deliver antibacterials into the bacteria. However, slight know-how about the interaction mechanism of these vesicles with the envelope of both Gram-negative and Gram-positive bacteria was acquired in the last years [3]. Herein, we developed a comprehensive study of the interacton of DOTAP/DOPE/DSPE-PEG liposomes with Gram-negative *Escherichia coli* and Gram-positive *Staphyloccocus epidermidis* aiming to bring a new insight into the potential of fusogenic cationic liposomes as vehicles for intracellular delivery in bacteria.

Materials and Methods: Liposomes composed by DOTAP: DOPE: DSPE-PEG2000 at a molar ratio 49.5:49.5:1 were produced by ethanol dilution method and size characterized by Dynamic Light Scattering (DLS). Fluorescence-labeled liposomes were produced by adding rhodamine-PE to the phospholipid mixture. *Escherichia coli* and *Staphyloccocus epidermidis* were exposed to 1, 5 and 10 mM lipid concentration. The interaction of liposomes with bacterial envelopes was then studied via: (i) quantification of rhodamine-PE in bacteria by flow cytometry and visualization of the lipid localization by confocal laser scanning microscopy (CLSM); (ii) co-localization studies of the SynaptoRed C2 membrane staining dye and rhodmine-PE in bacteria by CLSM; (iii) measurement of bacterial zeta-potential; and (iv) structural and morphological study of bacteria by transmission electron microscopy (TEM).

Results and Discussion: The internalization of rhodamine-PE in both *Escherichia coli* and *Staphyloccocus epidermidis* was negligible at 1.0 mM of lipids increasing significantly from 5.0 mM to 10 mM lipids. As expected, SynaptoRed C2 stained the membrane of both bacteria, showing a ring-like staining (Figure 1 A). Upon incubation of the bacteria with the liposomes, the SynaptoRed C2 dye was found in the cytosol (Figure 1 B) of the bacteria also stained with rhodamine-PE (Figure 1 C). These observations suggest that these dyes were internalized due to an increase on the envelopes' permeability upon liposomes-bacteria interaction. This hypothesis was supported by the verified depolarization of both bacterial surfaces, especially after incubation with liposomes containing the highest lipids concentration (10 mM). In addition, TEM study showed compromised envelopes in both bacteria upon exposure to 10 mM liposomal lipids. In particular, *Escherichia coli* presented clumping of the cytoplasm and cell lysis. In contrast, the thick peptidoglycan wall of *Staphyloccocus epidermidis* seems to prevent the rupture of cells structure.

Conclusions: We demonstrated that cationic liposomes increase the permeability of Gram-negative and Gram-positive envelopes in a dose-dependent manner. We found that accumulation of cationic liposomes on the bacterial surface can compromise the bacterial envelopes, rendering the bacteria permeable. This phenomenon is more extent in *Escherichia coli* envelope than in *Staphyloccocus epidermidis*. The permeabilization yielded by fusogenic cationic liposomes could be used to improve the penetration of antibacterials to replace antibiotics in both Gram-negative and Gram-positive bacteria.

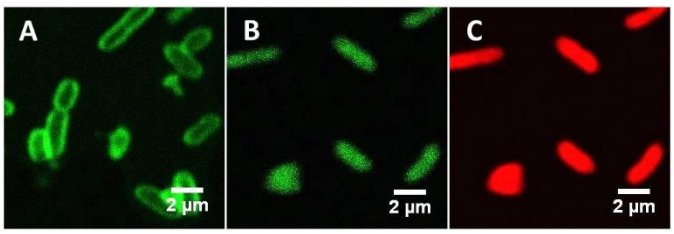

Figure 1. SynaptoRed C2 and liposomal rhodamine-PE localization in *Escherichia coli* upon liposomes-bacteria interaction. **A**- Control bacteria incubated in buffer and stained with SynaptoRed C2. **B**- SynaptoRed C2 staining in bacteria exposed 10 mM liposomal lipids. **C**- Rhodamine-PE staining in bacteria exposed 10 mM liposomal lipids.

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Tenofovir disoproxil fumarate/emtricitabine-loaded electrospun fibers for topical pre-exposure prophylaxis of vaginal HIV transmission

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Introduction: The global incidence of HIV remains at unacceptable high levels [1]. Women, particularly in the sub-Saharan region, are highly vulnerable to sexual transmission and new prevention strategies are deemed necessary. While oral pre-exposure prophylaxis (PrEP) with tenofovir disoproxil fumarate/emtricitabine (TDF/FTC; Truvada®, Gilead Sciences) is effective in men, mixed results have been observed for women, likely due to poor drug penetration of the lower female genital tract [2]. Thus, microbicide products containing TDF/FTC and administered directly in the vagina could help tackling such limitation and help avoiding early viral transmission events at the mucosal level. In this work, we developed TDF/FTC-loaded fibers and tested their pharmacokinetics (PK) as compared to oral Truvada®.

Materials and Methods: Hydrophobic and hydrophilic fibers were developed as potential vaginal delivery systems for TDF/FTC. The first were based on polycaprolactone (PCL) and the second composed by drug-loaded liposomes (DMPC:Chol:DOPE, 7:2:1) incorporated into a poly(vinyl alcohol) (PVA) matrix. Fibers were produced by electrospinning and characterized for: (i) size and morphology using scanning electron microscopy (SEM); (ii) structure and mechanical properties; (iii) drug loading and *in vitro* drug release (drugs were assayed by UV spectrophotometry); (iv) interaction with mucin by fluorescence quenching assay; and (v) cytotoxicity using the MTT metabolic activity assay. PK were further assessed in medroxyprogesterone-treated ICR mice. Drug levels in vaginal lavages, vaginal tissues and blood plasma were determined by LC-MS/MS after vaginal administration of fibers (70 μg/50 μg of TDF/FTC) and compared to the continuous treatment with daily oral Truvada® (62 mg/41 mg of TDF/FTC per kg).

Results and Discussion: PCL and liposomes/PVA fibers presented mean section diameter of roughly 700 nm and 150 nm, respectively (Figure 1). The long cylinder-shaped liposomes/PVA fibers featured discrete enlarged sections, likely corresponding to incorporated liposomes. Drug release in micellar medium at pH 4.5 was fast (within 15-30 min) and nearly complete for both types of fibers. Also, strong interaction with mucin indicates that fibers (particularly PCL ones) may feature high vaginal retention. The toxicity of drug-loaded fibers to CaSki and HEC-1-A genital cell lines was negligible. *In vivo* experiments showed that liposomes/PVA fibers were able to significantly enhance the genital concentrations of TDF, tenofovir (TFV; resulting from TDF hydrolysis) and FTC, as compared to PCL fibers and oral Truvada® (Figure 1). Relative bioavailability (F_{rel}) values of TFV and FTC were 4.0 and 29.4, respectively, in vaginal lavages as compared to Truvada® (TDF was not detected for oral treatment). PCL fibers also featured higher drug levels in lavages than oral Truvada® (F_{rel} values for TFV and FTC were 2.3 and 2.4, respectively). In all cases, drug levels were mostly undetectable at 4 h after administration. TDF, TFV and FTC levels in vaginal tissues were near the detection limit of the LC-MS/MS method for all groups, while systemic exposure was negligible for fibers.

Conclusions: Our results suggest that liposomes/PVA fibers may constitute an interesting system for the vaginal delivery of TDF/FTC in the context of topical PrEP.

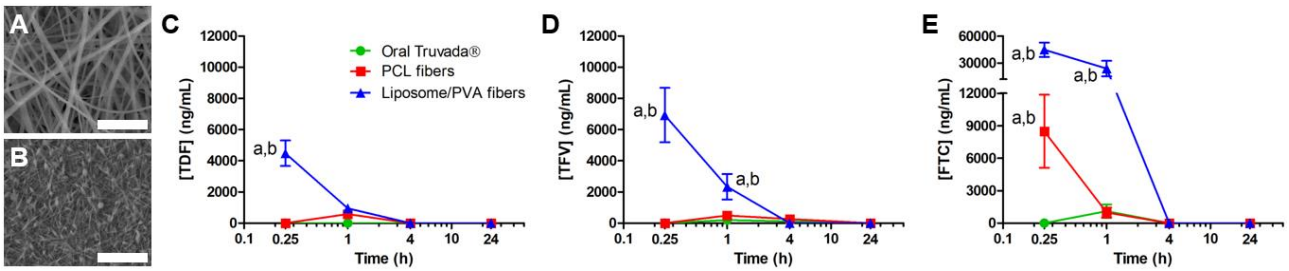

Figure 1. SEM images of (A) PCL and (B) liposomes/PVA fibers (scale bar = 10 μm). Levels of (C) TDF, (D) TFV and (E) FTC in lavages after vaginal administration of fibers or oral Truvada®. Results are presented as mean ± standard error of the mean (n = 5). (^{a}) and (^{b}) denote $p < 0.05$ when comparing fibers with Truvada® or between fibers, respectively (one-way ANOVA + Tukey's test).

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Microencapsulated isoniazid-loaded MIL-100-(Fe) nanoparticles in mannitol microspheres for pulmonary treatment of Tuberculosis

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Introduction

Tuberculosis (TB) is the principal motive of death caused by infectious disease in the World, affecting 1.8 million people every year. The habitual use, by oral route, of standard antiTB drugs has originated severe and widespread resistances. Metal organic frameworks (MOFs) are porous crystalline hybrid materials, constituted by inorganic units (cations, clusters) and polydentate organic ligands (carboxylates, azolates...) [1], which can act as drug reservoirs, shortening side effects and reducing the duration of the treatment. Especially, the mesoporous iron (III) trimesate, named MIL-100-(Fe) (MIL = Material of Institut Lavoisier) [2], has shown an absence of *in vivo* toxicity, besides exceptional loadings and controlled releases of diverse drugs [3]. In this work, we propose a new platform for pulmonary treatment of TB consisting of MIL-100-(Fe) nanoparticles (NPs) loaded with the first line antiTB drug isoniazid (H), microencapsulated in mannitol microspheres (Ma MS).

Materials and Methods

D-Mannitol, trimesic acid, $FeCl_3.6H_2O$ and isoniazid were acquired from Sigma-Aldrich.

MIL-100-(Fe) were synthetized by microwave-assisted hydrothermal synthesis [2]. Isoniazid was encapsulated in MIL-100-(Fe) NPs [H-MIL-100-(Fe)] by impregnation in water. MIL-100-(Fe) and H-MIL-100-(Fe) were dispersed in aqueous solutions of Ma and then, were spray-dried using a Buchi® Spray Dryer (Mini Spray Dryer B-290, Buchi®, Switzerland) [4] at the following conditions: T_{Inlet}: 160ºC, Aspirator: 70% and Nozzle cleaner: 5, getting dry powders. The obtained Ma MS were incubated in MilliQ water and phosphate buffered solution (PBS, pH=7.4) under bidimensional stirring at 37ºC. At different times (0, 1, 2, 4, 8, 24, 48 and 72 h), the released NPs were characterized by size and ζ-potential. To test if the MIL-100-(Fe) reaches deep lung, an *in vivo* study was carried out administering Ma MS (control MS) and Ma MS containing MIL-100-(Fe) [Ma-MIL-100 MS] to Wistar Kyoto female rats by intratracheal route. After 30 minutes, the rats were sacrified and their lungs were rinsed and fixed to do histological studies. The histological samples of lungs were examined by light field optical microscopy.

Results and Discussion

H was successfully encapsulated in MIL-100-(Fe), reaching an encapsulation rate of 28.9 ± 2.8 wt%. The dispersed MIL-100-(Fe) and H-MIL-100-(Fe) in water exhibited nanometric sizes (~130 nm) and negative surfaces (~ -25 mV). Ma MS are spherical particles of ~3 µm, suitable for deep pulmonary delivery. The NPs were released from these microspheres in aqueous media, maintaining their particle size and crystallinity in MilliQ water, while they were progressively degraded in PBS. A faster H release was detected under PBS, in agreement with a rapid NP degradation. In contrast, MIL-100-(Fe) NPs showed a high stability in water with only about 25% of the drug cargo released after 3 days. Finally, upon their pulmonary administration Ma-MIL-100 MS were uniformly distributed along all the lung (founding ~4% of the formulation), depositing the NPs mainly on the bronchioles surface and into the alveoli.

Conclusions

H-MIL-100-(Fe) NPs were successfully formulated in Ma MS, being effectively released from the MS in aqueous media. The MS presented adequate aerodynamic properties, so that the MIL-100-(Fe) NPs were released in the deep lung. The proposed pulmonary formulation paves the way to treat not only lung but also systemic diseases using MOFs.

Acknowledgments: This work was supported by the funding of the ISCIII (Projects FIS PS09/00816), Xunta de Galicia (Competitive Reference Groups, ED43LC2017/09-FEDER) and Ramon y Cajal funding (2014-15823).

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Microencapsulation of chitosan-based nanocapsules to gene therapy for pulmonary route

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Introduction

Chitosan (CS) nanocapsules (NCs) (CS NCs) are nanostructures made by a cover of CS, a lipidic interface of lecithin and a core of Miglyol® 812, that show interest for the administration of bioactive molecules by mucosal routes [1]. We propose their encapsulation into mannitol (Ma) microspheres (MS) (Ma MS) to obtain a vehicle with adequate morphological and aerodynamic properties for lung gene delivery. Aimed to improve their transfection efficiency, the CS NCs were coated with hyaluronic acid (HA) obtaining HA/CS NCs. In this work, microencapsulated CS NCs and HA/CS NCs loaded with the plasmid pCMV-βGal [2] (pCMV-βGal-CS NCs and pCMV-βGal-HA/CS NCs, respectively) were evaluated in rats to obtain evidence of lung transfection in vivo.

Materials and Methods

Ultrapure chitosan, hydrochloride salt (Protasan® UP CL 113); soybean lecithin (Epikuron® 145 V); Miglyol® (812 N); hyaluronic acid; pCMV-βGal; D-Mannitol; HPLC grade acetone and ethanol; MilliQ water. CS NCs were prepared by a solvent displacement procedure [3] which was adapted to incorporate HA and the plasmid. The morphology was determined by Transmission Electron Microscopy [TEM, Jem-2010 Electron Microscope, at 120 KV]. The size, polydispersity index and ζ-potential were determined using a Zetasizer Nano-ZS [Malvern Instruments, Malvern, UK]. NCs suspensions in mannitol solutions were atomized using a Buchi spray-dryer [Büchi® Mini Spray Dryer B-290, Switzerland]. The morphological characterization of the MS was performed by Scanning Electron Microscopy [SEM, Fesem Ultra-Plus, Zeiss]. Release studies of NCs from Ma MS was performed by incubating the powders in aqueous media (MilliQ water and PBS-Curosurf®), under magnetic stirring [Cimarec i Multipoint, Fisher Scientific, Spain]. The size and ζ-potential of released NCs were examinated at different times (0.5, 1, 2 and 4 hours), as were mentioned previously. The powders were administered intratracheally to rats using a Harvard inhaler [Harvard Apparatus]. The localization of the nanosystems in the lung was examited by confocal laser scanning microscopy (CLSM) [Leica TCS SP5 X, Germany]. The genetic expression was checked 72 hours after the administration of the powders by the histochemistry of 5-bromo-4-chloro-3-Indolyl-β-D-galactopyranoside (X-gal) [4].

Results and Discussion

Particle sizes of CS NCs (146 ± 0 nm) and HA/CS NCs (154 ± 1 nm) slightly increased when the plasmid was associated to their surfaces (151 ± 1 nm and 162 ± 1 nm, respectively). The NCs charge decreased when the HA was incorporated onto their surface (from +55 ± 0 mV to +34 ± 0 mV for CS NCs and HA/CS NCs, respectively). The associated plasmid slightly decreased the ζ-potential (+54 ± 0 mV and +29 ± 0 mV, respectively). NCs were microencapsulated in Ma MS of optimal size (2-3 μm) and morphology (spherical) for pulmonary administration. NCs released from MS in aqueous media, showed a size slightly higher than that of freshly prepared NCs; whereas their ζ-potentials did not change when MilliQ water was used as release medium, but decrease until negative values was observed when the medium was PBS-Curosurf®. The *"in vivo"* studies showed the capacity of the microencapsulated NCs to reach the alveolar region and to induce genetic expression, as shown by the presence of blue deposits in the lung tissue due to expression of the plasmid encoded beta-D-galactosidase.

Conclusions

Microencapsulated chitosan-based NCs showed the capacity of transport adequately plasmid to the lung, produce transfection and activity in the tissue. These micro-nanovehicles are promising for pulmonary gene delivery.

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Optical detection based on gold nanocomposites for the real-time monitoring of pathogenic bacteria infection progression

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The increase of antibiotic resistant bacteria is a rising concern worldwide. The extensive and routine use of first-line antimicrobials in medicine, agriculture, and farming over the last decades has led a major threat for the clinical practice. Resistance to first-line drugs to treat infections caused by *Staphylococcus aureus*, a common cause of severe infections in health facilities and the community, is widespread. The World Health Organization (WHO) has recognized antibiotic resistance as one of the major threats to global health and mankind. With the new generation of antibiotic becoming virtually ineffective, it is predicted that that multi-resistant bacteria will cause more deceases than cancer by the middle of the century, causing 10 million deaths worldwide by 2050 [1, 2]. Nanoparticles and antibiotics have been linked for a long time in order to achieve more effective drugs and in order to prevent the development of resistances. Targeted and triggered antimicrobial drug delivery can be achieved by using antibiotic-loaded nanoparticles funcionalized with specific targeting or triggering moieties [3]. In this regard, the reduction in the elapsed time between the diagnosis and the treatment and a longitudinal approach to detect and treat bacterial infection would be a huge advance in the prevention of antibiotic resistant bacteria. By analyzing the bacterial growth kinetics in real time using an optical imaging technology, and, at the same time, by treating the infection with nanoparticulated drug delivery systems would allow to adjust the use of a required amount of antibiotic only when needed.

In this project, we developed a bacteria detection system based on diferent gold nanocomposites (AuNCs). We propose that the state of aggregation of the AuNCs can serve as a signal output which can be monitorized in order to study the proliferation of bacteria. We hypothesized that gold nanoparticles functionalized with a positive charge cationic polyelectrolyte (poly-allylamine hydrochloride) could aggregate and alter their electromagnetic spectrum when coating the negative-charged bacterial wall.

Novel AuNCs were synthesized and tested in different media (i.e. PBS, TSB) against different concentrations (10^3-10^7 colony forming units (CFU)/ml) of Gram positive and Gram negative bacteria: *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (USA300) and *Pseudomonas aeruginosa*. Under certain conditions, we observed that the absorbance spectrum of the AuNCs was altered over time due to their interaction with bacteria. By comparing the absorbance at 520 nm and 600 nm we observed significant statistical differences between samples under the presence or absence of bacteria, managing to achieve a limit of detection around 10^6 CFU/ml. In addition, AuNCs covering bacteria were identified by scanning electron microscopy (SEM) and electron backscatter diffraction (EBSD) showing a successful supramolecular interaction (Figure 1).

Despite being a proof of concept, the idea behind this system could serve as a promising and simple method for the detection and identification of pathogens in clinical samples.

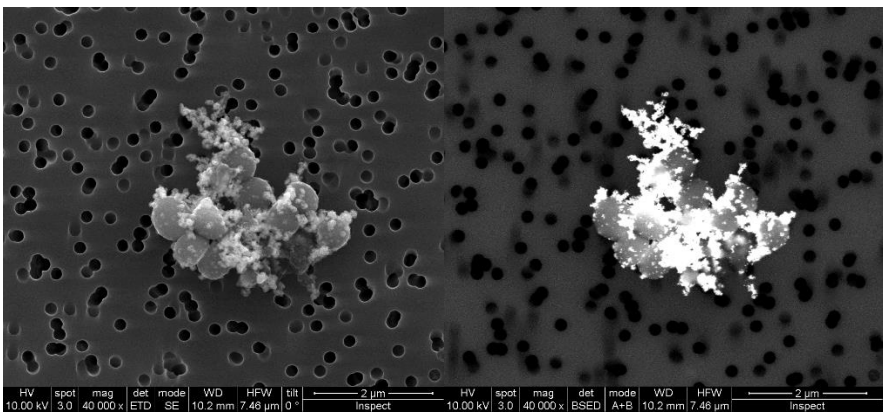

Figure 1. Staphylococus aureus coated with AuNPs (left image: SEM; right image: EBSD)

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Optimization and Characterization of Posaconazole Loaded Nanomicelles for Ocular Delivery

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1. Introduction

Ocular delivery of antifungal drugs has become more important due to increase in the incidence of fungal ocular diseases Posaconazole (PSC) is a highly lipophilic triazole group of antifungal drug [1]. Some case reports underlined that diluted commercial oral suspension of PSC has been topically used in the tresevere keratitis or sclerokeratitis [2,3]. However, the ocular delivery of PSC has not been examined yet. The aim of this study to optimize and characterise PSC loaded nanomicelles for ocular delivery.

2. Materials and Methods

2.1. Materials: PSC (Deva Pharmaceuticals, Turkey), TPGS-1000 Succinate, Pluronic F127 and F68 (BASF), PEG-PCL2000, PEG-PCL5000 and PEG-PLL2000 (synthesized in Yıldız Techinical University). Lipoid PE18:0/18:0-PEG2000(Lipoid GmbH). PTFE and RC 0.45µm filters (Sartorius).

2.2. Designing and Preparation of Nanomicelles Formulation: The nanomicelles were prepared by thin film hdyrated and O/W emulsion methods.Different types of copolymers or surfactants as either single or in combination were used to produce polymeric, surfactant and mixed micelles. The required amounts of copolymers/surfactants were calculated according to critical micelle concentration. For the optimized formulations, pH buffer and antimicrobial preservative were added to prevent ocular irritation and contamination. The effect of organic solvent type (acetonitrile, chloroform and acetone) has also examined on micelle formation and characterization.

2.3. Characterization of Nanomicelles

The mean particle size and zeta potential of nanomicelles were determined by Zetasizer Nano-ZS at 25±1°C. The morphology of nanomicelles was conducted by Atomic Force Microscopy (AFM) and PSC amount in nanomicelles (EE,%) was quantified by HPLC. The stability of PSC loaded nanomicelles in polyvinyl bottles was followed at 25±2ºC; 60±5% relative humidity (RH) and 40±2ºC-75±5%RH fro 3 months. PSC loaded micelles consisted of LipoidPE18:0/180-PEG2000 were hydrated by 5% mannitol solution and, then lyophilized.

3. Results and Discussion

3.1. Designing and Preparation of Nanomicelles Formulation

The micelle formation was not observed when the single copolymer was used, whereas single surfactant usage could form micelle due to the highly lipophilic character of the PSC. Unlike polymers, the surfactants used also increased the solubility of PSC and facilitated the micelle formation. The amount of surfactant and PSC affected the micelle formation for both surfactant and mixed micelles. Type of solvents had no effect on micelle formation.

3.2. Characterization of Nanomicelles

The particle size and zeta potential of all PSC loaded micelles were less than 20nm and in the range of -10-0mV, respectively. AFM images have revealed existence of the spherical nanomicelles in the optimized formulations. EE% of PSC in all the optimized formulations was over 85%.

The particle size particle size or zeta potential of micellar carriers was not affected by the use of different solvents, but was affected by the filter type. There were serious variations in particle size distribution when RC filter was used. All PSC loaded micelles produced with LipoidPE18:0/18:0-PEG2000 had stability problems in aqueous and lyophilized forms. PSC loaded micelles produced with TPGS were suitable for ocular administration in terms of particle size at day 180. There was no significant change in zeta potential after 180 days. When the pH values at the end of 180 days were evaluated due to the risk of ocular irritation, micelles at 25±2ºC-60±5%RH were applicable and those at 40±2ºC-75±5%RH were not suitable. In the quantitation studies, there wasn't significant difference between all versions of nanomicelles but the amount of PSC fell below 90%.

4. Conclusions

In our study, ocular drug delivery system of PSC, which hasn't been examined for ocular application so far, has been optimized. Optimized nanomicellar carriers significantly increased the solubility of PSC in water. These micelles could be considered as promising delivery system in ocular antifungal therapy, resulting in increase in ocular bioavailability of PSC.

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Optimization of soluble polypseudorotaxanes based nail lacquer for onychomycosis treatment

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Introduction: conventional topical administration of medicated nail lacquers has failed improving the efficacy of antifungal drugs in the local treatment of onychomycosis [1,2]. The dense and highly keratinized hydrophilic nail plaque structure and the formation of dehydrated film coating in nail surface difficult the drug penetration from solvent-based nail lacquer to the nail plaque. In last years, scientists have made an effort to develop new trans ungual delivery systems with better efficacy [1,2]. The use of hydroalcoholic dispersions of cyclodextrin/poloxamersoluble polypseudorotaxanes combined with penetration enhancers has demonstrated to have a great potential to promote the drug penetration in nail plaque [3-5]. The objective of this investigation is to optimize the composition of the nail lacquer based oncyclodextrin/poloxamer-soluble polypseudorotaxanes containing an 8% of ciclopirox base for the topical treatment of onychomycosis.

Materials and Methods

Nail lacquer was prepared by dispersing hydropypropyl-β-cyclodextrin, poloxamer 407, sodium dodecyl sulfate and ciclopirox base in different ethanol/water/ethyl acetate mixtures. Drug solubility in the base nail laque and ciclopirox permeability across bovine hoof membrane were determined using vertical Franz diffusion cells and compared with a marketed reference nail lacquer based on a hydro-alcoholic vehicle containing hydroxypropyl chitosan as film forming material.

Results and Discussion

Solubility results (Figure 1) indicate that ethanol concentrations greater than 45% in the mixture ethanol/water/ethyl acetate were necessary to achieve a concentration of cyclopyrox of 80 μg/ml, and that the presence of ethyl acetate is irrelevant for solubility. Laquers with different proportion of solvents (ethanol 49 to 59 %, water 40 to 49% and ethyl acetate 0,7 to 4%) were prepared and permeation studies across bovine hoof membrane were developed. Results shows that nail drug flux increases when a higer water proportion is used (figure 2). Finally, the selected formulation shows a higher nail drug flux when it is compared with the reference marketed lacquer.

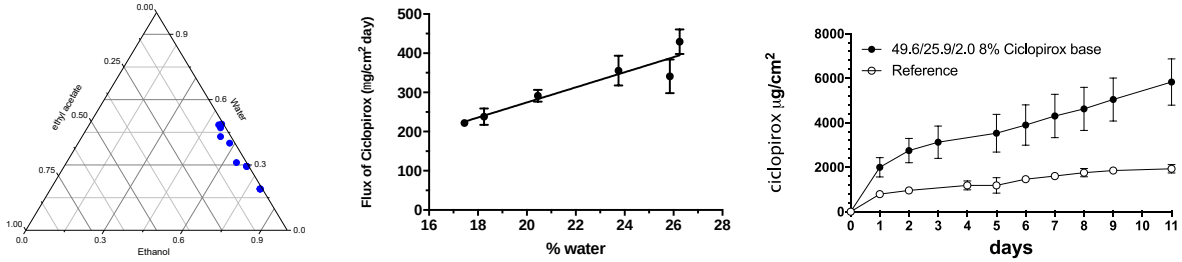

Figure 1. Left: Ternary diagram showing the area where solubilities ≥80μg/ml are achieved. Center: Relationship between the proportion of water in the lacquer and the nail drug flux. Rigth: Permeation kineticks of ciclopirox across the nail.

Conclusions

The composition of polypseudorotaxane nail lacquer has been optimized to achieve dose of 8% ciclopirox base. Results show that by properly selecting the base composition it is possible to obtain high diffusion profiles clearly superior to those obtained with the commercial reference lacquer.

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Pharmacokinetic studies and *in vitro* characterization of tacrolimus eyedrops

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Introduction and objectives: Atopic keratoconjunctivitis (AKC), or vernal keratoconjunctivitis (VKC), is an allergic disease that causes ocular surface damage produced by an inflammatory process [1]. Nowadays, there are not any commercial alternatives; thus, hospital pharmacy prepares these eye drops reformulating from parenteral drugs as magistral formulations. An improvement in the drug solubility complexing tacrolimus with Hydroxypropyl-β-cyclodextrin (HPßCD) and other copolymers was wanted to be developed in order to be able to remove the irritating compounds of the parenteral drug (Prograf®). The aim of this work was to enhance the solubility and evaluate the safety of topical tacrolimus solution, as well as study the tacrolimus formulations release and their ocular biopermanence.

Materials and Methods: Initially, tacrolimus phase solubility diagram in water with HPßCD was performed. It was studied the tacrolimus solubilization comparing the interaction between tacrolimus-HPßCD complex using water (MilliQ®), BSS® (Balanced Salt Solution, Alcon®) and Liquifilm® (Allergan) as copolymers. *Ex vivo* corneal permeability studies and Irritation Ocular Assay (HET-CAM) [2] were performed in order to evaluate the safety of the formulations over the ocular surface. *In vivo* studies were carried out on male Sprague-Dawley rats (250 g). Anesthetized animals were positioned into the PET/CT imaging bed and 7.5 µL of each formulation labelled with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) were instilled into the conjunctival sac eye. After the instillation, static PET frames at different times were acquired. Two animals (4 eyes) were tested for each formulation. *In vitro* release from the formulations and the control drug solutions (Prograf 0,03% and Tacrolimus suspension) in contact with Phosphate Buffered Saline (PBS) was estimated by using Franz diffusion cells and 0.2 µm cellulose acetate membranes (0.784 cm^2 membrane surface area).

Results and Discussion: The solubility diagram in water was A_P type and stability constant values of $K_{1:1}$=148.2 mol^{-1} and $K_{1:2}$=1.406 mol^{-1}. It can be seen a better tacrolimus solubility when it is combined the HPßCD and Liquifilm®. HET-CAM assay and *ex vivo* corneal permeability study did not show blood vessels injury after the addition of tacrolimus solution 5 minutes later contact and any considerable change in the corneal surface. The combined use of HPβCD and Liquifilm® show a tacrolimus solubility increase, it was reached an acceptable tacrolimus amount for the treatment of this disease (0.012 and 0.023%). *In vitro* release and *in vivo* biopermanence results are not concluded yet, so they are not analized.

Conclusion: Results reveal tacrolimus solubility improvement and irritation absence on the ocular surface. These changes in the formulation compounds would enhance the patient's adherence-to-treatment, reducing their eye discomfort.

Acknowledgments: X.G-O acknowledges the financial support of the Health Research Institute Foundation of Santiago de Compostela (FIDIS) (Predoctoral research fellowship).

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POSTER SESSIONS

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P01.4 Anti-inflammatory nanobiomaterial carrier for chondral repair (p.114)
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P05.4 Development of electrospun Silk Fibroin based asymmetric membranes for wound dressing applications (p.118)
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P06.4 Development of in situ forming hydrogel containing oxidized alginate and platelet rich plasma for wound healing applications (p.119)
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P07.4 Electrospun Nanostructured Wound Dressings for the Sustained Release of Natural Antimicrobial Compounds (p.120)
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P08.4 Electrospun-fibers for melatonin-controlled release (p.122)
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P12.4 Transplantation of cardiovascular progenitors with biomimetic microcarriers limit adverse remodeling in a rat myocardial infarction model (p.126)

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Anti-inflammatory nanobiomaterial carrier for chondral repair

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Introduction

This work contributes to developing an effective approach to manage the specific unmet clinical needs to treat knee chondral lesions. Knee chondral lesions gradually increase overtime resulting in the development of osteoarthritis (OA)[1]. Affected by 242 million people globally[2], OA eventually will lead to progressive total joint destruction. Over the years, many restorative techniques (e.g. autographs, allografts) have been used to enhance articular cartilage repair, however these clinical solutions fail to deliver an effective long-term treatment due to persistent inflammation. In the past decade, the fast advancement of nanotechnology has opened new possibilities to apply nano-engineered biomaterials to modulate several extracellular and intracellular biochemical and biophysical events. The work described here covers part of the European project RESTORE, where we have produced smart nanobiomaterials that can interfere with inflammation and evaluated their immune toxicity and cytotoxicity. The nanobiomaterials capacity to modulate cell behavior via chemical and physical stimuli to enhance cartilage repair and their integration into cuttting-edge 3D matrices that meet the needs of chondral lesions[4] will be further analyzed and discussed.

Materials and Methods:

Poly (lactic-co-glycolic acid) (PLGA) was used to produce PLGA nanoparticles (NPs) loaded with ibuprofen (anti-inflammatory drug) in order to respond to inflammatory threat. Empty and ibuprofen-loaded PLGA NPs were produced by the nanoprecipitation method. Both nanocarriers were characterized through nanoparticle size, PdI and zeta potential (ZP). Stability of PLGA NPs was studied over 72 hours at different pHs and in various cell media. Association efficacy and drug loading were quantified by validated RP-HPLC method. The sterility of empty PLGA NPs was evaluated considering bacterial contamination and endotoxin content. Primary human macrophages and dendritic cells were used to test NPs cytotoxicity through Resazurin and LDH assay and NPs immunotoxicity by FACS.

Results and Discussion:

Empty PLGA NPs were successfully produced by the nanoprecipitation method, obtaining nanoparticle size at around 173 ± 3 nm, PdI of 0.10 ± 0.04 and ZP of – 3.0 ± 0.4. No significant differences were obtained after ibuprofen encapsulation into PLGA NPs and this nanocarrier was considered stable over 72hours at different pHs and in various cell media. The AE of ibuprofen-loaded PLGA NPs was at aournd 75.6 ± 0.5 with a DL of 12.6 ± 0.1. The NP production was performed under sterile conditions, resulting in no bacterial contamination and endotoxin content, < 0.1 EU/mL. The cytotoxicity of empty PLGA NPs towards macrophages was tested in a range of NP concentrations (50, 100 or 200 μg/mL) and the results indicated that none of the concentrations tested were cytotoxic. On the other hand, preliminary results indicate that empty PLGA NPs do not lead to macrophage or dendritic cell activation, as levels of co-stimulatory molecular CD86 remained low.

Conclusions:

A stable nanocarrier composed of ibuprofen-loaded PLGA NPs was produced and characterized, obtaining good physico-chemical properties for further incorporation into 3D scaffolds or 3D cartilage microtissues engineered via 3D printing of nanocellulose and alginate bioink[5].

Acknowledgments: This work was financed by the project RESTORE- H2020-NMBP-TR-IND-2018-2020.

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Chitosan scaffols co-delivering BMP-2-MMP10 and BMP-2-alendronate for bone regeneration in osteoporosis

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Introduction

Osteoporosis (OP) is a disease characterized by a failed bone remodeling process. Consequently, the OP bone is characterized by high bone porosity, loss of bone mass, changes in the microstructure of the bones, high risk of fracture and delay in fracture consolidation. The situation is more complicated when a critical bone defect has to be regenerated because of the reduced self-repair capacity [1]. As previously described, combination of bone morphogenetic protein (BMP-2) and 17β-estradiol applied to a critical calvaria defect improved bone healing in OP rats. Nevertheless, this newly formed bone was less mineralized [2]. In this study we aim to improve tissue mineralization by the local and sustained released of variable BMP-2 and metallo-proteinase 10 (MMP10) combinations compared to BMP-2 combined with alendronate (ALD). The therapeutic molecules were formulated in microspheres and included in chitosan scaffolds as local systems for bone regeneration in OP. Scaffolds were characterized in terms of particle size, encapsulation efficiency (EE), porosity, drug release rate and osteogenic capacity.

Materials and Methods

Scaffolds preparation.- 4 mg of microspheres loaded with BMP-2, MMP10 or ALD were mixed with 30 µL of a 3% chitosan aqueous solution (CHT, Protasan® UP CL 213), crosslinked with 5% sodium tripolyphosphate (TPP) and freeze-dried. Microspheres were prepared by a double emulsion (w/o/w) method. Proteins loaded microspheres were prepared with a blend of RG 755 and RG 858 (9:1). The method to prepare the ALD microspheres was the same but using ALD in 1% of CHT as the internal phase. A 2.5% TPP in polyvinyl alcohol (PVA) solution was used as the external aqueous phase.

Cells culture.- To test the osteogenic differentiation, mesenchymal stem cells (MSCs) were seeded onto the scaffolds. The study was perfomermed using MSCs from two different species; cells isolated from the bone marrow of FVB mice (mMSCs) and from human bone marrow (hMSCs) (Lonza). At different culture times alkaline phosphatase (ALP) activity was checked. Then, cells were observed included in Paraplast®.

Animal model.- OP was induced in female mice by bilateral ovariectomy (OVX), four months latter a cranial critical size circular (4 mm) defect was practised as previously described [3]. Scaffolds were placed in the defect. The osteogenic response to the different treatments was evaluated by X-Ray after 6 and 12 weeks of implantation.

Results and Discussion

The mean volume diameter of BMP-2-MMP10 and ALD microspheres were 70.5 µm and 65.98 µm while their EE was 74.05 ± 20.7% and 71.9 % ± 12%, respectively. Scaffolds porosity was 94.6 ± 1.2%. Drug release profiles were characterized by a moderate burst effect for proteins and a very slow release of ALD. The in vivo response X-Ray images showed the combination of BPM-2 and MMP10 accelerated bone formation in the non-OP groups. Conversely, this improvement was not observed in the OP groups (Fig. 1). The evaluation of the treatments effect on bone mineralization is in process.

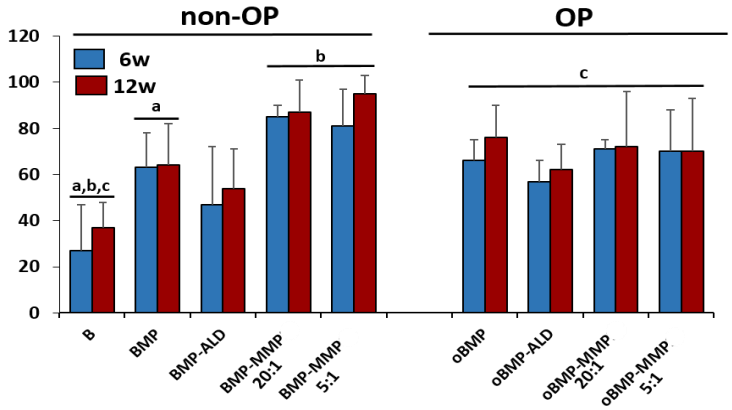

Figure 1. Bone regeneration in the different treatment groups. B (control), BMP (600 ng), BMP-ALD (75µg ALD), BMP-MMP-20:1 (30 ng MMP), BMP-MMP-5:1 (120 ng MMP). Data presented as means ± SD. $p<0.5$

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Chondroitin sulphate-functionalized PAMAM nanoparticles for application in rheumatoid arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory disease mainly characterized by joint synovial inflammation, and cartilage and bone tissues destruction. Many cytokines have been identified to be involved in the establishment and progression of the disease. Tumor necrosis (TNF)-alpha has been found to be a therapeutic target, while anti-TNF-α agents showed to induce long-term improvements in the RA symptoms and signs [1]. Despite recent advances in medical therapeutics, the available treatments are not completely safe and effective. Dendrimers presents several advantages as a drug delivery system which can allow considerable surface modification, being able to encapsulate, solubilise, stabilise, capture, retain and deliver in a specific target [2]. By its turn, the combination of dendrimers with synthetic and natural biodegradable polymers, which strongly interact with living cells, can increase the interaction between nano-systems and cells, thus enhancing its biological performance. In this work, we aim to develop an innovative therapeutic strategy that makes use of surface modified dendrimer nanoparticles to target the inflamed joint and treat RA. Poly(amidoamine) dendrimers (PAMAM) nanoparticles were functionalized with Chondroitin Sulphate (CS), lined with anti-TNF-α, to assist the receptor ligand-interaction, aiming to reduce the toxicity and provide anti-inflammatory properties. The chondroitin sulphate-functionalized PAMAM nanoparticles did not negatively affect the metabolic activity and proliferation of ATDC5 cell line. Moreover, all evaluated concentrations showed high internalization degrees, using the same cell line. Ongoing work comprises the evaluation of the inflammatory activity (e.g. ELISA) of CS/PAMAM- Anti-TNF-α.

Key words: Rheumatoid arthritis, Inflammation, PAMAM dendrimer, Chondroitin Sulphate and Anti-TNF-α

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Development of bioactive patches for the treatment of acute and chronic ulcers and wounds using 3D printing technology

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Introduction

Patches comprised of pectin, a non-toxic natural heteropolysaccharide were evaluated as occlusive hydrophilic patches to a wound bed as a mean to remove or control exudates.[1][2] To this end, manuka honey and propolis, known for their antimicrobial and wound healing properties, were incorporated to pectin and bioactive patches were fabricated by means of 3D bio-printing.

Materials and Methods

HM pectin from apple was combined with Manuka Honey to form 3D printable inks. Wound patches were printed using a 3D bioprinter (CELLINK® Inkredible, Sweden). Response Surface Methodology (RSM) was applied for the optimization of the inks (extrusion printing pressure, shrinkage minimization over drying, increased water uptake and minimization of disintegration of the dry patches upon conatct with aqueous media). Particles comprised of chitosan and cyclodextrin/propolisextract inclusion complexes bearing antimicrobial properties, were optimized and integrated to the produced patches. The bioprinted patches were assessed for their cytocompatibility, antimicrobial activity and *in vitro* wound healing properties. These studies were complemented with *ex vivo* skin adhesion measurements, relative surface hydrophobicity and opacity measurement, mechanical properties, visualization and spectroscopic techniques.

Results and Discussion

The *in vitro* wound healing studies revealed that the 3D-bioprinted patches enhanced the *in vitro* wound healing in terms of cell adhesion, migration and proliferation. Higher particle concentrations have an inhibitory effect, by obstructing cells from migrating due to intense sentimentation.

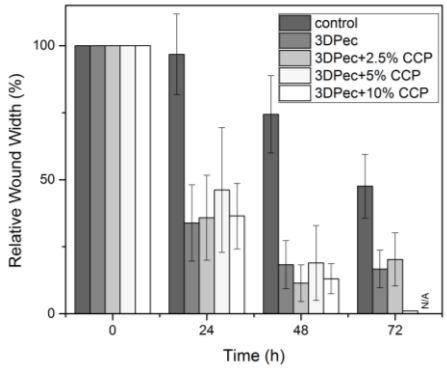

Figure 1. In vitro wound healing assay (dermal human fibroblasts). Relative Wound Width (%) vs Time (h) for 3D printed Pectin patches (3DPec) and 3D printed Pectin patches with different concentrations of Chitosan-Cyclodextrin/Propolis particles (CCP) (d=724±8 nm, PDI=0.42±0.11, ζ=+60.03±1.68 mV, Galic Acid Equivalent GAE=0.5±0.009% w/w).

Conclusions

Biodegradable 3D printable inks based on pectin, have been developed as a system for direct and indirect wound dressing applications, suitable for 3D printing technologies (e.g.3D pen and 3D printer). The 3D printable inks form water soluble free-standing transparent films upon drying whereas the antimicrobial and wound healing activity of the inks has been successfully enhanced by the addition of particles comprised of chitosan and cyclodextrin inclusion complexes with propolis extract.

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Development of electrospun Silk Fibroin based asymmetric membranes for wound dressing applications

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Nowadays, different wound dressings have been used to provide a protective barrier against external harmful agents and wound site dehydration. Among them (*e.g.* gauzes, hydrogels, sponges, membranes), the nanofibrous membranes have received an increased attention due to their high surface-to-volume ratio, interconnected pores and structural similarity to the native extracellular matrix [1]. Further, the nanofibers produced by using the electrospinning technique are the most explored since this methodology is simple, efficient, reproducible and versatile. Considering all these features, the researchers have recently produced nanofibrous asymmetric membranes, which possess a two-layered structure similar to the native skin layers (epidermis and dermis). In general, an asymmetric membrane displays (i) a dense and waterproof top layer that confers protection to the wound site in a similar way to the epidermis layer and (ii) a porous bottom layer that reproduces the dermis structure [1,2]. In this work, a new drug delivery system based on an electrospun asymmetric membrane (EAM) was produced through the electrospinning technique. The top layer was comprised of silk fibroin (SF) and poly(caprolactone) (PCL) to reproduce the properties of epidermis. SF is a fibrous protein that presents excellent biocompatibility, good water vapor permeability, biodegradability, mechanical strength, and minimal inflammatory reaction [3]. In turn, PCL is a hydrophobic synthetic polymer that exhibits a high mechanical strength [4]. On the other hand, the dermis-like bottom layer was manufactured with SF and hyaluronic acid (HA) loaded with an herbal drug (thymol (THY)). HA was selected since it provides a high capacity of hydration, water-sorption and water retention, as well as allows cell attachment, migration, and proliferation [4]. Additionally, THY was incorporated into bottom layer' nanofibers because it is an essential oil that displays antioxidant and antibacterial properties, which are crucial to enhance the wound healing process and avoid the occurrence of infections at the wound site [5]. The structural organization and mechanical properties exhibited by EAM are similar to those of the human native skin. Further, *in vitro* assays revealed that EAM promoted the cell adhesion, proliferation and spreading. Moreover, the THY release profile from bottom layer comprised a burst release in the first 8h, followed by a gradual release up to 24h, which is desired to confer an antioxidant and antibacterial effect at initial phase of the healing process. Overall, the obtained results demonstrated the appropriateness of the produced membrane for wound healing applications.

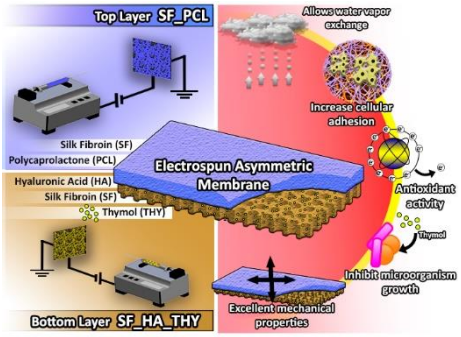

Figure 1. Illustrative representation of the electrospun asymmetric membrane production and its main properties.

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Development of *in situ* forming hydrogel containing oxidized alginate and platelet rich plasma for wound healing applications

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Introduction

The huge increase in the incidence of chronic wounds and the lack of effective treatments are making chronic wounds an important issue for health systems [1].Therefore, research in new therapeutical approaches to improve wound healing has become a major need. In this regard, the use of Platelet Rich Plasma (PRP) is a promising strategy, due to its anti-inflammatory and anti-fibrotic activity and to the presence of several cytokines and growth factors (GFs) involved in wound healing [2]. In order to improve the *in vivo* stability of PRP, it can be incorporated into drug delivery systems (DDS) that allow its controlled release, such as, alginate hydrogels. These formulations are able to absorb or donate water depending on the environment and they stimulate cell proliferation and migration due to the biomimetic nature, which can be beneficial in wound healing applications [3]. Taking that into account, an in situ hydrogel composed of PRP and oxidized alginate has been developed, to obtain a degradable hydrogel able to release cytokines and growth factors.

Materials and Methods

First, PRP was obtained centrifugating blood of healthy volunteers (approved by the Institutional Ethical Committee for Research Involving Human beings of the University of the Basque Country, procedure number M10/2019/080) . The level of several GFs in the PRP, suchs as, bFGF, EGF, IGF-1, PDGF-AA, PDGF-AB, PDGF-BB, VEGF, TGF-β, were determined using a multiplex ELISA assay.

On the other hand, alginate was oxidized with sodium peryodate, in order to modulate its hydrolysis rate. Alginate with three different oxidation degrees were obtained, 2,5 %, 5 % and 10 %. Their viscosity in different concentrations was analysed using a reometer, to dilucidate the optimal concentration to develop the hydrogels.

Hydrogels were developed mixing firstly PRP and alginate, and finally adding calcium sulphate in a proportion 1:1:0.1, respectively. The obtained hydrogels were characterized in terms of gelification time, hydrolitical degradation and PDGF-BB release.

Finally, the biocompatibility of the hydrogels was assessed directly and indirectly in fibroblasts and keratinocytes using a CCK-8 assay to determine the percentage of living cells in comparison to control.

Results and Discussion

The analysis of PRP showed different GF levels in PRP obtained from different volunteers, thus the PRP from different volunteers was mixed in order to create a pool and reduce the difference among PRPs. The GF levels obtained from the pooled PRP are summarized in Figure 1.

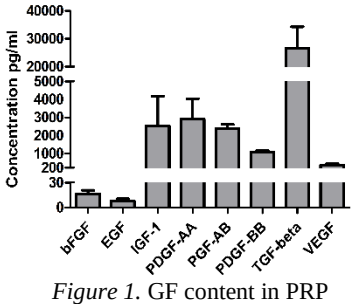

Figure 1. GF content in PRP

According to rheological studies, disolutions ranging from 0.3-0.5 Pa·s were choosen, and thus the alginates concentrations were 1,5 % for non-modified alginate, 3 % for alginate oxidized 2.5 % and 4 % for alginate oxidized 5 %. Regarding alginate oxidized 10 %, in order to obtain the desired viscosity an excessively high concentration was needed, and thus this oxidation degree was discarded.

Gelification time, was very similar in all the formulations, 6.35±0.2 min, 6.35±0.26 min and 4.18±0.25 min for hydrogels containing non-modified, 2.5 % oxidized and 5 % oxidized alginate respectively.

Regarding hydrolitical degradation of hydrogels containing non-modified alginate, 50 % of the hydrogel was degraded in the first 8 hours, and no more weightloss was observed for the rest of the study. Hydrogels containing oxidized alginate presented a higher weight loss, the remaining weight was 26.09±11.07 % and 22.93±7,31 % for hydrogels containing alginate oxidized 2.5 % and alginate oxidized 5 %, respectively.

Release studies showed a continuous PDGF-BB release for the 7 days that lasted the study. In the first hour, a release about 70 pg/ml was achieved in the three hydrogels. However, on day 7, the PDGF-BB release varied depending on the hydrogel, the obtained concentrations were 194.86±4.68 pg/ml, 258.64±20.52 pg/ml and 222.44±37 pg/ml for hydrogels containing non-modified alginate, 2.5 % oxidized alginate and 5 % oxidized alginate, respectively.

Finally, citotoxicity studies demonstrated that all the formulations were biocompatible, since cell viability was higher than 70 % in keratinocytes and fibroblasts treated with the hydrogels either directly or indirectly.

Conclusions

Overall, the developed hydrogels containing oxidized alginates presented appropriate characteristics to be used in wound healing, although their efficacy *in vitro* and *in vivo* should be assessed.

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Electrospun Nanostructured Wound Dressings for the Sustained Release of Natural Antimicrobial Compounds

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Wound healing is a complex health problem that should be adequately managed to achieve an adequate tissue regeneration avoiding or eliminating potential pathogenic infection. Drug-eluting wound dressings are used to locally release a drug to prevent or treat bacterial infections and promote wound healing. Some of the commercially available ones include different antimicrobial compounds (e.g. silver, gentamicin), though their potential contribution to the development of resistances and cytotoxicity limit their application [1, 2]. Natural compounds have emerged as an alternative to antiseptics and antibiotics due to their bactericidal properties through multiple mechanisms which are not prone to develop resistances. One of them is thymol, a natural monoterpenoid phenol present in thyme oil, widely-known as an efficient bactericidal and anti-inflammatory compound [3, 4]. On the other hand, polymeric nanofibrous scaffolds have attracted increasing attention for wound healing purposes since they can mimic the stroma of the tissue they are replacing [5]. The electrospinning technique is one of the most explored due to its simplicity, efficacy, reproducibility and versatility, producing polymeric meshes composed of ultra-fine fibers with diameters ranging from several micrometers down to few nanometers. The high surface area and nanoporosity make these structures particularly interesting for wound healing applications due to the promotion of homeostasis, fluid absorption and the ability of loading different bactericidal compounds in its structure [6]. Therefore, the use of natural compounds to avoid the emergence of resistances combined with novel wound dressing materials has arisen as a potential tool to successfully fulfil the complex requirements for wound healing.

In this work, asymmetric electrospun polycaprolactone (PCL)-based nanofibers were decorated with electrosprayed poly(lactic-co-glycolic acid) (PLGA) microparticles loading the antimicrobial compound thymol to develop drug eluting bactericidal dressings. These dressings were *in vitro* tested in bacteria and cell cultures to determine their ability to eradicate bacteria infections while being cytocompatible for human cells.

The electrospun nanostructured dressings decorated with thymol loaded PLGA microparticles obtained are depicted in Figure 1. PCL nanofibers formed a mesh in which nanofibers displayed mean diameters of 484 ± 83 nm, while PLGA microparticle diameter was of 1.634 ± 0.277 μm. PLGA microparticles preferentially attached to the nanofibers on which they were sprayed and they were not present on the other side of the scaffold, pointing to their inability to percolate across the scaffold. Thymol release from the synthesized scaffolds revealed an initial burst (≈ 60%) in the first hours to be then sustained until 24h (≈ 70 %), pointing to a thymol release kinetics that follows the Lindner and Lippold exponential model. Furthermore, the fabricated dressings successfully inhibited the *in vitro* growth of the model Gram positive bacteria tested, *Staphylococcus aureus* ATCC 25923, displaying clear inhibition zones in agar plates which were not found in not loaded scaffolds. In addition, human dermal fibroblasts and keratinocytes *in vitro* cultures after treatment with the scaffolds for 24h did not display cytotoxicity (viability ≥ 80%), though highly cytotoxic effects were observed when using free thymol (viability ≤ 10%), showing the importance of drug delivery systems for the controlled and/or sustained delivery of therapeutic compounds to achieve an efficient and targeted treatment.

Our results underline the importance of drug delivery systems for the successful treatment of infected wounds, and the relevance to customize the design of dressings considering the wound, the infection, and the bactericidal compound loaded and its release kinetics.

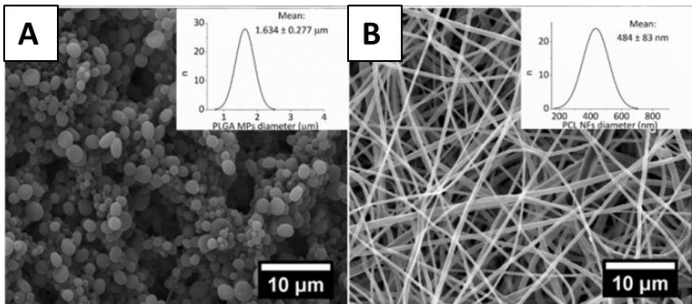

Figure 1. Electrospun nanostructured dressings decorated with PLGA microparticles. A) top-side view of electrospun dressings after 2h of microparticle electrospraying; B) bottom-side of the dressings after 2h of microparticle electrospraying.

Acknowledgments: This work was supported by the Spanish Ministry of Economy and Competitiveness (grant number CTQ2014-52384-R) and the EU (ERC Consolidator Grant program, ERC-2013-CoG-614715, NANOHEDONISM). CIBER-BBN is an initiative funded by the VI National R&D&i Plan 2008 -2011, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions and financed by the Instituto de Salud Carlos III (Spain) with assistance from the European Regional Development Fund.

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Electrospun-fibers for melatonin-controlled release

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Introduction

Regenerative medicine aims to improve or repair damaged tissues through artificially manufactured structures called scaffolds that will act as temporary bandages protecting and providing an optimal environment for the formation of new tissue. These scaffolds are intended to mimic the functions of the extracellular matrix, providing a suitable mechanical environment and promoting cell binding, proliferation, and differentiation [1]. The electrospinning technique allows the manufacture of ultrafine fibers of micro or nanometric size to mimic the extracellular matrix [2]. Many molecules, factors and cells regulate tissue repair. Melatonin is an anti-inflammatory agent known to regulate a variety of proinflammatory cytokines such as interleukin (IL) -1b, IL-6 and tumor necrosis factor-α preventing the translocation of nuclear kappa factor B to the nucleus and its binding to DNA. In addition, melatonin inhibits the production of adhesion molecules that promote leukocyte adhesion to endothelial cells, reducing oxidative stress or increasing levels of hydroxyproline and ascorbic acid, with demonstrated antifibrotic effect. In short, melatonin improves healing by significantly increasing angiogenesis and collagen synthesis [3]. The aim of this study was to manufacture melatonin loaded electrospun microfibers for dermal tissue regeneration by combining biodegradable poly-ε-caprolactone (PCL) and Soluplus, a well-known drug solubilizing agent. The combination of both polymers may help regulating drug release. Moreover, reinforcement of electrospun-fibers by means of poly(pseudo)rotaxane formation with α-cyclodextrins (α-CD) was investigated [4].

Materials and methods

The PCL and Soluplus®/ α-CD solutions were prepared by dissolving the neat polymer in hot chloroform (35–40 ºC) at different ratios. Melatonin was incorporated in the polymeric solutions at a final concentration of 2 %w/v. A climate-controlled electrospinning apparatus Yflow® Professional Electrospinning Machine (Yflow® S.D, Spain) was used for fabrication of all fibers. A series of experiments were carried out varying the electrospinning parameters to optimize the process. The applied electrical potentials ranged between 10 and 20 kV, the collection distance was set at 15.5 cm, the feeding rate selected was 1.0 mL/h for PCL/ Soluplus®/ α-CD solutions. The fibers were characterized by scanning electron microscopy to analyze the morphology of the fibers. The samples were gold-sputter coated under argon to render them electrically conductive prior to microscopy. The average diameter was determined by measuring the diameters of the fibers in SEM microphotographs, using the imageJ analysis software. The fibers were chemically characterized by X-ray powder diffraction, differential scanning calorimetry (DSC) and FT-IR spectrometry.

Results and discussion

The optimal fiber manufacturing process was obtained at 15 kV. Continuous and smooth fibers were successfully obtained with the solutions that contained a ratio of 4:3:1 of PCL:Soluplus®:α-CD and 1:1 PCL: Soluplus® in chloroform with a diameter of 2.96 ± 0.99 and 3.29 ± 0.44 µm, respectively (Figure 1). The micrometric fibers may resemble the natural extracellular matrix, promote cell adhesion and also allow the cell infiltration, and the gas and nutrient exchange.

Conclusion

Fibers covering a wide range of diameters can be obtained in the same mat, which may facilitate cell adhesion and infiltration. Moreover, melatonine release rate could be tuned by changing the polymers ratio.

Acknowledgements

Work funded by MINECO [SAF2017-83118-R], Agencia Estatal de Investigación (AEI) Spain, Xunta de Galicia [ED431C 2016/008], and FEDER (Spain).

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In vivo biocompatibility of soy protein and chitin sponge-like scaffolds derived from natural by-products

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1. - Introduction

Nowadays, with the increase in life´s expectancy and the growing number of people suffering from metabolic problems, chronic and degenerative diseases have become one of the most important healthcare challenges worldwide [1]. In that way, scaffolds have been widely employed because of their capacity to mimic the extracellular matrix (ECM) and the ability to host different cell populations on their porous microarchitecture [2, 3, 4]. However, the search for adequate sources of biomaterials represents one of the challenges to be faced in the field of tissue engineering and regenerative medicine. Here, we used soy protein and β-chitin obtained from food industry by-products to develop a brand new, sponge-like scaffold (SLS) and tested its biocompatibility for future biomedical applications.

2. - Materials and Methods

To study the effect of a previous conditioning step of the SLS, samples were divided in 3 different groups: lyophilized SLS without conditioning step, hydrated SLS, immersed into PBS for 5 min and dialyzed SLS, immersed into 1.6 L of Milli-Q water. Both the hydrated and the dialyzed samples were subjected to a final freeze-drying process. For the *in vivo* assay, the SLS were divided in four differet groups (n=3 samples per group): lyophilized, hydrated, dialyzed, and lipopolysaccharide (LPS) soaked SLS, as a positive control group for an inflammatory response. Wet SLS were implanted subcutaneously in C57BL/6 mice for 14 days. After 14 days, mice were euthanized. SLS and surrounding tissue was excised and fixed in 3.7% paraformaldehyde. Then, the biopsies were bisected and treated to be stained by H&E, Masson's trichrome (MT) and by anti-CD68, anti-CD163 and anti-ERG immunostainings.

3. – Results and discussion

H&E staining shows a predominant M2 macrophage profile, with an absence of gigant cell formations, promoting the deposition of collagen fibres inside the SLS and increasing the neovascularization process (***Figure 1***). However, both hydration and dialysis groups showed enhaced outcomes in all the tested parameters.

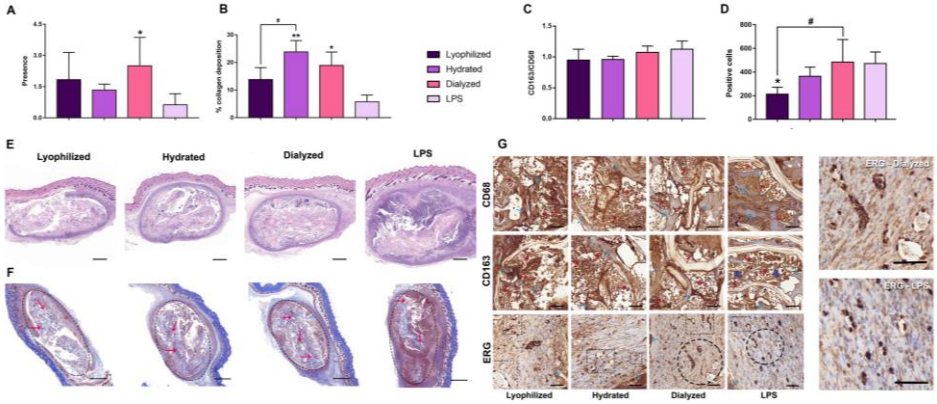

Figure 1. *In vivo* histopathological analysis. (A) Ratio between macrophages and polimorphonuclear neutrophils in the H&E staining. (B) Collagen deposition into the scaffold. (C) CD68 – CD163 positively stained cells. (D) ERG staining. * $p < 0.05$; ** $p < 0.01$ comparing to LPS group. # $p < 0.01$ between groups. (E) H&E staining. Scale bars are 500 µm. (F) Masson´s trichrome staining. Red arrows point out blue stained collagen fibres. Scale bars are 800 µm. (G) Images of the immunostainings. The dotted boxes show blood vessels in two zoomed images. Scale bars are 50 µm. Blue arrow shows scaffold fibres and red arrow shows positively marked cells.

4. – Conclusion

These results demonstrated that even if all the groups showed an excellent biocompatibility, a previous conditioning step (hydration or dialysis) before implantation is highly recommendable to maximize the soy protein and chitin SLS integration with the host's tissue.

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Intra-articular administration of nanoparticulated systems for synovial macrophages vectorization.

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Introduction. Osteoarthritis (OA) is a highly prevalent disease that curses with pain, articular stiffness and functional disability [1]. OA progression is directly associated to the stablishment of an inflammatory environment on the affected joint caused by the secretion of pro-inflamatory cytokines by activated macrophages. Due to the localized nature of OA, intra-articular administration of anti-inflamatory drugs has been proposed as an alternative to allow local high drug concentrations. However, current OA therapy do not promote an improvement in cartilage regeneration and are focused on the sympthomatology improvement [2]. β-Lapachone (βLap) is a lipophilic drug with recently pointed out inmunomodulatory activity that can be a candidate for OA treatment. The aim of this work is to obtain nanostructured lipid carriers (NLC) loaded with βLap suitable for intra-articular administration to be phagocytosed by activated macrophages [2-4].

Materials and methods. Drug solubility was assessed in different liquid and solid lipids at variable proportions. Based on experimental results an experimental design was established using Dataform®v3.1 software. NLC were prepared following the procedure described by Rouco et al [5] by a hot high shear homogeneization method using Transcutol HP and Compritol® 888 ATO as lipidic components and Tween and lecithin as surfactans. Obtained NLC were characterized in terms of size, PdI and ZP using a Zetasizer Nano-ZS, encapsulation efficiency and drug loading. InForm® v5.01 was used to model the experimental database by selecting lipidic components, % Tween and % lecithin as inputs and size, PdI, ZP, drug loading and encapsulation efficiency as outputs allowing to obtain the NLC composition able to produce the formulation with the desired characteristics. The effect of NLC mannose surface functionalization on NLC internalization was studied by adding stearylamine to the lipid blend and the consequent NLC incubation with a mannose solution (50 mM) [6]. Cell viability and internalization were evaluated using human macrophages. Cell uptake was studied using NLC labelled with cumarin-6, intracellular nanoparticles were measured using a fluorimetric method.

Results and discussion. The obtained NLC had similar values to those predicted by InForm® v5.01 (Figure 1A). NLC were smaller than 100 nm ensuring their phagocytosis by macrophages and, as shown in Figure 1A, ZP acquired significant negative values guaranteeing stability. NLC cell viability was close to the control and similar for plain and functionalized formulations (Figure 1B). Statistical analysis pointed out functionalizated NLC promoted higher internalization due to the presence of recognition factors as mannose receptors (Figure 1C). As expected, functionalizated NLC are larger and their ZP acquires positive values indicating surface was properly coated (Figure 1A).

Conclusion. The use of ANN allows to obtain the NLC composition able to show an adequate size, PdI and ZP loading the anti-inflamatory drug βLap. In addition, mannose functionalized-NLC improved macrophage internalization.

A

NLC	Size (nm)	ZP (mV)	PdI
Theoretical	116,55	-18,49	0,28
Optimized	77,75	-14,16	0,22
Functionalized	164,86	26,76	0,23

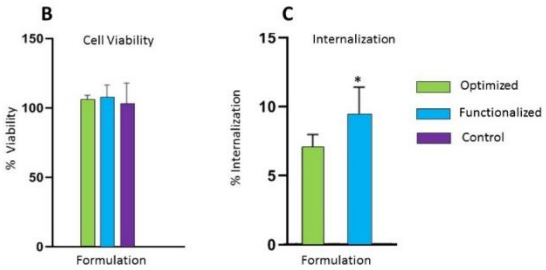

Figure 1. A) Theoretical, Optimized and Functionalized NLC properties after 14 days of storage. B) Macrophages viability after 24 hours of NLC administration. C) Macrophages internalization after 24 hours of NLC administration (*p < 0,05).

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Optimization of PLGA-nanoparticle loaded hydrogels for wound healing

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Introduction The skin is the main barrier in the body whose function is to protect it from infection and dehydration. When the barrier is damaged, a wound is formed, and the healing process begins. Acute wounds heal in 12 weeks, whereas chronic wounds need months [1]. Hydrogels are hydrophilic molecular networks produced by the physical or chemical cross-linking of polymers and have been used for wound dressings [2]. The hydrogel potential used in wound healing can be further explored with the loading of nanoparticles that allow controlled or sustained release of drugs. In this work, we propose the development of polyvinyl alcohol (PVA)/alginate hydrogels loaded with poly (lactic-co-glycolic acid**)** (PLGA) nanoparticles as carriers by Quality by Design using a Design of Experiments approach for the delivery of growth factors to enhance wound healing.

Methods Nanoparticles were produced by a water-in-oil-in-water double emulsion technique [3]. Hydrogels were produced by freeze-thawing. A multifactorial design 2 $^{(k-p)}$ was used to define the sample size and formulation composition. A total of 4 factors were studied; sodium alginate, glycerin, cycle duration time, and a number of cycles and 16 formulations were generated. The temperature was kept at -20ºC. Particle size and polydispersity index were assessed by Dynamic Light Scattering (DLS), and intermolecular interactions between nanoparticles and the htdrogel was evaluated by ATR-FTIR prior and after freeze-thawing.

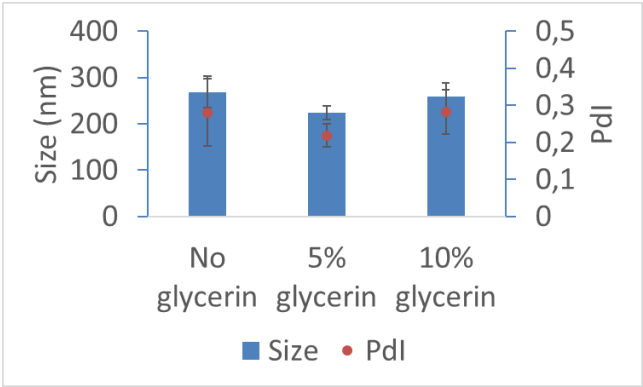

Figure 1. Size and polidispersity index of the PLGA-nanoparticles hydrogel freeze-thawed by 24 h.

Results and Discussion The hydrogels are formed upon freeze-thawing when the sodium alginate and PVA chains form a tight network due to the water crystals formed during freezing and hydrogen bonds formed during thawing [4]. It was observed that the freeze-thawing cycles of 12h and 24h did not affect the particle size of 266±39 nm and 269±34 nm, respectively (Fig. 1). Also, a narrow PdI index of 0.25 was observed throughout. Small particle size and narrow PdI values are desirable to mitigate the Ostwald ripening effect that results in particle aggregation and loss of formulation stability. The adition of 5% glycerin and 10% glycerin had no effect inn the particle size even after freeze thawing, however, it improved the rheology properties of the hydrogel. The ATR-FTIR analysis showed an interaction between PLGA and glycerin (1075 cm^{-1}) and an overall chemical stability.

Conclusions The multifactorial design 2 $^{(k-p)}$ allowed to understand the influence the formulation parameters on the overall charcateristcs of the PLGA-nanoparticles loaded hydrogel. The formulations will be further used to deliver growth factors and its *in vitro* and *in vivo* performance will be evaluated.

Acknowledgments: This work was financed by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operational Programme for Competitiveness and Internationalization (POCI), and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia (FCT) in the framework of the project POCI-01-0145-FEDER-032610 - PTDC/MEC-DER/32610/2017. It was also supported by FCT under the project UID/DTP/04567/2019.

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Transplantation of cardiovascular progenitors with biomimetic microcarriers limit adverse remodeling in a rat myocardial infarction model

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Introduction. Cell therapies have recently been used in combination with biomaterial delivery vehicles to enhance their therapeutic potential. In particular, hydrogels and cardiac patches have been explored in preclinical studies to deliver and retain cells in the infarcted myocardium [1,2]. However, their clinical translation has been limited due to difficulties injecting them through cardiac catheters. A tissue engineering approach that uses a biomimetic and biodegradable micro-platform as support for cardiovascular progenitors could be attractive for heart tissue repair. This system can be used as an injectable particulate scaffold for adherent cells due to their excellent injectability through minimally invasive catheter technology, capacity for three-dimensional stem cell support and controllable biodegradability [3,4]. The objective of this work was to test the efficacy of injectable biomimetic microparticles (MPs) to enhance the potential of cardiac progenitor cells (CPCs) in a rat myocardial infarction (MI) model. The mechanism contributing to their beneficial effects was also investigated.

Materials and Methods. Polymeric MPs were prepared using the multiple emulsion solvent evaporation method. Next, MPs were coated with collagen and poly-D-lysine (PDL) to provide particles with a favourable biomimetic microenvironment for cell adhesion. CPCs isolated from rat hearts were adhered to the MPs surface. The efficacy of the system was then evaluated *in vivo* in a chronic rat MI model. Myocardial infarction was induced by permanent occlusion of the left anterior descending coronary artery. A total of 33 animals with a left ventricular ejection fraction between 40 and 50% at day 5 post-MI were included in the study. One month post-MI, treatments were locally administered in 2 regions of the peri-infarcted myocardium. One month post-injection animals were sacrificed to perform the histological analysis of the hearts. Cell survival, cardiac function, ventricular remodeling and vascularization were analyzed. Finally, the contribution of CPCs-derived extracellular vesicles to cell therapeutic potential was investigated. To this end, the effect of extracellular vesicles from CPCs on cardiac fibroblasts was analyzed *in vitro*.

Results and Discussion. 5 um-diameter MPs were coated with collagen and PDL obtaining particles with a Z-potential of 15.3 mV. When combined with CPCs, complexes were formed in less than 1 hour. Interestingly, transplantation of CPCs adhered to MPs improved the long-term engraftment of transplanted cells for up to one month in a MI rat model. The improvement in cardiac cellular retention correlated with increased functional recovery. In consonance, better tissue repair/remodeling was observed in the animals treated with cells attached to MPs, which presented smaller infarct size and thicker right ventricular free wall than the animals treated with CPCs alone. The vascularization of the infarcted myocardium improved in animals treated with CPCs-MPs compared to the injection of CPCs alone. Finally, exosomes from CPCs decreased the expression of the pro-fibrotic marker transforming growth factor-beta 1 (TGF-β1) in cardiac fibroblasts.

Conclusion. The biomimetic microcarriers developed increased stem cell survival and cardiac function after MI through modulation of cardiac remodeling and vasculogenesis. CPCs derived extracellular vesicles play an important role in the antifibrotic effects of these cells.

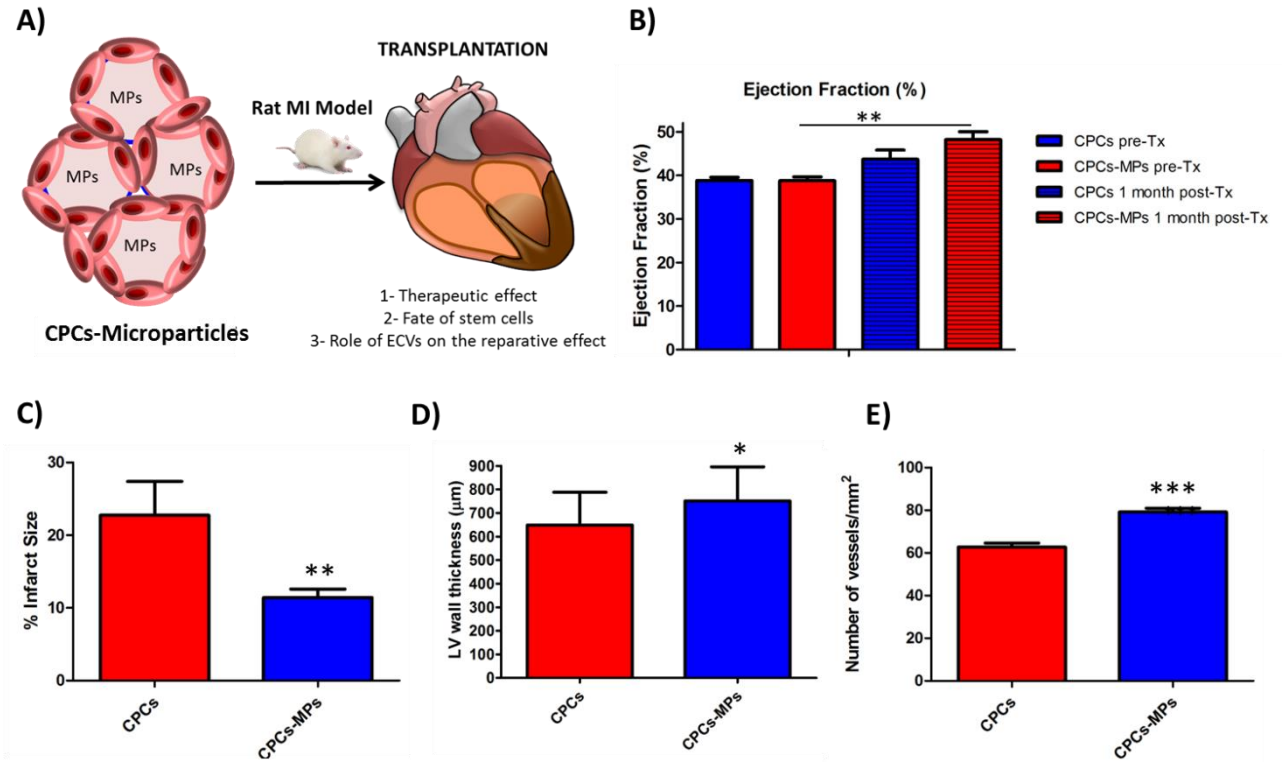

Figure 1. Transplantation of CPCs with biomimetic microcarriers limits adverse remodeling in a rat myocardial infarction model. A) Schematic representation of the cardiac tissue engineering strategy, B) CPCs-MPs improve cardiac function post myocardial infarction, C) CPCs-MPs reduce infarct size, D) CPCs-MPs showed greater left ventricular wall thickness and E) CPCs-MPs enhance vasculogenesis.

Acknowledgments: This work was supported by the Spanish Ministry of Economy and Competitiveness (SAF2017-83734-R).

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POSTER SESSIONS

Session 5: Targeted drug delivery and combination therapies

P01.5 An innovative liposomal hybrid molecule for the treatment of melanoma (p.133)
M. Matias, J.O. Pinho, A.P. Francisco, C.V. Eleutério, M.J. Perry, E. Mendes, J.D. Amaral, C.M.P. Rodrigues and M.M. Gaspar

P02.5 Bevacizumab loaded nanocapsules: cytotoxicity and internalization in lung and pancreatic cancer spheroids (p.134)
I. Fernández-Mariño, E. Tavukçuoğlu, H. Yanık, A. Şahin, G. Esendağlı, J. Crecente-Campo, M.J. Alonso

P03.5 Challenges on protein corona determination of polymeric nanocapsules (p.135)
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An innovative liposomal hybrid molecule for the treatment of melanoma

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Introduction: Melanoma is considered the most aggressive and deadly form of skin cancer, with increasing incidence and mortality worldwide [1]. Triazenes are well-known antitumor molecules, being dacarbazine and temozolamide (TMZ) two examples with clinical application in chemotherapy against melanoma, but with reduced efficacy. In the present study, new hybrid compounds were synthesized involving the combination of a triazene nucleus and melanocytotoxic phenols. Once these hybrid agents contain two distinct moieties covalently linked, they act through multiple mechanisms of action: 1) specificity to tyrosinase; and 2) toxic alkylating properties to melanoma cells [2]. The most active hybrid molecule (HM), which showed to be more potent towards human and murine melanoma cell lines than TMZ, was incorporated in long circulating liposomes (PEGylated liposomes). This nanothechnological strategy was employed to overcome the limitations of the current chemotherapy against melanoma and take advantage of the enhanced permeability of the tumor vasculature that allows liposomes to preferentially accumulate at tumor sites [1].

Methods: Liposomal formulations of HM were prepared by the dehydration-rehydration method, followed by extrusion. The antitumor activity of HM formulations was evaluated in a syngeneic murine melanoma model [3,4] and in a metastatic murine melanoma model, where B16F10 cells were injected subcutaneously and intravenously in C57Bl/6 mice, respectively. In addition, caspase 3/7 activity, tyrosinase activity, haemolytic activity, stability in human plasma and hepatic evaluation were determined by described methods [4,5,6].

Results and Discussion: The HM was efficiently incorporated (around 100%), in liposomes with a mean size of 100 nm and a polydispersity index below 0.1. Moreover, a high stability in human plasma without eliciting haemolytic activity in human red blood cells was obtained, and after intravenous administration in healthy mice, no hepatotoxic side effects were observed. Regarding pharmacologic activity, HM nanoformulations clearly demonstrated a notable reduction on the tumor progression, in comparison to free HM. More specifically, in the syngeneic murine melanoma model, it was achieved a remarkable antitumor activity for liposomal HM at a dose of 12 mg/kg, with a 7-fold reduction in terms of relative tumor volume (RTV) compared to control (Figure 1). Additionally, the therapeutic effect of a lower dose (6 mg/kg) led to an impaired tumor progression, although at a lower extent. Furthermore, for mice treated with free HM, the RTV was similar to control. In a metastatic melanoma model, liposomal HM presented 4-fold reduction of lung metastases compared to non-treated control mice, which was confirmed by histopathological analysis. Moreover, a strong correlation between tumor growth inhibition, increased caspase 3/7 activity and decreased tyrosinase activity in tumor protein extracts was observed for mice receiving HM nanoliposomes. Finally, in order to demonstrate the so desirable preferential accumulation in tumor sites rather than healthy tissues, as well as the accumulation in vital organs, the biodistribution profile studies of HM formulations are on course.

Conclusion: Overall, a new and highly effective therapy against melanoma has been successfully established.

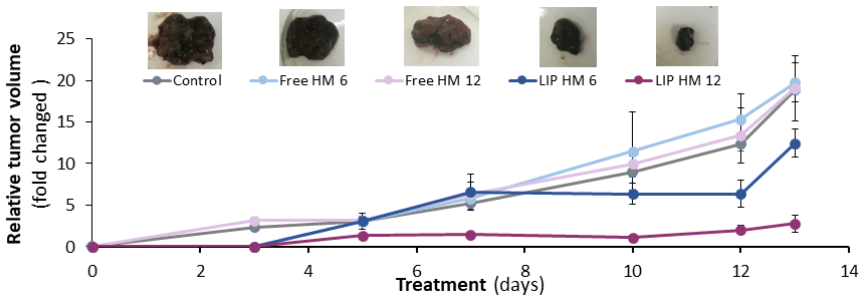

Figure 1. Influence of HM treatment [HM in free form (Free HM) or incorporated in liposomes (LIP HM) at doses of 6 and 12 mg/kg of body weight] in terms of relative tumor volume.

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Bevacizumab loaded nanocapsules: cytotoxicity and internalization in lung and pancreatic cancer spheroids

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Introduction. Monoclonal antibodies, due to their efficacy, safety and specificity, have become one of the most relevant cancer treatments in the last decades. However, these biomolecules show certain limitations such as a poor penetration, heterogeneous distribution in solid tumours and limited capacity to access intracellular locations [1]. In this sense, nanotechnology could facilitate the intracellular delivery of antibodies, allowing the target of oncoproteins, so far, inaccessible [2]. In this work, we have designed polymeric nanocapsules (NCs) for the intracellular delivery of antibodies and we have tested them in 2D and 3D co-cultures of lung and pancreatic cancer spheroids.

Materials and Methods. NCs were prepared by a self-emulsifying technique [3]. Two different polymers were employed as coating; hyaluronic acid (HA) and hydrophobized hyaluronic acid (HA-H). Bevacizumab was incorporated in the systems as monoclonal antibody model. Cyanine 5 was covalently attached to the hyaluronic acid to fluorescently label the systems.

In vitro assays were performed in 2D co-culture and 3D co-culture spheroids. NCI-H441 (lung cancer cell line) or CFPAC-1 (pancreatic cancer cell line), THP-1 (monocytic cell line) and SW982 (fibroblast cell line) were used in the experiments in a ratio of 1:0.5:0.25, respectively. Nanosystems cytotoxicity and internalization were evaluated by flow cytometry. Internalization was also confirmed by fluorescence microscopy.

Results and Discussion. Two prototypes of NCs with a different polymeric coating (HA and HA-H) were developed and characterized, showing similar physicochemical properties. HA NCs showed a particle size of 139 ± 11 nm, PDI 0.35 and negative zeta potential of -9 ± 2 mV; whereas HA-H NCs showed particle size of 123 ± 2 nm, PDI 0.25 and negative zeta potential of -17 ± 2 mV.

The results of the *in vitro* experiments showed that HA and HA-H NCs have a low cytotoxicity profile after 24 h of incubation in NCI-H441and CFPAC-1 spheroids, at concentrations as high as 6.0 mg/mL. Internalization assays performed in 2D models showed a higher uptake for HA-H NCs in co-cultures of both NCI-H441 and CFPAC-1, with almost double fluorescent positive cells, as compared to HA NCs. In contrast, internalization assays in 3D spheroids models showed around 70% of fluorescent positive cells for both nanosystems. The differences in the organization of cells in 2D and 3D co-cultures might explain the results of these experiments.

Conclusions: HA and HA-H nanosystems loaded with Bevacizumab have shown a low toxicity profile in 3D co-cultures spheroids with NCI-H441 and CFPAC-1 cells. Furthermore, the internalization of HA and HA-H nanosystems was remarkably high for 3D spheroids cells, without differences between myeloid and cancer cells. Future *in vitro* assays will prove the potential of these systems for the intracellular delivery of monoclonal antibodies.

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Challenges on protein corona determination of polymeric nanocapsules

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Introduction: Polymeric nanocapsules (NCs), made of an oily core and a polymeric shell, are known for being versatile nanocarriers for the delivery of both hydrophobic and hydrophilic drugs, protecting them from degradation and modulating their biodistribution profile and body clearance [1]. However, when these systems are intravenously administered, they spontaneously become coated by a protein layer, called protein corona. Adsorbed proteins can affect NCs targeting and circulation time, toxicity and physical stability [2]. To elucidate the biological fate of different nanocarriers, a proper protein corona characterization is needed. Nevertheless, protein corona experiments are usually not straightforward and present many limitations. In this work, protein coronae of different NCs were compared using different experimental approaches.

Materials and Methods: Different negatively charged polymers, oils and surfactants were employed to prepare the NCs. SYPRO® Ruby protein gel stain was purchased from Bio-Rad (United States). Citrated human plasma was obtained from peripheral blood donations of healthy volunteers. NCs were prepared by solvent displacement (SD) and self-emulsifying (SE) techniques. Briefly, all the inner organic components of the SD NCs were dissolved in ethanol and injected into the polymer-containing aqueous phase [1]. Afterwards, ethanol was removed from the formulation and replaced by water. SE NCs were prepared by injecting a polymer and surfactant aqueous solution into the mixture of the lipophilic components.

NCs were incubated in human plasma for 1 h. Then, NCs were isolated by different techniques: ultracentrifugation (UC), asymmetrical flow field-flow fractionation (AF4) and tangential flow filtration (TFF). Protein content was analyzed through fluorescent-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Results and Discussion: Two SD NCs with a polymeric shell made of hyaluronic acid (HA) and polysialic acid (PSA) were prepared, showing a particle size of 160 ± 3 nm and 151 ± 2 nm, PDI of 0.12 and 0.11, and a Z-potential of -37 ± 1 mV and -49 ± 2 mV, respectively. Similarly, HA and PSA NCs were prepared by SE technique showing a particle size of 146 ± 6 nm and 151 ± 8 nm, PDI of 0.33 and 0.32, and a Z-potential of -10 ± 1 mV and -9 ± 3 mV. Incubation with 90% citrated human plasma led to zeta-potential close to neutrality but did not change their size. NCs were stable in 90% human plasma for, at least, 8 hours. Surface area was calculated based on nanoparticle tracking analysis and was kept constant for all the prototypes. Separation of NCs-protein corona from the remaining free proteins was optimized for each mentioned technique. SDS-PAGE showed different protein fingerprints depending on NCs nature, such as a strong human serum albumin reduction in certain cases.

Conclusions: Experimental procedures to determine protein corona need to be optimized depending on the nature of the nanosystems. UC, AF4 and TFF were found to be valid methods for the separation of NCs-protein corona from free proteins. However, each method showed different limitations. Differences between the protein corona of NCs could be detected through fluorescent-stained SDS-PAGE, however, further experiments including mass spectrometry should be performed to unequivocally identify and quantify all the involved proteins.

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Combinatory therapeutic strategy to metastatic colorectal cancer treatment using functionalized CEA-targeted nanoparticles

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Introduction: Colorectal cancer (CRC) is one of the most common and deadliest cancer in the world, mainly due to distant metastasis events. Despite the improvements on the chemotherapy, 5-fluorouracil (5-FU), remains one of the most effective drugs commonly used to treat CRC (1, 2). However, this anti-metabolite has several limitations, due to its low local bioavailability and high toxicity. Nevertheless, treatment failure might be also due to chemoresistance associated with the immunesuppressive tumor microenvironment (TME), which promotes cancer cell immune escape and impairs treatment outcome (3). To overcome these drawbacks, nanomedicine might be used as a promising strategy to provide an effective, controlled and targeted therapy. Nanoparticles (NPs) are one of the most studied vehicles in drug delivery and their ability to link targeting moieties (e.g. an antibody) on surface allow a specific targeting. This targeted therapy might be useful to decrease secondary effects of chemotherapy and improve treatments. Carcinoembryonic antigen (CEA) is one of the most interesting candidates to target CRC cells, because is a glycoprotein cell surface overexpressed in most colorectal tumors (4). The aim of this work is to develop an innovative combinatory therapy based on immunochemotherapy targeted nanoparticles (NPs) for Intravenous (IV) administration. Two different targeted NPs will be developed. The first nanosystem is planned with tropism to CRC cells expressing the CEA, carrying the chemotherapeutic agent 5-FU. The other NP formulation will target an immunesuppressive molecule highly expressed at the colorectal TME, blocking its activity, while delivering a pro-inflammatory cytokine. The work here presented is focused in the first formulation.

Material and Methods: Polymeric NPs were produced through double emulsion technique, with poly (lactic-co-glycolic acid) (PLGA) and PLGA-poly (ethylene glycol) (PLGA-PEG) polymers and loaded with 5-FU, followed by functionalization with a monoclonal antibody targeting CEA, through the chemically link carbodiimide chemistry. Physical-chemical properties were tested for its average size and Polydispersity Index (PdI), assessed by Dynamic Light Scattering (DLS), while surface charge was assessed through Laser Doppler Anemometry (LDA). Morphology as well as association efficacy (AE) and drug loading (DL) were evaluated by TEM and HPLC, respectively.

Results and Discussion: Physical-chemical characteristics of polymeric NPs were assessed, and it was observed that the average of particle size is 141.4±5.35 nm, which is described as ideal to escape renal filtration, biliary excretion and also accumulate in tumor using EPR effects. PdI was up to 0.1, confirming a monodisperse population. The developed NPs were closed of neutrality charged (-3.51±0.46 mV) which is described as desired charge due to high tendency of accumulation at the tumor site (5). The encapsulation efficacy (AE) was also evaluated being around 30% of 5-FU encapsulated into the NPs with 1.5 % of drug loading. Due to the low hydrophilicity of 5-FU, low values of AE were expected. The shape is another important feature since spherical particles tend to be more easily internalized. Functionalized and non-functionalized NPs will be assessed by TEM to confirm the spherical shape of polymeric NPs, followed by *in vitro* studies.

Conclusions: 5-FU-loaded PLGA-PEG NPs were produced and functionalized with an antibody targeting CRC cells CEA antigen. Importantly, according to the literature, the physical-chemical characteristics of these NPs, namely low size, low PdI and surface charge are described as ideal for IV administration to permeate through the tumor vasculature (5). The low values of AE and DL will be improved changing the ratio drug/polymer or using microfluidics techniques to improve drug encapsulation. As future work, we intend to also develop functionalized NPs carrying a pro-inflammatory cytokine to target the imnunesuppressive TME. After NPs functionalization, further *in vitro*, *ex vivo* and *in vivo* assays will be performed to assess their distribution and internalization, as well as their impact on cancer and immune cell profile. Therefore, this combinatory strategy will have a dual role acting on cancer cells and recruiting immune cells, changing the TME activity, improving the outcome of chemotherapy and modulating the tumor immunosuppressive environment.

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Copper-based complex liposomes as an alternative for solid tumors treatment

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Introduction: Cancer is undoubtedly a worldwide challenge in terms of clinical management. Particularly, colon cancer and melanoma are complex and aggressive diseases that require novel therapeutic strategies to improve clinical outcomes. In this context, metallodrugs, more specifically copper-based complexes, such as [Cu(phen)Cl_2] (Cuphen), have emerged as highly promising anticancer agents [1-3]. Lipid-based nanosystems, namely liposomes, may be used as delivery tools for anticancer drugs, promoting the preferential accumulation at tumor regions through the enhanced permeation and retention (EPR) effect [4]. Additionally, solid tumors microenvironment is slightly acidic compared to healthy tissues [5]. Therefore, a pH-triggered Cuphen release from long circulating liposomes at tumor sites constituted the strategy in the present work.

Materials and Methods: The antiproliferative activity of Cuphen and the ligand phenanthroline was assessed by MTT assay in several human and murine tumor cell lines, at 48 and 72 h. In order to enhance the accumulation at tumor sites and promote a local pH-dependent Cuphen release, this copper complex was associated to long circulating liposomes with pH-sensitive properties. Liposomes with the lipid compositions DMPC:Chol:DSPE-PEG (F1), DMPC:CHEMS:DSPE-PEG (F2) and DOPE:CHEMS:DMPC:DSPE-PEG (F3) were prepared by the dehydration-rehydration method [1,2]. Liposomes were characterized in terms of loading capacity, incorporation efficiency (I.E.), mean size, polydispersity index, and zeta potential. To validate the pH-sensitive properties, all nanoformulations were incubated in different pH conditions at 37ºC for 90 min, and the metallodrug still associated to liposomes was quantified. To assess the antitumor effect of Cuphen in both free and liposomal forms, syngeneic murine models of colon cancer and melanoma were accomplished [2].

Results and Discussion: Both phenanthroline and Cuphen displayed antiproliferative effects against the tested tumor cell lines, with IC_{50} values <10 µM and <6 µM, respectively. The developed Cuphen nanoformulations F1, F2 and F3 presented Cuphen loadings of 19, 30, 31 nmol/mL corresponding to I.E. of 68, 84 and 97%, respectively. All nanoformulations were very homogeneous in terms of mean size (<125 nm) and zeta potential (around -3 mV). The pH-sensitivity test revealed an enhanced Cuphen release from DOPE-containing liposomes (F3) as a function of pH decrease (from 6 to 4.5) that ranged from 47 to 94%, respectively. For F2, the respective obtained values were 36 (pH 6) and 88% (pH 4.5). In contrast, F1 did not display such release behavior. Following these promising results, the therapeutic efficacy of Cuphen formulations was evaluated *in vivo*. In the melanoma syngeneic murine model, Cuphen nanoformulations F1 and F2 significantly reduced melanoma tumor progression (3 and 2-fold) compared to control group and to mice receiving free Cuphen, respectively, with no hepatic toxic effects. Furthermore, in the syngeneic murine colon cancer model, F3 displayed a high antitumor activity, with significant tumor progression reductions (4.7, 2 and 4.5-fold), compared to control mice and groups receiving Cuphen and phenanthroline in free forms, respectively. Also, as in the case of melanoma murine model, no hepatic toxic side effects were observed for all treated groups, proving the safety of Cuphen formulations for *in vivo* administration.

Conclusions: The results obtained in this work demonstrate that the association of Cuphen to long circulating liposomes with and without pH-sensitive properties is highly advantageous for the treatment of solid tumors. These nanformulations constitute an effective alternative to the current available therapies in clinical use. The elucidation of Cuphen mechanism of action is on course.

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Decorated sphingomyelin nanosystems for selective targeting of metastatic lung cancer

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Introduction. Despite the important advances reached in the last years, cancer survival is severely compromised by the onset of metastasis. In the case of lung cancer, it has a 5-year mean survival below 5% for a metastatic stage. Therefore, it is extremely important to identify novel biomarkers for developing innovative therapeutic strategies to specifically tackle metastatic cancer. For this particular purpose, our group has performed a molecular analysis of disseminated lung cancer cells to identify novel biomarkers that can be exploited for the selective targeting of sphingomyelin nanosystems (SNs) to metastatic cancer cells. We have selected TAS1R3 (Figure 1a) and confirmed that it is highly expressed in metastatic non small cell lung cancer samples but not in healthy tissue [1]. On view of this, we studied different strategies to decorate SNs for the specific targeting to TAS1R3, the low molecular weight molecule lactisole, a polyclonal antibody (anti-T1R3), and an aptamer (TAS49F, selected and provided by the company Aptus Biotech S.L.).

Methods. Sphingomyelin nanosystems (SNs) were prepared by ethanol injection and characterized in terms of their morphology and physicochemical properties by Transmision Electron Microscopy (TEM), Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA). Lactisole was modified with a lipid chain (C16-18-COOH, Galchimia, Spain) and incorporated to SNs by its addition to the organic phase prior de formation of nanosystems. The incorporation of lactisole was confirmed and quantified by High-Performance Liquid Chromatography (HPLC). SNs were formulated with DSPE-PEG-NHS (Nanocs, USA) and anti-T1R3 (Abcam, United Kingdom) and TAS49F-NH_2 (Aptus Biotech, Spain) were associated to the surface of SNs by NHS-ester crosslinking. The reaction was followed by western blot and gel electrophoresis, and the association efficiency was measured by micro-BCA and Quant-iT™ OliGreen® ssDNA, respectively. *In vitro* assays, cell toxicity and cell internalization, were performed using lung cancer cell lines and fluorescent SNs (labelled with TopFluor®, Avanti Polar Lipids, USA).

Results. SNs had a mean size of 85 ± 7 nm with a narrow distribution (PDI 0.10) and slightly negative surface charge (-15 ± 4 mV). The lipid derivative lactisole was succesfully incorporated to SNs reaching an 80% of association. Lactisole-SNs showed a good colloidal stability in different media. Confocal microscopy and flow cytometry experiments showed an efficient interaction of Lact-SNs with metastatic cancer cell lines (Figure 1b). In addition, western blot and gel electrophoresis confirmed that SNs were surface decorated with anti-T1R3 antibody and TAS49F aptamer, respectively. Both formulations were tested *in vitro* to study their toxicity and their interaction with TAS1R3 receptor.

Conclusions. We have successfully associated different types of ligands for targeting a novel biomarker identified by our group, TAS1R3. All formulations can be efficiently internalized in metastatic lung cancer cell lines and have a great potential for the selective treatment of metastases. Further experiments will be planned to investigate the therapeutic potential of the developed formulations.

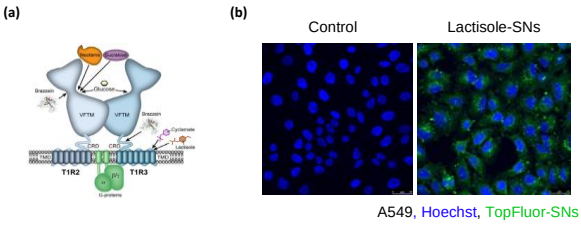

Figure 1. (a) Scheme of the TAS1R3 transmembrane receptor. (b) Stability of Lactisole-SNs in PBS and saline serum. (c) Internalization of Lactisole-SNs into lung cancer cells.

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Design and *in vitro* characterization of controlled release idebenone-loaded PLGA microparticles for LHON treatment

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Introduction and objectives. Idebenone has shown potential as a good therapeutic alternative in the treatment of Leber's Hereditary Optic Neuropathy (LHON) [1]. Nevertheless, conventional pharmaceutical forms have shown low effectiveness and high variability due to the difficulty to achieve effective concentrations in the eye [2]. PLGA microparticles as novel delivery systems could successfully encapsulate hydrophobic drugs [3]. The aim of this work is to obtain and assess different idebenone-loaded PLGA microparticles for intravitreal injection as a LHON alternative treatment.

Materials. Idebenone and Sodium hyaluronate were provided by Acofarma® (Spain). Resomer RG 502 25G, Resomer RG 503 25G and Resomer 503 H 25G (PLGA) were supplied by Evonik® (Germany). Polyvinil alcohol (PVA) (Sigma Aldrich, Germany), Methilene chloride (DCM) (Analar Normapur®), Phosphate Buffered Saline (PBS), Agua Milli-Q® Millipore (Merck®), Agua Elix Millipore (Merck®), Viscking Synthetic Dialysis Membranes (Medicell Membranes Ltd.) were also used. The rest of products are analytical grade and all solvents comply with the limits established by USP XXII and European Pharmacopoeia.

Methods. Idebenone-loaded PLGA microparticles were prepared by emulsion/solvent evaporation method. Different compositions were tested in order to select the formulation with better characteristics. Evaluation and characterization were carried out in terms of size, particle size distribution, morphology, production yield (PY), encapsulation efficacy (EE) and loading capacity (LC), as well as its *in vitro* release profile and ocular toxicity.

Particle size and size distribution were measured by a light scatering technique, while morphology was evaluated by scanning electron microscopy (SEM). Production yield (PY), encapsulation efficacy (EE) and loading capacity (LC) were obtained by experimental procedures. *In vitro* release profile was assessed by Franz diffusion cells and measured by UV-Vis spectrophotometry, while ocular toxicity was evaluated by HET-CAM test. Additional assays may be made in order to support previous tests.

Results and Discussion. Resulting microparticles show spherical appearance and smooth surface. Sieves with predetermined sizes were used to preestablish size distribution. PY, EE and LC values reveal good production properties. The *in vitro* release study shows an idebenone sustained release, with a kinetic profile based on bioerosion and drug diffusion processes, observing a good mathematical adjustment ($R^2 > 0.99$) and a 24h latency period. No formulation shows ocular irritation on HET-CAM test (Irritation Score = 0). Supplementary results from additional assays may be shown in order to endorse main results.

Conclusions. The microencapsulation technique described in this work is useful for the encapsulation of hydrophobic drugs and their subsequent controlled release for, at least, four weeks, maintaining their biological activity.

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Design of nanoparticles from self-assembling peptide epitopes as a new cancer vaccine against Non-Small Cell Lung Cancer

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Introduction

Researches has shown that immunotherapy is one the most specific treatments for Non-Small Cell Lung Carcinoma (NSCLC) and can result long-lasting remissions. Self-assembling peptide epitopes can form nanostrustures with high antigen density that provide effective immune responses against cancer cells, being compelling materials for the design of a new generation of more succesful cancer vaccines[1]. Self-assembly domains in the peptides are typically not immunogenic[2], but can be linked to MAGE-A3 epitopes, cancer specific antigens present in 55% of the NSCLC patients. KVAELVHFL (Tc) is an antigenic epitope of MAGE-A3 that induces specific CD8+ responses. AKFVAAWTLKA (Th) is a T-helper cell epitope inducing CD4+ cell responses[3]. In this study, we hypothesized that by adding a self-assembling (SA) sequence to the N terminal of these epitopes, nanoparticles could be spontaneously generated from these peptides, forming structures with enhanced immunogenicity.

Materials and Methods

Peptides with different sequences were obtained from China Peptides. The critical aggregation concentration (CAC) of the self-assembling peptides epitopes were determined using the Nile red assay. Nanoparticles of self-assembling peptide epitopes were prepared by a solvent displacement method and characterized with photon correlation spectroscopy, nanoparticle tracking analysis and scanning electron microscopy. Circular dichroism spectra of the nanoparticles in PBS were obtained with a double beam CD spectrometer (JASSCO J-1100) at a wavelength range from 180-250 nm. The stability studies were performed in cell medium (RPMI) supplemented with 10% foetal bovine serum. Hemolysis assays were done on fresh human blood samples from healthy volunteer donors using NPs at 1, 10 and 20µM concentrations.

Results and Discussion

The average CAC of SA-Tc was 15.98µM, wherease for the mixture of SA-Tc and SA-Th it was 8.35µM; all these numbers are in the range of other self-assembling peptides. The average hydrodynamic size of SA-Tc and the mixture of SA-Tc and SA-Th peptides was 140±18 nm and 153±19 nm, respectively. The PDI for both nanoparticles were below 0.2. The surface zeta potential of the SA-Tc and the mixture of SA-Tc and SA-Th nanoparticles was +20,8±3,9 mV and +23,7±2,5 mV, respectively. Nanoparticle tracking analysis analysis showed that nanoformulations were homogenous particle populations with a concentration of 10^9 particles/ml. The circular dichroism spectra of the nanoparticles in PBS had one minimum at 215 nm, indicating that these peptides form ß-sheets within the structure of the nanoparticles. The size of the nanoparticles was monitored for 8 hours in physiologic conditions and no significant changes in particle size were observed. The peptide nanoparticles were compatible with human red blood cells when they were tested for hemolysis.

Conclusions

Self-assembling peptide epitopes can form nanoparticles with suitable characteristics to generate potent immune responses. Future studies will characterize these nanomedicines in immune cell models and *in vivo* in NSCLC models.

Acknowledgements

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Drug release and drug permeability from double layer coated PLGA nanoparticles loaded with celecoxib

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Introduction

Nanoparticles (NP) have a great potential for the treatment and diagnosis of cancer. Among the advantages of NP their ability to control drug release and targeting are of paramount importance [1]. NP can be administered through different routes but many anticancer applications use a systemic IV administration where prolonged circulation in the bloodstream is mandatory to achieve NP accumulation in the tumor by the well-known EPR effect. Another alternative is the intratumoral administration of drug-loaded NP. Besides, NP can be surface decorated to achieve their targeting towards disease cells within the tumor. In this project, celecoxib (CX)-loaded poly(lactic-co-glycolic acid) (PLGA) NP were coated with chitosan (CS) and hyaluronic acid (HA) in order to achieve particle internalization through binding to the CD-44 receptor overexpressed in the membrane of some epithelial cancer cells. The effect of CX nanoencapsulation on drug permeability and release has been evaluated *in vitro*.

Materials and Methods

CX-loaded PLGA NP were prepared by solvent evaporation and surface decorated with CS and HA through a layer-by-layer deposition method. Drug release was studied in a dissolution equipment type 2 (Pharmatest PTS III). NP were loaded into 10 mL dialysis bags made from Visking dialysis tubing with a MWCO limit of 14000 Da. The bags were attached vertically to the paddle sticks and immersed in 900 ml of the dissolution media (PBS) at 75 rpm for 18 days. The experiment was also carried out with empty NP and a saturated aqueous solution of CX in PBS as controls. The permeability study was performed through a Parallel Artificial Membrane Permeability Assay (PAMPA) (Millipore). This test was carried out with free CX in PBS (5% DMSO was used as a co-solvent) and CX-loaded NP suspension. Incubation times were adjusted to maintain the concentration gradient and permeability coefficients were obtained according to manufacturer guidelines. The amounts of CX in both experiments were monitored by an adapted HPLC method [2].

Results and Discussion

Drug release results are shown in figure 1. NP have the ability to restrict drug release. During nearly 18 days CX permeated from the PBS solution through the dialysis membrane following a two-step pattern. After a lag time of 36 h, CX passed at a constant rate (0.45 %/h) from 36 h to 86h and a slower second step (0.16 %/h) until 417 h. In contrast, permeability rates were reduced when CX was incorporated in NP. After a lag phase lasting 10 h, the first step took place at 0.25%/h and the second at 0.072%/h. PAMPA experiments also showed the ability of NP to restrict drug permeability (Pe in solution: $1x10^{-7}$cm/s; (Pe in NPs: $3.4x10^{-8}$ cm/s).

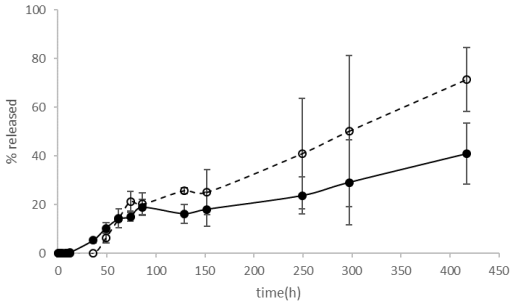

Figure 1. Drug release from the dialysis bags into the dissolution media. (closed circles/continuous line:CX loaded NP; empty circles/dashed line: CX solution)

Conclusions

NP represent a feasible approach for the intratumoral injection of CX in order to achieve a controlled drug release within the tumor site.

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Entrapping Docetaxel and Doxorubicin Prodrugs within the Hydrogel Matrix of Discoidal Polymeric Nanoconstructs for Cancer Treatment

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High loading and specific release of therapeutic agents with the tumor microenvironment are key factors in the development of novel nano-based therapeutic systems against cancer. Here, Discoidal Polymeric Nanoconstructs (DPNs) are employed to load and systemically deliver both hydrophilic and hydrophobic therapeutic molecules. DPNs appear as disks, with a diameter of about 1 μm and a height of 400 nm, and a mechanical stiffness that can be modulated from about 10 kPa to several tens of MPa. Soft DPNs, presenting a stiffness of about 10 kPa, were shown to circulate longer, more effectively avoid the sequestration by hepatic and splenic immune cells, and accumulate at higher doses in the tumor vasculature as compared to rigid DPNs [1]. DPNs were synthetized by mixing together hydrophobic – poly(lactic-co-glycolic acid) (PLGA), and hydrophilic – polyethylene glycol diacrylate (PEG-diacrylate), polymer chains using a top-down, template-based fabrication process [1]. Therapeutic molecules can be incorporated within the polymer matrix of soft DPNs following two different loading approaches: *direct loading*, where the molecules of interest are directly mixed together with the constituting polymers while forming the actual DPN polymer matrix; *absorption loading*, where the molecules of interest are introduced within an already formed DPN polymer matrix via capillary suction. In the latter case, DPNs were first lyophilized and then re-hydrated in a highly concentration solution of the molecules of interest. Indeed, only therapeutic molecules that can be dispersed in organic solvents can be incorporated via direct loading; whereas, only therapeutic molecules that can be dispersed in water can be incorporated via absorption loading.

Following this notion, three prodrugs were realized, namely oleic-Docetaxel (O-DTXL); linoleic-Doxorubicin (L-DOX) and PEG-Docetaxel (PEG-DTXL). O-DTXL was incorporated via both direct and absorption loading within the polymeric matrix. Conversely, the more hydrophilic derivatives L-DOX and PEG-DTXL were loaded via absorption. The resulting DPNs were characterized for their size and stability by dynamic light scattering and Multisizer Coulter Counter. Moreover, electron microscopy (SEM and TEM) were used to reconstruct the actual morphology of the particles (**Figure.1A-B**). The prodrug encapsulation efficiencies and release kinetics were examined using HPLC. The cytotoxicity of the prodrug-loaded DPNs was performed via a conventional MTT assay on two cell lines, namely MDA-MB-231 and U87-MG. With the absorption loading. it was observed that O-DTXL formed small micelle-like particles, returning encapsulation efficiencies as high as 20%. Differently, free DTXL and O-DTXL loaded directly within the DPN matrix returned low encapsulation efficiencies of about 0.1 and 2%, respectively. The more hydrophilic L-DOX and PEG-DTXL were associated with a 6% and 15% of encapsulation efficiency, respectively. In vitro cytotoxicity tests confirmed that hydrophilic prodrugs are more efficient than O-DTXL mostly for their faster release rates (**Figure.1C**).

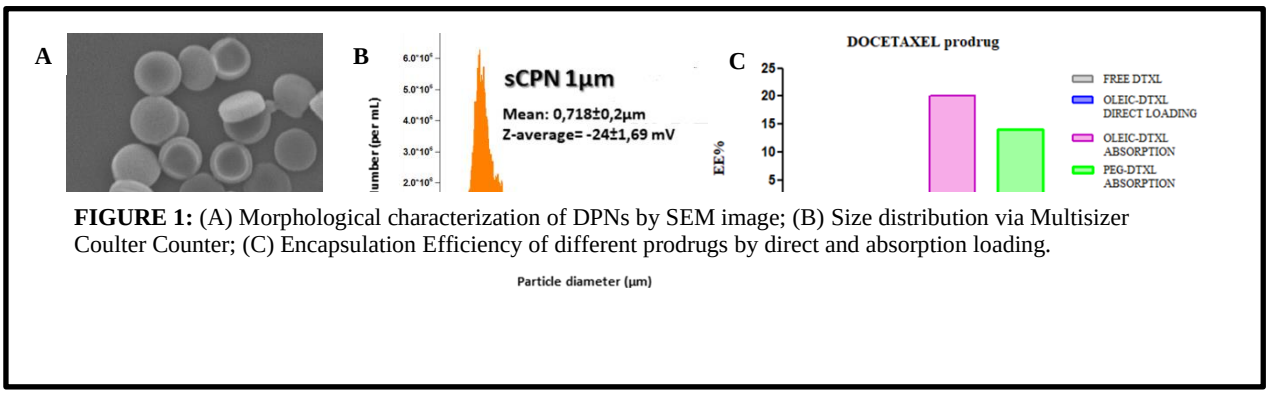

FIGURE 1: (A) Morphological characterization of DPNs by SEM image; (B) Size distribution via Multisizer Coulter Counter; (C) Encapsulation Efficiency of different prodrugs by direct and absorption loading.

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Evaluation of nanosystems based on biopolymers: Fucoidan from the edible brown seaweed *Laminaria japonica* and Chitosan

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Introduction: Macroalgae have great potential in several fields such as biomedicine, cosmetic and nutraceutic, and seaweeds are a source of bioactive compounds with biological activities [1,2]. *Laminaria japonica* is an edible brown macroalgae, having fucoidan as the main polysaccharide in its composition, and being comprised by oligosaccharides and sulphate groups. Another biopolymer, chitosan (CS), composed by *N*-acetylglucosamine and D-glucosamine units, has been widely proposed for biomedical applications due to its low toxicity and biocompatibility in several delivery routes [3]. These polysaccharides have been reported as advantageous matrixes for the production of microparticles and nanoparticles, which have been proposed as drug delivery systems [4] with therapeutic value [5]. The composition and structure of fucoidan from brown seaweeds have been associated to several widely studied biological properties, such as antioxidant, antitumoral, anticoagulant, antiviral activities [2]. In parallel, the pharmaceutical field has been exploring for years the use of particulate systems to transport bioactive compounds while providing their protection, enabling the association of high drug doses and the decrease of side effects [3]. Nanocarriers are the most popular drug delivery systems nowadays and several methods are described for their production [6]. The aim of this work was to prepare nanosystems formed by fucoidan from *Laminaria japonica* and chitosan, using a technique of polyelectrolyte complexation. Different fucoidan/chitosan (FUC/CS) weight ratios were tested and physicochemical properties (size and zeta potential) were studied.

Materials and Methods: Fucoidan from *Laminaria japonica* (Sigma-Aldrich, Spain) and chitosan (Sigma-Aldrich, Germany) were solubilised in ultrapure water and acetic acid (1%, v/v), respectively, and filtered before use (0.45 µm and 5-13 µm, respectively). Polyelectrolyte complexation was used to prepare FUC/CS nanoparticles, varying FUC/CS mass ratio from 1/4 to 4/1 [7]. In brief, the technique requires two polymers with opposite charges, which upon contact, form the nanoparticles by electrostatic interaction. The adjustment of the pH of polymeric dispersions to a value of 4 prior to nanoparticle preparation was tested as variable, thus ensuring chitosan's protonation. All nanoparticle formulations were characterised regarding their size and zeta potential by diluting the samples in deionised water (Zetasizer Nano ZS, Malvern Panalytical, UK).

Results and discussion: The nanoparticles were prepared successfully. FUC/CS nanoparticles produced without pH adjustment resulted in sizes around 400 nm. Curiously, the adjustment of the pH of the polymers led to nanoparticles with higher size, around 800 nm. This suggests the possible occurrence of stronger interaction between the polymers, the increased size being consequence of the presence of more polymeric chains in the nanoparticles. In both cases, the variation of the polymeric ratio had no impact on the resulting size and it was also observed that the nanoparticles FUC/CS = 1/4 resulted in very high sizes, up to 1000 nm. Regarding the zeta potential, the adjustment of pH had not a significant impact on the parameter. Nanoparticles of FUC/CS = 4/1 presented a moderate positive charge between +10 and +15 mV. The opposite formulation (FUC/CS = 4/1) had zeta potential within +47 and +54 mV, reflecting the higher amount of chitosan present in the nanoparticles. In fact, this tendency was observed throughout the set of formulations from FUC/CS = 4/1 to 1/4, with an increase of zeta potential accompanying the increase of chitosan in the nanoparticles. All formulations had a positively charged surface, which indicates higher charge density of chitosan.

Conclusions: Nanoparticles formulated using fucoidan from *Laminaria japonica* and chitosan were evaluated. The adjustment of the pH of polymeric solutions to 4 did not have a positive impact, generating an increase of nanoparticles' size, despite no effect was seen on zeta potential. On the other hand, the high charge density of chitosan resulted in the formation of nanoparticles with positive charge, independently of the used polymeric ratio. The next step will comprise the association of a model drug, envisaging a drug delivery application.

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Evaluation of the lyoprotective effects of lactose and mannitol: application to sildenafil citrate liposomes

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Introduction

Passive lung targeting is currently a priority for several drugs including sildenafil citrate (SC). Liposomes have been shown to be safe for pulmonary administration [1] and relevant advances in liposomal formulations are being achieved. Nevertheless, therapeutic application is still challenged by the instability of liposomes, reason why lyophilization is recommended to extend their self-life. The inclusion of lyoprotectant agents is a key point [2], but only lactose and mannitol are currently approved as additives for pulmonary formulations. The aim of this study was to evaluate the lyoprotective ability of lactose (L) and mannitol (M) for lyophilization of SC liposomes and to compare the results with the standard sucrose (S) and trehalose (T).

Materials and Methods

SC liposomes were prepared from egg phosphatidylcholine (EPC) and cholesterol (Chol) mixtures by direct sonication of components [3]. 4% w/w of M, L, S, or T was added to the liposome suspension before lyophilization. Freeze-drying cycles with and without annealing and with and without secondary drying were carried out. Visual appearance, redisolution time and remaining moisture (RM) in cakes were registered. Drug entrapment efficiency (EE%), hydrodynamic diameter (Dh), polydipersion index (PDI) and zeta potential, of fresh and rehydrated liposomes were analyzed and compared. Morphologic characterization was performed by scaning electronic microcopy (SEM). The thermal behaviour of samples with different additives was studied by DSC using a STARe software package.

Results and Discussion

After primary drying without annealing, similar values of RM where found for L, S, or T and lower values for M, although statistical significance was not achieved. Annealing, hardly improved the results of RM% for L, S and T and the opposite effect was observed for M, due to crystalization. It was observed that secondary drying at 20±2º C performed with samples showing RM≥ 5% lead to the collapse of cakes, irrespectively of the size of liposomes, the additive used and the protocol applied. Uniform and homogeneous cakes, however, were obtained when samples with RM ≤ 4 % underwent a secondary drying at this temperature. It was also observed that removing the unfrozen water occurs slowly, particularly when performed at temperature under 20º C. Accordingly, a RM cut-off of 4 % and a temperature of 20º C were selected for the secondary drying. Without annealing, secondary drying was faster for M than the rest of additives which confirm the beneficial effects of M as bulking agent. On the other hand the most relevant changes in Dh and PDI of rehydrated liposomes was registered in samples with M, followed by T and then S or L, the latter showing the most benefical effects for lyoprotection. DSC results confirmed the favourable lyoprotective effects of L and S, compared to T and M. Figure 1 shows SEM images of lyophilized cakes.

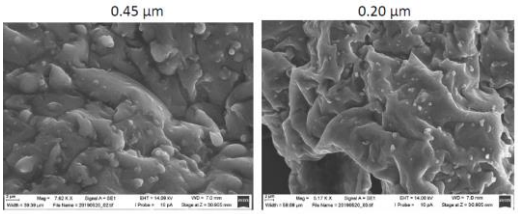

Figure 1. SEM images of lyophilized cakes with S as lyoprotectant

Conclusions.

Mannitol showed the best effects as bulking agent but sucrose and trehalose were the most effective as lyoprotective agents for sildenafil citrate liposomes.

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Functionalized Magnetic Nanoparticles: smart nanocarriers for drugs, oligonucleotides and antibodies for the treatment and diagnosis of cancer

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Nanotechnology has received tremendous attention, in particular from the field of cancer research. In this sense, magnetic nanoparticles (MNP) offer a versatile platform due to the combination of their magnetic properties and the tunability of the surface with different coatings and active molecules [1]. Particularly, MNP can be used as contrast agents in magnetic resonance imaging and therapeutics through hyperthermia, if an alternating magnetic field is applied [2]. Furthermore, molecules of interest can be attached to the surface using tailored linkers allowing their release in the tumoral environment (smart nanoparticles) [1].

In this regard, we propose a system based on MNP synthesized by the co-precipitation method, affording structures with a core of maghemite of around 14 nm. The structures were coated with dextran-based molecules and further modified with fluorophores (Cy5), drugs (gemcitabine), oligonucleotides (miRNA34a) or antibodies (MMP14) (Fig. 1). To control the release of therapeutic molecules inside tumoral cells, we have used linkers containing disulfide bonds, which are prone to rapid cleavage in tumor tissues due to the presence of glutathione in higher concentrations in malignant cells compared to normal cells and it is in higher concentration intracellularly compared to the extracellular environment [2].

All the nanoparticles obtained were fully characterized using standard techniques (e.g. DLS, TEM) and their efficacy was tested *in vitro* in a variety of pancreatic cancer cell lines (PANC-1, BxPC-3 and MiaPaca-2). Cell viability, cell cycle, cell death and internalization assays were studied to evaluate their potential as drug delivery systems. Cytotoxic assays were done with and without the application of an alternating magnetic field to evaluate the effect of the hyperthermia in cells. Furthermore, MNP functionalized with Cy5 and MMP14 antibody were also tested *in vivo* in a lung cancer mouse model.

MNP functionalized with gemcitabine had a remarkable effect on the reduction of cell viability in pancreatic cancer cell lines. Interestingly, their activity could be increased by the incorporation of miRNA34a to the nanoparticle. Additionally, the combination of this multifunctionalized nanocarrier with hyperthermia showed a synergistic effect in the reduction of cell viability. Apart from that, MNP functionalized with Cy5 and the antibody MMP14 targeted the tumoral environment in mouse lung cancer model, probably due to the EPR effect.

In summary, we have prepared and assessed functionalized magnetic nanoparticles using an easy to scale-up process with an enormous potential for both diagnosis and treatment of cancer.

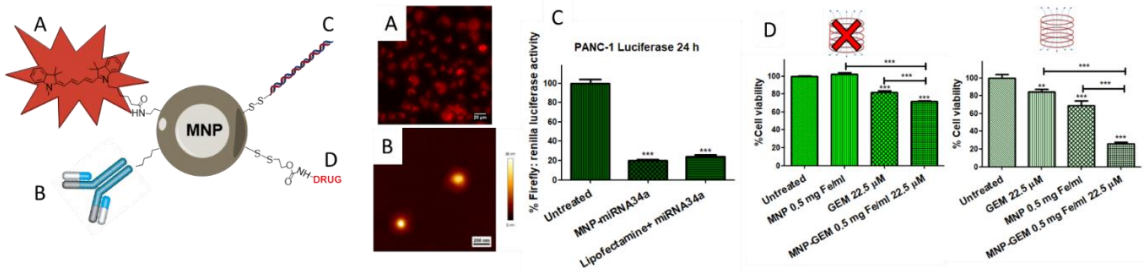

Figure 1. Schematic representation of a multifuncionalized MNP. A) MNP-Cy5. Fluorescence image in PANC-1 cells. B) MNP-MMP14. AFM image that confirms the presence MMP14 around MNP. C) MNP-miRNA34a. Luciferase assay that confirms the correct functionalization. D) MNP-GEM. Viability assay with and without the application of an alternating magnetic field.

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Glutathione modified PLGA nanoparticles for CNS drug delivery

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Introduction

Designing a treatment for central nervous system (CNS) pathologies has the ever-present challenge of overcoming the blood brain barrier (BBB). Nanoparticles (NPs) have shown to be a viable resource in improving the access to the CNS and reducing side effects of active compounds. These capabilities can be further enhanced via surface modification with a ligand [1, 2]. Hence, glutathione modified rhodamine B loaded-PLGA nanoparticles were formulated in order to study their ability in crossing the BBB.

Materials and Methods

Rhodamine B loaded-PLGA 502 nanoparticles (RhNPs) were obtained through a solvent extraction-evaporation method from an O/W emulsion and later functionalized with glutathione via an amidic bond (Gl-RhNPs). NPs were characterized regarding size, encapsulation efficiency (EE) and, in vitro release. Male Wistar rats (225-270 g) were administered with RhNPs and Gl-RhNPs at a 50 mg/kg dose in the tail´s vein. Control animals received saline. After 1 hour, rats were sacrificed and their brain, liver and kidneys were extracted, frozen and, later analysed. Analysis was done by homogenizing the weighted organs with 5 mL CH_2Cl_2, they were then incubated for 1 hour and centrifuged at 6,000 rpm for 10 min. Fluorescence was measured at λ_{ex} = 555 nm and λ_{em} = 575 nm.

Results

Both NPs formulations showed homogenous sizes, around 200 nm, and low EE. Rhodamine B release after 1 hour was less than 15% for both formualtions. Animals receiving Gl-RhNPs showed an average amount of rhodamine B 3 times higher than those receiving RhNPs (Figure 1). Regarding liver and kidneys, the amount measured was similar, being slightly higher with Gl-RhNPs.

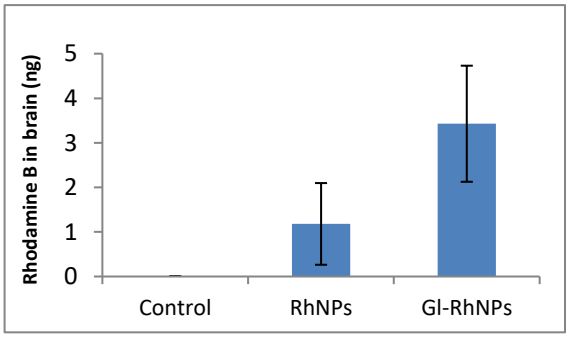

Figure 1. Average amounts of rhodamine B (ng) in the brain of male Wistar rats.

Discussion and Conclusions

Both NP formulations were able to reach the CNS. Taking into account that Rhodamine B cannot cross the BBB by itself [3] the passage of NPs indicates the potential of these nanosystems to facilitate the passage of drugs which exhibit difficulty to access to the CNS.

While further studies are required to reach definite conclusions, the glutathione functionalized PLGA-NPs showed better access to the CNS than the non-functionalized NPs, thereby resulting in an interesting approach for the development of functionalized drug delivery systems to treat CNS disorders.

Acknowledgments: This work was partially supported by the UCM research group "Formulation and bioavailability of new medications" (910939).

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Improving breast cancer conventional chemotherapy using CBD-PLGA-microparticles

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Introduction: In the last decades, cannabidiol (CBD) has attracted a great deal of interest in cancer disease, due to its ability to inhibit the proliferation, migration and invasion of cancer cells [1]. The anticancer activity of CBD as monotherapy is highly established in breast cancer. In other cancer types (like glioma or gastrointestinal malignancies) CBD has also shown to increase the activity of conventional chemotherapeutics [2]. However, this has not been evaluated in breast cancer. Despite the potential therapeutic interest of CBD, its high lipophilicity and stability problems hamper the development of effective formulations. Microencapsulation could resolve this challenge [3].

In this work, we evaluated, for the first time, the anticancer activity of CBD in solution and CBD-loaded-polymeric-microparticles (CBD-Mps) when combined with doxorubicin (DOX) and paclitaxel (PTX) in breast cancer models.

Materials and Methods: MCF-7 (oestrogen receptor positive) and MDA-MB-231 (triple negative) cells were selected as breast cancer models. Three treatment strategies were used to evaluate the *in vitro* effect of CBD in solution (CBD_{sol}) on PTX and DOX antitumor activity: i) pre-administration protocol to determine if CBD at suboptimal concentrations sensitises breast cancer cells (cells were pre-treated with CBD and then treated with PTX or DOX), ii) co-administration protocol to evaluate synergism (cells were treated with CBD+PTX or CBD+DOX) and iii) pre+co-administration protocol (cells were pre-treated with suboptimal concentrations of CBD and then with CBD+PTX or CBD+DOX). CBD-Mps were prepared by the oil-in-water emulsion–solvent evaporation technique using PLGA-RG-502 as polymer. Mps were characterised by determining particle size, morphology, drug loading and drug release. The antitumor efficacy of CBD-Mps as monotherapy or in combination with DOX and PTX was tested *in vitro* in both cells lines. Finally, MDA-MB-231 derived tumours grafted on the chorioallantoic membrane of the chick fertilised eggs (in *ovo* model) were used to evaluate the antitumor efficacy of this formulation administered either as monotherapy or in combination with PTX (pre+co-administration protocol).

Results: Regarding combination studies of CBD_{sol}**,** suboptimal concentrations of this cannabinoid (cell death <10%) sensitised both MCF-7and MDA-MB-231 cells to PTX and DOX, decreasing the inhibitory concentration 50 values of these conventional antineoplastics. The co-administration of CBD+PTX or CBD+DOX was also useful, showing an additive or synergistic effect in all tested combinations (combination indexes ≤1). Developed microparticles exhibited a particle size around 24μm, high encapsulation efficiency (94.62±4.62%), a CBD content of 8.601±0.42mg /100 mg Mps and a controlled drug release for a month. Administered as monotherapy, this formulation showed an extended antiproliferative activity for at least 10 days in both MCF-7 and MDA-MB-231 cells. Due to this long term antitumor activity, CBD-Mps allow the combination of both pre and -co-administration protocols with a single administration. In fact, following this strategy CBD-Mps significantly enhanced the antiproliferative activity of both PTX and DOX in MCF-7 and MDA-MB-231 cells; demonstrating a similar or even better efficacy than CBD_{sol} daily administered at the same concentration. Finally, CBD-Mps (single administered) also showed to improve significantly the *in ovo* antitumor efficacy of PTX, reporting a slightly better efficacy than CBD_{sol} daily administered. However, significant differences between both CBD treatments were not detected.

Conclusions: This works evidences for the first time the potential use of CBD in combination with PTX and DOX for the treatment of both hormonal receptor positive and triple negative breast tumours. Moreover, the use of CBD-Mps is of particular interest by optimising the antitumor activity of this agent.

Acknowledgments: This work was supported by Complutense Research Fund (Ref. FEI16/83) and by the Complutense University UCM research group "Formulation and bioavailability of new drug products" (Ref. UCM-910939). A.I. Fraguas-Sánchez has been granted with a research fellowship (Ref: FPU 14/06441) from the Spanish Ministry of Education.

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Influence of cholesterol fraction on the physic and biopharmaceutical properties of sildenafil citrate liposomes

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Introduction

Cholestrol (Chol) plays a relevant function of regulating the physical properties of liposomes by controlling the membrane fluidity and the mechanical strength of the lipid bilayer [1]. Besides, it has been reported that increasing the Chol reduces the ion binding and modifies the surface charge of the membrane [2]. According to this, the molar fraction of Chol in the lipid bilayer likely influences the biopharmaceutics of drug loaded into liposomes. The aim of this study was to evaluate the influence of Chol on the characteristics of sildenafil citrate (SC) liposomes and its effect on the in vitro drug delivery, using different biorelevant fluids.

Materials and Methods

SC liposomes were prepared from egg phosphatidylcholine (EPC) and Chol mixtures at different molar fraction (18%, 30% or 45%), by direct sonication of components, following a previously described method [3]. Multiple filtration through 0.20 µm was performed for size homogenization and a pH gradient was applied for remote drug loading. Drug entrapment efficiency (EE%), hydrodynamic diameter (Dh), polydipersion index (PDI) and zeta potential, as well as redox potential and turbidity of samples were determined. The composition of the lipid bilayer in liposomes prepared from different EPC and Chol mixtures where determined by enzymatic methods.

In vitro drug delivery assays were performed using biorelevant media representative of serum and broncalveolar fluid. Analytical quantification of SC in samples was preformed by HPLC. Different kinetic models were explored for data fitting of the in vitro drug delivery profiles.

Results and Discussion

Table 1 and figure 1 show the result obtained for liposomes prepared from different EPC-Chol mixtures.

% mol Cholesterol	EE (%)	Dh (nm)	PDI	Zeta (mV)
18	62.29± 0.20	173.6± 3.1	0.12± 0.03	-17.0± 11.1
30	54.90±11.43	206.0± 35.3	0.18± 0.09	-25.1± 18.3
45	55.02± 5.21	199.2± 14.2	0.14± 0.02	-12.7 ± 1.3

Table 1. Influence of EPC-Chol mixtures on the characteristics of liposomes obtained by direct sonication of components

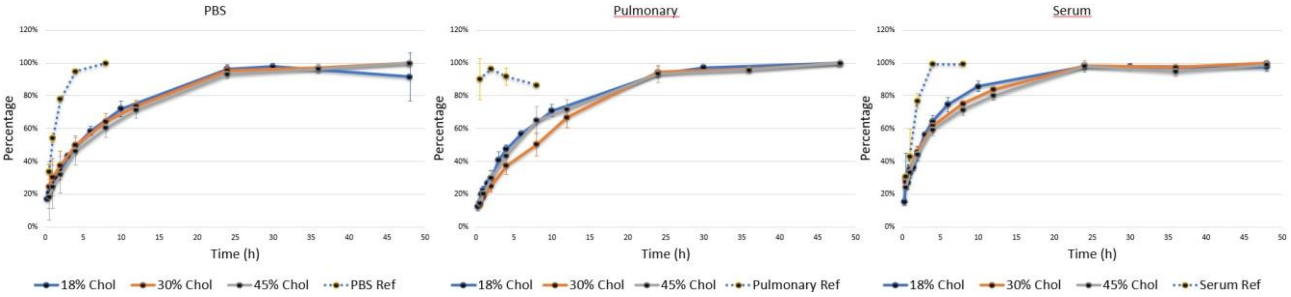

Figure 1. In vitro drug delivery profiles obtained using different biorelevant media

Conclusions.

Liposomes produced a sustained release of SC, irrespectively of Chol molar fraction in the lipid bilayer. The drug release exhibited the highest rate for plasma simulant fluid, followed by PBS and pulmonary simulant fluid.

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Influence of lyoprotective agents on the biocompatibility and cell internalization of liposomes

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Introduction

Lyophilization is the main approach used to extend the self-life of formulations based on liposomes, but this process involving freezing and drying may produce disruptive effects on their structure. Literature data provide information about the selection of lyoprotectants according to liposome composition or loaded drug [1], but studies on this topic considering the administration route proposed for the liposomal formulation are not available. In this study, liposomes for pulmonary delivery were produced. Since lactose (L) is the additive approved and most used for this administration route, the influence of L on the biocompatibility and cell internalization of liposomes was evaluated and compared to sucrose (S), a standard lyoprectant agent.

Materials and Methods

Liposomes were prepared from egg phosphatidylcholine (EPC) and Chol mixtures by direct sonication of components and filtration though 0.20 µm or 0.45 µm was performed for size homogenization. Unloaded liposomes (B), sildenafil citrate liposomes (SC) and coumarin liposomes (C) were prepared and analyzed. C was used as fluorescent probe. L or S (4% w/w) were added to SC and C liposomes to evaluate the influence of additives on biocompatibility (SC+L and SC+S) and cell internalization (C+L and C+S). Normal Human Dermal Fibroblasts (NHDF) were used as cell model to study the effect of B and SC liposomes with and without additive on cellular growth by the tetrazolium-based colorimetric assay (MTT) [2]. Confocal laser scanning microscopy (CLSM) was used to study the cell internalization of C liposomes with and without additive. Imaging analysis were performed with a Zeiss LSM710 laser scanning confocal microscope.

Results and Discussion

The Results from MTT assay (Figure 1) showed that B liposomes as well as SC liposomes, with or without additive, did not produce cytotoxic effects. When compared to control (K-), B and SC liposomes did not show statitical differences, irrespectively of size and additive used.

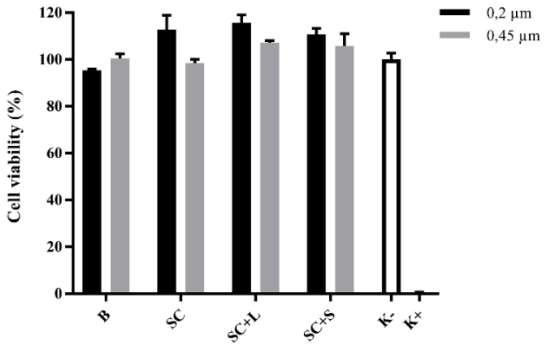

Figure 1. Biocompatibility of liposomes without additive (B and SC) and with lyoprotectant (SC+L and SC+S)

Regarding internalization, the images obtained by confocal laser microscopy from samples without additive and samples with L or S showed the ability of liposomes to enter the cell, irrespectively of size and the presence of additive.

Conclusions.

According to these results the use of lactose or sucrose for stability improvement of liposomes during the lyophilisation process is feasible, in terms of cell biocompatibility and internalization.

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Lipid Nanoparticles for Benznidazole Oral Delivery Targeting to the Lymphatic System to Treat Chagas Disease

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According to the WHO there are 8 million persons chronically infected by Chagas disease (CD) resulting in 12.000 reported deaths annually. CD is one of the most significant health problems of Latin America. However, nowadays it has been reported worldwide.[1]

Benznidazole (BNZ) is the only approved drug in US (30/08/2017) as the first option treatment for CD but can only administered for 14 days or less, while it is known that at least 2-3 months of treatment are required for full efficacy.[2] This approval for short-term CD therapy is due to the severe side effects associated with extended use, which severely impacts the applicability of BZ as a universal CD treatment.

CD is caused by *Trypanosoma cruzi* (*Tc*) parasite and their reservoirs have been localized into the Lymphatic System (LS).[3] Poor compound distribution into the LS could be the reason why extended BNZ treatment is required and why reactivation of the parasites in the chronic stage is commonly found.

Therefore, this project is developing BNZ formulations based on solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) to reduce compound exposure in system circulation to facilitate BNZ distribution into the LS. This could reduce BNZ efficacious dose (at present 5-7mg/kg/day), reduce its side effects, and possibly contribute with the treatment shortening.

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Liposomes as a tool to potentiate *in vitro* and *in vivo* the anti-tumor effect of new coordination compounds

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Introduction. Melanoma derives from the malignant transformation of melanocytes and it is known for its aggressiveness and poor prognosis since conventional treatments often fail [1].-Metal-based drugs have demonstrated great potential being also assumed that they might be less toxic for healthy cells, in comparison to tumor cells [2, 3, 4]. Therefore, following the area of research of Correia and co-workers [5], organic compounds and metallodrugs were synthesized and screened for their antiproliferative activities towards tumor cells. Three of them demonstrated high cytotoxic properties in human and murine melanoma cell lines. Aiming to improve their *in vivo* biodistribution profile, the selected compounds were incorporated in long-circulating liposomes with also pH-sensitive properties.

Methods. Dioleoyl phosphatidyl ethanolamine (DOPE), cholesteryl hemisuccinate (CHEMS) and distearoyl phosphatidyl ethanolamine covalently linked to Poly (ethyleneglycol) (DSPE-PEG) were selected for liposomes preparation [4]. Taking into account the hydrophobic properties of the selected compounds, a neutral fluid phospholipid, dioleoyl phosphatidylcholine (DOPC), was also included in the lipid mixture. Nanoliposomes were characterized in terms of incorporation parameters, mean diameter size and surface charge. The antiproliferative properties of the selected compounds towards tumor cell lines were evaluated in the free and liposomal forms by the MTT assay [4, 6]. The safety of developed liposomal formulations was also assessed by hemolytic activity in Red Blood Cells (RBCs) and following i.v. administration in healthy CD1 mice [3]. The proof of concept was evaluated in a xenograft murine melanoma model [4].

Results and discussion. The selected compounds were efficiently associated to liposomes with incorporation efficiencies (I.E.) ranging from 60 to 70%. Their mean size ranged from 100 to 135 nm which is crucial taking into account the fact that these carriers might be able to accumulate in regions of enhanced vascular permeability, characteristic of certain tumor pathologies. The cytotoxic properties of the compounds were preserved after incorporation in liposomes with IC_{50} values in the μM range (5 to 9 μM) particularly for one of the selected compounds. The absence of hemolytic activity evaluated in RBCs (< 5%) either in free or liposomal forms ensured their safety for parenteral administration. These results were also confirmed after i.v. injection of the most cytotoxic compound in healthy mice. The proof of concept, in a xenograft murine melanoma model in now under course.

Conclusions. The obtained results constitute a promising approach for melanoma therapy. The use of a pH-sensitive long-circulating liposomal formulation that is able to induce a pH-triggered release of the incorporated cytotoxic metallodrug, in acidic microenvironments such as the ones observed at tumor sites, constituted the rational of the present work.

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Mannosylated nanovaccines and inhibition of the immune-suppressing microenvironment sensitizes melanoma to immune checkpoint modulators

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Introduction: A low response rate, acquired resistance, and severe side effects have limited the clinical outcomes of immune checkpoint therapy [1]. Currently, it is broadly accepted that melanoma therapy will benefit from integrated complementary approaches, which can inhibit tumor immune-suppression and enhance immunity in an orchestrated manner. We hypothesized that the combinatorial therapy with anti-PD-1/anti-OX-40 (αPD-1/αOX40) – to inhibit tumor immune-suppression and to boost T-cell activity, respectively – could be improved by cancer vaccination, through an improved 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-targeted mannosylated cancer nanovaccines containing major histocompatibility complex class I and class II melanoma MART-1 antigens and Toll-like receptor ligands as immune adjuvants. Animals were immunized subcutaneously in two independent studies using two different melanoma models.

Results: Although the double combination of mannosylated nanovaccines with αPD-1/αOX40 resulted in higher T-cell infiltration into tumors at early stage, it did not enhance the inhibition of tumor growth compared to αPD-1/αOX40 alone, which was attributed to an increased infiltration of myeloid-derived suppressor cells. Combining the double therapy with ibrutinib, a myeloid-derived suppressor cell inhibitor, led to a remarkable tumor remission and a prolonged survival in melanoma-bearing mice. This trivalent combination showed a superior antitumor efficacy in two independent studies using two different melanoma models. More than two months since tumor inoculation, the survival was around 70% in the groups treated with the trivalent combination, whereas the groups treated with αPD-1/αOX40 and ibrutinib showed only about 20% of survival.

Conclusions: The synergy between the mannosylated nanovaccines, ibrutinib and αPD-1/αOX40 provides essential insights to devise alternative regimens to improve the efficacy of immune checkpoint modulators in solid tumors by regulating the endogenous immune response.

Acknowledgments: This work was supported by the Fundação para a Ciência e Tecnologia-Ministério da Ciência, Tecnologia e Ensino Superior (FCT-MCTES) and the Israeli Ministry of Health under the framework of EuroNanoMed-II. The project also received support from the Melanoma Research Alliance, the European Research Council (ERC), the Israel Cancer Research Fund, and the European Structural & Investment Funds through the COMPETE Programme and the National Funds through the FCT-MCTES. This work was partially funded by "la Caixa" Health Research 2018 call, Project proposal HR18-00589.

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Microencapsulation of Infliximab in a PEOT/PBT multiblock copolymer

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Introduction. Systemic administration of Infliximab in rheumatoid arthritis results in high efficacy and maintenance of clinical remission, nevertheless, its adverse effects limit it use as first-line therapy. Moreover, local administration of antibodies by intraarticular injection has showed a high clearance rate from the joint[1]. Microencapsulation could be an excellence approach to overcome these limitations due to the increase the retention time of the formulation into the joint and its prolonged release of the drug in the site of action [2]. Double emulsion/solvent evaporation is one of the most used methods to prepare microcapsules whereas ultrasonic atomization has not been so widely explored. Nonetheless, due to its mild conditions, ultrasonic atomization seems to be an excellent methodology to microencapsulate proteins and labile drugs preserving its stability[3]. This work aims to fabricate infliximab-loaded microcapsules made of poly(ethylene-oxide-terephthalate)-poly(butylene-terephthalate)(70%PEOT/30%PBT) copolymer using double emulsion/evaporation method and ultrasonic atomization, in order to compare the formulations features (particle size, encapsulation efficiency, *in vitro* delivery profile, infliximab stability) after microencapsulation using both methods.

Materials and Methods. Microcapsules were fabricated using a dual feed coaxial ultrasonic nozzle. An infliximab aqueous solution and a polymeric dispersion in CH_2Cl_2 were infused through the inner and outer channel, respectively. Then, microdrops were collected over a 1.5 % PVA stirring solution. Subsequently, solvent was removed and microparticles were isolated. Moreover, microcapsules were prepared following a modified double emulsion/evaporation method. In short, 1 ml of an Infliximab solution was emulsified with 5 ml of PEOT/PBT dispersion in CH_2Cl_2 for 3 minutes. Then, the primary emulsion was emulsified for 10 minutes in PVA 1,25 % and solvent was removed at room temperature. Finally, microcapsules were collected by centrifugation and lyophilized. Microparticle formulations were characterized in terms of particle size (light scattering), morphology (SEM), encapsulation efficiency, release and physical state of the drug and polymer after microencapsulation (DSC and FTIR). Structural stability of infliximab after microencapsulation was analysed by SDS-PAGE and second derivative UV spectroscopy. Cell compatibility of infliximab-loaded and empty microcapsules was tested in RAW 264.7 macrophages.

Results and Discussion. Process yield of the microencapsulation process was higher for ultrasonic atomization (84.2–94.5 %) than for double-emulsion method (68,7–79,2 %). Regarding about particle size, there are a great difference between both formulation process. Whereas the ultrasonic generated microcapsules have a mean size of 37.962±0.110 μm, microcapsules obtained by double emulsion/evaporation method shown a narrower distribution with a mean size of 1,393±0.002 μm. An optimal formulation with an encapsulation efficiency of 80.2% could be obtained by ultrasonic atomization. SEM micrographs showed a core-shell structure and a spherical and smooth surface. Infliximab-loaded and blank microcapsules, polymer and infliximab were analysed by FTIR and DSC showing the absence of physical changes in the polymer conformation after microencapsulation process. SDS-PAGE showed a single band at 150 KDa under non-reducing conditions and two bans at 50 and 25 kDa under reducing environment, confirming structural integrity of Infliximab. No shift of second-derivative UV spectrum was observed for the peaks corresponding to phenylalanine and tyrosine/tryptophan, suggesting that tertiary structure of loaded-infliximab was preserved after microencapsulation process. *In vitro* release followed the Korsmeyer-Peppas kinetic model ($Q=0.04787*t0.346$;$R^2=0.9990$), suggesting a Fickian-difussion release. After an initial burst of over 6% in the first 1.5 hours, 60.3% of loaded-infliximab was delivered in a sustained manner for 2 months. High cell viability rates (95-100%) were achieved after 24 h of incubation with microcapsules. Actually, further studies are in progress to characterize the microcapsules prepared by double-emulsion/evaporation process.

Conclusions. Biocompatible infliximab-loaded microcapsules were successfully obtained by ultrasonic atomization with suitable features for intra-articular administration in terms of morphology and particle size. Further analysis should be performed in order to verify that infliximab maintains its structure and biological activity after microencapsulation.

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Morin-loaded PLGA-phe2 nanoparticles: Accessing the CNS?

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Introduction:

Flavonoids like morin could be used to prevent cellular damage caused by reactive oxygen species (ROS) due to their antioxidant capacity. This together with their anti-inflammatory effects makes them potential candidates to treat neurological disorders such as Alzheimer´s disease [1]. However, flavonoids and other polyphenols experience rapid biotransformation and exhibit low capacity to cross the blood-brain barrier (BBB) [2]. Polymeric nanoparticles (NPs) are a good strategy to increase their passage through the BBB as well as the attachment to their surfaces of different molecules, like phenylalanine, which exhibit affinity for cerebral tissues [3]. In this work we have studied the biodistribution in rat brain and other organs of two new morin-loaded PLGA nanoparticle (Np) formulations prepared with and without functionalization with phenylalanine (phe2).

Materials and Methods:

Two Np formulations (M-NP and M-phe2-NP) were prepared with PLGA 502 by the solvent evaporation method and loaded with morin hydrate. PLGA in formulation M-phe2-NP was bound to phe2 by carbodiimide reaction before the preparation of the NPs. Characterization of NPs was carried out by determining particle size, encapsulation efficiency (EE), in vitro release and tissue biodistribution.

For biodistribution studies the same NP formulations were prepared but loaded with rhodamine B (Rh-B) (R-NP and R-phe2-NP). These studies were performed in male Wistar rats (225-270 g) with administration of the NPs in the rat tail's vein. Animals were sacrificed 1 h and 2 h after administration. Quantification of the fluorescence tracer (Rh-B) was performed in liver, brain and kidney samples.

Results and discussion:

Both morin formulations showed similar sizes; 190-200 nm for M-NP and 180-196 nm for M-phe2-NP, respectively being adequate for crossing the BBB. EE of both morin-loaded NP formulations was around 80%. In the biodistribution studies, brain measurements showed fluorescence with both formulations. Taking into consideration that Rh-B cannot cross the BBB by itself [4], the measured fluorescence can only be attributed to the presence of NPs in the brain, where the levels of formulation R-phe2-NP are 3.3 times higher than those achieved by R-NP at 1 hour, and 3.1 times higher after 2 hours. However, the high variability found with formulation R-NP (with very low fluorescence in some samples), does not allow us to reach conclusive results for this formulation. As expected, Rh-B levels found in the liver were the highest as it accumulates in this organ [4]. For this, the fluorescence detected probably corresponds to both encapsulated and free Rh-B. Neither liver nor kidney samples showed significant differences between R-NP and R-phe2-NP formulations.

Conclusions:

NPs prepared with PLGA and functionalized with diphenylalanine peptide (phe2) can improve the passage of drugs such as morin across the BBB. Morin-loaded PLGA NPs functionalized with phe-2 showed high EE and adequate particle size distribution resulting in suitable carriers to search for neuroprotective effects.

Acknowledgments: This work was supported by the UCM research group "Formulation and bioavailability of new medications" (910939).

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New polymeric nanocomplexes against glioblastoma initiating cells

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Most tumors have a population of cells resistant to conventional antitumor treatments. These cells, which are also capable of regenerating a tumor, are called cancer stem cells (CSCs) or tumor-initiating cells. Their main characteristics are dedifferentiation, self-renewal, heterogeneity, migration and the ability to metastasize. To eliminate this population, drugs such as rapamycin, cyclopamine or trans-retinoic acid are currently been used in the clinics or investigated in clinical trials. Besides, gene therapy strategies aimed at modulating the genes involved in CSC maintainance are currently being investigated. These gene therapies were most commonly delivered by viral vectors, but due to their immunogenicity, these are being replaced for polymeric vectors [1].

The objective of our work was to design and synthesize new polymeric vectors for gene therapy. Different polymers based on polyphosphazenes have been synthesized using click-chemistry, as previously reported by our group [2]. The polymers were characterized by ^{31}P, ^{1}H NMR, COSY and HSQC. The complexes were prepared by electrostatic interaction between the polymers and a model nucleic acid that induce enhanced green fluorescent protein and luciferase expression when it is transfected. Two types of particles were obtained: one composed by cationic polymer and nucleic acid and another composed by cationic polymer, anionic polymer and nucleic acid. The nanocomplexes were characterized for their particle size (by Dynamic Light Scattering), surface charge (by laser doppler anemometry) and plasmid association (by an electrophoresis migration assay). Their toxicity and transfection in human glioblastoma cells (U87MG) were tested in vitro by the MTS and a luminescence assay, respectively. Once the formulation was optimized, the plasmid was substituted by a Bone Morphogenic Protein-4 (BMP-4) encoding plasmid, which induces the differentiation of CSCs to less malignant phenotype. Their efficacy was tested by clonogenicity assay in two glioblastoma cell lines.

All the prototypes obtained showed a size of around 100 nm, positive charge (+40mV) and efficient association of the nucleic acid. In vitro tests demonstrated that the association of the anionic polymer improves, significantly, the toxicity and the transfection as compared to the naked plasmid and the systems composed of only cationic polymer and the plasmid. The two cell lines treated with the nanocomplexes inducing a BMP-4 transgene showed a reduction in cell clonogenesis as compared with the untreated cells, indicating a possible biological effect reducing the CSCs traits.

In conclusion, the nanocomplexes that combine cationic and anionic polymer showed a good transfection in tumor cells, and when loaded with a BMP-4 expressing plasmid, can drive CSC differentiation towards a less aggressive phenotype.

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Novel liposomes for therapy of neurodegenerative diseases

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Introduction: One of the most worrying problems from our ageing society is early onset of age-related neurological diseases, either due to external environmental or genetic predisposition [1]. These diseases mostly develop in the brain [2], which implies that therapeutic molecules must overcome the blood brain barrier (BBB). Curcumin possesses neuroprotective and anti-inflammatory properties [3], along with a capability to be a metal ion chelator and inhibit the deposition of amyloid beta [4], features of Alzheimer's disease. This phytochemical cannot however cross the BBB, is not stable in the bloodstream and is cytotoxic at high concentrations [3]. Liposomes can encapsulate lipophilic molecules like curcumin, and deliver them directly to the brain, concentrating these bioactive compounds, with little to no setbacks [5].

Materials and Methods: A novel type of liposomes (Exo-Lip), with a mixture of phospholipids mimicking the natural-occurring exosomes' lipid composition, was produced and characterized by DLS (size, surface charge), composition (FTIR). Its capacity to encapsulate curcumin was quantified by taking advantage of its inherent fluorescence. Cytotoxicity was evaluated in two different cell lines (resazurin conversion assay and trypan blue exclusion assay), hemolysis was evaluated by absorbance using pig erythrocytes and whole organism toxicity was studied with zebrafish embryotoxicity assays. Neuroprotection was evaluated *in vitro* using a human neuronal cell line.

Results and Discussion: Exo-Lip nanocarriers have adequate physicochemical properties to cross the human BBB and are not cytotoxic to tested cell lines. They were compared to a more studied liposomal system constituted of DODAB and monoolein (MO). Moreover, neuronal cells uptake encapsulated curcumin, which had a neuroprotective effect, by reducing ROS production and increasing cell viability after t-BHP insult. Furthermore, encapsulated curcumin was effectively internalized in zebrafish embryos, an informative model system, with little side effects.

Conclusions It was possible to successfully produce biocompatible, bioactive nanocarrier systems for delivery of lipophilic curcumin, mimicking the natural-occurring exosomes' lipid composition.

This strategy will potentially contribute to reduce disease progression and alleviate its symptoms, providing a better life for both the patient and the caregivers.

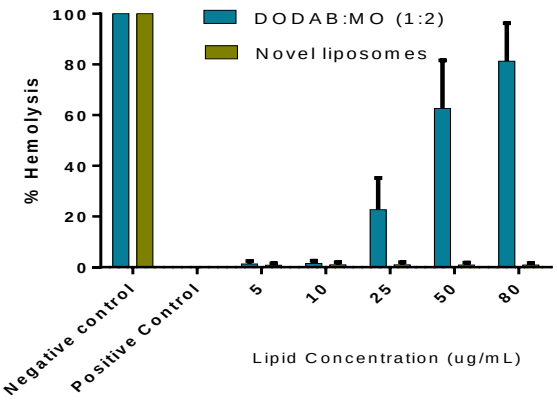

Figure 1. Comparison of hemolysis induced by DODAB:MO and Exo-Lip liposomes

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Optimization of Three-Dimensional Breast Cancer Culture Platforms for the Advanced Preclinical Evaluation of Polymer Therapeutics

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The conjugation of anti-cancer drugs to polymers [1] provides particular advantages when compared to the "free" form of the drug, such as enhanced passive tumor targeting and altered pharmacokinetics [1] thanks, in part, to the enhanced control over biodistribution and drug release. Polymer therapeutics-based approaches can take advantage of microenvironmental stimuli, such as pH, redox status, and the elevated levels of certain enzymes, to promote the specific release of drugs from the polymer backbone at the tumor site [2]. *In vitro* characterization of polymer therapeutics commonly employs traditional two-dimensional (2D) cell cultures; however, these platforms do not fully recapitulate important *in vivo* tumor characteristics, including nutrient and oxygen gradients, cell-cell interactions, and cell-extracellular matrix interactions [3]. Therefore, we have developed optimized three-dimensional (3D) breast cancer culture systems as a platform for the robust characterization of novel anti-cancer polymer therapeutics.

We established and optimized patient-derived breast cancer organoid and spheroid cultures [4] derived from four different breast cancer subtypes growing in basement membrane matrix extract (BME) (See Figure 1). We characterized breast cancer subtype-defining receptors (ER, PR, ErbB2) by Western blotting in organoids and spheroids, monolayer-cultured cells, and primary tumor tissue. As part of the characterization, we also compared the levels of reactive oxygen species (ROS) and the lysosomal cysteine protease cathepsin B between 2D and 3D cell line-derived cultures. We also optimized the use of BME-embedded organoids and spheroids to study toxicity using a polyglutamic acid (PGA) conjugate of the conventional chemotherapeutic agent doxorubicin (PGA-Dox) that employs an acid cleavable hydrazone linker, and internalization profiles of polymer therapeutics by confocal microscopy using fluorescently-labeled unconjugated PGA.

Our current data confirm the maintenance of ER, PR, ErbB2 expression in breast cancer spheroids compared to monolayer cultures; however, 3D cultures of MCF7, MDA-MB-453 and ZR75 cell lines displayed significantly (p-value < 0.05) increased ROS levels compared to monolayer cultures, while cathepsin B levels tended to decrease (p-value 0.09) in 3D ZR75 cultures when compared to monolayer culture. We observed the internalization and lysosomal colocalization of PGA-Dox in spheroids and determined an IC50 value 10-fold higher in 3D culture than in 2D culture for PGA-Dox and 6.5-fold higher for free Dox.

Our findings highlight the need to employ 3D culture conditions to generate robust characterization data for polymer therapeutics-based anti-cancer strategies. We now aim to apply our 3D models derived from cell lines and patient samples to test both single-drug and combination in the hope that this platform will accelerate clinical translation.

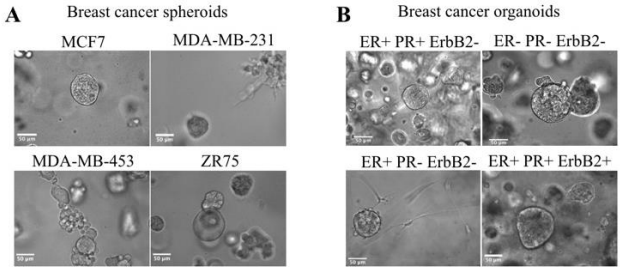

Figure 1. Representative images of breast cancer 3D models. A. Cell-line derived breast cancer spheroids. Scale bar = 50 μm. B. Patient-derived breast cancer organoids. Scale bar = 50 μm

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Peptide-associated nanomedicine to interfere senescence escape and avoid relapse in breast cancer

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Introduction: Cellular senescence can be identified by the irreversible arrest of cell cycle due to potential oncogenic stress. However, senescent cells induced by chemotherapy can escape this state to a more persistent form of cancer cells, which have been linked to cancer resistance to treatment and cancer relapse [1]. Therefore, our research has been focusing on exploring several biological compounds that can prevent the senescence escape or eliminate the accumulated senescent cells from the body. We have previously explored the role of the peptide 4N1K for prevention of senescence escape and studied the mechanism of action [2]. Due to the biopharmaceutical limitations of peptide delivery, we proposed here the association of 4N1K to biodegradable and biocompatible nanosystems. We selected for this purpose emulsions composed by vitamin E and sphingomyelin (SM). These sphingomyelin nanosystems (SNs) are highly stable overtime and in different biological fluids.

Materials and Methods: 4N1K was modified with a C18-PEG-COOH chain to increase its hydrophobicity and integration in SNs. Peptide-associated 4N1K nanosystems (SNs-Ks) were prepared by the ethanol injection method and characterized for their physicochemical properties through Dynamic Light Scattering (DLS), Laser Doppler Anemometry (LDA), Nanotrafficking Analysis (NTA), Multi-angle light scattering (MALS) and Atomic Force Microscopy (AFM). The efficient association of 4N1K was determined by High Pressure Liquid Chromatography (HPLC). *In vitro* studies were carried out in breast cancer MCF7 proliferative and senescent cells, to determine the activity of the proposed nanoformulation. Chemotherapy induced senescence (CIS) was generated through treatment of breast cancer cells MCF7 with Doxorubicin for 96h. After that, cell emergence was induced using 10% FBS serum. Cellular uptake of SNs-Ks was assessed by confocal microscope and flow cytometry after incorporating of fluoresent Top-Fluor SM into the formulation. Moreover, the stability of prepared formulation was evaluated in different biological fluids.

Results and discussion: SNs-Ks were successfully prepared with a mean size 106.1 ± 18.4 nm, PdI of 0.2, a +54 ±3.9mV zeta potential, and have high association efficiency (81.65% ±2.32). MALS and NTA results have a close similarity with results obtained by DLS, proving the monodispersity of the prepared nanosystems. The size of SNs-Ks grows slightly in RPMI with 1%FBS however remain in the acceptable range (271 ± 9 nm). Flow cytometer results show 83% of cells uptake the SNs-Ks after only 10 minutes incubation, and confocal images were also obtained proving a higher internalization of SNs-Ks with respect to SNs, which could be due to the presence of the peptide at the surface. Regarding the activity of SNs-Ks, they induced an effect in proliferating and senescent cells at a concentration 10 times less compared to the peptide alone.

Conclusions: Small and monodisperse nanosystems decorated with 4N1K peptide were successfully prepared and prevented senescence escape in MCF7 cells through their senolytic effect.

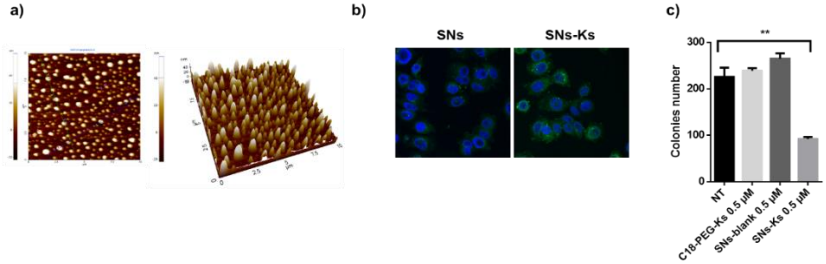

Figure 1. a) AFM photos of SNs-Ks in 2D and 3D. b) Cellular uptake observed under the confocal microscope of SNs (green channel) upon 2h incubation at 37ºC in MCF7 breast cancer cells (cell nuclei stained with DAPI, blue channel). c) MCF7 colony forming assay; cells were treated or non-treated (NT) with SNs to examine their toxicity.

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Peptide-decorated nanosystems for the targeted delivery of anticancer drugs

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Introduction Cancer has become a major public health problem, positioning itself as the second leading cause of death in the world [1]. Improving the way in which therapeutic molecules are delivered to the tumor is one of the major challenges today to get more efficiency anticancer therapies [2]. Surface-decoration with peptides is a promising alternative to improve the access of nanocarriers and the associated therapeutics to the cancer cells. In this work, we propose the functionalization of a biocompatible type of nanostructures, sphingomyelin nanosystems (SNs) with two peptide ligands able to interact with specific cell surface biomarkers. We aim to improve the interaction of SNs with the targeted cells and to prove the versatility of our approach for the development of targeted nanopharmaceuticals.

Materials and methods Uroguanylin (UroG, 16 aminoacids), a natural ligand of the Guanylyl Cyclase C (GCC) receptor constitutively expressed in primary and metastatic colorectal cancer tumors and a peptide fragment from the leptin hormone (LAPIK, 5 aminoacids) with antagonist function over the leptin receptor (LepR) similarly overexpressed cancer cells, were selected to decorate the nanocarrier. In order to facilitate the peptide incorporation into the nanosystem both peptides, UroG and LAPIK, were modified with a hydrophobic chain (C_{18}-PEG_{12}-COOH and C_{18}-PEG_6-COOH, respectively). While LAPIK was custom synthesized by a biotechnology company, UroG conjugation was conveniently designed by creating a covalent link to the hydrophobic chain through an amide linker. Proper characterization for the UroG derivative (UroGm) was performed by HPLC, NMR, and MALDI-TOF techniques. Decorated nanosystems (LAPIK-SNs and UroGm-SNs) were prepared by the ethanol injection method and fully characterized (in terms of size, surface charge, and particle concentration) using a Zetasizer NanoZS® and a Nanosight NS3000 System. The possibility to upload the targeted nanosystems with a cytostatic drug (etoposide) was additionally evaluated and its encapsulation was analyzed by HPLC. Morphologic characterization was assessed by Scanning Transmission Electron Microscopy. *In vitro* studies were carried out in comparison with the non-decorated SNs to confirm the targeting activity of both peptide ligands.

Results UroG conjugation to the hydrophobic chain (UroGm) was successfully achieved and confirmed by NMR, MALDI-TOF and HPLC (with approximately 75% conjugation yield). Both peptide derivatives, UroGm-SNs and LAPIK-SNs, were efficiency associated to SNs, confirmed by NMR and HPLC respectively, and the resulting surface-decorated nanocarriers show a nanometric size below 150nm, a narrow distribution, and a negative surface charge. Internalization assays clearly suggest, in both cases, an improved internalization due to the functionalization of SNs with the ligands (Fig 1 a,b). Therapeutic activity of UroGm was analyzed *in vitro* showing a dose-dependent effect directly related to the decrease in proliferative capacity.

Conclusions Overall, we have surface-decorated our nanosystems with two different peptides that specifically binds cell surface receptors. In both cases, an improved accumulation of the functionalized nanosystems was observed upon incubation with the metastatic colorectal cancer cell line SW620. In conclusion, our results indicate that SNs can be successfully decorated with peptides as a targeting strategy for the development of more efficient anticancer therapies.

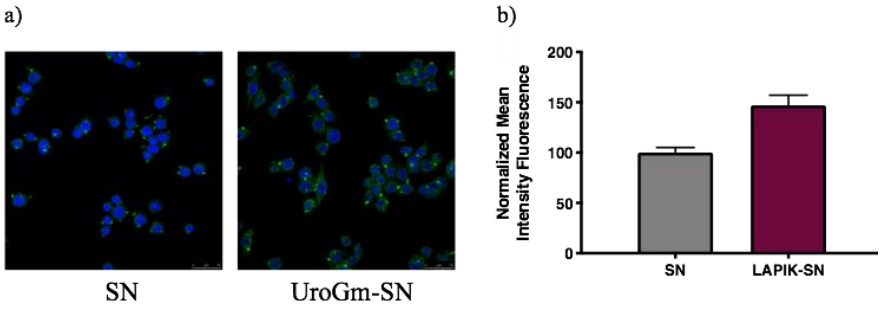

Figure 1. (a) Confocal microscope images showing the improvement in the internalization for the decorated nanosystems. (b) Increased in the internalization of the LAPIK-SN determined by flow cytometry.

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Precision targeting of colorectal cancer cells using YIGSR laminin peptide

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Introduction

Cancer cells from various sources have been reported to express high levels of the 67 kDa laminin receptor, the entity responsible for the interactions between cells and laminin of the extracellular matrix [1]. Specifically, the pentapeptide TyrIle-Gly-Ser-Arg (YIGSR), derived from laminin b1 chain, has been identified as the laminin binding site to its cell surface receptor. The CMCht/PAMAM can successfully act as drug delivery vehicles, tracking systems (attaching FITC) and be engineered to show specific moieties at the surface [2].We hypothesized that attachment of YIGSR on the surface of previously developed CMCht/PAMAM dendrimer nanoparticles (NP) would lead to their preferential uptake by metastatic cancer cells over fibroblasts or endothelial cells.

Materials and Methods

CMCht/PAMAM previously synthetized by our group were modified using simple EDC chemistry. After synthesis, nanoparticles were characterized using TEM. Sizes and surface changes of all nanoparticles were measured using zeta potential and DLS technologies. The specific internalization of peptide-linked CMCht/PAMAM nanoparticles by colorectal cancer cells' was assessed in standard 2D culture flasks in a co-culture of live stained HCT-116 cancer cells (RED) and L929 fibroblasts (BLUE).

Results and Discussion

Herein, we describe a simple method to covalently link the YIGSR peptide to our dendrimer nanoparticles. Characterization techniques such as DLS show that the size of the modified nanoparticles increased when compared to the non-modified nanoparticles, with an increase from 54 to 130 nm (average). Also, the surface charge measured with Zeta Sizer were less negative compared to the unmodified NPs, indicating a reduction in the number of carboxylic acid groups on NP surface following peptide binding as well as the addition of the positively-charged arginine of the peptide sequence. Regarding NP's internalization, confocal studies after 24h of culture suggest in fact a targeted internalization by cancer cells, possibly overexpressing the laminin receptor. Quantitative studies as well as 3D studies are ongoing to confirm these promising findings.

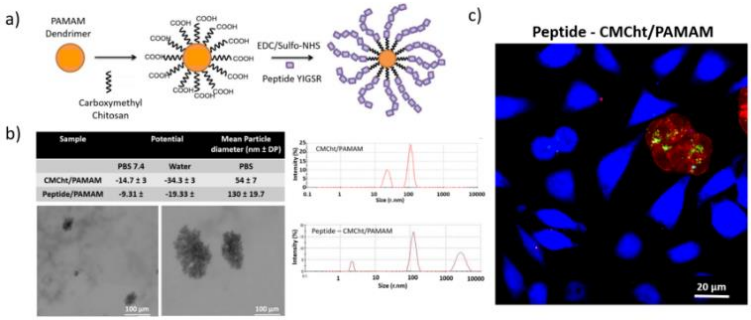

Figure 1. a) Schematic representation of CMCht/PAMAM dendrimer modification with the peptide; b) Characterization results of DLS, Zeta Sizer and TEM; c) Confocal microscope image of targeted internalization of CMCht/PAMAM nanoparticles in a co-culture of L929 fibroblasts (BLUE) and HCT-116 cancer cells (RED).

Conclusions

The preliminary results confirm the successful modification of CMCht/PAMAM dendrimer nanoparticles with the peptide using simple EDC chemistry. Confocal images endorse the possibility of preferential internalization of peptide-modified NP in HCT-116 cancer cells. Moreover, we hope to improve the therapy efficiency for disseminated metastatic cancer without the currently drawbacks of low drug accumulation in target sites.

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Quantification of the coating polymer on nanocapsules for drug delivery via Asymmetric Flow Field-Flow Fractionation

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Introduction

The surface properties of nanocarriers are crucial in determining many of their characteristics, including their stability, specificity, maximum drug loading and ability to cross biological barriers. Despite its importance, accurate studies of the coating polymers are hindered by the lack of adequate analysis techniques. Asymmetric Flow Field-Flow Fractionation (AF4) is a separation and characterization technique whose popularity in the field of nanomedicine [1] has rapidly increased in the last few years, thanks to its gentle separation conditions, broad working range and versatility. Here AF4 will be applied to the characterization of the polymeric coating of several nanocapsules, determining its coverage and stability in different experimental conditions.

Materials and Methods

Three different nanosystems have been studied, namely Self-Emulsifying Nanocapsules (SE-NC), Solvent-Displacement Pegylated Nanocapsules (SD-Peg-NC) and Solvent-Displacement non-Pegylated Nanocapsules (SD-NC). The nanosystems were prepared with methods previously described in the literature [2], [3]. Different combinations of coating polymers have been tried. The unreacted polymer was separated from the coated nanocapsules by AF4 and quantified by RI detection, adapting an existing method [4]. Fluorescent labelling of the polymers with off-line detection was used to reduce the LOD of the technique.

Results and Discussion

The fractionation method has to be carefully devised in order to permit the quantification of the unreacted polymer while, at the same time, prevent the dissociation of the bound one. Under the right conditions, an efficient baseline separation could be achieved in most of the systems studied (v. Figure 1).

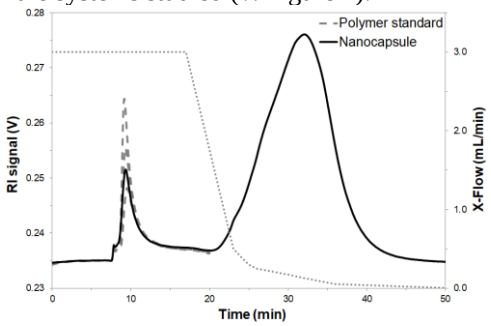

Figure 1. Fractograms of polymer-coated nanocapsules (solid line) and polymer standards (dashed lines). The polymer elutes at 10 min, the nanocapsules at 20-40 min. The corresponding Cross-Flow program is superimposed to the curves (dotted line).

The composition of the incubation and fractionation media is of the uttermost importance in the stabilization of the polymer coating, whereas the size and shape of the nanocapsules are generally constant, independently from the medium. While RI detection provides satisfying results in most cases, the use of fluorescent labels proved necessary in the characterization of particularly complex formulations.

Conclusions

AF4 can be successfully applied to the quantification of the coating polymer in nanocapsule formulations for drug delivery. The analysis method developed here can be adapted to characterize different kinds of nanosystems. First attempts at relating the stability of the polymer coating to both the composition of the nanocarrier and the external medium were made. As a future perspective, the information retrieved by AF4 about the surface properties of the nanocarriers could be used to predict their biological fate.

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Reprogramming Tumor Associated Macrophages to treat Lung Cancer with Immunostimulant-loaded nanocapsules

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Introduction: Among different immune cells in the tumor microenviroment, tumor-associated macrophages (TAM) are the most abundant cell type. These cells present an immunosuppressive phenotype with pro-tumoral functions (M2-like) [1]. Certain immunostimulants are known to repolarize M2-like into antitumor macrophages (M1), but also to boost strong immune responses, which may cause systemic toxicity. To avoid undesired effects, these drugs can be encapsulated into nanocarriers, to direct them straight towards TAMs. In this work, we have co-encapsulated two different immunostimulants in the same nanostructure. Seven prototypes of nanocapsules (NCs) with the same oily core but with different polymeric shells were evaluated regarding their toxicity and *in vitro* ability to repolarize macrophages. Finally, a preliminary *in vivo* study was done in mice to evaluate the capacity of these nanosystems to treat lung cancer.

Materials and Methods: NCs were produced by solvent displacement technique by injecting an ethanolic phase into an aqueous phase [2]. Seven different polymers were tested to analyze the influence of the polymeric shell in their *in vitro* interaction with cells. *In vivo* studies were carried out in murine heterotopic lung cancer model (CMT167) with the cells injected subcutaneously. The drug-loaded NCs were injected intratumorally.

Results: NCs showed a particle size in the range of 85-150 nm and zeta potential between -48 and +62 mV, varying according to their composition. The encapsulation of the lipophilic drug was in the range of 7-19 % for the different prototypes. The systems were stable under storage conditions, in the fridge, for at least six months and in cell culture media for 72 hours.

Primary human macrophages M0, exposed to non-toxic doses of NCs loaded with one immunostimulant, acquired a M1-like phenotype, releasing significant levels of pro-inflammatory cytokines, such as IL-6, CCL5 or CXCL10. At lower doses, five out of seven prototypes showed better results than the free drug, with a tendency of the positively charged prototypes to increase CXCL10 production. A new functional assay of macrophage ability to kill cancer cells upon treatment with the nanosystems shows a higher efficacy for two out of the three positively charged NCs.

For the *in vivo* experiments, we have compared the antitumoral efficacy of NCs loaded with one and two different immunostimulants, to explore the synergistic effects. These NCs loaded with two immunostimulants showed slightly increased particle size and decreased zeta potential in comparison with non-loaded NCs. In terms of efficacy, the NCs loaded with a combination of two immunostimulants showed the highest inhibition of the tumor growth.

Conclusions: Immunostimulants-loaded NCs showed the capacity to repolarize TAM and to reduce tumor progression when injected intratumorally in a murine lung cancer model.

Acknowledgments: This work was supported by MINECO- PCIN-2017-129/ AEI, under the frame of EuroNanoMed III (2-INTRATARGET project), the Worldwide Cancer Research, UK, and the AECC ("Asociación Española contra el Cáncer, Spain). Our research group has the recognition as Competitive Reference Groups, Consellería de Educación e Ordenación Universitaria, Xunta de Galicia, Ref: ED431C 2017/09. Carmen Fernández Varela acknowledges a predoctoral grant from the Spanish Ministry of Science, Innovation and Universities (grant reference PRE2018-086253).

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Shape-defined PLGA microPlates depot for the local delivery of anti-inflammatory molecules

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Biomaterial depots represent an attractive alternative to systemic delivery in that may allow to increase the drug in the target site, reduce toxicity, minimize the therapeutic dose and number of administrations [1]. Over the years, nanoparticles, microparticles, implants of poly(D,L-lactide-co-glycolide (PLGA) have been demonstrated for diverse biomedical applications. Yet, initial burst release and optimal modulation of the release profiles limit their clinical use. In general, larger particles achieve longer release profile because they have the tendency to form a stable depot in the injection site and can store large amounts of therapeutic agents [2]. Here, a top-down approach is employed for synthetizing shape-defined PLGA microPlates (μPLs) for the sustained release of two anti-inflammatory molecules, namely curcumin (CURC) and dexamethasone (DEX). Two different shape-defined PLGA particles with a 20 x 20 μm square base, and height of 5 μm (short μPLs) [2] or 10 μm (tall μPLs) were developed. Different amounts of PLGA were used during the synthesis in order to modulate the Young's modulus and mitigate the initial burst drugs release, without affecting the μPL geometry. The modulation of Young's modulus is a fundamental feature in tissue depots for promoting μPLs integration with the surrounding tissue (see **Figure 1**). Short μPLs, realized with 5 mg of PLGA, and tall μPLs, realized with 15 mg of PLGA, show a stiffness value of about 2.08 ± 0.5 annd of 3.1 ± 0.9 MPa, respectively,.similar to cartilage [3]. The fine-tuning of the μPL geometry and PLGA amounts provides the opportunity to modulate the diffusion of CURC and DEX out of the μPL matrix, with a continuous release up to 10 days. The anti-inflammatory activity of μPLs loaded CURC and DEX was tested *in vitro* on LPS-stimulated rat macrophages (BMDMs). Results showed that both drugs released from μPLs reduced the expression of the inflammatory cytokines on BMDMs at both concentrations tested. CURC loaded μPLs (CURC-μPLs) were applied on skin burn murine model. A single application of CURC-μPLs promoted a fast recovery after UVB irradiation compared to multiple free CURC applications. On the other hand, *in vivo* DEX loaded μPLs (DEX-μPLs) therapeutic efficacy was assessed in a mechanically-induced Osteoarthitis mouse model. A single intra-articular injection of DEX- μPLs holistically protected both the articular cartilage and the broader synovial health, through 4 weeks of rigorous mechanical overloading of the joints. In addition, it was observed that μPLs alone can facilitate the protection the articular cartilage surface by acting as cushion. In conclusion, a top-down fabrication strategy allows us to synthetize shaped-defined μPLs for both the mechanical protection and the sustained delivery of anti-inflammatory molecules, by simply tailoring their geometry (particle height) and PLGA density.

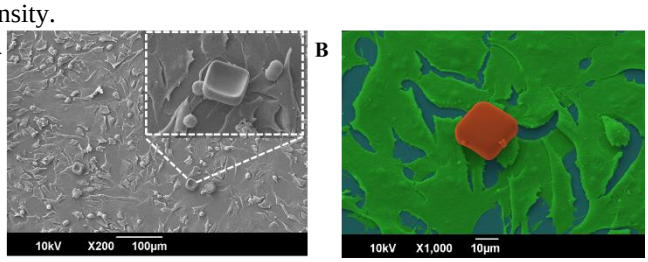

Figure 1. A 30° tilted view of a SEM image of chondocytes incubated with μPLs. In the lateral inset, a magnified image shows cells interacting with μPLs; B. false-color SEM image of a μPL (red) deposited and not internalized over a layer chondrocytes (green)

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Targeted nanoparticle-based vaccine as a powerfull platform to improve immune modulation therapy in colorectal cancer

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Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the fourth cause of cancer death worldwide. This work is focused on the development of a multifunctional CRC immunotherapy based on a nanoplatform rationally designed to deliver combinations of neoantigens and immune regulators to modulate host immunity against CRC.

Poly(lactic-co-glycolic) (PLGA) nanoparticles (NP) were prepared by the double emulsion solvent evaporation method. To potentiate a targeted delivery, NP surface was modified according to targeted cells: 1) mannose to target CD206 at dendritic cells (DC) and 2) a peptide motif to target $\alpha_v\beta_3$ receptors at tumor and vascular cells within the tumor microenvironment (TME). NP were characterized in terms of size, zeta potential and surface morphology. CRC neoantigens, adjuvants, immune modulator loadings were quantified by fluorescence. Immature DC and CRC cells were used to evaluate the *in vitro* NP cytotoxicity and NP cellular uptake profile by flow cytometry. The *in vivo* NP uptake by myeloid antigen presenting cells and the expression of maturation and co-stimulatory molecules at the DC cell surface within draining lymph nodes were also evaluated by flow cytometry. The immunotherapeutic efficacy of our multivalent nanovaccine was assessed, alone and in combination with immune modulators and/or a tumor-associated macrophages (TAM) inhibitor, in the immune-competent CRC model using MC38 cells.

NP presented a mean diameter close to 200 nm, low polydispersity index, neutral surface charge, spherical shape, and high loadings for neoantigens, adjuvants, and immune modulators. No cytotoxic effects were observed on immature DC and CRC cells up to 48 h of incubation. NP were extensively internalized by immature DC *in vitro* and by migratory DC *in vivo*, but also by CRC cells. Nanovaccines induced DC activation and maturation, significantly increasing the expression of CD86 and MHC I markers. A noteworthy tumor remission was observed in tumor-bearing mice treated with the double or triple combination therapy including the nanovaccine, the TME-targeted NP, and/or the TAM inhibitor.

This innovative approach discloses the synergy among the targeted nanovaccine and immune modulatory therapies within TME, which overall outcome may constitute a real hope for patients with metastatic CRC disease.

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The influence of lipid bilayer thickness on the microfluidic assembly of small unilamellar liposomes

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Introduction

Liposomes are an important and versatile platform for drug and gene delivery [1]. While in many aspects liposome fabrication is considered a mature technology, a fine control of their size is still limited by the fact that most processes are bulk-based, actuating on the sample at the macroscale level. This results in poor control of the composition, temperature and stress gradients at the nanoscale level during processing, often resulting in poor control of liposome sizes and polydisperse populations. Microfluidic devices can be used for precise and fast mixing and are thus a promising approach to overcome these issues. Along these lines, Jahn et al [2], have developed an elegant approach by combining solvent exchange with microfluidic hydrodynamic focusing to tune the sizes of DMPC:Cholesterol (1:1) liposomes. By manipulating the flow rate ratio (FRR) between a lipid-alcohol solution (flowing in the middle channel) and an aqueous buffer (flowing through the side channels), the width over which the hydrodynamic focusing occurs is also manipulated, influencing the mixing time, and therefore influencing also the assembly time of the liposomes in a homogeneous way as the two solvents mix. Thus, by manipulating the FRR, the size of the liposomes could be tuned within a range of ca. 100-250 nm, achieving smaller sizes when increasing the extent of the hydrodynamic focusing (i.e. increasing the FRR). In this work, we expanded this initial work first by implementing it on a commercial microfluidic device, and secondly, by trying to understand the role of the lipid type on the resulting liposome sizes. As such, we explored the size-controlled formation of liposomes constituted by 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and DLPC-DMPC mixtures.

Materials and Methods

For the liposome fabrication we used a commercial cross-shaped channel chip with square cross-section of 100 µm (Microfluidic ChipShop, ref: 02-0757-0166-02). The lipids were purchased from Avanti Polar Lipids and dissolved in ethanol. The side fluid was water. The flow conditions were varied by adjusting the total flow rate and flow rate ratio. Particle characterization is performed routinely with DLS.

Results and Discussion

Overall, we confirmed the general trend of a liposome size decrease with an increase in the FRR, as observed also by others [2-3]. Interestingly the sizes of DMPC liposomes are significantly smaller than those for DLPC. DMPC liposomes reach a size plateau at high FRR of ca. 50 nm, while DLPC liposomes reach a size of ca. 110 nm. Also interesting is the fact that the DLPC:DMPC mixtures reach an intermediate plateau size of ca. 70 nm. Liposome formation is typically preceded by bilayer disk aggregates that are formed as intermediate structures [4]. Such disks tend to grow with time, and when a critical size is reached, they fold into a liposome. We interpret these results by accounting that the larger bilayer thickness associated with DMPC must have a larger energy penalty associated with the disk edge, hence limiting DMPC bilayer disk growth and forcing their closure into vesicles at smaller sizes.

Conclusions

We observe that with a commercial microfluidic device we were able to tune the sizes of liposomes in a range from ~200 to 50 nm, depending on the flow conditions employed on the microfluidic device and dependent on the liposome composition. Both the observations of vesicle size control using microfluidics, along with the inherent size differences associated with vesicle bilayer thickness are important considerations when expanding the technology for liposomes for encapsulation of drugs and nucleic acids.

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Uptake of celecoxib PLGA-microparticles by macrophages to improve the anti-inflammatory response

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Introduction

Inflammation is the response to a tissue injury that activates the immune system in which macrophages (McFc) play a major role [1]. Biodegradable PLGA microparticles (MPs) loaded with anti-inflammatory agents targeted to McFc can be a potential approach to treat chronical inflammatory diseases. Several physicohemical characteristics of the MPs (composition, size, shape and surface) are necesary to achieve their uptake by McFc [2]. Celecoxib (CXB), a selective cyclooxygenase-2 (COX-2) inhibitor, is an effective anti-inflammatory agent. The aim of this work is to develop a new formulation of CXB-loaded PLGA microparticles able to target macrophages in order to improve the anti-inflammatory effects of CXB.

Material and methods

PLGA microparticles loaded with fluorescein (MPS-F) and CXB (MPS-CXB) were developed by the solvent extraction-evaporation method from an O/W emulsion. Fluorescein (F) and CXB were incorporated at 5% and 10%, respectively in the formulations. Blank microparticles (MPS-B) were also prepared. Mean diameter and particle size distribution were analyzed by laser diffraction. Morphology and surface characteristics were analyzed using scanning electron microscopy (SEM). *In vitro* release of CBX from the MPs was carried out at 37±0.5 °C in PBS (pH=7.4). *In vitro* evaluation of internalization into McFc and the anti-inflammatory effect were studied on a RAW 266.7 mouse macrophage cell line. Analysis of the uptake of MPS-F was performed by fluorescence microscopy. For the anti-inflammatory effect, cells were incubated for 72 hours with formulations MPS-B and MPS-CXB, and CXB solution. Quantification of the inflammatory mediator TNF-α was analyzed by ELISA. To evaluate the inhibition of gene expression, PCR-RT analysis was performed.

Results and Discussion

All formulations developed presented narrow size distributions with mean particle sizes <6 μm, being suitable for McFc uptake [3]. Encapsulation efficiency of CXB in formulation MPS-CXB was 95.64±1.08%. *In vitro* release of CXB from formulation MPS-CXB shows a rapid initial release for the first 72 h, followed by a slow release during 60 days. Phagocytosis studies show that MPS-F were uptaked by macrophages after 3 h incubation. *Figure 1* shows the results obtained for TNF-α, in which non statistically significant diferences were found between control (Mock) and blank MPS indicating that the presence of blank MPS did not modify the concentration of the inflammatory mediator. The presence of CXB produced an important reduction in TNF-α value. In addition, the best results were obtained when CXB was encapsulated within MPS. This could be due to the fact that microparticles release the drug inside the McFc, resulting in a direct inhibition in the formation of TNF-α. These results are in agreement with the ones obtained by PCR-RT, in which the decrease of the TNF-α signal is a consequence of the inhibition at the gene level.

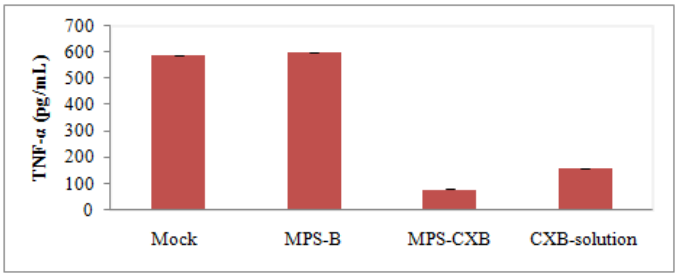

Figure 1. TNF- α values obtained after 72 h incubations of macropahges with MPS-B, MPS-CXB and CXB solution.

Conclusions

The extraction-evaporation method used was suitable to obtain microparticles of adequate size for their uptake by macrophages. Encapsulation of celecoxib whitin PLGA microparticles could be a valuable tool to improve the anti-inflammatory response, probably due to the fact that drug release occurs inside the macrophages.

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Validation of a multifunctional nanodevice for cancer theranostic

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Introduction NanoChemBio team has a robust know-how in the field of nanotechnology. They have prepared a broad portfolio of nanoparticles of different nature that has been broadly used in nanomedicine. They have developed a series of normalized protocols for multifunctionalization of these nanoparticles based on orthogonal protection strategies. These engineered nanoparticles have been successfully conjugated to small molecules (drugs and sensors), proteins and nucleic acids (oligonucleotides, plasmid DNA, siRNA, and miRNA) without altering the properties and activity of the bioactive cargoes.

Aim: To validate an effective, safe and non-toxic nanosystem based on the use of synthetic nanospheres that are multivalent and trifunctionalized with (i) a drug, (ii) a diagnostic tracker and (iii) a ligand for targeted delivery.

Results and Discussion: (i) Protocols have been successfully developed to generate these theranostic nanoparticles in a reproducible manner and to determine their physico-chemical properties. (ii) Efficiency of cellular uptake and cell viability in a panel of breast cancer cell lines have been successfully achieved (analysis by flow cytometry and confocal microscopy). (iii) physicochemical characterization protocols have been carried out (iv) Tumour development and tracking of nanodevices were analysed by NIR fluorescence imaging. Interestingly, the IC50 of standard anticancer drug is higher than the IC50 of nanoparticle-bound drug. From the in vivo studies, there are three main achievements worth highlighting: (1) location of the multifunctionalized nanoparticles within the tumour area; (2) tumour sizes were markedly reduced and (3), in contrast to free drug, nanoparticle-bound drug did not induce any toxicity in the mice.

Conclusions: A prototype of this theranostic nanodevice to treat breast cancer and to monitor treatment efficiency together with to determine localization of the tumour focus and, what is even more important, to locate metastatic foci has been developed. Further studies are carried out to scale-up the production of these nanodevice and to study deeply their in vivo nanotoxicity.

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Zebrafish can be a reliable model to study critical properties of novel cancer nanomedicines

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Introduction. Cancer is responsible for millions of deaths each year, mostly because of metastatic processes derived from the primary tumor [1]. New therapies are being developed to inhibit this metastatic process, many of them based on the use of nanomedicines, for the transport of anticancer drugs and more sophisticated molecules such as gene therapies [2]. The use of biodegradable and biocompatible nanosystems represents a promising alternative for the development of innovative cancer nanomedicines. However, relevant experiments, regarding their toxicological profile and ability to interact with the targeted cancer cells, are key to determine the full potential of novel cancer nanomedicines. Conventionally, 2D cell culture models have been used to study the characteristics of new cancer drugs, but they are inadequate because of some limitations that they entail such as the absence of environmental interactions. We propose the use of zebrafish (ZF) as a better model to evaluate the interaction of nanosystems in a living organism and with xenotransplanted cancer cells. ZF has a high grade of homology with the orthologous human disease-related genes, and has been extensively used for the toxicological evaluation of chemicals [3]. Some recent works highlight the potential of ZF for the study of novel anticancer nanomedicines [4].

Materials and Methods. In this work, cationic nanosystems (NSC) formulated through the ethanol injection method were used. As a model biomolecule, and considering the interest of gene therapy for the treatment of cancer, we have next associated a plasmid. Prior to *in vivo* assays, *in vitro* experiments were performed in different cancer cell lines to check the toxicity, internalization, and the transfection efficiency of plasmid-loaded NSC (pNSC), to compare next with *in vivo* results. *In vivo* assays were performed in 48 hours post fertilization ZF embryos. NSC and pNSC were incubated in the water to check the toxicity and internalization into ZF cells. Microinjection in the duct of Cuvier was performed to study the permanence of NSC and pNSC in ZF circulation. After microinjection of U-87 MG cells stained with DiL, xenotrasplanted ZF were used to determine the ability of NSC and pNSC to interact with cancer cells *in vivo*.

Results and Discussion. *In vitro* tests have demonstrated the capacity of NSC and pNSC to interact and transfect tumor cells, preserving their viability. Toxicity tests *in vivo* showed relevant differences among NSC and pNSC (54% and 19% mortality 72h post-incubation, respectivelly), most probably due to changes in the surface charge after plasmid association. NSC were able to interact with zebrafish cells both after addition into water and microinjection into the duct of Cuvier, but the internalization was higher in this latter case. Importantly, we observed co-localization between the fluorescent (*Figure 1.A*) signals corresponding to the pNSC and the U-87 MG cancer cells xenotrasplanted in fish (*Figure 1.B*).

Conclusions. ZF is a promising model for preclinical evaluation of innovative cancer nanomedicines as an intermediate model between cell culture models and murine models. The use of ZF *in vivo* assays allowed us studying toxicity, distribution, and interaction of NSC and pNSC with cancer cells.

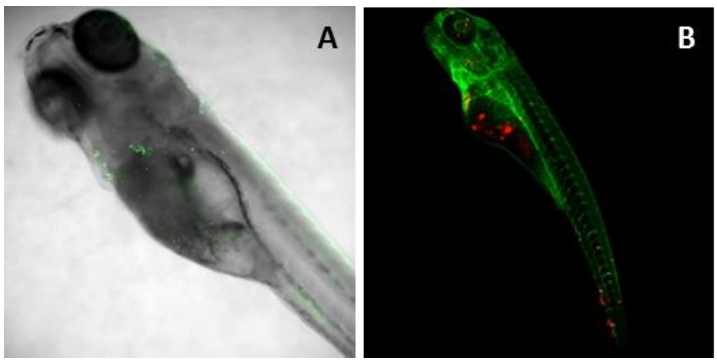

Figure 1. ZF *in vivo* assays: internalization of pNSC incubated in water (*A*); and co-localization of pNSC and cancer cells (*B*).

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POSTER SESSIONS

Session 7: Delivery of biomacromolecules and oligonucleotides

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Antigen-conjugated chitosan nanocapsules for targeting dendritic cells

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Introduction: *Streptococcus pneumoniae* is an opportunistic pathogen that colonizes human upper respiratory tract and causes pneumonia, meningitis and septicemia. This pathogen presents cell membrane proteins such as PsaA (pneumococcal surface adhesin A) involved in adhesion and nasopharyngeal colonization processes [1]. On the other hand, chitosan nanosystems have been described as interesting tools for antigen delivery, enhancing the immunogenicity of nasally administered vaccines [2]. Antigen-loaded vaccine delivery systems should reach to nasal subepithelial lymphoid follicles (diffuse NALT) for their uptake by dendritic cells (DCs) and activate specific T cells for produce an adaptive immune response [3] . The objective of this work is the synthesis, characterization and stability studies (biological media and mucus) of chitosan nanocapsules with a thiol-maleimide coupling of PsaA antigen on their surface, as well as the study of T lymphocyte activation after nanocapsules presentation by DCs.

Materials and methods: Chitosan-maleimide and PsaA-S-Acetylthioacetate (SATA) coupling was performed according to references and the resulting conjugate was characterized by 1H-NMR and circular dichroism. Nanocapsules were prepared by solvent displacement technique and characterized by Dynamic Light Scattering, Nanoparticle Tracking Analysis and Scanning Electron Microscopy. Quantification of association efficiency of PsaA to chitosan nanocapsules was measured by colorimetric BCA assay. Colloidal stability was studied in simulated nasal fluid in the presence/absence of mucin, using freshly prepared and freeze-dried formulations. Human monocyte-derived dendritic cells were generated form buffy coats using GM-CSF and IL4. Their morphology and viability were evaluated (7-AAD and MTS) after their incubation with blank and antigen-conjugated chitosan nanocapsules. Flow cytometry analysis (FACS) were also carried out for the quantification of $CD4^+/CD25^+$ and $CD8^+/CD28^+$ signals in human peripheral blood mononuclear cell (PBMC) derived T lymphocytes.

Results and discussion: 1H-NMR and circular dichroism confirmed the efficient conjugation between chitosan-maleimide and PsaA-SATA. Nanocapsules prepared with antigen-conjugated chitosan presented a homogeneous distribution of spherical shaped particles, with size of 266±32 nm and a positive charge of +30±1 mV. Association rates were three times higher compared to nanocapsules encapsulating PsaA without polymer-protein conjugation. Good stability results were achieved after 24 hours incubation at 37°C in simulated nasal fluid, however, gradual aggregation/immobilization of the nanocapsules occurred in the presence of mucin. Dendritic cells were isolated from monocytes and their viability were demonstrated at nanocapsules concentrations up to of 200 ug/ml. The FACs study examined the ability to activate allogenic T lymphocytes by mature DC in the absence and presence of antigen-conjugated nanocapsules. The maturation of pre-incubated inmature DC in the presence of antigen-conjugated nanocapsules produced antigen presenting cells (APC) with higher capacity to activate CD4 (CD4+/CD25+, 19% activation) and lower to CD8 T lymphocytes (CD8+/CD28+, 17% activation) compared to inmature DCs (CD4+/CD25+ 16% and CD8+/CD28+, 18% activation).

Conclusion: The thiol-maleimide conjugation between the polymer and the antigen produced nanocapsules with high stability and greatly improved association efficiency, enabling surface presentation of PsaA for immune cell recognition. Complementary studies on cytokine secretion profiles and as well the evaluation of antigen-specific responses are currently underway to further evaluate the potential of these nanocapsulaes as vaccine delivery systems.

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Biocompatible iron (III) carboxylate Metal-Organic Frameworks as promising RNA nanocarriers

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Introduction

Despite the great interest of RNA therapeutics, the development of a successful gene delivery process is still a major challenge.[1] The novelty of RNA-based therapies resides on the combination of the potential down-regulation of the gene expression by small-interfering RNA (siRNA), inhibiting the expression of mutant proteins, together with the major tumour suppressor role of microRNA (miRNA) in cancer.[2,3] We propose here a new and efficient nucleic acid entrapping into the mesoporosity of nanoscaled biocompatible iron(III) carboxylate metal-organic frameworks (nanoMOFs) using an easy and biofriendly method.[4,5]

Materials and Methods

The iron-carboxylate nanoMOFs were synthesized by microwave-assisted hydrothermal synthesis as previously reported.[5,6] For the RNA encapsulation, the RNA solution at 0.1 $mg{\cdot}mL^{-1}$ was added to the nanoMOFs suspension at 1 $mg{\cdot}mL^{-1}$ keeping a molar ratio 20:1 during 1h. The physicochemical properties, stability and siRNA entrapment were evaluated by X-Ray diffraction, N_2 adsorption, dynamic light scattering and fluorescence/infrared spectroscopy. Colorectal cells were maintained in supplemented DMEM, performing a cytotoxicity and uptake assays by MTT and fluorescence confocal microscopy, respectively.

Results and Discussion

The RNA was efficiently located within the important nanoMOF porosity obtaining an encapsulation efficacy ~71 or ~85% after only 1 h. Specific interactions between the RNA phosphate groups and the iron metal sites of the nanoMOFs were established, preserving the characteristic crystalline structure and its colloidal stability properties resulting in a significant reduction of the MOF porosity. In addition, a lack of cytotoxicity and hemolytic effect were observed in human colorectal cells and human red blood cells in contact with the RNA@nanoMOFs. These nanosystems were rapidly internalized after 4h by cells, enabling to protect and release the genetic material in the cytoplasm, leading to an effective *in vitro* gene activity.

Conclusions

A complete sequence of nucleic acids was successfully incorporated into the high mesoporosity of biocompatible iron(III) carboxylates nanocarriers. These nanovectors were able to both protect and release the genetic material in the cytoplasm, leading to efficient transfection activities. These promising outcomes pave the way for developing an efficient gene delivery platform based on biocompatible porous nanoMOFs.

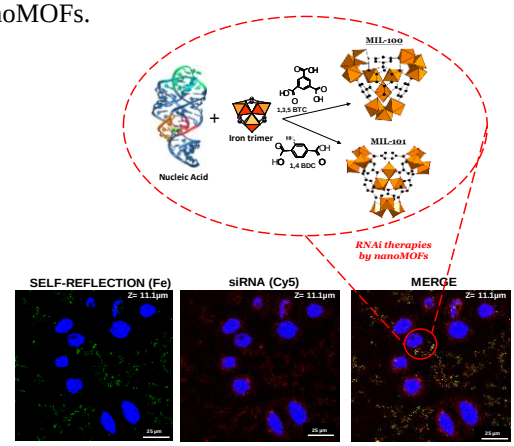

Figure 1. A) Nucleic acid association scheme: iron carboxylate nanoMOFs & siRNA; B) Fluorescence confocal microscopy images of Sw480 cells incubated with Cy5-siRNA@nanoMOFs. In green, nanoMOFs directly observed by the iron self-reflection, in red the Cy5-labelled siRNA and in blue the nucleus stained with DAPI. The scale bar corresponds

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Delivery of nucleic acid mimics (NAMs) in bacteria mediated by lipoplexes

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Introduction: Treatment of septicaemia is threatened by the bacterial resistance to antibiotics. Nucleic acid mimics (NAMs) able to inhibit bacterial genes are emerging as an alternative to traditional antibiotics. However, NAMs cannot passively permeate the multi-layered bacterial envelope [1]. Therefore, liposomes were considered as possible carriers to facilitate the internalization of NAMs in bacteria. Locked nucleic acids (LNA) and 2´-OMethylRNA (2´OMe) were evaluated as model NAMs and formulated into DOTAP-DOPE liposomes, forming lipoplexes. To improve the colloidal stability of the lipoplexes, PEGylated lipids (DSPE-PEG) were added to the formulation [2, 3]. We investigated the complexation and colloidal stability of these lipoplexes in human serum, which is of relevance in the context of intravenous administration. The potential of these lipoplexes to fuse with the envelope of Gram-negative and Gram-positive bacteria and deliver the NAMs into the bacterial cytosol was then assessed.
Materials and Methods: Fluorescence correlation spectroscopy (FCS) and nanoparticle tracking analysis were used to respectively study the complexation and colloidal stability of lipoplexes, with and without DSPE-PEG post-PEGylation, directly in human serum, over time. Thereafter, the interaction of liposomes with the cell envelope of *Escherichia coli* and *Staphylococcus aureus* was studied via a lipid mixing assay. Fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM) analysis then enabled the evaluation of the extent of NAMs internalization into the referred bacteria.
Results and Discussion: Our data showed good complexation stability of the NAMs in the human serum for the PEGylated liposomes. Liposomes were able to fuse with both the Gram-negative and Gram-positive bacteria tested. Accordingly, the confocal images taken after the FISH assay demonstrated a successful internalization of the NAMs in both bacteria (Figure 1).
Conclusions: We demonstrated that DOTAP-DOPE liposomes may fuse with both Gram-negative and Gram-positive bacteria and successfully deliver NAMs in both bacterial types. In addition, NAMs remain stably complexed within the lipoplexes even when incubated in human serum, which is promising for intravenous administration of the lipoplexes to target septicaemia.

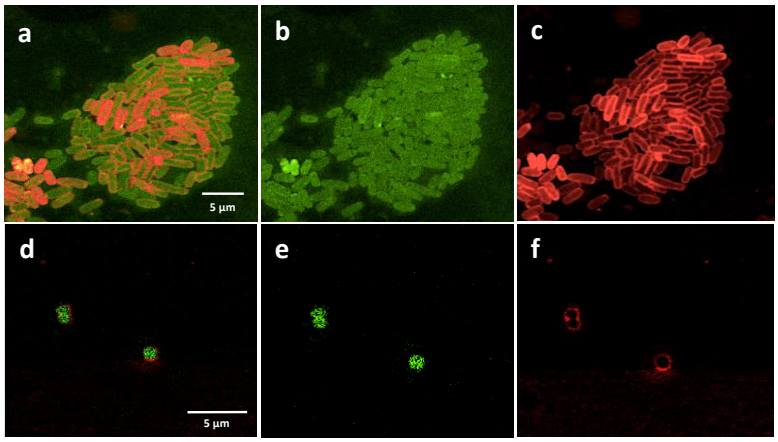

Figure 1. Representative CLSM images of *E. coli* **(a-c)** and *S. aureus* **(d-f)**. Both bacteria were incubated for 1h with lipoplexes containing HiLyte^{TM} Fluor 488 labeled NAMs (green). Bacterial membranes were labeled with DiD dye (red). **(a,d)** represent merged channels and **(b,c,e,f)** represent separate channels.

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Designing Efficient siRNA Delivery Systems for Cancer Immunotherapy

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Introduction: The ability of small interfering RNAs (siRNAs) to specifically and enzymatically silence gene-expression at pre-translational level makes it a potential therapeutic molecule for various diseases including cancer [1]. Especially, cancer immunotherapy can take advantage of siRNAs for silencing cancer-specific undruggable genes to potentiate immune-activation and tumor eradication [1]. The two main challenges include, (a) the identification of right combination of genes to obtain potent and sustained anti-tumor immune response, and (b) intracellular delivery of the therapeutic siRNA. The free unmodified siRNA is easily degraded in the body and hence require chemical or delivery-system mediated stabilization for its *in vivo* activity [2]. Desirable physico-chemical properties for a clinically translatable delivery system includes, (a) safe material; (b) ability to self-assemble into uniform nano-metric objects; (c) high siRNA loading; (d) structural stability in physiological conditions; (e) protection of siRNA from nuclease-mediated degradation; (f) high cellular uptake in the target tissue; (g) endosomal escape and release of siRNA cargo in the cytoplasm [2-4]. Here, a rational design approach was applied to engineer RNA nanosystems with above mentioned properties for efficient siRNA delivery.

Materials and Methods: A natural, biocompatible polymer was chemically modified with a hydrocarbon tail, cationic side chain and endosomal escape facilitator in a one-pot amide bond forming reaction. The resulting polymer was purified by dialysis (7000 kDa cut-off membrane), lyophilized and used for preparing RNA loaded formulations. Transfer RNA (tRNA) was used as a model for siRNA. The formulations were prepared either by simple mixing of the components (to form nanocomplexes, **NCPs**) or through solvent-displacement technique, where an excess of an aqueous phase was added to an ethanolic phase to form lipid-polymer hybrid nanocapsules (**LPH-NCs**) [5,6]. Both types of nanosystems were characterized for its size (dynamic light scattering, DLS), zeta-potential, RNA loading (agarose gel electrophoresis), ability to release RNA (heparin competition and RNA displacement assay) and stability in physiological media (cell culture medium and serum).

Results and Discussion: The chemically modified polymer showed enhanced RNA loading, ability to self-assemble into nanosystems (size in water: 150 – 300nm for NCPs and 120 – 200nm for LPH-NCs), negative or positive zeta-potential depending on the presence/ absence of a coating and the nature of the coating material. LPH-NCs released the RNA after incubation with heparin (~10 molar excess) indicating the ability to release siRNA upon suitable stimulus. LPH-NCs also showed enhanced stability in physiological media compared to NCPs.

Conclusions: LPH-NCs prepared with modified polymer showed favorable physico-chemical properties making it a promising candidate for *in-vitro* and *in-vivo* evaluation. Further biological assessment will reveal the true potential of this formulation. Depending on the *in-vitro* transfection results, the composition and physico-chemical characteristics can be modulated to enhance RNA delivery. Rational design of RNA formulations would not only enable the preparation of efficient and safe nano-systems with multi-functional properties, but it also provides the ability to tailor the system to suit our needs.

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Development of high drug loaded filaments of HPMC for 3D printing

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Introduction

During the last decade, 3D printing technology has enabled the design of new drug delivery systems using polymers which can be molten. Several studies deal with drug-loaded filaments in which drug and excipient melting points are similar [1], [2] or drug melting point is much higher than excipient melting point. Affinisol™ is a HPMC which can be molten (melting point 135ºC-200ºC) [3]. Metformine's melting point is 270ºC. No studies were found in which filaments have a drug load higher than 10% using different types of hydroxypropyl methylcellulose HPMC [1].

The aim of this study is to prepare a filament of HPMC with high drug load and suitable properties to be used in 3D printing.

Matherials and methods

HPMC (Affinisol™ 15cP) was donated by The Dow Chemical Company (Midland, MI); metformine was donated by Pharmhispania S.A. Pharmaceuticals (Barcelona, Spain), magnesium stearate, polyethylene glycol 6000 (PEG 6000) and triethyl citrate. Magnesium stearate was used as a lubricant [4]. PEGs are used as plasticizers, decreasing the blend's melting point [4]. Triethyl citrate was used as plasticizer too [4].

The compositions of the formulations evaluated in this study are listed in Table 1. The theoretical drug content of the blends was 50% w/w. The blend of drug and excipients was kept in a vacuum desiccator for 24 h and then was extruded using a single-screw filament extruder (Noztek Pro Desktop Filament Extruder, Noztec, UK) in order to obtain the drug loaded filament (extrusion temperature 150-160 ºC). The flow speed was measured for each blend.

Blend	MF (%)	AFF (%)	ES (%)	PEG 6000 (%)	TEC (%)	Extrusion T (ºC)	Flow speed (cm/min)
1	50	50				150-160	1,1
2	50	45	5			160	3,33
3	50	45		5		150	-
4	50	40	5	5		150	7,9
5	50	40	5		5	150	6,3
6	50	37,5	5	7,5		150	3
7	50	35	5	10		150-160	-
8	50	35	7,5	7,5		150	2,58
9	50	44	3	3		150	1,75
10	50	35	7,5		7,5	150	53,3

Table 1. Composition of the filaments [Metformine (MF), Affinisol™ (AFF), magnesium stearate (ES), triethyl citrate (TEC)].

Results and Discussion

As it is shown in Table 1, flow speed of filaments 1 and 9 was very low. Batches containing at least 5% ES show an improved flow speed. Filaments 3 and 7 suggest that blends without ES or with high difference in percentage between ES and PEG 6000 made the filament production impossible. Blends with TEC presented high flow speed but the obtained filaments were very fragile and, moreover, filament obtained with blend 10 was burnt. Filaments made of blends 4, 6 and 8 presented better properties such as flow speed and flexibility.

Conclusions

Filaments containing AFF have been prepared with a drug load of 50%. ES improves the flow of blends with MF and AFF, and PEG 6000 improves the flexibility of filaments. The filament that presented better properties to be used in 3D printing corresponds to blend 4 (50:40:5:5 ratio of MF:AFF:ES:PEG600).

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Development of SLNs as mRNA and pDNA delivery systems

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Introduction. The main challenge for an efficient nucleic acid-based therapy is "delivery". The design of appropriate delivery systems is a key point to overcome all the extra- and intracellular barriers that hamper the transfection process. Solid Lipid Nanoparticles (SLNs) are regarded as one of the most versatile and effective vector [1]. The aim of the present work was to evaluate the influence of formulation factors in the development of SLNs as plasmid DNA (pDNA) or messenger RNA (mRNA) delivery systems. The efficacy and long-term stability of the vectors were assesed.

Materials and Methods. Two different formulations of SLN (SLN1 and SLN2) were prepared by the emulsification-evaporation method [2]. SLN1 had DOTAP as cationic lipid whereas SLN2 had a mix of DOTAP and DODAP. pDNA and mRNA encoding EGFP were used as nucleic acids, and were complexed with protamine (PROT), dextran (DX) or hyaluronic acid (HA) and SLNs. pDNA and mRNA vectors were stored at 4ºC during 7 months. Vectors were characterized at different times in terms of size, zeta potential and the binding, protection and release capacity of the nucleic acids from the delivery system. The efficacy of transfection in human retinal pigmented epithelial cells (ARPE-19) was also determined at different storage times and intracellular disposition of Cy5 labeled mRNA was analyzed.

Results and Discussion. The size of freshly prepared pDNA-systems ranged from 165 to 200 nm, and the superficial charge from +30 to +45mV. In the case of mRNA-vectors, the size was above 200 nm (200-350 nm) and the superficial charge ranged from +18 to +37 mV (Fig 1A). The electrophoresis gels showed that all the vectors were able to bind and release the nucleic acids. The percentage of transfected cells was almost 100% with mRNA-systems, but lower with pDNA (40%) probably due to the limiting step of nuclear entry [3]. This major barrier can be overcome during mitosis, but ARPE-19 cells present low division rate. All mRNA formulations showed the highest transfection efficacy at 24-48 h, and at 96 h the protein expression decreased notably, whereas pDNA formulations showed the maximum transfection at 72-96 h, and it lasted at least 11 days. The synthesis of the encoded protein with mRNA is fast and its expression is temporary, which makes it a more predictable molecule than DNA; however, mRNA provides short-term transfection.

The long-term stability study showed that in the case of pDNA, a significant increase in the particle size occurred in DX-vectors, regardless of the type of SLN; transfection efficacy of all vectors remained stable during the seven months of storage. In the case of mRNA, size and zeta potential were stable, except for HA-SLN2 and PROT-SLN2, and transfection decreased significantly with SLN2-vectors. DODAP is a pH sensitive lipid that becomes cationic at the acidic endosomal pH, resulting in the release of the genetic cargo into the cytoplasm. However, the lower capacity of the lipid DODAP to condense the genetic material (it has lower number of positive charges) could be responsible of the lower stability of SLN2-based vectors, especially for mRNA. Fig 1B shows the influence of PROT, HA and DX on mRNA condensation.

Conclusion. This study shows that the transfection efficacy, nucleic acid delivery and stability of SLN-vectors are highly influenced by formulation factors, especially for mRNA. An appropriate design of the formulations, adapted to the type of nucleic acid, will allow achieving the desired protein expression profile depending on the therapeutic application.

A

FORMULATION		SIZE (nm)		ZETA POTENTIAL (mV)		EFFICACY OF TRANSFECTION (%)	
		t0	t7months	t0	t7months	t0	t7months
mRNA	*DX-SLN1*	246,77±1,31	205,13±1,70	37,23±0,98	33,68±1,86	96,95±0,54	85,87±0,55
	DX-SLN2	205,13±1,70	252,90±6,16	36,47±0,33	33,58±0,63	72,82±0,47	4,12±0,35
	HA-SLN1	202,40±2,19	255,67±6,15	27,45±0,58	34,13±0,69	96,56±0,33	91,66±0,94
	HA-SLN2	349,17±9,88	Aggregate	18,45±0,87	3,27±3,31	52,75±0,31	11,92±1,67
	PROT-SLN1	251,60±6,46	258,20±11,92	28,83±0,65	30,78±1,61	96,84±0,27	58,26±2,34
	PROT-SLN2	261,70±3,98	260,90±10,55	25,37±0,63	17,83±0,45	26,62±0,13	3,80±0,36
DNA	*DX-SLN1*	176,40±0,35	245,20±10,22	45,42±2,68	45,95±1,81	24,70±0,69	36,79±4,25
	DX-SLN2	165,80±1,71	262,10±15,57	42,22±0,92	36,18±0,82	35,96±0,15	32,22±2,32
	HA-SLN1	201,17±1,27	211,97±1,92	29,83±1,08	32,85±1,42	45,78±2,73	46,94±0,77
	HA-SLN2	194,23±0,81	225,43±7,25	35,60±1,93	34,10±0,52	38,51±1,61	34,68±1,31

B

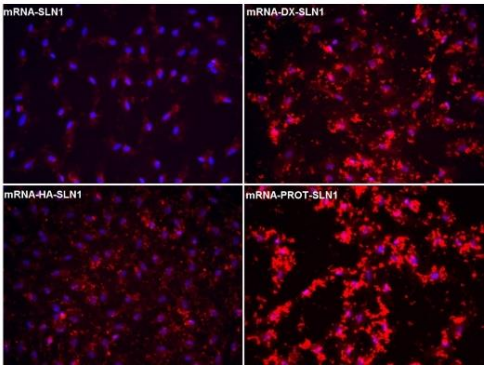

Figure 2. A:efficacy of transfection, size and zeta potential. B: intracellular distribution of Cy5-mRNA vectors with SLN1.

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Enveloped polymeric nanocomplexes (ENCPs) for the intracellular delivery of Bevacizumab

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Introduction:

Different strategies have been followed in order to improve the limited tumor accumulation of the monoclonal antibody (mAb) bevacizumab (BVZ) and avoid its described resistances mechanisms [1]. The use of nanocarriers could help BVZ reaching the VEGF-A protein (BVZ target) present in the extracellular matrix and surface of the cancer cells as well as the intracellular pool of VEGF-A [2]. Overall, this could be translated into an inhibition of angiogenesis and cell migration [3]. Moreover, the association of BVZ to the nanocarrier might reduce the "binding site barrier" effect [2,4].

Materials and Methods:
BVZ was kindly donated by mAbxience (Spain). Hyaluronic acid, (HA, 47-57 kDa) was purchased from Lifecore biomedical (United States). Ethyl Lauryl Arginate (LAE) was a gift from Vedeqsa (Spain). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (United States).

Nanocomplexes were prepared by the injection technique in order to use a reproducible and simple method. For a properly physicochemical characterization, the Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA) were used. The nanocomplexes stability in simulated biological fluids was studied in PBS supplemented with FBS.

Results and Discussion: Polymeric nanocomplexes enveloped by two polymeric layers were formed according to the self-assembling technique. The core of the nanocomplex is composed of the cationic surfactant Lauryl Arginate (LAE) and BVZ. Two different coating layers, the external one being hyaluronic acid, were assembled around the core in order to prevent the stability of the ENCPs in biological media. The obtained nanosystem had a size of 182 ± 3 nm, a negative surface charge (-27 ± 1 mV) and a BVZ concentration of 185 µg/mL. They were stable upon incubation for at least 24 hours in PBS supplemented with fetal bovine serum.

Conclusions: Polymeric ENCPs fulfill the standards in terms of particle size, zeta potential, drug loading and stability required for intravenous administration.

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Evaluation of anti-inflammatory effect of quercetin liposomes in hepatic ischemia and reperfusion injury

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Introduction

Ischemia and reperfusion injury (IRI) is a common complication from liver surgeries and transplantation due to inflammation and oxidative stress that can lead to organ failure [1]. To minimize IRI severe effects, non-steroid anti-inflammatory drugs (NSAID) and corticosteroids are currently used in clinic. Nevertheless, they present a limited efficacy, being required alternative therapeutic approaches. Quercetin is known for its anti-inflammatory and antioxidant properties [2]. However, it presents a low *in vivo* efficacy due to its poor bioavailability and fast liver metabolism [3]. Here, we aim at improving treatment efficacy through the design of quercetin long-circulating liposomes that will preferentially target inflamed liver areas in IRI.

Materials and Methods

Quercetin long-circulating liposomes were prepared by film hydration method [1], and characterized according to hydrodynamic diameter, polydispersity index and superficial charge. The lipid content was determined through the Rouser method and the incorporated drug quantified by HPLC. Drug release assays were performed at 37 °C and nanosystems' stability was evaluated at 4 °C for 30 days. The *in vitro* model of IRI was implemented in a hypoxia chamber and overall cell viability evaluated by the MTS assay. mRNA expression levels of inflammatory biomarkers, namely tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and interleukin 10 (IL-10) were assessed by RT-qPCR. The *in vivo* hepatic model of IRI was developed in Wistar rats, where a laparotom was performed to expose the liver hilum and a vascular microclamp was used to interrupt the blood supply to the three cephalic lobes for 20 min. Ischemia onset was verified visually by the change in the liver coloration into a paler shade and reperfusion initiated with the removal of the microclamp. 6 h or 24 h after the reperfusion started, blood was collected by cardiac puncture for transaminase level evaluation, rats were sacrificed by an isofluran overdose and liver was stored for histochemical analysis and evaluation of TNF-α expression level by RT-qPCR.

Results and Discussion

The optimized quercetin long circulating liposomal formulations (Q-Lip) presented a drug to lipid ratio of 31 ± 3 µg/µmol, with a size under 0.13 µm with low polydispersity and zeta potential values around zero (mV) at pH 6.0. The developed nanosystems are stable up to 1 month at 4 °C, either containing EggPC or SoyPC as the main lipid in the bilayer. Q-Lip were able to maintain cellular viability at high concentrations (up to 500 µM) even in the absence of oxygen and nutrients. The anti-inflammatory effect of the optimized nanoformulations was evaluated using our customized IRI *in vitro* model. Incubation of cells in the presence of Q-Lip allowed to reduce the expression of inflammatory biomarkers, revealing a potential anti-inflammatory effect of the developed nanosystem. This potential was confirmed in our *in vivo* rat model, where quercetin liposomes injected 24 h before the surgical procedure led to a decrease of serum transaminase levels, TNF-α expression and improving the liver recovery after the surgery.

Conclusions

Long circulating liposomes were able to successfully incorporate quercetin, which increased cellular viability over the free drug. The effect of nanoformulation was evaluated *in vitro* and *in vivo* and in both models the capacity of suppressing inflammation was observed. This result shows that quercetin liposomal formulations may become a promising strategy for hepatic IRI treatment.

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Evaluation of polymeric nanoparticles as gene delivery systems in 2D and 3D glioblastoma models

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Introduction: Glioblastoma multiforme is one of the most aggressive brain tumors with a median survival of 15 months. It mainly affects patients over 60 years. The first line treatment combines surgical recession, chemotherapy and radiotherapy, but this process is still inefficient with small medical benefits and severe side-effects[1,2]. Gene therapies hold a great promise in being able to target specific pathways inside glioblastoma cells by the introduction of growth-regulating or tumor suppressors sequences (siRNA/miRNA) which are rendered therapeutically effective by a delivery system[3]. Polymeric nanocarriers offer a combination of high biocompatibility and delivery efficacy, protecting the genetic material from a fast clearance and enzymatic degradation and promoting their interaction with the target cells.

Materials and Methods: Protamine:dextran nanoparticles were formulated by the ionic-gelation method. Their physicochemical properties were studied by Photon Correlation Spectroscopy and Laser Doppler Anemometry. Their morphology was analyzed by TEM. The stability was examined for one month under storage (4ºC) and physiological conditions (37ºC, pH 7.4). The association of different nucleic acids to the nanoparticles was studied by agarose gel electrophoresis. *In vitro* studies on nanocarrier toxicity were carried out in U87MG cells and spheroids by proliferation and cell death assays. Moreover, the cellular uptake of the fluorescently labelled-nanosystems was examined by Confocal Microscopy and quantified by Flow Cytometry. The transfection study with different doses of pDNA-loaded nanoparticles was carried out with a plasmid encoding the enhanced green fluorescent and Luciferase protein (pEGFP_Luc) in 2D and 3D cell culture models. The protein expression was examined by fluorescence microscopy.

Results: Protamine nanoparticles present spherical morphology, size below 200 nm, positive surface charge (+36 ± 7 mV) and high encapsulation of nucleic acids (>90%). They are stable for one moth at 4ºC and in simulated physiological media for 4 hours. The viability studies indicated low/non-toxicity for these polymeric nanoparticles in both culture models. Moreover, cellular uptake studies showed an efficient internalization of TAMRA-labelled nanosystems reaching the inner cell compartments. The quantification by flow cytometry confirmed that 99% of the glioma cells were positive for the presence of the nanosystems. Additionally, we performed internalization studies employing nanoparticles loaded with fluorescently labelled siRNA. The preliminary transfection assay of nanoparticles loaded with pEGFP_Luc was analyzed by the green fluorescent emitted by the EGFP protein. This result showed an efficient capacity of protamine nanoparticles to transfect glioma cells for doses greater than 1 ug/well.

Discussion and Conclusion: The combination of protamine and dextran by the ionic cross-linking technique allowed to obtain nanoparticles without using high energy forces. Their physicochemical properties made them suitable for the association and protection of different genetic cargos both at 4ºC and 37ºC. This nanocarriers presented low toxicity for U87MG cells and spheroids. Moreover, they were efficiently internalized in both cell models indicating their potential as gene delivery systems. Finally, protamine:dextran nanoparticles promoted an efficient transfection using a model pDNA in glioblastoma cells which confirmed that this formulation could be considered a promising gene nanocarrier for the glioblastoma treatment by gene therapy.

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Evaluation of the effect of ionic liquids as cryoprotectants on the stability of polymer nanoparticles

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Introduction: Ionic liquids (ILs) are salts, that are liquid below 100 °C and may act as water substitutes [1]. Then, they may be considered potential excipients to act as cryoprotectants in the freeze-drying of nanoformulations mitigating freezing stress [2]. Hence, the aim of this work was to evaluate the effect of ILs as cryoprotectants on the stability of polymer nanoparticles after freeze-drying.

Materials and Methods: Poly(lactic-co-glycolic acid) (PLGA) 50:50 was used to prepare nanoparticles by a W/O/W double emulsion technique [1]. The inner phase was an aqueous solution of bovine serum albumin (BSA) at 20 mg/mL with 5 %(w/v) of the ILs. Six ILs were studied as cryoprotectants, three choline-aminoacid ILs, [Cho][Phe] and [Cho][Glu], already used in other study of our group [1], and (2-hydroxyethyl) trimethylammonium glycinate [Cho][Gly], and three imidazole-based ILs, 1-ethyl-3-methylimidazolium bromide [C2mim][Br], 1-butyl-3-methylimidazolium bromide [C4mim][Br] and 1-hexyl-3-methylimidazolium bromide [C6mim][Br]. Trehalose was used as control. The samples were characterized about their association efficiency (AE) and loading capacity (LC). After and before the freeze-thawing and freeze-drying, it was calculated the particle size ratio and the retention efficiency (RE).

Results and Discussion: The BSA nanoparticles showed similar AE between the nanocarriers produced in the presence of the ILs and the nanoparticles without them or without trehalose, presenting values close to 80 % (**Table 1**). The nanoparticles presented LC values around 50 % and 26 % for choline-based ILs and imidazole-based ILs, respectively (**Table 1**). Additionally, the choline-based ILs seem to be more suited as cryoprotectants, since after freeze-thawing and freeze-drying, they showed higher RE and suitable particle size ratio, when compared with the imidazole-based ILs (**Table 1**). Thus, at the studied percentages of ILs, 5 % (w/v), results indicate that the ILs keep the stability of the produced nanoparticles, specially the choline-based ILs.

Table 1. Association efficiency (AE) and loading capacity (LC) of the BSA-loaded PLGA nanoparticles and particle size ratio and retention efficiency (RE) of the nanoparticles after freeze-thawing and freeze-drying. n=3, mean ± SD.

Cryoprotectant	BSA nanoparticles		Freeze-thawing		Freeze-drying	
	AE (%)	LC (%)	Ratio	RE (%)	Ratio	RE (%)
Without cryoprotectant	88.0 ± 1.5	78.9 ± 1.0	2.8 ± 0.4	99.4 ± 0.4	2.8 ± 0.8	98.3 ± 0.7
Trehalose	80.9 ± 0.6	78.9 ± 1.1	1.1 ± 0.1	88.4 ± 1.1	1.1 ± 0.1	89.4 ± 0.5
[Cho][Phe]	90.8 ± 0.7	50.1 ± 1.0	1.4 ± 0.4	92.8 ± 0.6	1.4 ± 0.5	91.6 ± 0.4
[Cho][Glu]	89.3 ± 0.2	45.7 ± 0.3	2.0 ± 0.3	79.3 ± 1.2	1.6 ± 0.4	75.8 ± 0.8
[Cho][Gly]	95.2 ± 0.3	53.1 ± 0.9	1.3 ± 0.2	96.8 ± 1.5	1.2 ± 0.2	94.6 ± 0.6
[C2mim][Br]	90.2 ± 1.3	26.7 ± 0.4	1.8 ± 0.2	66.3 ± 1.0	2.3 ± 0.5	65.9 ± 0.6
[C4mim][Br]	75.3 ± 0.5	27.5 ± 0.2	1.5 ± 0.5	68.5 ± 0.5	2.1 ± 0.5	70.8 ± 0.7
[C6mim][Br]	77.1 ± 0.5	25.5 ± 0.5	1.7 ± 0.2	55.5 ± 1.3	1.9 ± 0.3	56.5 ± 0.9

Conclusion: Overall, this work showed that ILs may be used as cryoprotectants since they showed the ability to preserve the nanoparticles after freeze-drugs. Further studies will focus on the assessment of the performance of the developed delivery systems.

Acknowledgments: This work was supported by Fundação para a Ciência e Tecnologia, I.P., through funding UID/DTP/04567/2019. It was also financed by FEDER funds through the COMPETE 2020-POCI and by Portuguese funds through FCT in the framework of the project POCI-01-0145-FEDER-032610 - PTDC/MEC-DER/32610/2017. A.J. and R.C. would like to thank ALIES for the grant PADDIC 2018-2019.

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Fluorescence Cross-Correlation Spectroscopy as a tool to evaluate the assembly of cationic liposome-coated polycation-DNA complexes (lipopolyplexes) for gene therapy

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Introduction: Gene delivery vectors hold great promise for the development of therapeutics. While viral vectors can provide successful gene transfection in vivo, safety concerns associated with them make non-viral vectors a more appealing alternative for future therapies. However, the in vivo transfection efficiency of non-viral vectors has been very limited so far, and part of this issue is due to an incomplete understanding of the optimal parameters needed in these nano-formulations. A clear example of this issue are complexes of polycations, lipids and nucleic acids (usually called lipopolyplexes). While lipopolyplexes have been widely proposed as useful nanotherapeutics [1], to the best of our knowledge, no work has yet evaluated in a quantitative manner the degree of success in obtaining these three-component structures. The aim of this work is to employ Fluorescence Cross-Correlation Spectroscopy (FCCS) to evaluate the assembly of lipopolyplexes prepared under different conditions.[2]

Materials and Methods: Polymer-DNA complexes (polyplexes) with different Polymer N/DNA P ratios (1-3) were prepared by mixing polylysine and plasmid DNA in different proportions in water. Then, cationic liposomes with varying degrees of PEGylation were added to the prepared polyplexes to try to obtain different lipopolyplex formulations, studying a range of lipid N/ DNA P between 0.5 and 5. The obtained particles were characterized by Dynamic Light Scattering (DLS), Z Potential, Transmission Electron Microscopy (TEM) and FCCS (employing an Atto 488-labeled polymer and a Texas Red-labeled lipid).

Results and Discussion: In this work, we have prepared a library of different lipid-polylysine-DNA strucures, characterizing them by DLS, Z Potential, TEM and FCCS. FCCS allows the evaluation particle diffusion in two spectrally-separated channels, revealing the presence or absence of interactions between the differently-labeled components[3]. Employing Atto 488-labeled polylysine and Texas Red-labeled lipids, we have shown for the first time that FCCS allows us to evaluate the degree of co-localization of the polymeric and lipidic components in a quantitative manner. This degree of cross-correlation was found to vary greatly with different ratios of the main components, as well as with the presence or absence of a polyethylene glycol (PEG)-grafted lipid, which is expected to have significant implications for their therapeutic use.

Conclusions: The results here obtained show that FCCS can become a powerful tool in the context of nanoformulation design, providing new insights that cannot be achieved by traditional characterization techniques.

Acknowledgments: This research is supported by Microfluidic Layer-by-layer Assembly of Cationic Liposome - Nucleic Acid Nanoparticles for Gene Delivery project (032520) co-funded by FCT and the ERDF through COMPETE2020. JLP is supported by a post-doctoral fellowship from the Ramón Areces Foundation.

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Herbicide-loaded chitosan-solid lipid microparticles: a smart strategy towards safer agrochemical products

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Introduction: Pesticides are widely used in agricultural production, often developed through very strict regulation processes to function with reasonable certainty and minimal impact on human health and the environment [1]. Therefore, exploring novel applications for approved excipients with a history of safe use in pharmacy and cosmetics is a smart strategy to obtain safer agrochemicals. Pesticide microformulations have had great application as an effective and viable option for pest management to surpass the limitations of conventional products. The motivation behind the advanced pesticide microformulations is to develop products with less harmful effects to the environment along with enhanced biodegradability. These advancements can be an effective means to reduce environmental damage on a long-term basis. It is recognized that only 0.1% of pesticides reached the target pests, while 99.9% leaked into the surrounding environment [2]. Therefore, the aim of this study was to formulate and characterize hybrid microparticles based on solid lipids and of chitosan (CS) to produce a less toxic herbicidal formulation for the effective and safe control of weeds in agriculture.

Materials and Methods: For this purpose, solid-lipid microparticles (SLM) were formulated with different solid lipids (e.g. stearic acid; octadecylamine; gelucire 48/16; Compritol ATO; Lipocirel A SG; Precirol ato 5; Geleol; cetyl palmitate; stearyl acohol) and coated with different CS amounts (concentrations from 0.25 to 1.5% w/v were studied). The SLM were prepared using an herbicide-containing oil phase, while and aqueous phase was prepared by dissolving an appropriate amount of sodium lauryl sulfate (SLS) in purified water, followed by heating at the same temperature of the oil phase. Microparticles were then obtained by a hot emulsification method where the aqueous phase was added dropwise to the melted oil phase and then homogenized using a high-shear laboratory mixer (Ultra-Turrax®, IKA-Labortechnik) [3]. Microparticles were coated with CS, at a CS:SLM ratio of 1:4 for 1 h at room temperature, with a gentle stirring. Formulations were characterized for particle size distribution and zeta potential, as well as colloidal stability.

Results and Discussion: Compritol proved to be the best solid lipid for herbicide encapsulation. Microparticle size was always ≤9.4±4.4 μm, increasing uo to with increasing CS concentrations. Coating originated a reservoir-type of structure (Figure 1), which is supported by drastic change of Zeta potential from negative to highly positive values (+20.0±3.0 mV) after coating with CS.

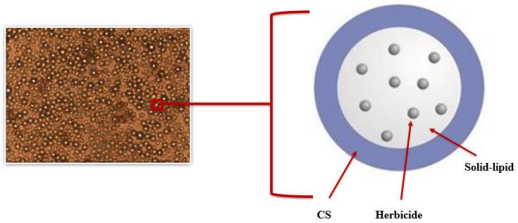

Figure 1. Structure of herbicide-loaded chitosan-solid-lipid microparticles (100x magnification)

Conclusions

These preliminary studies of a herbicide-loaded chitosan-solid-lipid microparticles shows the feasibility and the potential of pharmaceutical excipients as as alternative ingredients for eco-friendly herbicide formulations.

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High-throughput screening of a combinatorial polymer library of nanoparticles for controlled delivery of messenger RNA

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Introduction. Messenger RNA (mRNA) has been regarded as a highly promising tool for gene therapy owing to its transient nature and biodegradability, which offers the possibility of controlling protein production by modulating mRNA pharmacokinetics [1]. Degradable polymers such as polyamidoamines and poly(β-amino ester)s have emerged as biocompatible options to enhance transfection efficiency compared to conventional transfection agents [2, 3]. Their chemical versatility has enabled the rapid generation of libraries by combining different amines and acrylates/acrylamides *via* Michael addition. Herein, we designed a high-throughput screening strategy to identify formulations capable of transfecting mRNA with superior performance than conventional transfection agents. Moreover, we incorporated a photocleavable diacrylate moiety to render these polymers sensitive to ultraviolet (UV) radiation. Our hypothesis was that the formulated nanoparticles would accentuate mRNA release to the cytoplasm "on demand", by facilitating its escape from endolysosomes upon UV irradiation [4].

Materials and Methods. We screened transfection efficiency of these formulations using mRNA encoding Cre recombinase that induced GFP expression in reporter fibroblasts upon recombination. From the 158 tested compounds, we selected 7 hits for subsequent investigations. After purification by dialysis (MWCO = 2 kDa), these hits were tested for mRNA transfection across several cell types (dermal fibroblasts, keratinocytes, endothelial cells, macrophages). In addition, the effect of UV radiation in transfection efficiency was assessed after exposing cells in the presence of polyplexes for 10 min (λ = 365 nm, 10 mW/cm^2). Optimised polyplexes were characterised by NMR, DLS, NTA and ζ-potential measurements.

Results and Discussion. Our high-throughput screening strategy identified 7 hits capable of transfecting Cre mRNA with comparable efficiency to Lipofectamine® 2000 in the Cre reporter fibroblasts. However, when transfecting GFP mRNA, only one hit had comparable transfection efficiency to Lipofectamine® 2000 across the tested cell types, with superior results in dermal fibroblasts. Transfection efficiency was not associated with particle number or size, but rather with physicochemical properties of the starting monomers. The incorporation of alkyl chains and hydroxyl groups in the lead candidate contributed to an improved polyplex stability and biocompatibility, whilst maintaining transfection efficiency due to the presence of tertiary amines that have high buffering capacity. On the other hand, although the remaining hits had a greater number of amines, their positive charge resulted in greater cytotoxicity. Although our hit was shown to be photosensitive, UV light activation did not translate in higher protein expression. On the contrary, UV irradiation reduced mRNA release from the polyplex after heparin replacement assay. This effect was attributed to crosslinking between the degraded polymer and the complexed mRNA.

Conclusions. Our high-throughput screening pipeline identified one formulation whose polymer composition enhanced mRNA transfection in fibroblasts, compared to commercially available transfection agents. Our results also emphasised the need to characterise transfection efficiency depending on the mRNA cargo.

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Rational design of a lyophilization process for Nanostructured Lipid Carriers

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Introduction

Nanostructured Lipid Carriers (NLC) are known as the second generation of lipid nanoparticles. They are composed by a blend of solid and liquid lipids, maintaining their solid state also at body temperature [1]. However, nanoparticles are often prepared as suspensions and water removal by methods such as lyophilization is required to obtain a solid dosage form [2]. Hence, this work proposes the use of Artificial Intelligence tools, with utility in the development of other pharmaceutical processes [3], on the lyophilization procedure optimization for NLC.

Materials and Methods

NLC were prepared by hot high shear homogenization. Several cryoprotectants (trehalose, sucrose, mannitol, glucose, fructose, lactose and sorbitol) were added in proportions ranging from 2.5 to 20% to the obtained formulations. Suspensions were frozen (liquid nitrogen or -80°C freezer) and lyophilized for 24-48 hours. Reconstitution of dried powders was performed by manual shaking, followed by sonication. NLC suspensions and reconstituted liophilisates were characterized in terms of particle size, polydispersity index and surface charge. Results are expressed as increase in particle size (Δ size), polydispersity index (Δ PdI) and zeta potential (Δ ZP) of reconstituted powders compared to NLC suspensions. The database obtained was modeled using two Artificial Intelligence (AI) softwares, FormRules® v4.03 (Neurofuzzy logic, NFL) and INForm® v5.01 (Artificial Neural Networks, ANN/Genetic algorithms) to analyze the impact of the lyophilization process parameters on NLC characteristics and to estimate the optimal lyophilization conditions, respectively.

Results and Discussion

The values of Δ size, Δ PdI and Δ ZP ranged among 23±1-157±4 nm, 0.06±0.01-0.51±0.05 and -5±3-7±7 mV respectively. Besides, models for Δ size and Δ PdI exhibited, in all cases, R^2 values above 70% and calculated f values higher than f critical, indicating a good model predictability [3]. Nevertheless, AI tools failed to model Δ ZP, probably due to the high similarity of the obtained values. In a first approach, NFL was employed to analyze the effect of freezing speed, the cryoprotectant selected and the amount used on Δ size and Δ PdI. NFL logic models obtained showed that both parameters depended on all the variables studied. Information extracted from "IF-THEN" rules indicated that trehalose and sorbitol can be employed at any tested percentage in order to achieve a low increase in both size and PdI. On the other hand, glucose, fructose and lactose should be employed at a concentration up to 10% while mannitol and sucrose should be used above 10%. Moreover, fast freezing with liquid nitrogen is preferable in order to achieve optimal results (low Δ size and Δ PdI).

In a second and more detailed approach, the influence of cryoprotectants molecular weight (MW), freezing speed and osmolarity on the parameters above mentioned was investigated. The obtained models showed that all the parameters studied affected Δ size and Δ PdI, as in the previous case. However, according to "IF-THEN" rules, MW and osmolarity seem to play a more relevant role. Regarding optimal lyophilization conditions, INForm® models predicted that values of just 49 nm and 0.11 would be obtained for Δ size and Δ PdI in lyophilisates obtained using a 6% of trehalose and liquid nitrogen for the freezing step.

Conclusions

"IF-THEN" rules obtained by NFL have proven to be useful to understand the effect of the different variables analyzed on the characteristics of NLC dried powders obtained by lyophilization. Furthermore, ANN/genetic algorithms models allow for the estimation of the lyophilization conditions required to achieve NLC dried powders with optimal characteristics.

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Shedding light on the isolation of extracellular vesicles for application as therapeutic agents

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Introduction: Extracellular vesicles (EVs) are membrane bodies secreted by all cells that play an important role in paracrine communication [1,2]. In recent years, EVs have been attracting the interest of many scientists as they are emerging as potential candidates to develop new therapeutics for many diseases [3]. However, the emergent nature of the field leaves several related issues to be elucidated. In this work, we focus on providing insights into a fundamental aspect, namely the isolation method that leads to greater recovery and purity as well as more functional vesicles. The two most commonly used methods, ultracentrifugation (UC) and size-exclusion chromatography (SEC), were compared [4]. This study includes the isolation and separate analysis of different subpopulations of EVs: microvesicles (MVs) and exosomes, an area still quite unexplored in most studies.

Methods: As our main purpose was to develop a reliable method for isolation of EVs with potential for cardiac repair, EVs were obtained from rat cardiac progenitor cells (CPCs) provided by Coretherapix SLU [5]. CPCs were cultured using DMEM/F12 and neurobasal medium (1:1) supplemented with 10% FBS, 1% P/S, 1% L-glutamine, 1% B27, 0.5% N2 and 50 μM β-mercaptoethanol as well as several cytokines. For isolation of EVs, CPCs medium was changed to: i) complete medium, ii) medium without FBS, B27 and N2 (serum components) or iii) basal medium. Conditions ii) and iii) respond to the incompatibility of several chromatographic columns with serum-enriched samples. At 24h and 48h, conditioned medium was harvested and MVs and exosomes were isolated by UC or SEC. Characterization of different EVs subpopulations was performed by scanning electron microscopy (SEM), measure of total protein content and presence of specific markers of different EVs subpopulations (Alix, CD63 and calnexin).

Results and discussion: SEM imaging revealed a heterogeneous population of 150-500 μm spherical MVs and 100-150 μm cup-shaped exosomes (Figure 1A).Culturing CPCs in 'starving' conditions with the absence of supplements and/or serum led to a marked reduction in the production of EVs. In particular, at 24h a 2.6-fold and 4.1-fold increase in the production of MVs and a 5.1-fold and 3.5-fold increase in the production of exosomes were observed when cells were cultured in complete medium compared to complete starvation and serum-depleted medium, respectively. At 48h, differences were more pronounced with an insignificant amount of EVs isolated when medium lacked some components compared to a slight increase in EVs production in cells cultured in complete medium (Figure 1B). Apart from the low production, culturing the CPCs in starvation would require a profound analysis of the cells undifferentiated phenotype and vesicles content, as these could be dramatically affected. Therefore, culturing CPCs in absence of serum or any supplement was discarded and SEC was adapted to serum-compatible columns. Regarding the isolation method, preliminary results indicate that similar recovery was obtained when EVs were isolated by UC and SEC (Figure 1C). Current studies focus on elucidating the purity of the EVs samples obtained by both isolation protocols and characterizing subpopulations enriched in MVs or exosomes. A functional study is now being performed to shed conclusive light on the optimal isolation method for future efficacy studies.

Conclusions: Altogether, this study helps to provide useful insights into the isolation method that leads to a greater and higher functionality of EVs for future potential application as therapeutic agents.

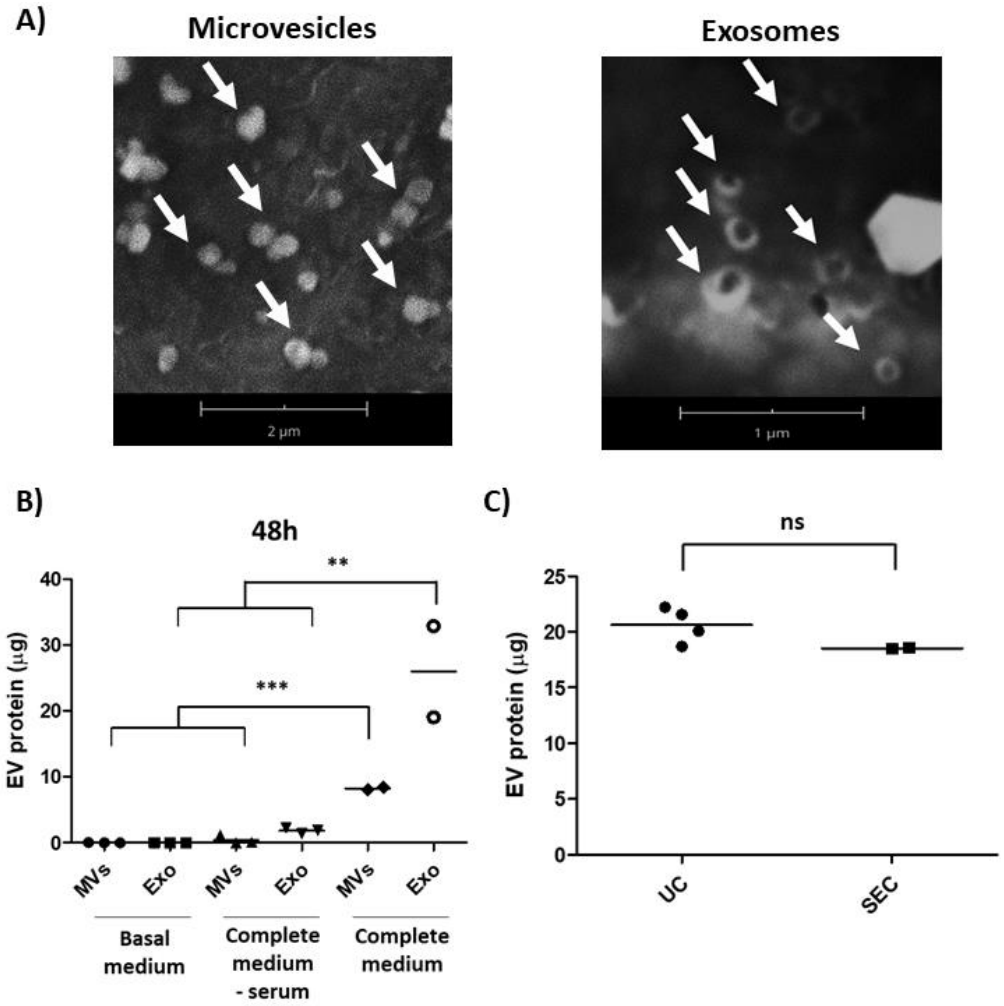

Figure 1. Characterization of EVs. A) Representative SEM images of MVs and exosomes (arrows) isolated by UC. B) Quantification of MVs and exosomes (Exo) protein content isolated by UC after 48h of cell culture in basal medium, complete medium without serum or complete medium. C) Quantification of total EVs protein obtained by UC and SEC. ** $p < 0.01$, *** $p < 0.001$ and ns: not significant.

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Short-term effect of a non-viral vector containing a plasmid encoding alfa-Galactosidase A on liver transaminases in a knock-out model of Fabry disease

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Introduction: Over the years, one of the most significant challenges of gene therapy has been the effective and safe delivery of the genetic material to its target. Advantages of viral vectors include the high cellular uptake, the high transduction efficacy and long-term gene expression. By contrast, safety concerns are considered major drawbacks, and the risk of immunogenicity has been previously reported as an important hurdle in gene therapy medicinal products development [1]. In fact, certain limitations preclude universal applicability of gene therapy with viral vectos, including transient liver transaminase elevations due to the immune responses [2]. On the contrary, one of the main advantages of non-viral vectors is the low immunogenicity. The main objective of this work was to assess the potential risk of immunogenicity of a solid lipid nanoparticle (SLN) based-vector by measuring the glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in the liver after the intravenous administration to a knock-out (KO) model of Fabry disease [3].

Materials and Methods: The non-viral vector was prepared with SLN, dextran (Mw of 3.26 KDa), protamine and a plasmid DNA (pDNA) containing the gene encoding the enzyme alfa-Galactosidase A (α-Gal A) (pR-M10-α-Gal A), defective in Fabry disease [4]. Particle size and surface charge were 262±6 nm and +38±2 mV, respectively. After intravenous administration of a single dose (60 µg of pDNA) through the tail vein to KO Fabry mice, the animals were sacrificed and the activity of GOT and GPT were measured in the liver with a comercial kit assay (Merck/Sigma-Aldrich Spain).

Results and Discussion: Figure 1 features the activity of transaminases GOT and GPT in the liver of KO mice untreated and treated with the SLN-based vector. As the figure shows, the vector did not increase ($p<0.05$) the GOT and GPT activity, and therefore it not induce hepatic cell destruction. It is important to take into account that one of the target organs for the treatment of Fabry disease with this formulation is the liver, where the transgen α-Gal A is intended to be expressed in order to be later released to blood circulation. Actually, in previous studies, this vector was able to transfect mainly the liver and increase the hepatic α-Gal A activity in wild type and Fabry mice, respectively.

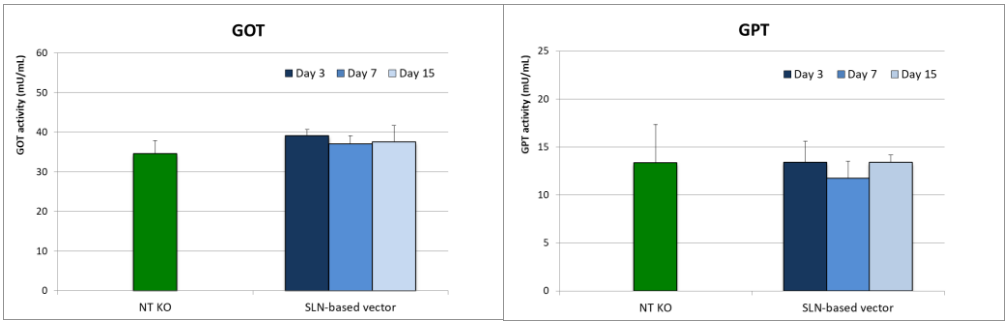

Figure 1. GOT and GPT activity in the liver of mice untreated and treated with the SLN-based vector. NT KO: non-treated knock-out mice

Conclusion: The SLN-based vector did not induce elevation of the GOT and GPT activity after intravenous administration to Fabry mice, indicative of lack of development of immune response.

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Size-controlled formation of monoolein-DDAB nanoparticles using microfluidics

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Introduction: Monoolein (MO) is an ubiquitous lipid in the production of drug delivery formulations mostly because of its propensity to form cubosome nanoparticles (NPs) [1-2]. Cubosomes constitute dispersions of lipid bicontinuous cubic phases in water typically stabilized with an amphiphilic polymer[3]. Its interior interconnected matrix of lipid bilayers separating two independent interconnected networks of water channels make them excellent candidates for encapsulation and delivery of both hydrophobic and hydrophilic drugs. Cubosomes are typically prepared either by fragmenting the cubic liquid crystal in excess water using high energy or using solvent-exchange in which the lipid is first dissolved in a water-miscible solvent and then mixed with water and polymer stabilizer. In both cases poor experimental control at the microscale limits the fine tuning of the particle properties and results in NPs with broad size distributions. In this work, we employ the solvent-exchange method using a microfluidic device achieving rapid and controlled mixing at the microscale and obtaining cubosomes of tunable size and low polydispersity. Simultaneously, we study the effect of the incorporation of didodecyldimethylammonium bromide (DDAB) on the charge and structural properties of the lipid NPs.

Materials and Methods: A cross-shaped microfluidic device is used. The lipids used are MO and DDAB. The stabilizer is the pluronic F127 block copolymer. The ethanol-MO-DDAB solution is flowed through the central inlet, being squeezed/focused by two side streams of water-F127. As the lipid-ethanol solution narrows, ethanol and water are mixed in a controlled way by diffusion, leading to formation of the lipid NPs. Particle characterization is performed with DLS.

Results and Discussion: By manipulating the flow rate ratio (Q_R) between the two solutions we manipulate the width in which the hydrodynamic focusing occurs, influencing the assembly time in a homogeneous way (Fig). Thus, by manipulating the flow rate ratio, we were able to tune the size of the cubosome NPs, achieving smaller sizes when increasing the extent of the hydrodynamic focusing (i.e. increasing the flow rate ratio). This is valid for initial MO concentrations up tp 2 wt%, above which the size control is lost. Interestingly, when replacing 10 % of MO with DDAB, the size dependence with Q_R is lost, but the system becomes more tolerant to increasingly higher amounts of lipid in the initial solution. Now, even for 7 wt% initial concentration the size polydispersity is small. These results suggest that for pure MO-F127 cubosomes, the size is directly dependent on the exchange rate of solvent molecules, like found for microfluidic-assisted liposome synthesis [4]. When DDAB is included, the size does not depend on the solvent exchange-rate any longer, but controlled sizes with low polydispersity are still obtained.

Conclusions: By allowing a precise control over the mixing and exchange between the two solvents, microfluidics promises to become an effective approach to further tailor the structure and efficiency of drug delivery systems. Here we show that this tunability is further dependent on the lipid composition. Neat MO particles are very dependent on the flow conditions, while MO-DDAB systems are less sensitive but still result in particles with low polydispersity. Controlling NP size and monodispersity is a relevant step towards the design of new and more efficient formulations.

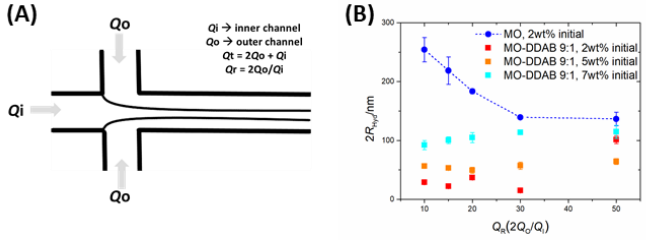

Figure 1. (A) Schematization of the hydrodynamic focusing flow. (B) Hydrodynamic diameter ($2R_{Hyd}$) dependence of MO and MO-DDAB formulations as a function of the flow rate ratio Q_R.

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Stability evaluation of sulphated locust bean gum-coated lipid nanocapsules

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Introduction: Drug delivery systems have been increasingly proposed in therapeutics, providing therapeutic efficacy improvement in varied ways [1]. Lipid nanocapsules (LNC) are versatile nanocarriers, as they enable the association of lipophilic molecules in an oily core, which is surrounded by a polymer shell that provides protection from potential degradation [2]. Overcoming multidrug resistance and biological barriers are two potential strategies for the use of LNC, with promising results [3]. The aim of this work was to prepare locust bean gum (LBG)-based LNC and evaluate their stability upon storage at 4 ºC, for 30 days. The solvent displacement technique is the most used method to prepare LNC [4], requiring an oily phase that will comprise the core, and an aqueous phase which will provide the coating and functions as a shell. LBG is a neutral galactomannan that has been finding interesting applications in drug delivery [5]. Due to its neutral character, it cannot be used in particle preparation methods involving electrostatic interactions. Therefore, a charged derivative of LBG was synthesised in this work (sulphated LBG, LBGS), to successfully produce LNC [6]. **Materials and Methods**: For the preparation of LBGS, commercial LBG (M_w = 589100 Da [6], Industrial Farense, Portugal) was purified, by dispersion in water, at 85 ºC, followed by centrifugation (22000 xg, 20 ºC, 1 h) to remove the protein content. The supernatant was precipitated with ethanol 96º, concentrated by centrifugation under the same conditions, and then sulphated by a SO_3.DMF complex, prepared from chlorosulfonic acid (Merck, Germany) and dry dimethylformamide (VWR, Portugal) [6]. LNC were prepared by solvent displacement technique. Briefly, an organic phase made of 1,2-dioleoyloxy-3-trimethylammoniumpropanchloride (DOTAP) dissolved in ethanol, Miglyol® 812 and and acetone was poured over an aqueous phase containing LBGS. Finally, the organic solvents were removed under vacuum to a final volume of 10 mL. Variable concentrations of DOTAP (0.05% and 0.1%, w/v) and LBGS (0.2% to 2.0%, w/v) were tested. After production, LNC were stored at 4 ºC, and their physicochemical stability in terms of size and polydispersion index was evaluated over a period of 30 days. **Results and Discussion**: LBGS was successfully prepared, which was confirmed by FTIR. Eight LNC formulations were prepared using the synthesised LBGS, overall showing sizes of approximately 200 nm and ζ-potential around -80 mV. Additionally, the results show monodisperse populations (PdI < 0.2), indicating that varying LBG and DOTAP amounts did not impact on the physicochemical parameters of LNC. Thus, the formulations containing 0.2% and 0.5% (w/v) of LBGS and 0.05% and 0.1% (w/v) of DOTAP were selected to assess stability. Upon storage at 4 ºC and considering the measurement at day 1 as a reference, the size of nanocapsules was maintained in all formulations, which was also observed for PdI.As for the ζ-potential, the nanocapsules maintained the strong negative charges, above -70 mV, no variations being perceived during the 30 days of the experiment, indicating stability of this parameter. **Conclusions**: LBGS was sucessfully prepared and used to coat the LNC, acting as a polymeric as shell. The nanocapsules were observed to maintain their physicochemical characteristics upon 30 days of storage at 4 ºC. Ongoing studies involving encapsulation of a model drug, curcumin, are under investigation. Cytotoxicity assays in epithelial cells are also envisaged.

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Subcutaneous administration of nanostructured lipid systems containing alpha elosulfase. Biodistribution studies in mice

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Introduction

Mucopolysaccharidosis (MPS) are a group of rare metabolic disease caused by deficiency of lysosomal enzymes. Mucopolysaccharidosis IVA or Moquio A, the deficit of intra-lysosomal enzyme leads to a progressive accumulation of keratan sulfate and chondroitin 6-sulfate predominantly in specific tissues such as cartilage and connective tissues, cardiac valves, cornea, etc [1]. Currently, one of the two available therapies for MPS IVA is the intravenous administration of the recombinant GALNS enzyme (elosulfase alfa) to patients weekly (so-called Enzyme Replacement Therapy, ERT) [2]. The major disadvantage of the ERT is inefficient distribution of the enzyme and therefore, the difficulty in achieving therapeutic concentrations in primary affected tissues (bone and brain. Also, ERT treatments can produce drug-related hypersensitivity and anaphylactic reactions. Lipid nanoparticles have shown a great potential to enhance their permanence in blood and tissues [3]. Recently, we developed a new type of nanoestructured lipid carriers (NLC) as a carrier for the delivery of elosulfase alfa and showed their ability to immobilize the therapeutic enzyme [4].

As a follow-up, the main goal of this work was to evaluate determine the *in vivo* biodistribution in mice of stable NLC for the delivery of elosulfase alpha to lysosomes as a potential therapy for treating MPS IVA.

Materials and methods

NLCs containing elosulfase alfa were prepared according to PCT/ EP2019/ 068629 [4]. For the study, the NLC resuspended in Milli Q water were stained with 1,1′-dioctadecyl-3,3,3’, 3’-tetrachloyl dihydrobocyanine perchlorate (DiD, Thermo). Biodistribution studies were carried out by triplicate in wild-type mice (60±10 g). Animal studies were approved by the Animal Care Committee of Xunta de Galicia (15010/2019/005).

Each animal received 300 µL of NLC dispersion at a concentration of 100 mg/ml in a 1% of taurocholate solution by subcutaneous solution. After 24 hours, the animals were anesthetized and euthanatized and organs were removed. Sections of the organs were visualized using a JEOL TEM 1011 microscope and fluorescence microscope Leica TCS-SP8 after fixation and staining.

Results and discussion

The images obtained by confocal microscopy were all fused to obtain a complete section of the tissue (Photoshop CC software). In addition, the expansion of plans to different objectives magnification (20X, 4X, 63X, and 100X) were included. Images show that NLC were distributed in all the tissues studied including the brain and bone tissue. The TEM images confirmed also that NLCs were able to cross the blood-brain barrier and reach bone, lug, liver, spleen kindey and skeletal muscle.

Conclusions

Results indicated that NLCs containing elosulfase alpha were capable to reach and penetrate into the main tissues that are usually affected in Morquio syndrome. NLC have shown to overcome the blood brain barrier. Hence, this enzyme delivery system is promising for the development of enzyme carriers to improve the therapeutic effects of MPS IVA.

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Tailor made Oligonucleotide-hybrid-nanosystems: development and characterization

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Introduction. Gene silencing has emerged as a promising tool for treating complex diseases such as cancer or systemic metabolic disorders [1]. Despite the huge potential of synthetic oligonucleotides as gene silencing molecules, their efficient administration remains a challenge to beat due to their low stability, small cell internalization and lack of specificity [2]. Among synthetic oligonucleotides GapmeRs are antisense oligonucleotides comprised of a synthetic single strand containing a central block of DNA nucleotides flanked by locked nucleic acids on each side. This structure provides several advantages as increased stability and decreased size when compared to other synthetic oligonucleotides [3]. Nanoparticulated systems (NPs) allow for the incorporation of several therapeutic molecules on their structure that, owing to their surface modification abilities, can be specificly released on the target cells. NPs pegylation is a widely used strategy to avoid opsonisation, reduce its clearance and increase their blood circulation permanence time [4]. The main objective of this work is to develop pegylated hybrid nanosystems with specific surface properties able to incorporate GapmeRs.

Materials and Methods. Polymer-lipid hybrid nanoparticles were prepared by a modified nanoprecipitation method. Briefly, PLGA or PLGA+DOTAP were dissolved in acetone to which a small volume of an aqueous solution of protamine or protamine+GapmeRs was added and mixed. Inmediately, this mixture was poured on an aqueous solution containing DSPE-PEG with or without lecithin. The obtained NPs were characterized in terms of size, Polydispersity Index (PdI) and zeta potential. GapmeRs were condensed on the protamine solution for 40 minutes previously to formulation. Fluorencently labelled GapmeR was used to analyze NPs encapsulation efficiency. To do so, NPs suspensions were filtered through 10 KDa MWCO and fluorescence was measured on a plate reader. Oligonucleotide concentration was assessed by means of the correspondent calibration curve. All experiments were carried out al least in triplicate.

Results and Discussion. Pegylated hybrid nanoparticles were successfully obtained without the need of using synthetic surfactants. The synthesis of nanoparticles incorporating only DOTAP (0:100 Lec:DOTAP) led to the development of highly positive NPs (+26.22 ± 3.75) of 147 ± 13.3 nm and PdI 0.07. On the other hand, when DOTAP was replaced by lecithin (100:0 Lec:DOTAP) highly negative NPs were obtained (-24.03 ± 0.92) of 147.7 ± 12.8 nm and PdI 0.10. Moreover, when both lipids were combined on the same proportion (50:50 Lec:DOTAP) neutral NPs were obtained (-3.62 ± 3.86) of 155.6 ± 0.71 nm and PdI 0.07. All nanoparticles showed high GapmeR encapsulation efficiency being 84,47 ± 0,52 % for 0:100; 83,27 ± 1,14 % for 100:0 and 84,55 ± 0,41 % for 50:50 Lec:DOTAP. Interestingly, the incorportation of GapmeRs modified NPs surface charge as shown in Figure 1. While not much effect was observed for negatively charged NPs, neutral and positively charged ones significantly decreased their zeta potencial with the incorpotation of GapmeR. The differences in surface charge of NPs are expected to modulate cellular uptake and intracellular localization [5], which could condition gene silencing efficiency, an effect that currently remains unknown.

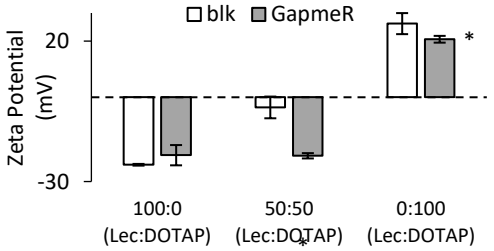

Figure 1. Zeta potential of empty (blk) and loaded (GapmeR) nanoparticle formulations. * Denotes statistical differences compared to blk formulations ($p \leq 0.05$).

Conclusions. Hybrid nanoparticles with remarkably low polydispersity index were obtained**.** All formulations successfully incorporate GapmeRs with high encapsulation efficiency. A portfolio of NPs with variable surface charge was developed that will serve in future work to elucidate the effect of NPs surface charge on gene therapy efficiency.

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Targeted cathelicidin nanomedicines as novel glucoregulor for diabetes therapy

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Type 1 Diabetes (T1D) is one of the most common chronic diseases in children and has increased incidence over the past 50 years [1]. T1D is caused by the autoimmune destruction of the insulin producing pancreatic β cells, resulting in the requirement of lifelong administration of insulin to control glycemia, with no cure available. Therefore, approaches aiming to restore endogenous pancreatic function through β cell regeneration are an alternative treatment option. Cathelicidin, also known as LL-37, is an antimicrobial peptide, recognized for a pleiotropic role in the innate immune system, has revealed a promising role T1D treatment due to its ability to increase β cell function and neogenesis [2]. LLKKK18 is a synthetic peptide, derived from the sequence of cathelicidin, with a smaller size sequence and with various conserved activities of LL-37, but its potential therapeutic role in islet function is still unexplored. The enzymatic degradation of peptides decreases their bioavailability and in that sense, loading on a drug delivery system allows protection from degradation and a sustained drug delivery, avoiding systemic toxicity. One of the most successfully used drug delivery systems are polylactic-co-glycolic acid (PLGA) nanoparticles (NPs). Being biocompatible, biodegradable and FDA approved, are also easily functionalized with a ligand that targets a particular receptor in a cell type. In β cells, a widely described receptor is the glucagon-like peptide-1 receptor (GLP-1R), although also found in other organs [3]. In this work, we aim to develop a nanoformulation able to recover β cell mass and pancreatic function by the targeted delivery of LLKKK18 to β cells by functionalizing the NPs with exenatide, a GPL-1R agonist, with a higher avidity in binding the GLP-1R than its natural ligand, GLP-1.

LLKKK18-loaded PLGA NPs were prepared following a w/o/w double emulsion solvent evaporation method [4]. These NPs were characterized in terms of size, zeta potential and polydispersity using dynamic light scattering (DLS); size distribution and NP concentration using nanoparticle-tracking analysis (NTA). Furthermore, the obtained association efficiency (AE) and drug loading (DL) of LLKKK18 were determined indirectly by measuring the unloaded LLKKK18 using MicroBCA, RP-HPLC and fluorescamine, and directly after dissolving the lyophilized NPs in dimethyl sulfoxide. The study of the peptide cumulative release from the NPs was performed using a variation from the Franz cells, in which the lower compartment, containing the NPs in phosphate buffer (PB, pH=7.4) under magnetic stirring, is separated from the upper compartment, containing the same buffer, separated by a 12 kDa dialysis membrane, incubated for a week at 37 ºC. The effects from the blank or LLKKK18-loaded NPs on the cell viability of L929 fibroblast cell line were also evaluated using the MTT reduction assay after 24 h incubation with the NPs.

The obtained results from DLS and NTA were in agreement, indicating a mean hydrodynamic size around 150 and 140 nm, for blank and LLKKK18-loaded NPs, respectively. The narrow size distribution was maintained, with and without peptide loading, at 0.10. AE and DL were determined around 70 – 88 % and 0.7 – 0.9 %, respectively and resulting -3.1 mV zeta potential. Although with an almost neutral zeta potential, NPs were stable in aqueous solutions up to 20 days at 4 ºC in PB. The preliminary *in vitro* drug release study indicated a sustained and slow drug release of the peptide from the PLGA NPs. Furthermore, from the viability assay, a PLGA concentration without cytotoxic effects was determined, and the loading with different LLKKK18 concentrations will next be tested. In the near future, the NPs will also be functionalized with exenatide using the thiol-maleimide "click" chemistry. The ability of the formulation, or free peptide, to promote glucose-mediated insulin release will be addressed using an insulinoma cell line INS-1E and the functionality of NPs in targeting specifically β cells while avoiding other cell types will also be evaluated.

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POSTER SESSIONS

Session 8: Oral delivery of drugs and other active ingredients

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Bioengineered glucose-responsive nanoparticles in artificial pancreas for Diabetes therapy

Joana Moreira Marques[1;2;3*], Rute Nunes[1;2], Helena Florindo[4], Domingos Ferreira[5], Bruno Sarmento[1;2;6]

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Introduction: Type 1 Diabetes *mellitus* (T1DM) is a chronic auto-immune disease characterized by the lack of insulin secretion due to the destruction of insulin-producing pancreatic β-cells, accounting for 10% of DM cases [1,2]. T1DM is not preventable and most of the cases are lately diagnosed after a massive destruction of the pancreatic β-cells [3]. The only definitive cure consists in replacing the destroyed pancreas; however, the number of available pancreas or islets is limited, in addition to the need of a lifelong immunosuppression therapy [4,5]. Induced pluripotent stem cells (iPSCs), differentiated into β and α-cells, are promising candidates to replace pancreatic islets, to mimic the pancreatic closed loop and regulate insulin secretion [6]. The goal of this project is to develop a biomimetic pancreas, comprising differentiated iPSCs immobilized in a biofunctional matrix and pH-responsive nanoparticles (NPs) encapsulating exenatide, sensitive to glucose levels. The sensitiveness to glucose will be accomplished by the incorporation of the enzyme glucose oxidase (GOx) in the system. In the presence of high levels of glucose, GOx degrades the molecule into gluconic acid, decreasing the surrounding pH (~5). pH-sensitive engineered NPs will respond to the decrease of the environmental pH by releasing their cargo. In the present work, the development and characterization of pH-sensitive NPs is addressed.

Methods: Formulations of pH-sensitive NPs were produced by using different ratios of PLGA and polymethacrylates. NPs were prepared by a modified solvent emulsification-evaporation method based on water-in-oil-in-water double-emusion technique. Different amounts of polymer (ranging from 20 mg to 50 mg) were dissolved in ethyl acetate. After, Milli-Q water or exenatide aqueous solution were added (to produce empty or loaded NPs, respectively) and the mixture was homogenized by ultrasound for 30 s with 70% amplitude, forming the primary emulstion. Then, surfactant solution (1% (w/v) Poloxamer 407 in water) was added and mixed in the same sonication conditions. Different sonication times (in the second sonication step) ranging from 15 s to 30 s were also tested. The second emulsion was then added to 7.5 mL of surfactant and left under magnetic stirring for 3h, to allow organic solvent evaporation. Three replicates were produced for each formulation and NPs were characterized regarding average size and size distribution by dynamic light scattering, and zeta potential by laser Doppler electrophoresis. For the measurements, samples were diluted with 10 mM sodium chloride to an appropriate final concentration.

Results: The different NPs formulations showed a hydrodynamic size ranging from 90 to 100 nm, and a narrow size distribution (< 0.160), showing a monodisperse population. Concerning surface charge, NPs presented zeta potential values in the range of 30 to 40 mV for formulations with higher ratios of polymethacrylates while the surface charge decreased until -7 mV for NPs with high proportions of PLGA. Moreover, exenatide loading and association efficiency are still under optimization. In order to increase NPs size, different polymer amounts and sonication times were tested and the best formulation tested so far showed an average size around 160 nm, a low polidispersity index (0.140) and surface charge around -6 mV.

Conclusions: In summary, pH-sensitive polymeric NPs were successfully produced by the referred methodology and have the potential to be used in the artificial pancreas system to be developed as a diabetes therapy. Nevertheless, more tests still need to be performed regarding drug encapsulation.

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Continuous microfluidic assembly of insulin-loaded pH response nano-in-microparticles: towards to insulin oral delivery

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Introduction

Oral delivery offers enoumerous advantages that overcome the main drawbacks from other routes of administration namely parenteral route. However, oral administration of proteins encounters different challenges through the gastrointestinal tract, such as the protein activity inactivation by gastric pH and enzymes, as well as poor permeability in the intestinal epithelium [1]. The use of delivery systems such as liposomes can surpass these drawbacks. Liposomes [2] are lipid vesicles mostly produced by bulk methods, which can require further post-processing steps, as high pressure extrusion, in order to obtain a better control of size and polydispersity index (PDI). Recently, a glass-capillary based microfluidics facilitated continuous production of liposomes have drawn increasing attention due to its controllable liposomes size/morphology manipulation and high batch-to-batch reproducibility, whereas the commonly applied polydimethylsiloxane (PDMS) based microfluidics chips has low resistence towards organic solvents, which may set limitations for its further application in the synthesis of nanoparticles [3]. In this work, an advanced nano-in-microparticle system, towards to insulin oral delivery, that protects the protein against the acidic conditions of the gastric environment and enables an improved protein transport across of the intestinal epithelium, was designed and developed.

Materials and Methods

Egg-phosphatidylcholine and distearoylphosphatidylethanolamine-poly(ethyleneglycol) 2000 were obtained from Lipoid (Germany). Cholesterol, recombinant human insulin (rhIns), medium viscosity chitosan (Mw= 190 000–310 000 Da, 75–85% deacetylated), were purchased from Sigma-Aldrich (USA). Poloxomer 407 (Kolliphor® 407) was purchased from BASF (Germany). MF grade of hydroxypropyl methylcellulose acetate succinate was purchased from ShinEtsu (Japan). Liposomes were produced by nanoprecipitation using a glass-capillary microfluidic device, whereas nano-in-microparticles were obtained through a glass-capillary based single-emulsion production. The nanoparticles were characterized in terms of their morphology using DLS and cryo-TEM., whereas the nano-in-microparticles were characterized using confocal fluorescence microscopy and attenuated total reflectance Fourier transformed infrared spectrometry. rhIns was quantified using a high-performance liquid-chromatography. Finally, the interaction between nanoparticles and cells were qualitatively and quantitatively evaluated by confocal microscope and flow cytometry.

Results and Discussion

Insulin-loaded liposomes, with a size of 144 ± 23 nm and a mean PDI of 0.130 ± 0.003, were obtained. Chitosan was physically adsorbed in order to improve the mucoadhesion of nanoparticles, and the yielded nanohybrids were sequentially encapsulated in a pH-responsive polymer (MF) micro-matrix, as such, enabling a nanoparticles protection from the hush gastric conditions, obtaining microparticles with a size of 19 ± 1 µm. *In vitro* release assays showed no insulin release at gastric environment (pH 1.2). The confocal microscopy was performed in order to evaluate the insulin-loaded nano-in-microparticles behavior under gastric and intestinal conditions.

Conclusions

A multistage oral insulin delivery system using a continuous two-step microfluidics process was successfully designed, developed and optimized. Common liposome batch production drawbacks were overcome, and a narrow size distribution was obtained, turning this into a promising method towards the proteins oral delivery.

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Development and optimization of Orally Dissolving Films targeting pediatrics and patients suffering from dysphagia

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ABSTRACT

Orally dissolving films (ODFs) have received much attention as potential drug delivery systems for oral administration of drugs for pediatric patients. With their unique properties and advantages, the technology offers improved patient compliance and wider acceptability, eliminated fear of choking, ease of administration and dosing convenience, without the requirement of water. The aim of this study was the development of ODF formulations with suitable physicochemical and mechanical properties as a potential dosage form for pediatric use using anti-epileptic model drug (topiramate). ODFs were prepared using HPMC (hydroxy propyl methyl cellulose), guar gum, in combination with plasticizers such as glycerin and sorbitol as well as other excipients. Films were prepared via solvent casting method and then produced ODFs were evaluated for mechanical properties, disintegration time, dissolution time and dosage form uniformity. Initial studies focused on screening of different film-forming polymers used for the preparation of orally dissolving films in order to optimize and propose suitable polymers and plasticizers with a suitable manufacturing technique. The work also sought to improve the loading capacity and drug content uniformity of both hydrophilic (topiramate) drug into ODFs. Loading capacity of topiramate load reached 58.95% with 25 mg topiramate per film (6 cm^2). Results demonstrated good disintegration time of below 60 seconds and dosage from uniformity which was assessed using weight variation and content uniformity. In conclusion, the ultimate goal of any drug delivery system is successful delivery of the drug to the body; however, patient compliance should not be overlooked. ODFs provide convenient drug delivery systems not only for special populations with swallowing difficulties, such as children and the elderly, but also for all patients. Therefore, the outcome of this research project could be a starting point for further work to optimize and assess ODFs for delivering other drugs via this formulation.

Development of Water-in-Water (W/W) emulsions based on alginate and caseine for drug delivery.

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Introduction

Water-in-water (W/W) emulsions are liquid/liquid dispersions of two immiscible aqueous phases, in absence of both oil and surfactant. These emulsions are formed in aqueous mixtures of two hydrophilic polymers, when segregative phase separation is induced by thermodynamic incompatibility between the two polymers [1]. In recent years, the study of water-water interfaces and the stabilization of W/W emulsions [2] has become a topic of remarkable interest, as these emulsions achieve the multiphase compartmentalization of conventional emulsions and, due to their high water content and the absence of surfactant and oil component, have a greater biocompatibility, in addition to the possibility of effectively incorporating biomolecules, hydrophilic drugs and microorganisms [1]. It should be noted that W/W emulsions can be precursors for the preparation of microgels, nanogels and other polymeric nanostructures (nano-aggregates, nanocapsules and nanospheres) [3], for potential pharmaceutical applications.

The objective of this work has been the development of protein-polysaccharide W/W emulsions in the water/casein/alginate system. Clindamycin hydrochloride (2 wt%) has been incorporated as a water-soluble model drug and its diffusion to a receptor medium has been studied.

Materials and Methods

Materials. Sodium Alginate (Sigma), Sodium Casein from Bovine Milk (Sigma), Clindamycin Hydrochloride (Fagron), and MilliQ Water.

Methods. The emulsions were prepared from mixtures of an aqueous solution of alginate (containing clindamycin) and casein in phosphate buffer solution (PBS pH 7.4). The solutions were mixed using an Ultraturrax T8 homogenizer (IKA) at 25,000 rpm, to form the emulsion drops. All samples were stored in a thermoregulated bath at 25°C. Emulsions were characterized by optical microscopy, using ImageJ image analysis software. Drug release studies were performed on the Vision® G2 Elite 8 ™ Dissolution Test equipment, using dialysis bags (Cellu SepT3® MWCO 12000-14000 Da hydrophilic membrane), and 150 mL of 1X PBS as receptor solution, at 37°C, with a stirring of 250 rpm for 24 hours. Four replicates were tested for each formulation. The quantification of clindamycin in the samples was performed by HPLC (Shimadzu equipment, Nexera X2) with UV detection.

Results and Discussion

The formation and stabilization of Water-in-Water (W/W) emulsions have been investigated in the pseudoternary system composed of water, casein and alginate.The emulsions selected were: A2.5C2.5, A2.75C2.5, C12.5A0.5; C13.75A0.25, where the letters refer to the composition (A: alginate, C: casein) and the order of incorporation; the numbers indicate the quantitative composition. Clindamycin (2 wt%) was succesfully incorporated to the emulsions. The samples presented similar droplet sizes, in the range of 5-10 μm. Emulsions stability was determined micro and macroscopically for 1 month at 25°C, with and without drug. Clindamycin loaded emulsions showed a higher stability, that could be attributed to the ionic contribution of the drug to the repulsive interactions between polymers.

The emulsions showed a slower release of clindamycin over time in comparison to clindamycin in PBS solution, clindamycin in casein solution or clindamycin in alginate solution (Fig. 1). The results obtained suggested that the drug is mainly dispersed in the inner phase.

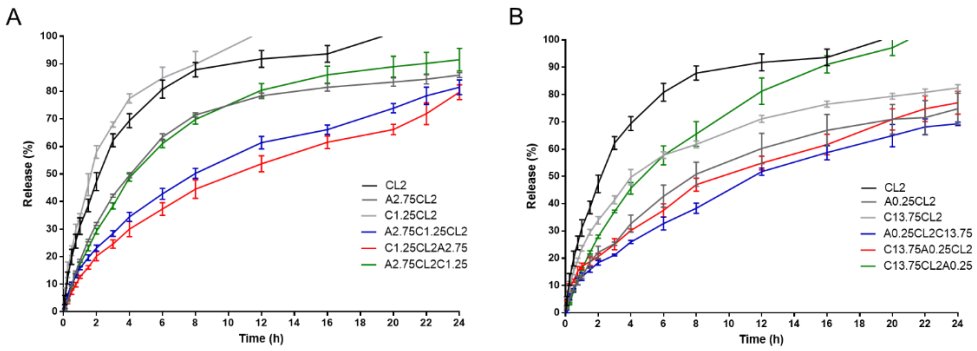

Figure 1. Clindamycin release profiles from solutions and W/W emulsions at 37ºC (A) Clindamicyn released from solutions and casein in alginate emulsions, and (B) Clindamicyn released from solutions and alginate in casein emulsions. Values are means of n = 4 ± SEM.

Conclusions

W/W emulsions based on alginate and casein containing clindamycin (2%) have been developed. Phase separation was not observed in a period of time of 1 month. These W/W emulsions can be considered as potential novel drug delivery systems due to their biocompatibility (absence of oil and surfactant) and the slower diffusion of actives compared to polymeric solutions.

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Evaluation of quercetin-loaded nanoparticles in a *C. elegans* model of type II diabetes

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Introduction

Quercetin is a dietary flavonoid present in some fruits and vegetables with antioxidant, anti-obesity, anti-diabetic and anti-inflammatory properties. Nevertheless its use in therapeutic is highly hampered by its hydrophobic character as well as by a very low oral bioavailability [1]. One possible strategy to minimize these drawbacks may be its encapsulation in zein nanoparticles.

Zein is a class of prolamine protein found in corn that is classified as Generally Recognized as Safe (GRAS) by the FDA. Likely, nanoparticles based on this protein, may be obtained with simple procedures in an aqueous environment [2]. Another important advantage would be, as for many other proteins, its capability to interact with a number of compounds in a non specific way [3]. The aim of this work was to evaluate the effect of quercetin-loaded nanoparticles on the fat accumulation by an *in vivo* model of type II diabetes based on *Caenorhabditis elegans* [4].

Materials and Methods

Zein nanospheres and nanocapsules were prepared by a desolvation method [2]. For this purpose, a hydroalcoholic solution of the protein, quercetin and, eventually, the oily phase (in case of nanocapsules) was mixed with water. The resulting nanocarriers were purified and, finally, dried by Spray-drying. For the preparation of gastroresistant nanocarriers, the just formed nanoparticles were incubated with a commercial polymer previously to their purification and drying. In all cases, nanoparticles were characterized by determining their size, polydispersity index (PDI), zeta potential and payload. The morphology of the nanoparticles was examined by SEM.

For the *in vivo* evaluation in *C. elegans*, the effect in the fat accumulation of the free and encapsulated flavonoid was evaluated. N2 strain was used, age-sincronized in L1 larval stage, fed with Nematode Growth Medium (NGM) with *Escherichia coli OP50*, supplemented with glucose (0.5% w/v). The fat accumulation was determined using Red Nile stainig and Orlistat was used as a positive control of fat reduction.

Results and Discussion

Zein-based nanoparticles displayed a mean size ranging from 220 to 265 nm. The co-encapsulation of an oily phase and/or the coating of nanoparticles with the gastroresistant polymer increased the mean size of the resulting nanocarriers. In all cases, the polydispersity of the different batches was low (below 0.3) and the nanoparticles possessed a moderate negative charge.

In the *in vivo* study, the ingestion of zein-based nanoparticles by nematodes was confirmed by the presence of fluorescently labeled nanoparticles observed in the pharynx and through the gut of worms 2h post administration. On the other hand, quercetin (at a concentration of 50µM) reduced the accumulation of fat in the worms (about 7.1%). This decrease was found to be significantly higher when nanoencapsulated in zein nanospheres (about 12.5%). This fact would be related to the increasing of bioavailability of quercetin by zein nanospheres. On the contrary, when quercetin was encapsulated in zein nanocapsules, no differences with the control nematodes were found. This finding would be related to the presence of oil in the nanodevices. Finally, the coating of nanospheres with a gastroresistant polymer annihilate the fat-lowering effect of quercetin. This finding would be related to the mildly acid conditions (pH 4-5) that can be found in the intestinal lumen of *C. elegans* [5].

Conclusions

Quercetin reduces the accumulation of fat in *C. elegans* when growth in a glucose supplemented medium. This effect increased two-times when the flavonoid was encapsulated in zein nanospheres. However, its formulation in oily nanocapsules or the coating of nanospheres with a gastroresistant polymer negatively affected its fat-lowering effect.

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Evaluation of the mucus-permeating properties of polymeric-based nanocarriers

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Introduction

The development of mucus-permeating nanoparticles may be an adequate strategy to improve the bioavailability of poorly absorbed drugs. For this purpose, the decoration of the surface of nanoparticles with hydrophilic polymers (i.e., PEG) has been proposed [1]. The objective was to evaluate the mucus-permeating properties of polymeric-based nanocarriers, including nanospheres and nanocapsules, and the effect of their coating with PEG.

Materials and Methods

Polymeric nanoparticles (NP) were produced by a desolvation method and dried by lyophilization. In order to produce the oily loaded nanoparticles (M-NP), the oil was added before the formation of the nanoparticles. Whereas, PEG was incorporated to the formulation after the formation of nanoparticles by simple incubation in an aqueous environment (NP-P and M-NP-P).

In order to characterise the physico-chemical properties of all the formulations the size, the polydispersity index (PDI) and the zeta potential of the nanoparticles were determined. To evaluate the surface hydrophobicity of the formulations the Rose Bengal test was carried out [2]. The diffusion of nanoparticles through pig intestinal mucus, as an in vitro measurement of their mucus-permeating properties, was assessed by the Multiple Particle Tracking (MPT) technique [3].

Results and Discussion

Polymeric nanoparticles (NP) were 170 nm in size with a PDI of 0.1, displaying a zeta potential of -45 mV. These nanoparticles presented a hydrophobic surface with a poor capability to diffuse in mucus. A similar behaviour was observed for M-NP, evidenced of an important mucoadhesiva character.

On the other hand, when nanoparticles were decorated with PEG (NP-P and M-NP-P), there was a reduction in the hydrophobicity value and an increase in the ability of these nanoparticles to diffuse in pig mucus.

Figure 1 shows fluorescence microscopy images of intestinal samples from animals receiving orally either M-NP (Figure 1A) or M-NP-P (Figure 1B) fluorescently labelled with Lumogen red. The biodistribution of these formulations was radically different. M-NP was observed in the protective mucus layer, whereas M-NP-P was able of crossing this barrier and reach the intestinal epithelium.

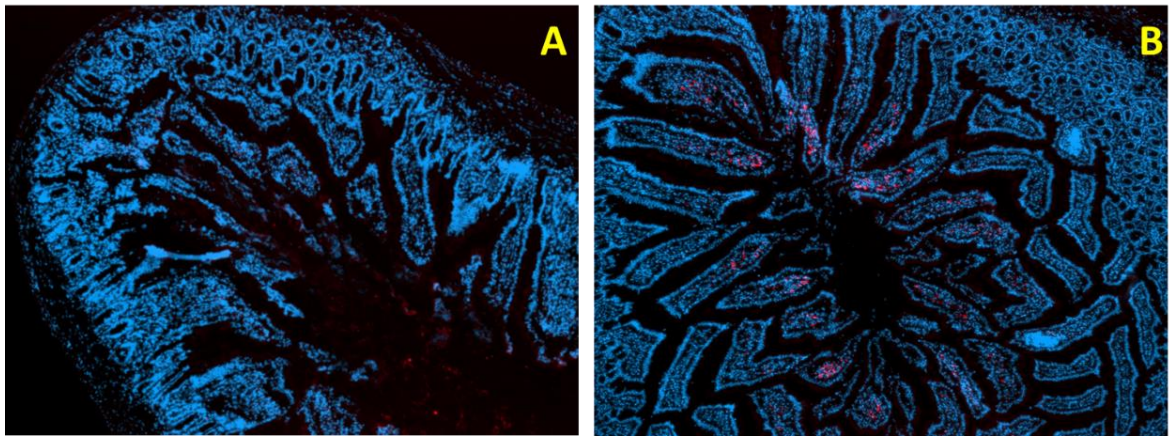

Figure 1. Fluorescence microscopy images of duodenum 2 hours after the oral administration of M-NP (A) or M-NP-P (B).

Conclusions

Polymeric-based nanocarriers can be an ideal system to deliver orally drugs with poor solubility. The inclusion of an oil in the matrix of the nanoparticle decreases the capability of the formulation to diffuse in mucus. Contrarily, the decoration of nanoparticles with PEG result in an increase in the mucus-permeating properties of the nanocarrier.

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Metal-Organic Frameworks as detoxifying agents

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Introduction: In human health risk assessment, ingestion of food and water is considered a major route of exposure to many contaminants. Moreover, the therapeutic misadventures, illicit drug ingestion or attempted suicide by using of harmful substances is a major worldwide public health problem that causes both a significant cost and severe health problems, even death. Unfortunately, for the vast majority of these poisoning, there are no specific pharmacological antidotes and currently available detoxification methods are weak and poorly prescribed (*e.g.* gastric lavage, ipecacuanha). However, they are accused of being ineffective and even of causing unnecessary complications, inducing severe adverse effects, which limit their use. Therefore, a fast and effective detoxification treatment remains a challenge. In general, it is desirable to use adsorbents in the form of powders exhibiting a larger surface area. These materials should act like "chemical sponges" selectively adsorbing the toxins in the gastrointestinal (GI) tract, and thus preventing the uptake in the blood and subsequent distribution to target organs.

In this context, a new class of crystalline porous materials, known as Metal-Organic Frameworks (MOFs), has attracted an increasing attention from academic and industrial domains. Compared to classical adsorbents such as organic (carbons) or inorganic solids (zeolites or silica), MOFs present several advantages (versatile composition, large structural variability, a very important porosity, etc.), making them excellent candidates for an efficient detoxification.

Materials and Methods: In this study, we target the oral detoxification of the extensively used and challenging aspirin (ASA, used as toxin drug) with two biocompatible MOFs (MIL-127 and MIL-125-NH_2). [1,2] Aside from their biocompatibility, these materials exhibit a high porosity associated with an important adsorption capacity and are stable at different pH values, even under GI conditions.

Results and Discussion: We have first evaluated the *in vitro* stability of MOFs and its ASA encapsulation capacity under GI conditions. The chemical and structural integrity of MOFs were confirmed after incubation in GI. On the other hand, the MOFs adsorption capacity was compared with activated charcoal, which is actually used in oral intoxications. Although the activated charcoal works better as detoxifying agent than MOFs in gastric conditions, it releases a significant amount of the adsorbed ASA when it passes to intestinal conditions. In contrast, MOFs are able to retain its ASA cargo along the GI tract. Further, when orally administered upon an ASA overdose in an animal model, MOFs are able to reduce the salicylate GI adsorption and toxicity more than 40-fold (avoiding histological damage) while exhibiting exceptional GI stability, poor intestinal permeation, and safety. Finally, an *ex vivo* intestinal model was used to assess MIL-127 bypass across the intestinal barrier, demonstrating a lack of intestinal absorption (probably because of their large particle size), high structural and chemical stability and poor intestinal permeation of the MOF and its constitutive ligand.

Conclusions. Both tested MOFs, which combine exceptional GI stability and important drug adsorbent capacity, are promising safe and efficient oral detoxification agents. These results open fascinating perspectives for the safe and efficient treatment of poisoning and accidental intoxication using biocompatible MOFs.

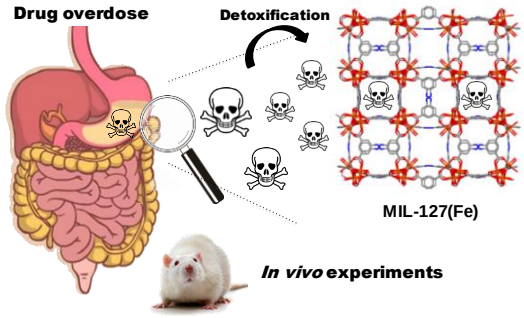

Figure 1. Scheme of the use of MOF as detoxifying agents

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Pollen grains for oral insulin administration

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Introduction: Oral route presents several difficulties for the administration of macromolecular drugs, in terms of poor stability and low permeability. Polymeric nanocarriers have shown to be promising vehicles for transmucosal delivery, due to their capacity to protect drugs and because their improved interaction with mucosal barriers. Among the investigated biomaterials, protamine is known for its good penetration properties, and that is why protamine nanocapsules (NCs) have been reported as potential carriers for oral peptide delivery. However, nanoparticles are often removed from the mucosa by the natural protective mechanisms of the gastrointestinal tract. In order to solve this problem, our approach is the exploration of prufied pollen grains (PG) as biomimetic mucointeractive platforms to improve the interaction of nanocarriers in contact with biological barriers.

Methods: Pollen of sunflower (*Helianthus annuus*) was treated by several consecutive washing steps to remove potentially allergenic components. PG were purified, producing hollow structures. For the optimization of nanocarriers to pollen grains, non-biodegradable fluorescent nanospheres with different size, surface charge and surface modifications were used. In the next step, protamine nanocapsules loaded with bovine insulin were developed and evaluated *in vivo* in normoglycemic and diabetic rats. [1]. On the day of the experiment, rats were fasted for 4 h and with free access to water. Insulin-loaded protamine NCs loaded in PG or insulin-loaded protamine NCs loaded alone were administered by oral gavage, and the blood glucose levels were monitorized at diferent time points (30 min, 1, 2 up to 8 h) after administration.

Results: After the purification process, we were able to obtain allergen-free PG with a hollow structure and a specific surface morphology. Nanospheres with different particle sizes (100 and 200 nm) and with positive and negative surface charge showed different affinities when they added over PG, nevertheless these differences did not affect to high capacity for association, (final loading of ~0.8 mg nanospheres/mg PG) and showed a similar sustained release profile over 8 hours. Protamine nanocapsules showed a profile coherent with their size and surface charge (~0.6 mg nanocapsule/mg PG). For the *in vivo* experiments with bovine insulin-loaded protamine nanocapsules, we obtained an association efficiency of 55%. Subcutaneous administration of insulin solution (2 IU/Kg), was employed as positive control, producing a decrease of 60% in blood glucose levels after 1 h. In our preliminary studies, the nanocarriers associated with bovine insulin in combination with PG at a dose of 100 IU/ kg, were able to reduce the blood glucose levels after 4 hours of administration while nanocapsules alone had no effect on glucose levels. This delayed in the effect of the insulin in decrease glucose levels on blood could be attributed to the slow transit and enhanced residence time of the developed pollen/nanocarrier-based delivery carriers in the small intestine.

Conclusion: We have developed a multy-step delivery platform based on pollen grains, able to enhance the mucosal interaction, stability and retention of polymeric nanocarriers, allowing for improved efficacy upon oral administration.

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Targeted release of therapeutic bioagents from enteric microparticulated formulations.

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In recent years, the development of new enteric drug delivery dosage forms based on polymethacrylates has increased. Eudragit® RS100 is a controlled release positively charged biocompatible copolymer based on poly(ethylacrylate-methyl-methacrylate-chlorotrimethylammonioethyl-methacrylate) cointaining around 4-7% of quaternary ammonium groups. This protects the cargo against gastric degradation but being insoluble at physiological pH values, it swells and becomes permeable to water, producing a sustained release over time.[1]

The encapsulation of bioactive agents in vectors (i.e., microparticles, capsules, mixed matrices, etc.) made of this polymer offers extra advantages: it is a mucoadhesive polymer, increasing the bioavailability of the therapeutic agent released. In addition, after releasing the cargo its erosion-resulting fragments are excreted without reaching systemic circulation.[2]

Several therapeutic enzymes have been approved by the FDA for the treatment of cystic fibrosis, leukemia, cancer treatment, etc.[3] But, it is known, that orally administered proteins may undergo unfolding or inactivation due to the acidic pH environment, thus, a protective coating is necessary to preserve their activity.

It is known the beneficial effects of probiotics in pathologies such as irritable bowel syndrome, necrotizing enterocolitis, colorectal cancer, etc.[4]. In the current dosage form, bacteria are not protected from gastric degradation with the consequent reduction of cell viability even for acidic bacteria such as *Lactobacillus acidophilus*.

In this work, the encapsulation of bioactive agents for colonic delivery in acid-resistant microcapsules is presented. On the one hand, the encapsulation of a model protein and, on the other hand, the encapsulation of model bacteria as an example of the administration of probiotics.

Bovine Serum Albumin (BSA) and Horseradish Peroxidase (HRP) were encapsulated in Eudragit® RS100 microparticles by the double emulsion-solvent evaporation method. Protein encapsulation efficiency was calculated by the BCA method. The stability of proteins was studied by circular dichroism and fluorescence, The biological activity of the HRP protein before and after simulated digestion was measured by the ABTS test.

Escherichia coli and *Lactobacillus acidophilus* were encapsulated in Eudragit® RS100 microparticles by a modified high-internal-phase double emulsion. The bacterial loading was calculated by TGA. The influence of reagents and encapsulations conditions were evaluated by contacting bacteria with each condition to test the potential viability loss. The viability of encapsulated bacteria was studied after simulated digestion. Long term cell viability was investigated after storage for 4 days, 1 and 5 months.

Microparticles have been characterized by scanning electron miscroscopy. *In vitro* cytotoxicity of the resulting microparticles was evaluated in Caco2-TC7 cell line, as an *in vitro* model of intestinal barrier.

BSA microparticles with an average diameter of 172.9 µm and an 88.4% of protein encapsulation and HRP microparticles with an average diameter of 178.2 µm and a 95.8% of protein encapsulation, were synthesized. The distribution of protein was homogeneous within the polymeric matrix. Both proteins were not structurally affected by the encapsulation process maintaining their secondary structure. The 100% biological activity of the proteins was confirmed by the ABTS method.

E. coli and *L. acidophilus* were encapsulated in particles of an average diameter of 212.0 and 293.6 µm, respectively, facilitating the potential intestinal mucosa attachment on large surfaces. It was proved that encapsulated bacteria resisted simulated acid conditions and released the load under simulated intestinal conditions. *In vitro* released bacteria excced the recommended therapeutic dose $>10^6$ CFU/mL. Long-term viability studies revealed that the microencapsulated bacteria were viable up to 5 months.

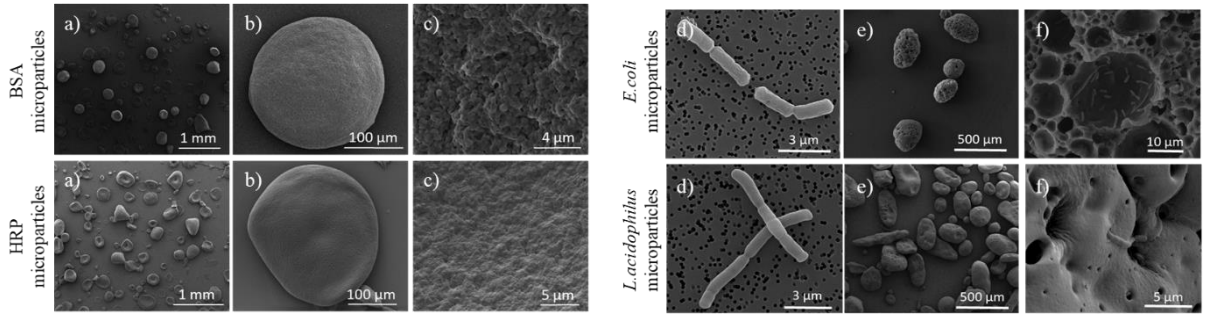

Figure 1. SEM characterization of: a-b-c) protein loaded microparticles, c-d-e) bacteria loadedmicroparticles.

- Polymetacrylate microparticles have been successfully synthesized by two modified methods using double emulsion.
- Model proteins and model bacteria have been encapsulated without being affected by acidic conditions and being potentially possible their release in the colon.
- Both microparticles showed high cytocompatibility at the doses tested.

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Thiamine-poly(anhydride) coating zein nanoparticles for oral insulin delivery. *In vivo* evaluation in *Caenorhabditis elegans*

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Introduction

One possible strategy to promote the oral bioavailability of biomacromolecules (i.e. protein and peptides) when administrated by the oral route may be their encapsulation in nanoparticles with mucus-permeating properties. In order to obtain these nanocarriers, one alternative would be to modify their surface with hydrophilic materials to facilitate their diffusion in mucus [1].

In the other hand, *Caenorhabditis elegans* (*C. elegans*) has been used as a model to study the hyperglycemic conditions in diabetes. In this context, high glucose levels in the culture media of *C. elegans* increased fat accumulation [2]. Besides, many of the components of the signaling pathway of insulin are well conserved from nematodes to humans. Therefore, the *C. elegans* may be an adequate *in vivo* model to evaluate non-invasive systems for oral insulin delivery, such as polymer-based nanoparticles. The aim of this work was to prepare and evaluate zein nanoparticles coated with a poly(anhydride)-thiamine conjugate (GT) as carriers for the oral delivery of insulin. For this purpose, the effect of this nanocarrier on the lipid metabolism of *C. elegans* under high glucose conditions was also investigated.

Materials and Methods

Nanoparticles were prepared from a hydroalcoholic solution of zein and insulin, by desolvation with water. Then, nanoparticles were incubated with the polymer conjugate GT, purified by tangential filtration and, finally, dried. The resulting nanoparticles were characterized and the insulin loading was calculated by RP-HPLC [3]. For *in vivo* evaluation, N2 Bristol wild-type *C. elegans* (Caenorhabditis Genetics Center, USA) were cultured at 20 ºC on NGM (Nematode Growth Medium) agar with *Escherichia coli* OP50 under high glucose conditions (50 mM). Synchronized L1 nematodes were cultivated on NGM plates containing 50 UI/mg of pure insulin or insulin-loading GT-NPZ at 20 ºC by 48 h. Orlistat supplemented plates (6 µg/mL) was used as fat reduction control. The fat content determination in the nematodes was realized by the fixative-based Nile red method [4].

Results and Discussion

The resulting nanoparticles displayed a mean size of about 250 nm, a negative surface charge and a payload of 80 µg insulin per mg nanoparticles. The presence of the coating layer on the surface of nanoparticle was put in evidence by a more negative surface charge and, particularly, by a significant capability to diffuse in intestinal mucus (more than 5-times) when compared with naked ones.

Initially, *C. elegans* were incubated with GT-NPZ fluorescently labelled with Lumogen® Red. The ingestion of zein nanoparticles by nematodes was confirmed by the presence of fluorescence in the pharynx and through the gut of worms. When the nematodes were fed with nanoencapsuled insulin, the fat content of the nematodes was about 18% lower than those treated with free insulin or empty nanoparticles. Moreover, the fat accumulation in the nematodes decreased by increasing the GT-to-zein ratio employed in the preparation of nanoparticles. In *C. elegans*, the effect human insulin is mediated by a *daf-2*/insulin receptor that controls the levels of fat accumulation [5]. All of these results suggest that the encapsulation of insulin in nanoparticles would promote the absorption and, thus, the interaction of the protein with its specific receptor (daf-2/insulin).

Conclusions

GT-coated zein nanoparticles may be used as oral nanocarries for insulin delivery. In the *in vivo C. elegans* model, these nanoparticles decreased significantly fat accumulation. In order to confirm this potential, further studies are planning on the *in vivo* rat model.

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Zein nanoparticles as potencial oral carriers for resveratrol

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Introduction: Resveratrol (RSV) is a promising candidate for incorporation on large human consumption products in order to take advantage of its pleiotropic effects on human health [1]. However, the use of RSV in the food industry has been hampered by its low aqueous solubility, poor stability and extensive metabolization by CYP 450, resulting in poor bioavailability and, consequently, hindering its efficacy. The use of nanotechnology-based systems may help to overcome many of the formulation challenges associated with RSV [2]. In this work, nanoparticles (NPs) based on the corn protein zein were developed in order to increase the bioavailability of RSV, potentially allowing its inclusion in different food products.

Methods: RSV-loaded zein NPs were produced by nanoprecipitation. The size and size distribution of NPs were characterized by dynamic light scattering and zeta potential by laser Doppler electrophoresis. The surface morphology of zein NPs was assessed by scanning electron microscopy (SEM). The stability of liquid suspensions of NPs was assessed after 30 days of storage at 4 ºC. The process of freeze-drying of zein NPs was optimized by using different cryoprotectants in order to improve NPs characteristics after resuspension in liquids. The toxicity of unloaded and RSV-loaded zein NPs was assessed in Caco-2 and HT29-MTX cell lines by MTT assay. Further, the permeability of RSV, either free or associated to zein NPs, was tested in Caco-2 cell and Caco-2/HT29-MTX cell co-culture monolayer models.

Results: Unloaded and RSV-loaded NPs presented average diameter values in the range of 120-180 nm, narrow size distribution (<0.150) and charge of around +20 mV. The association efficiency of the drug was greater than 77% for different initial drug loads. SEM images of zein NPs revealed a round shape and smooth surface. Liquid suspensions of zein NPs were stable for at least one month when stored at 4 ºC. The freeze-drying of zein NPs, using sucrose or trehalose as cryoprotectants, allowed an easier resuspension of NPs in liquids without significantly changes to the initial colloidal properties. RSV-loaded NPs presented no cytotoxicity to the colorectal cell lines Caco-2 and HT29-MTX (CC50 values >100µM of drug concentration). Finally, results from permeability studies across colorectal cell lines suggest that zein NPs may have some impact in the metabolism of RSV by intestinal cells (*Figure 1*).

Conclusions: Overall, the proposed RSV-loaded zein NPs present the potential to be used in the development of therapeutic or functional food products.

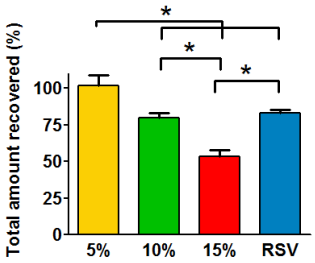

Figure 1. Total amount of RSV (expressed as % of the initial amount added to the apical compartment) recovered from membranes, apical and basolateral compartments of Caco-2 model after permeability experiments. Results are presented as mean ± SD (n=3). (*) denotes a significant difference (p<0.05).

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